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Modulation of Ethylene Synthesis in Acotyledonous Soybean and Wheat Seedlings

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Abstract. The characteristics of ethylene production and ACC conversion in 8-day-old soybean seedlings were examined and a relationship between cytochrome P-450 activity and ethylene-forming enzyme (EFE) activity was found. An atmosphere containing 10% carbon monoxide (CO) significantly inhibited ethylene production and ACC conversion in control soybean seedlings, but had only a slight effect on soybean seedlings treated with uniconazole. Foliar application of triclopyr, a pyridine analogue of the phenoxy herbicides, significantly increased ethylene production and ACC conversion in control, but not in uniconazole-treated seedlings. Triclopyr treatment also resulted in a three-fold increase in extractable cytochrome P-450 of 5-day-old etiolated soybeans. At equimolar concentrations tetcyclacis was more effective than uniconazole in reducing shoot elongation and endogenous ethylene production. Although uniconazole and tetcyclacis did not inhibit ACC conversion in nonherbicide-treated soybean seedlings, they did prevent the observed increase in ACC-dependent EFE activity following triclopyr application. However, the rate of ACC conversion in etiolated soybean segments was sensitive to uniconazole, and tetcyclacis inhibited the rate of ACC conversion by 2.6-fold in etiolated soybean segments within 4 h after treatment. Microsomal membranes were isolated from 5-day-old naphthalic anhydride-treated etiolated wheat shoots as this tissue contains much higher cytochrome P-450 levels than soybean shoots. Optical difference spectroscopy demonstrated that ACC generated binding spectrum characteristic of a reverse-type-I cytochrome P-450 substrate when combined with reduced microsomes. In vitro conversion of ACC to ethylene by microsomal membranes was NADPH-dependent, inhibited by CO, and had an apparent K_m and V_{max} of 45 μM and 0.345 nl/mg protein/h, respectively. These results suggest that cytochrome P-450-mediated monooxygenase reactions may be intimately involved in the conversion of ACC to ethylene in young soybean and wheat seedlings.

Triazole and norbornadiazetine-type plant growth retardants inhibit cytochrome P-450-dependent oxidation reactions during gibberellin synthesis (Rademacher et al. 1987). These compounds also inhibit ethylene production due to impaired conversion of ACC to ethylene (Grossmann et al. 1989, Sauerbrey et al. 1988). Uniconazole [(E)-(p-chlorophenyl)-4,4-dimethyl-2-(1,2,4,-triazol-1-yl)-1-penten-3-ol] is a potent member of the triazole family, which have been described as "plant multiprotectants" because of their ability to protect plants from several environmental and chemical stresses (Fletcher and Hofstra 1988). Kraus et al. (1991) reported that uniconazole reduced basal and stress-induced ethylene production by inhibiting ethylene-forming enzyme (EFE) activity in wheat and acotyledonous soybean seedlings. They also reported that soybean appears to have two distinguishable EFE systems, since uniconazole inhibited EFE activity in the seedling, but not in the cotyledon. Although many systems with apparent EFE activity have been described (Yang and Hoffman 1984), only recently has a member of the hydroxylase enzymes been isolated and demonstrated to satisfy all characteristics of authentic EFE activity (Ververidis and John 1991).

Cytochrome P-450 is a generic name for a group of hemoprotein enzymes whose properties have been reviewed recently (Donaldson and Luster 1991). They form carbon monoxide (CO) complexes which have a major absorption band at 450 nm and catalyze the monooxygenase reaction of a wide variety of substrates. Cytochrome P-450-mediated re-

actions require molecular oxygen and NADPH, and the enxyme system is bound, generally to the smooth endoplasmic reticulum. Cytochrome P-450 levels are reported to be high in young plant tissue, whereas almost absent in senescing tissue (Hendry 1986).

Tetcyclacis (5-(4-chlorophenyl)-3,4,5,9,10-petaaza-tetra-cyclo-4,5,1,0,0-dodeca-3,9-diene) is a norbornadiazetine-type compound which inhibits gibberellin biosynthesis (Rademacher et al. 1987) and is also cited as an inhibitor of other cytochrome P-450-mediated reactions (Cole and Owen 1987). An atmosphere of 10/20/70% carbon monoxide, oxygen, and nitrogen, respectively, has been demonstrated to effectively inhibit cytochrome P-450-mediated reactions (Fonne-Pfister et al. 1988). It has been reported that herbicides with auxinic activity, such as 2,4,-D, induce increased cytochrome P-450 activity in Jerusalem artichoke tubers (Adele et al. 1981, Reichhart et al. 1980). Cytochrome P-450 substrates and inhibitors generate unique and characteristically different optical binding spectra as described by Jefcoate (1978). Wiggins and Baldwin (1984) reported that triazole compounds generate type II binding spectra which indicates that these compounds are cytochrome P-450 inhibitors.

The present study investigates the relationship between ethylene production, EFE activity, and cytochrome P-450 using inhibitors (uniconazole, tetcyclacis, and carbon monoxide) and a promoter (triclopyr) or cytochrome P-450 activity.

Materials and Methods

Plant Material and Chemical Treatments

Soybean (*Glycine max.* L cv. Bicentennial) seeds (6) were sown in vermiculite in a double styrofoam cup system (Asare-Boamah et al. 1986) and drenched with 250 ml of 0.4 mg/L uniconazole, $2.7 \cdot 10^{-6}$ mol/L tetcyclacis (BASF, Limburgerhof, Germany), or water. Plants were grown in a controlled environment room with day/night lighting and temperatures of 16/8 h and 25/20°C, respectively, for 8 days (expanding unifoliate stage) with watering as required. Light from Sylvania metarc lamps, reached 200 $\mu E \cdot m^{-2} \cdot S^{-1}$ irradiance at plant level. For cytochrome P-450 studies, wheat (*Triticum aestavum* cv. Frederick) and soybeans were sown in vermiculite in flats, thoroughly soaked with the solutions described above, and grown in the dark at 24°C for 5 days. Naphthalic anhydride was applied to wheat seed as a coating (0.5 g/100 g seed) before planting. Dark- and light-grown soybean seedlings were foliar sprayed with triclopyr [(3,5,6-trichloro-2-pyridinyl)oxy acetic acid] at a rate equivalent to 4.4 g.a.i./ha at 5 and 8 days of age, respectively, and controls sprayed with water.

Inhibition Studies

Light-grown acotyledonous soybean seedlings were placed in 50-ml test tubes (two plants/tube) containing 3 ml of distilled water. Tubes were flushed with air and sealed. In one set, 5 ml of air was removed and replaced with 100% CO and, to another set, 0.5 μmol of ACC were added and the tubes allowed to incubate 2 h prior to adding CO. The tubes were incubated 1 h and then ethylene was measured.

Inhibition of ACC conversion to ethylene by tetcyclacis was investigated using etiolated acotyledonous soybeans. The seedlings were cut into 1-cm pieces and 1 g of tissue was floated in 5 ml of water in 50-ml flasks. Tetcyclacis (final concentration, 1×10^{-4} mol/L) was added to the flasks and incubated on a shaker (100 rpm) for 4 h before the addition of 0.5 μmol of ACC. The flasks were then sealed and incubated 1 h before measuring ethylene.

Ethylene Quantification

Samples were incubated in the dark at 24°C until a detectable quantity (12 ppb) of ethylene was present (1–4 h), then a 3-ml gas sample was withdrawn from the tubes or flasks, and the ethylene content of the sample was determined using a Hewlett-Packard gas chromatograph as previously described (Kraus et al. 1991). The injector, column, and detector temperatures were 60°, 85°, and 250°C, respectively, and the helium carrier gas flow was 24 ml/min.

Cytochrome P-450 Extraction, Quantification, and Binding Spectra

Cytochrome P-450 was extracted from etiolated 5-day-old acotyledonous soybeans or naphthalic anhydride-treated wheat as described by McFadden et al. (1990). Addition of 1% (wt/vol) polyvinylpolypyrollidone (PVPP) to the extraction buffer prior to grinding appeared to stabilize the enzyme. The cytochrome P-450 levels in the microsomal fraction (1.0 mg protein/ml) were measured with a Beckman DU 65 spectrophotometer as described by Estabrook and Werringloer (1978) using an extinction coefficient of 91 $cm^{-1} \cdot mM^{-1}$. Spectral changes characteristic of substrate binding to cytochrome P-450 were measured according to Jefcoate (1978) using 1 ml of 1 mg protein/ml microsomal suspension and ACC dissolved in the microsomal suspension buffer (pH 7.4) at micromolar concentrations of 12, 36, 72, and 144.

In Vitro NADPH-Dependent ACC Conversion

In vitro conversion of ACC to ethylene was assayed according to the methods of McFadden et al. (1990) using microsomal membranes isolated from 5-day-old naphthalic anhydride-treated, etiolated wheat seedling shoots. Each sample tube contained 1 mg microsomal protein in a final volume of 1 ml (pH 7.4). ACC was dissolved in the microsomal suspension buffer and added to the tubes to achieve final micromolar concentrations of 20, 40, 50, 100, 160, 200, 500, and 1000. Sealed test tubes (15 ml) were incubated at 30°C with shaking (100 rpm) for 2 h after which time 3 ml of head space gas was withdrawn and analyzed for ethylene content. The microsomal membranes were bubbled with 100% carbon monoxide for 1 min or NADPH was withheld to test the dependence of the reaction on oxygen and NADPH.

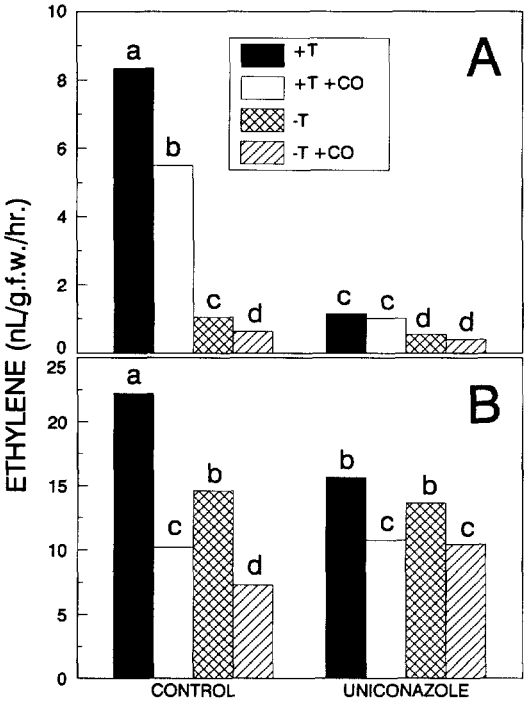

Fig. 1. Effect of 10% carbon monoxide (CO) on basal (−T) and triclopyr-induced (+T) (**A**) ethylene production and (**B**) exogenous ACC (0.5 μmol) conversion from acotyledonous 8-day-old soybeans. Triclopyr (4.4 g.a.i./ha) was applied to the control and uniconazole-treated soybeans 5 h prior to the experiment. Treatment means marked by a different letter are significantly different ($p = 0.05$, $N = 6$).

Results and Discussion

Inhibition of Ethylene Production and ACC Conversion by Carbon Monoxide

An atmosphere containing 10% CO significantly inhibited basal and triclopyr-induced ethylene production in the control soybean seedlings, but did not significantly reduce ethylene production in uniconazole-treated soybeans (Fig. 1A). Inhibition of basal and triclopyr-induced ethylene production by uniconazole indicates that uniconazole and CO may inhibit ethylene biosynthesis via a similar mechanism.

Uniconazole did not inhibit the basal rate of exogenous ACC conversion to ethylene. However, the significantly increased rate of ACC conversion induced by triclopyr was prevented by uniconazole (Fig. 1B). These results demonstrate that triclopyr causes an increase in EFE activity in the control soybean seedlings and that this stimulated activity is inhibited by uniconazole. Carbon monoxide inhibited the exogenous ACC conversion rate at least 50% in both the triclopyr-treated and nontriclopyr-treated control seedlings, while having a lesser (27–30%), but significant, effect on the comparable uniconazole-treated seedlings (Fig. 1B). Yang and Hoffman (1984) proposed that the conversion of ACC to ethylene may be accomplished by a N-hydroxylation reaction, and Grossmann et al. (1989) suggested that cytochrome P-450 may be involved in this reaction. CO and heterocyclic nitrogen-containing plant growth retardants (Rademacher et al. 1987) are known cytochrome P-450 inhibitors. These results demonstrate that both compounds inhibit ethylene synthesis and a relationship between cytochrome P-450 activity and ethylene synthesis is indicated.

Effect of Tetcyclacis on Ethylene Production and ACC Conversion

Equimolar concentrations of tetcyclacis and uniconazole applied to soybean seeds as a soil drench resulted in similar anatomical changes. They reduced shoot elongation and stimulated rooting (Fig. 2). Tetcyclacis treatment inhibited shoot elongation more than uniconazole, indicating that tetcyclasis is a more potent inhibitor of gibberellin biosynthesis. Tetcyclacis and uniconazole treatment inhibited the basal rate of ethylene production from acotyledonous soybean seedlings by 60% compared to the control seedlings (Fig. 3A). Triclopyr-induced ethylene production was reduced six- and 15-fold by uniconazole and tetcyclacis, respectively (Fig. 3A). Hence, tetcyclacis is also a more potent inhibitor of "stress" ethylene production than uniconazole. Uniconazole and tetcyclacis did not inhibit the basal rate of ACC conversion (Fig. 3B), but prevented the triclopyr-induced increase in ACC conversion in light-grown acotyledonous soybean seedlings.

Tetcyclacis is reported to inhibit cytochrome P-450-mediated herbicide metabolism by 45% in segments of etiolated wheat shoots within 6 h after application (McFadden et al. 1989). The effect of tetcyclacis on the conversion of ACC in etiolated segments of soybeans seedlings was investigated. Within 4 h after the addition of tetcyclacis ($1 \cdot 10^{-4}$ mol/L final concentration) the conversion of exogenous ACC was inhibited 2.6- and 1.5-fold in the control and uniconazole-treated etiolated soybean segments, respectively (Fig. 4). These results are similar to those of McFadden et al. 1989) who reported that tetcyclasis inhibited cytochrome P-450-dependent diclofop metabolism by 45% within 6 h after treatment. A more rapid inhibition of the conversion of ACC to ethylene by tetcyclacis would suggest direct inhibition of the enzyme(s) responsi-

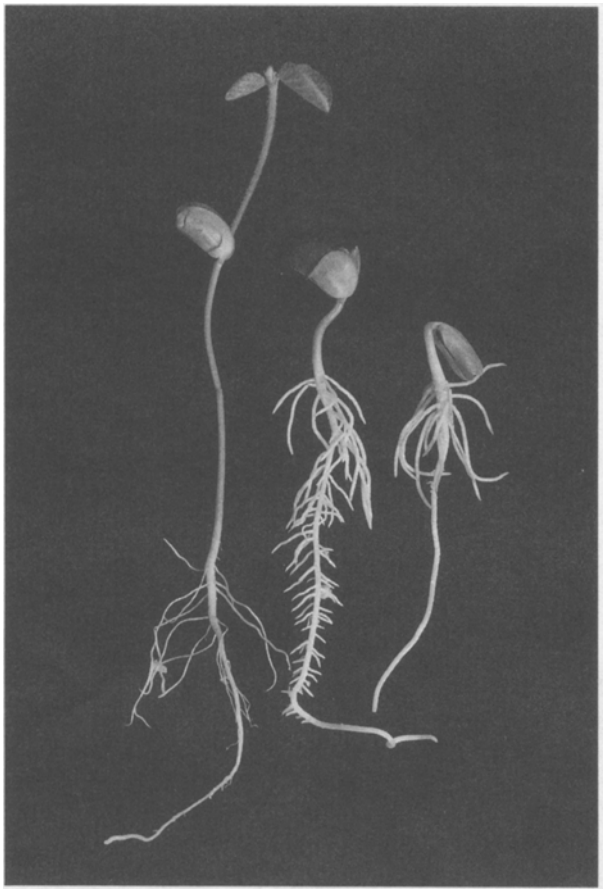

Fig. 2. Tetcyclacis and uniconazole-mediated anatomical changes in 8-day-old soybean seedlings. Soybean seeds were soil drenched with $2.7 \cdot 10^{-6}$ mol/L tetcyclasis and uniconazole solutions. From left to right: control, uniconazole-, and tetcyclacis-treated seedlings.

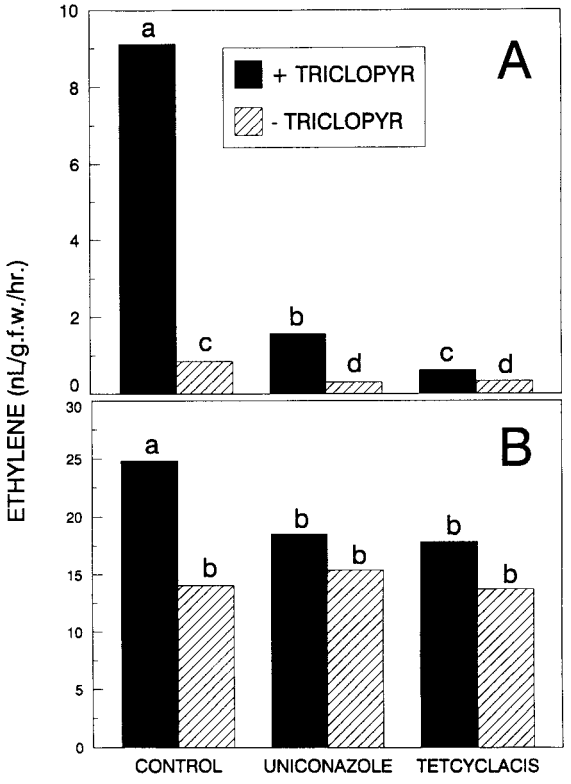

Fig. 3. Influence of $2.7 \cdot 10^{-6}$ mol/L soil drench applications of tetcyclacis and uniconazole on basal (−T) and triclopyr-induced (+T) **(A)** ethylene production and **(B)** exogenous ACC (0.5 μmol) conversion from acotyledonous soybean seedlings. Triclopyr (4.4 g.a.i./ha) was applied to the control, tetcyclacis-, and uniconazole-treated soybeans 5 h prior to the experiment. Treatment means marked by different letters are significantly different ($p = 0.05$, $N = 3$).

ble for the conversion; however, uptake and movement to the site of action may explain the relatively delayed action. It was observed that the rate of ACC conversion was inhibited 25% by uniconazole in etiolated tissue (Fig. 4). However, in light-grown seedlings, the basal rate of ACC conversion was not inhibited by uniconazole (Figs. 1B and 3B). This suggests that uniconazole is a more efficient EFE inhibitor in etiolated than in light-grown tissue, and the reason for this should be examined. Etiolated soybean segments had a higher rate of ACC conversion (40 nl/g fresh wt/h) than light-grown soybean seedlings (22–25 nl/g fresh wt/h) (Figs. 1B, 3B, and 4). The higher rate of ACC conversion in the etiolated tissue may reflect higher inherent EFE activity or could be due to higher ACC uptake by the etiolated segments compared to the light-grown seedlings.

Cytochrome P-450 Levels

The cytochrome P-450 levels in etiolated soybean seedlings were investigated to determine if triclopyr treatment induced increased levels of the enzyme. However, it was technically difficult to extract and quantify the cytochrome P-450 levels in soybean seedlings due to the low levels present. The observations that the triazoles inhibit cytochrome P-450-mediated gibberellin synthesis (Rademacher et al. 1987) and that wheat is reported to have cytochrome P-450 levels 10 times higher than soybeans (West 1980) may explain the requirement, observed in our laboratory, for 10-fold higher uniconazole applications on wheat to achieve shoot retardation similar to that of soybeans. This apparent relationship between cytochrome P-450 levels and the uniconazole concentration required to result in similar shoot retardation of wheat and soy-

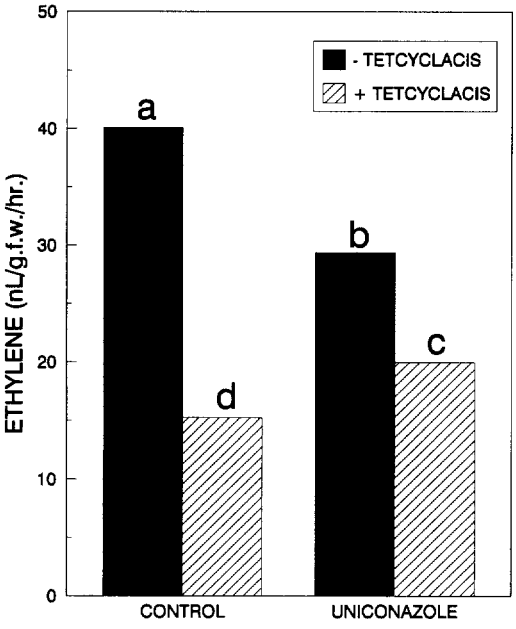

Fig. 4. Effect of tetcyclasis ($1 \cdot 10^{-4}$ mol/L) on exogenous ACC (0.5 μmol) conversion in etiolated control and uniconazole-treated soybean seedling segments 4 h after tetcyclacis application. Treatment means marked by different letters are significantly different ($p = 0.05$, $N = 4$).

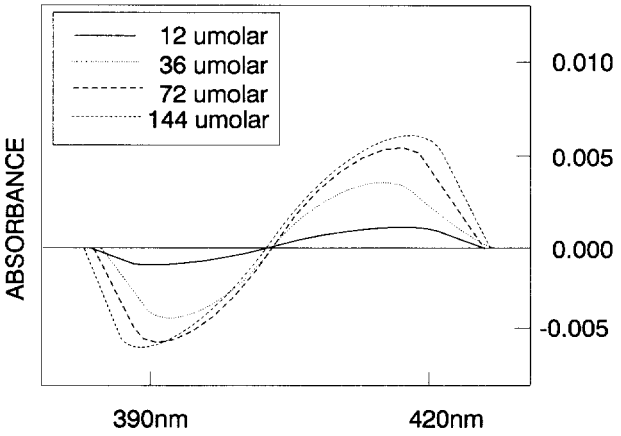

Fig. 5. ACC binding spectra recorded using dithionite-reduced microsomal membranes isolated from naphthalic anhydride-treated etiolated 5-day-old wheat shoots. ACC concentrations were 12, 36, 72, and 144 μmol and the microsomal suspension contained 1 mg protein/ml.

beans is noteworthy. Cytochrome P-450 levels in 5-day-old etiolated soybean seedlings increased from a basal level of 0.031 mmol/mg to a triclopyr-induced level of 0.085 nmol/mg microsomal protein ($N = 3$). These results are similar to those of Hendry and Jones (1984) who reported that 80-h-old mung bean seedlings had cytochrome P-450 levels of 0.025 nmol/mg microsomal protein and support previous findings that herbicides with auxinic activity increase cytochrome P-450 levels (Adele et al. 1981). Several other stresses, such as mechanical wounding and fungi which are known to increase ethylene production, have also been shown to increase cytochrome P-450 levels in plants (Hendry and Jones 1984).

Due to the inherently higher cytochrome P-450 level in wheat, all subsequent extraction of the enzyme was performed using etiolated wheat shoots. When cytochrome P-450 was extracted according to the methods of McFadden et al. (1990), the levels tended to be variable and often low. It was found that the addition of 1% (wt/vol) PVPP to the extraction buffer dramatically increased the microsomal cytochrome P-450 levels and reduced the degradation of the enzyme to cytochrome P-420 (Donaldson and Luster 1991), which is found in the postmicrosomal supernatant fraction (data not shown). When PVPP was used the level of cytochrome P-450 extracted from the wheat shoots was 0.237 nmol/mg protein, which is similar to the level (0.21) previously reported for wheat shoots (McFadden et al. 1989).

Cytochrome P-450-Binding Spectra

Substrates and inhibitors of cytochrome P-450 usually generate characteristic binding spectra (Jefcoate 1978). Inhibitors of cytochrome P-450, such as the triazoles, produce type II spectra due to direct binding of the heterocyclic nitrogen to the heme (Wiggins and Baldwin 1984). Due to binding near the catalytic site, hydrocarbon-based substrates generally produce reverse type I spectra, characterized by a trough at 390 nm and a peak at 420 nm (Jefcoate 1978). It has been stated that binding spectra results are often poor in quality and difficult to interpret; therefore, caution must be exercised (Jefcoate 1978, McFadden et al. 1989). ACC generated a binding spectrum characteristic of reverse type I substrates when combined with dithionite-reduced microsomes isolated from wheat shoots (Fig. 5). It is important to note that pure ACC solutions did not have any absorbance spectra in the wavelength range scanned, and binding spectra similar to wheat was obtained with soybean microsomes; however, the amplitude of the peak at 420 nm was greatly reduced (data not shown). It was also observed that as the ACC concentration increased the amplitude of the binding spectrum

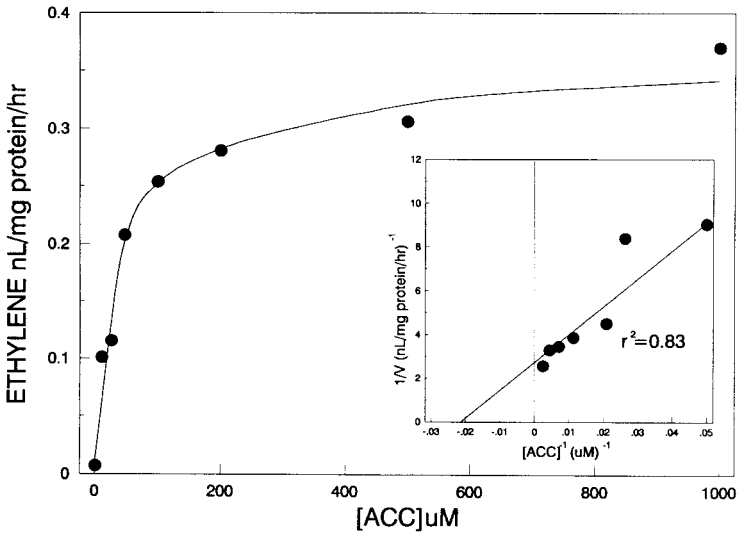

Fig. 6. Lineweaver-Burk plot of in vitro ethylene production from microsomal membranes isolated from naphthalic-anhydride-treated etiolated wheat shoots. The membranes (1.0 mg protein in 1.0 ml final volume) were supplied with ACC and 2 mM NADPH and incubated 2 h at 30°C with shaking 100 rpm). The data presented is from the average of three experiments.

changed proportionately and reached a maximum when the ACC concentration exceeded 72 μmol (Fig. 5). These results suggest that ACC interacts with cytochrome P-450 in a way indicative of it being a substrate.

In Vitro NADPH-Dependent ACC Metabolism

Cytochrome P-450-mediated conversion of ACC to ethylene by microsomal membranes isolated from naphthalic anhydride-treated wheat shoots was investigated. The isolated microsomal membranes converted ACC to ethylene with a V_{max} of 0.34 nl/mg microsomal protein/h when supplied with NADPH (Fig. 6). Ethylene was not produced in the absence of NADPH or when the microsomal membranes were bubbled with CO. These results suggest that cytochrome P-450 may be involved in the observed conversion of ACC to ethylene as cytochrome P-450-mediated reactions require oxygen and NADPH and are inhibited by CO (McFadden et al. 1990). EFE activity is reported to be lost upon tissue homogenization (Yang and Hoffman 1984). In our case it appears that the microsomal membranes are capable of converting ACC to ethylene if they are supplied with NADPH, as synthesis of NADPH is lost upon homogenization. The apparent K_m of the in vitro membrane-associated EFE activity was approximately 45 μM (Fig. 6). Because it was difficult to consistently obtain detectable ethylene levels at very low ACC concentrations using the present assay method, the K_m may actually be lower than 45 μM. These results are in close agreement with the apparent K_m reported for EFE in vivo (Yang and Hoffman 1984). Further studies on the cytochrome P-450 enzyme system and its relationship to EFE activity in plants are required, and the stereospecificity of this reaction should be investigated.

Yang and Hoffman (1984) proposed that ACC could be oxidized by a N-hydroxylase to form N-hydroxy-ACC. N-Hydroxylation is a very common reaction catalyzed by cytochrome P-450 enzymes and has been demonstrated recently in plant tissues (Halkier and Moller 1991). Yang and Hoffman (1984) also proposed that N-hydroxy-ACC would be unstable and fragment to yield ethylene and cyanoformic acid. The results of this study suggest that a cytochrome P-450-mediated reaction may be responsible for the N-hydroxylation of ACC to ultimately yield ethylene in young plant tissues. The EFE activity described in this investigation appears to be different than that reported by Ververidis and John (1991) who recovered an enzyme with authentic EFE activity from melon fruit (*Cucumis melo*). However, it is probable that different enzymes with EFE activity exist in different plant tissue. This likelihood is supported by our observation (Kraus et al. 1991) that uniconazole inhibited ACC conversion in soybean seedlings but not in soybean cotyledons.

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