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# Effect of 1-Aminocyclopropane-1-Carboxylic Acid on the Production of Ethylene in Senescing Flowers of *Ipomoea tricolor* Cav.[1]

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## ABSTRACT

**Application of 1-aminocyclopropane-1-carboxylic acid (ACC) to rib segments excised from flowers of *Ipomoea tricolor* Cav. resulted in the formation of $C_2H_4$ in greater quantities than produced under natural conditions. The ability of ACC to enhance $C_2H_4$ production was independent of the physiological age of the tissue and its capacity to synthesize $C_2H_4$ without applied ACC. When ACC was fed to rib segments that had been treated with [$^{14}$C]methionine, incorporation of radioactivity into $C_2H_4$ was reduced by 80%. Aminoethoxyvinylglycine and aminooxyacetic acid inhibited $C_2H_4$ production in rib segments of *I. tricolor* but had no effect on ACC-enhanced $C_2H_4$ production. Protoplasts obtained from flower tissue of *I. tricolor* did not form $C_2H_4$, even when incubated with methionine or selenomethionine. They produced $C_2H_4$ upon incubation with ACC, however. ACC-dependent $C_2H_4$ production in protoplasts was inhibited by *n*-propyl gallate, AgCl, $CoCl_2$, KCN, $Na_2S$, and $NaN_3$. ACC-dependent $C_2H_4$ synthesis in rib segments and protoplasts was dependent on $O_2$, the $K_m$ for $O_2$ being 1.0 to 1.4% (v/v). These results confirm the following pathway for $C_2H_4$ biosynthesis in *I. tricolor*: methionine [selenomethionine] → *S*-adenosylmethionine [selenoadenosylmethionine] → ACC → $C_2H_4$.**

A number of hypotheses have been proposed concerning the mechanism by which methionine, a precursor of $C_2H_4$ in higher plants, is converted to $C_2H_4$ (for a review, see ref. 17). Lieberman (17) proposed that methionine was directly converted to $C_2H_4$ by a mechanism involving free radicals. Burg (5), on the other hand, suggested that SAM[4], formed through activation of methionine by ATP, was the first intermediate in the pathway of $C_2H_4$ biosynthesis from methionine. Evidence supporting this hypothesis was supplied by Adams and Yang (1) and Konze and Kende (15). Konze and Kende (15) based their conclusions on the similarity of the kinetic properties of the $C_2H_4$-synthesizing system *in vivo* and of the SAM-forming enzyme *in vitro*, with methionine and selenomethionine as substrates. Using these same substrates, they also supplied evidence that direct conversion of methionine to $C_2H_4$ through a reaction with free radicals was not the mechanism by which $C_2H_4$ was generated *in vivo* (15). Recently, ACC was identified as a further intermediate in the pathway of $C_2H_4$ biosynthesis (2, 4, 19). The final step in the pathway of $C_2H_4$ biosynthesis, the conversion of ACC to $C_2H_4$, was investigated in apple tissue (2) and in homogenates of etiolated pea seedlings (16). In both instances, it was found to require $O_2$. The mechanism of this reaction has not yet been elucidated.

The addition of ACC to tissues which do not form $C_2H_4$ under natural conditions results in high rates of $C_2H_4$ production (4, 6, 12, 18). In studies summarized in this paper, we investigated the conversion of ACC to $C_2H_4$ in flower tissue of *Ipomoea tricolor*, where the pathway of $C_2H_4$ synthesis has been studied extensively in our laboratory (10, 14, 15), and in protoplasts derived from *Ipomoea* flower tissue.

## MATERIALS AND METHODS

**Chemicals.** L-[U-$^{14}$C]Methionine (250–260 mCi/mmol) was purchased from New England Nuclear; Cellulysin and ACC were from Calbiochem-Behring Corp., La Jolla, Calif., $CoCl_2$ and KCN were from Baker Chem. Co.; $NaN_3$, $(NH_4)_2SO_4$, and mannitol were from Mallinckrodt; Ficoll was from Pharmacia Fine Chemicals; L-methionine was from Nutritional Biochemicals; AgCl was from Allied Chemical Corp., New York; and all other chemicals were from Sigma Chemical Co. AVG was a gift from Dr. M. Lieberman (United States Department of Agriculture, Beltsville, Md.).

**Plant Material.** Morning-glory plants (*I. tricolor* Cav., cv. Heavenly Blue) were grown as described previously (9). Rib segments were excised from flower buds according to Kende and Hanson(13). The following designations were used to describe the age of the flower tissue: day 0, day of opening and fading of the flower; day −1, 1 day before flower opening; day −2, 2 days before flower opening.

**Incubation of Plant Material.** Rib segments were excised between 4:00 and 6:00 PM and were placed in 5-cm Petri dishes containing three discs of Whatman No. 1 filter paper and 1.7 ml $H_2O$ or test solution. The dishes were kept overnight in the growth cabinet in which the plants were grown. The following morning, the rib segments were removed from the Petri dishes, and batches of 10 to 13 segments were transferred to 25-ml Erlenmeyer flasks containing three discs of Whatman No. 1 filter paper and 0.8 ml $H_2O$ or test solution. After 1 h, the flasks were flushed with $C_2H_4$-free air for 2 min, sealed with serum-vial caps, and placed in the dark at 27 C.

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[4] Abbreviations: SAM: *S*-adenosylmethionine; AAA: aminooxyacetic acid; ACC: 1-aminocyclopropane-1-carboxylic acid; AVG: aminoethoxyvinylglycine.

**Determination of $C_2H_4$ Formation.** $C_2H_4$ was measured by gas chromatography as described previously (13).

**Measurement of Rolling Up of Rib Segments.** Angular measurements ($\alpha$) were taken during rolling up of the rib segments by aligning the sides of the segments in the flask alongside a protractor as described by Hanson and Kende (9). When segments were flat and unrolled, $\alpha$ was scored as 180°; as rolling up occurred, $\alpha$ increased to 360°.

**Measurement of Radioactivity in $C_2H_4$.** A 25-ml gas sample of [$^{14}$C]$C_2H_4$ evolved by senescing rib segments was withdrawn from each incubation flask with a 50-ml disposable syringe, while 25 ml saturated ammonium sulfate were injected into the flask with a second syringe. After the 25-ml gas sample was withdrawn, the needle of the syringe was replaced with a serum-vial cap. Two 1-ml samples were taken from each syringe, and the concentration of $C_2H_4$ was determined by gas chromatography. One ml ice-cold 0.25 M mercuric perchlorate in 2 M $HClO_4$ was injected into each syringe. The syringes were agitated on a wrist-action shaker at 5 C for 2 h. Samples from the gas phase of the syringes were analyzed for $C_2H_4$ to make sure that all of the $C_2H_4$ had been absorbed by the mercuric perchlorate. The serum-vial caps were removed, and the mercuric perchlorate was transferred from the syringes into scintillation vials. The syringes were rinsed with 15 ml scintillation fluid (Biofluor, New England Nuclear) which was also added to the scintillation vials. The radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer, model 3255 (Packard Instruments, Downers Grove, Ill.).

**Preparation of Protoplasts.** The bases of 50 flower buds were obtained on day −1 by removing the colored petals, ribs, and pedicels from the flowers. The reproductive parts then were removed from the center of the bases. The bases were dipped in 70% (v/v) ethanol, washed three times in sterile 0.6 M mannitol, and cut into fine strips. The strips were transferred to four 8-cm Petri dishes each containing 20 ml filter-sterilized 2% (w/v) Cellulysin in 0.6 M mannitol with 50 mM mercaptoethanolamine-HCl and 30 mM Na-Mes buffer (pH 5.5). The suspension was incubated and gently shaken for 11 h at 30 C. The protoplast suspension was filtered through three layers of cheesecloth and then through a 80-μm mesh nylon net. The protoplasts were collected by centrifugation at 300*g* for 20 min, washed with 0.6 M mannitol, and recentrifuged. The protoplasts then were diluted with 0.6 M mannitol, and recentrifuged. The protoplasts then were diluted with 0.6 M mannitol and 20% (w/v) Ficoll [purified according to Galbraith and Northcote (7)] in 0.6 M mannitol to give a final concentration of 10% (w/v) Ficoll in 20 ml. A gradient was formed by layering 10 ml 8, 6, and 0% (w/v) Ficoll in 0.6 M mannitol over the protoplast suspension. The intact protoplasts were floated to the interphase between 0 and 6% (w/v) Ficoll by centrifuging at 300*g* for 20 min. These protoplasts were harvested with a Pasteur pipette, washed with 0.6 M mannitol, and recentrifuged. The protoplasts were diluted with 0.6 M mannitol to yield the required concentration of the protoplasts. The protoplasts were viewed under the microscope at various stages during their preparation and during the subsequent experiments. Healthy protoplasts were scored on the basis of their ability to take up neutral red into their vacuoles.

## RESULTS

**Stimulation of $C_2H_4$ Production by ACC in Rib Segments of *I. tricolor*.** The level of $C_2H_4$ production by flowers of *I. tricolor* depends on the physiological age of the flower (13). $C_2H_4$ production from rib segments of flower buds was high on day −2 and low on day −1 (Fig. 1). On day 0, $C_2H_4$ production from the rib segments increased starting around 2:00 PM and was accompanied by a rolling up of the rib segments resembling the rolling up of ribs in the senescing flower. When rib segments excised from flower buds on days −2 and −1 and from flowers on day 0 were incubated on 1 mM ACC, $C_2H_4$ production was greatly enhanced irrespective of the physiological age of the tissue (Fig. 1). The segments treated with ACC rolled up at an earlier stage than those incubated on water alone (data not shown). The stimulation of $C_2H_4$ formation by ACC in rib segments on day 0 was saturated at 15 mM ACC (Table I). There was no inhibition of either $C_2H_4$ production or rolling up when rib segments were incubated on day 0 on concentrations of ACC exceeding 15 mM.

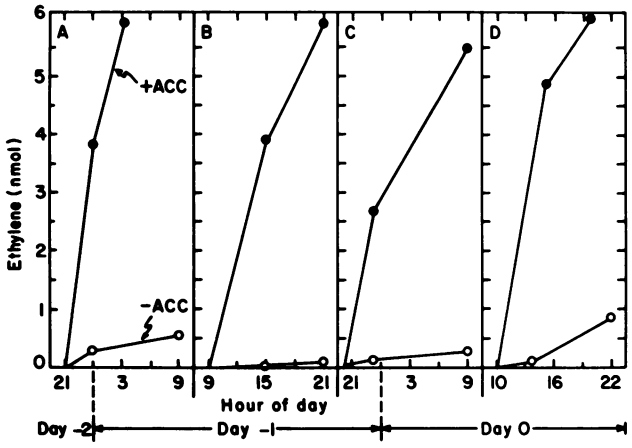


FIG. 1. Effect of ACC on $C_2H_4$ production in rib segments of flowers or flower buds of *I. tricolor* on days −2, −1, and 0. The rib segments were excised, incubated on distilled $H_2O$ and transferred, in batches of 13 segments, to 25-ml Erlenmeyer flasks containing $H_2O$ or 1 mM ACC. The flasks were left open for 1 h, flushed with $C_2H_4$-free air, closed with serum-vial caps, and incubated in the dark at 27 C. A: Rib segments were excised from flower buds on day −2 between 4:00 and 5:00 PM, incubated on $H_2O$ until 8:00 PM, and transferred to $H_2O$ or ACC; B: rib segments of flower buds were excised on day −2 between 5:00 and 6:00 PM, incubated on $H_2O$ overnight, and transferred to $H_2O$ or ACC in the morning of day −1; C: rib segments of flower buds were excised on day −1 between 4:00 and 5:00 PM, incubated on $H_2O$ until 8:00 PM, and transferred to $H_2O$ or ACC; D: rib segments of flower buds were excised on day −1 between 5:00 and 6:00 PM, incubated on $H_2O$ overnight, and transferred to $H_2O$ or ACC in the morning of day 0.

Table I. *Effect of Different ACC Concentrations on Formation of $C_2H_4$ from Rib Segments of Flowers of I. tricolor*

Rib segments were isolated between 5:00 and 6:00 PM on day −1 and were incubated on distilled $H_2O$ overnight and through the morning of day 0. Between 2:30 and 3:00 PM on day 0, they were transferred to 25-ml Erlenmeyer flasks and incubated on $H_2O$ or different concentrations of ACC. After 1 h, the flasks were flushed with $C_2H_4$-free air and closed with serum-vial caps. $C_2H_4$ production was monitored for 3 h.

| Concentration of ACC | $C_2H_4$ Produced | | |
|---|---|---|---|
| | 1 h | 2 h | 3 h |
| *mM* | *nmol/13 segments* | | |
| 0 | 0.01 | 0.14 | 0.26 |
| 1 | 0.58 | 1.21 | 1.79 |
| 10 | 1.32 | 2.62 | 3.55 |
| 15 | 1.68 | 3.31 | 4.41 |
| 20 | 1.62 | 3.00 | 4.18 |
| 25 | 1.54 | 3.01 | 4.20 |
| 30 | 1.53 | 3.18 | 4.31 |

**Effect of ACC on Conversion of [$^{14}$C]Methionine to [$^{14}$C]$C_2H_4$.** The conversion of [$^{14}$C]methionine to [$^{14}$C]$C_2H_4$ in the presence and absence of unlabeled ACC was studied to determine whether or not ACC was an intermediate in the *in vivo* production of $C_2H_4$ from methionine. Rib segments isolated from flower buds on day

−1 were incubated on [$^{14}$C]methionine overnight, rinsed the next morning in distilled $H_2O$ to remove excess [$^{14}$C]methionine, and incubated on $H_2O$ or ACC for 4 h. $C_2H_4$, which was produced at a constant rate during the 4-h incubation period, was trapped in mercuric perchlorate, and its radioactivity was determined (Table II). ACC enhanced $C_2H_4$ production by about 12-fold, as compared to the $H_2O$-treated control. The level of radioactivity in $C_2H_4$ produced by ACC-treated tissue was 5 times lower than that in $C_2H_4$ produced by rib segments incubated on $H_2O$ alone. The specific radioactivity of $C_2H_4$ from rib segments incubated on $H_2O$ was about 80 times higher than that of $C_2H_4$ from rib segments incubated on ACC. The application of exogenous ACC to rib segments reduced the incorporation of radioactivity from [$^{14}$C]-methionine into [$^{14}$C]$C_2H_4$ by 80%, indicating that ACC was an intermediate in the pathway of $C_2H_4$ synthesis from methionine.

**Effect of AVG and AAA on ACC-Enhanced $C_2H_4$ Production in Rib Segments of *I. tricolor*.** The rhizobitoxine analog AVG inhibits $C_2H_4$ production in many plant tissues (17). AVG also inhibits the conversion of SAM to ACC by an enzyme obtained from tomato fruit (4). AVG, at a concentration which inhibited the natural production of $C_2H_4$ by rib segments, did not inhibit ACC-enhanced $C_2H_4$ production in the same tissue when the segments were pretreated with AVG for 3 h prior to incubation on 1 mM ACC (Fig. 2). Pretreatment of the rib segments with AVG for 18 to 19 h prior to incubation on ACC + AVG decreased $C_2H_4$ production by about 30% compared to $C_2H_4$ synthesis in tissue, which was incubated on $H_2O$ only for 18 to 19 h prior to incubation with ACC (data not shown). AAA also inhibited the production of $C_2H_4$ during the senescence of rib segments of *I. tricolor* (Fig. 3A), 50% inhibition being obtained at 0.1 mM AAA. AAA delayed, but did not inhibit the rolling up and senescence of the flower tissue (Fig. 3B), indicating that AAA did not act as a general poison. AVG had a similar effect on $C_2H_4$ production and senescence in *I. tricolor* (13). AAA had no effect on ACC-enhanced $C_2H_4$ production in rib segments of flowers from *I. tricolor* (results not shown).

**Effect of $O_2$ on ACC-enhanced $C_2H_4$ Production by Rib Segments of *I. tricolor*.** $C_2H_4$ production by plant tissues is inhibited under anaerobic conditions (8). The conversion of methionine to ACC is not dependent on $O_2$ (2). The requirement of $O_2$ for ACC-enhanced $C_2H_4$ production was investigated. Erlenmeyer flasks containing 10 rib segments and preincubated on 1 mM ACC for 2 h were evacuated and refilled with argon. Using a syringe, the appropriate volume of argon was removed and replaced with $O_2$ to give the desired $O_2$ concentration. ACC-enhanced $C_2H_4$ production increased as the concentration of $O_2$ was increased in the flasks (Fig. 4A). The rate of $C_2H_4$ production for each $O_2$ concentration was highest during the period immediately after the injection of $O_2$. The $K_m$ for $O_2$ was found to be 1% (v/v) when the initial rates of $C_2H_4$ production (0–10 min, Fig. 4A) were plotted in a Lineweaver-Burk plot (Fig. 4B). When the overall rates (0–100 min, Fig. 4A) were used for the Lineweaver-Burk plot, the $K_m$ for $O_2$ was 1.4% (Fig. 4B). Concentrations of 50 and 100% (v/v) $O_2$ partially inhibited $C_2H_4$ production by rib segments (Fig. 4B).

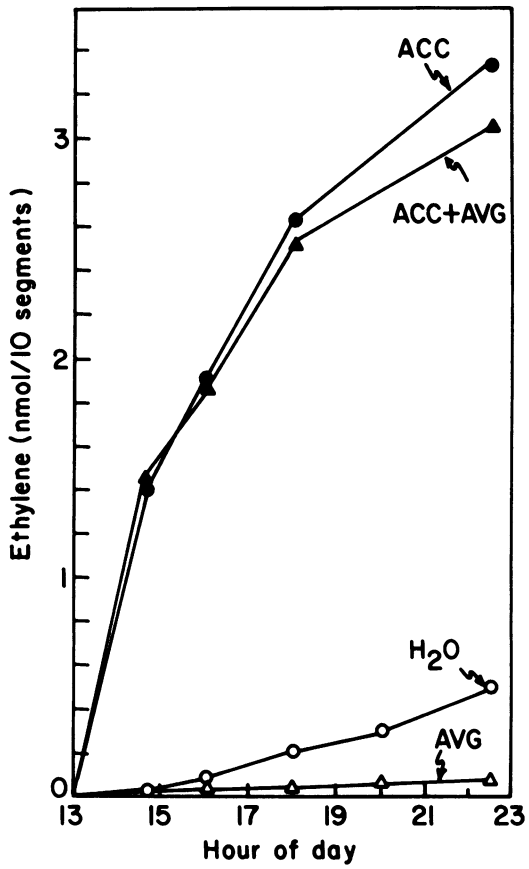


FIG. 2. Effect of AVG on ACC-enhanced $C_2H_4$ production of rib segments of *I. tricolor*. Rib segments were excised from flower buds on day −1 between 4:00 and 5:00 PM, incubated on $H_2O$ overnight, and transferred to 5-cm Petri dishes containing $H_2O$ or 0.1 mM AVG in the morning of day 0. The segments were incubated on $H_2O$ or AVG for 3 h and then transferred, in batches of 10 segments, to 25-ml Erlenmeyer flasks containing either 1 mM ACC, $H_2O$, 0.1 mM AVG, or 0.1 mM AVG + 1 mM ACC. The flasks were left open for 1 h, flushed with $C_2H_4$-free air, closed with serum-vial caps, and incubated in the dark at 27 C. $H_2O$ → $H_2O$ (○); $H_2O$ → ACC (●); AVG → AVG (△); AVG → ACC and AVG (▲).

Table II. *Effect of ACC on Incorporation of Radioactivity from [$^{14}$C]-Methionine into [$^{14}$C]$C_2H_4$ in Rib Segments of I. tricolor*

Rib segments were excised between 5:00 and 6:00 PM on day −1 and incubated overnight and through the morning of day 0 in a Petri dish containing a disc of Whatman No. 1 filter paper and 2.8 ml 10.4 μM L-[U-$^{14}$C]methionine (256 Ci/mol). Between 2:00 and 2:30 PM on day 0, the segments were rinsed to remove [$^{14}$C]methionine from the surface of the tissue and blotted, and batches of 10 segments were incubated in 25-ml Erlenmeyer flasks containing a disc of Whatman No. 1 filter paper and 0.8 ml distilled $H_2O$ or 15 mM ACC. Each Erlenmeyer flask contained a small vial with a filter-paper wick and saturated $Ba(OH)_2$ to trap $CO_2$. The flasks remained open for 1 h, then were flushed with $C_2H_4$-free air, and sealed with serum-vial caps. The $C_2H_4$ evolved between 4:30 and 8:30 PM was collected, and the radioactivity in this $C_2H_4$ was determined.

| Incubation Medium | $C_2H_4$ Formed | Total Radioactivity in $C_2H_4$ | Specific Radioactivity |
|---|---|---|---|
| | *pmol/10 segments* | *pCi* | *Ci/mol* |
| $H_2O$ | 243 | 201 | 0.83 |
| 15 mM ACC | 2978 | 41 | 0.01 |

**$C_2H_4$ Production by Protoplasts Obtained from *I. tricolor* Flower Buds.** To investigate the effect of ACC at the cellular level, ACC was fed to protoplasts prepared from the bases of flower buds which had been excised on day −1. The protoplasts did not form any significant amount of $C_2H_4$, even when incubated with 5 mM L-methionine or 5 mM DL-selenomethionine for 3 h. However, $C_2H_4$ was formed when protoplasts were incubated on ACC (Fig. 5). $C_2H_4$ was produced from ACC by intact but not by osmotically ruptured protoplasts. ACC-induced $C_2H_4$ production in protoplasts was saturated at 5 to 10 mM ACC and had a $K_m$ of 0.5 mM. $O_2$ was also required for the production of $C_2H_4$ from ACC in protoplasts (Table III). The $O_2$ requirement for ACC-dependent $C_2H_4$ production by protoplasts might have been due to an energy-requiring uptake system. To test whether the intra-

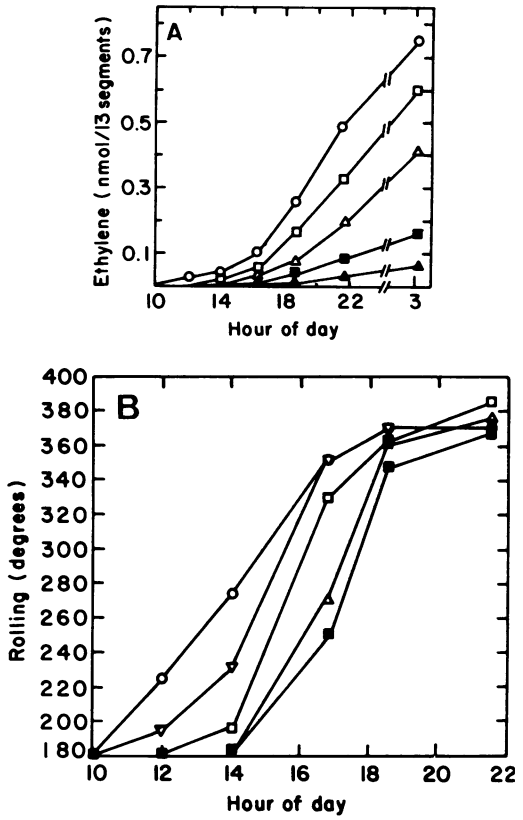


FIG. 3. A: $C_2H_4$ production by rib segments of *I. tricolor* flowers on day 0 treated with different concentrations of AAA. Rib segments were excised from flower buds on day −1 between 4:00 and 5:00 PM and incubated overnight on $H_2O$ (○), 33 μM AAA (□), 0.1 mM AAA (△), 0.33 mM AAA (■), and 1 mM AAA (▲). In the morning of day 0, the segments were transferred to 25-ml Erlenmeyer flasks containing the same concentrations of AAA. The flasks were left open for 2 h, flushed with $C_2H_4$-free air, closed with serum-vial caps, and incubated in the dark. B: The effect of different concentrations of AAA on senescence (rolling up) of rib segments on day 0. The rib segments were treated as described under Figure 3A, and the degree of rolling up was assessed by aligning the sides of the segments in the flasks alongside a protractor. (○), $H_2O$; (▽), 10 μM AAA; (□), 33 μM AAA; (△), 0.1 mM AAA; (■), 0.33 mM AAA. Rolling up at 1 mM AAA was almost identical to that at 0.33 mM AAA.

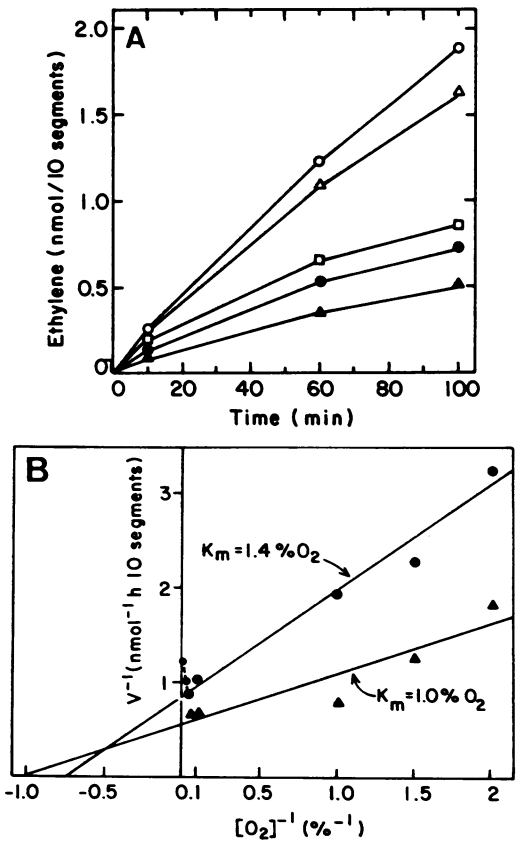


FIG. 4. A: Effect of different concentrations of $O_2$ on $C_2H_4$ production by rib segments of *I. tricolor* flowers incubated on ACC. Rib segments were excised from flower buds on day −1 between 4:00 and 5:00 PM and incubated on $H_2O$ overnight. In the morning of day 0, the segments were transferred to 25-ml Erlenmeyer flasks containing 1 mM ACC. After 2 h, the flasks were closed with serum-vial caps, evacuated to 100 μm mercury pressure 10 times, and refilled each time with argon. The appropriate volume of argon was removed with a syringe and was replaced with $O_2$ to yield final concentrations of 0.5 (▲), 0.67 (●), 1.0 (□), 10 (△), and 20 (○)% (v/v) $O_2$. B: Lineweaver-Burk plot of the rates of $C_2H_4$ production by rib segments of *I. tricolor* flowers incubated on day 0 with ACC in the presence of different concentrations of $O_2$ (▲——▲), points calculated from rates of $C_2H_4$ production between 0 to 10 min (A); (●——●), points calculated from rates of $C_2H_4$ production between 0 to 100 min. In addition to the $O_2$ concentrations given in A, points obtained at 50 and 100% $O_2$ are also plotted.

cellular conversion of ACC to $C_2H_4$ was $O_2$-dependent, protoplasts were preincubated with ACC in the presence of $O_2$ and then washed free of exogenous ACC. $C_2H_4$ production by these preloaded protoplasts was indeed found to require $O_2$. In this case, no inhibitory effect of $O_2$ was observed at higher $O_2$ concentrations (Table III). The lack of $C_2H_4$ production in the absence of $O_2$ was not due to irreversible damage since protoplasts preloaded with ACC started to produce $C_2H_4$ when they were brought back to air after 2 h under $N_2$ (data not shown).

AgCl, at a concentration of 0.1 mM, 1 mM KCN, and 1 mM *n*-propyl gallate inhibited ACC-enhanced $C_2H_4$ production in protoplasts by more than 90% (Table IV). $NaN_3$ and $Na_2S$, both at concentrations of 1 mM, and $CoCl_2$, at a concentration of 0.1 mM, also inhibited ACC-induced $C_2H_4$ production, but only by 40 to 54%. The protoplasts remained intact in the presence of these inhibitors and retained their ability to take up neutral red into their vacuoles.

## DISCUSSION

Applied ACC enhanced $C_2H_4$ production in rib segments from flower tissue of *I. tricolor* irrespective of the physiological age of the tissue and in protoplasts isolated from the bases of flower buds. ACC-enhanced $C_2H_4$ production was saturable with respect to ACC concentration in both rib segments and protoplasts (Table I; Fig. 5). $C_2H_4$ production in response to ACC was also saturable in pea stem sections (12). However, $C_2H_4$ production was not saturable with respect to ACC concentration in an ACC-dependent, cell-free $C_2H_4$-forming system obtained from etiolated pea seedlings (16). This discrepancy could be explained by assuming that the *in-vitro*, ACC-dependent $C_2H_4$-forming system from peas is not the same that operates *in vivo* or that saturation of $C_2H_4$ formation in pea stem sections reflects the presence of a saturable uptake system for ACC.

Conversion of ACC to $C_2H_4$ in rib segments and protoplasts of *I. tricolor* was $O_2$-dependent (Table III; Fig. 4) as was also found in the case of apple tissue (2) and soybean leaves (19). Through the use of protoplasts preloaded with ACC in air, the possibility could be excluded that $O_2$ was required for the uptake of ACC only, rather than for the conversion of ACC to $C_2H_4$. The cell-free $C_2H_4$-forming system from etiolated pea seedlings also required

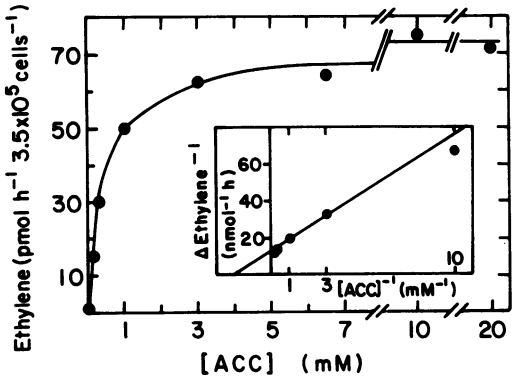


FIG. 5. $C_2H_4$ production from protoplasts of *I. tricolor* incubated on different concentrations of ACC. Protoplasts ($3.5 \times 10^5$) in 0.6 M mannitol were incubated with 0, 0.1, 0.3, 1, 3, 6.5, 10, and 20 mM ACC in a total volume of 300 μl in sealed 10-ml test tubes for 90 min at 28 C. A Lineweaver-Burk plot derived from this experiment is shown in the inset.

Table III. *$C_2H_4$ Production by Protoplasts Obtained from Flower Bases of I. tricolor Preloaded or Incubated with ACC in Presence of Different Concentrations of $O_2$*

Protoplasts ($5 \times 10^6$) in 2 ml 0.6 M mannitol were incubated in 15 mM ACC for 45 min at 28 C. These protoplasts then were washed free of extracellular ACC by centrifugation in a Ficoll gradient followed by a wash in 0.6 M mannitol. Another batch of protoplasts was used directly without preincubation on ACC. These protoplasts and the preloaded protoplasts ($5 \times 10^5$) were placed in 10-ml serum vials in a total volume of 300 μl 0.6 M mannitol, and the vials were closed with serum-vial caps. ACC (15 mM) was added to the protoplasts that had not been preloaded. The serum vials were flushed with $N_2$ for 2 min. For 50 and 100% $O_2$, the $N_2$ was replaced with these concentrations of $O_2$, for 20% $O_2$ with air. For all other concentrations of $O_2$, the appropriate volume of $N_2$ was removed with a syringe and was replaced with $O_2$ to yield the desired concentration of $O_2$. The protoplasts were incubated at 28 C, and the amount of $C_2H_4$ formed was determined after 1 and 2 h.

| Oxygen Concn (v/v) | $C_2H_4$ Formed | | | |
|---|---|---|---|---|
| | Protoplasts Preloaded with ACC | | Protoplasts Incubated with ACC | |
| | 1 h | 2 h | 1 h | 2 h |
| % | *pmol/5 × $10^5$ protoplasts* | | | |
| 0 | <2 | <2 | <2 | <2 |
| 2 | 4 | 6 | 14 | 48 |
| 5 | 10 | 16 | 34 | 85 |
| 10 | 19 | 33 | 78 | 178 |
| 20 | 45 | 81 | 172 | 366 |
| 50 | 159 | 223 | 293 | 549 |
| 100 | 223 | 302 | 252 | 618 |

$O_2$ (16). The role of $O_2$ in $C_2H_4$ formation from ACC is not known. $O_2$ may be either directly involved in $C_2H_4$ synthesis or may be required for the formation of $H_2O_2$, the presence of which appears to be necessary for $C_2H_4$ production in homogenates of etiolated pea seedlings (16). $O_2$ dependency may also be due to a requirement for ATP in the conversion of ACC to $C_2H_4$; ATP may be needed directly for the synthesis of $C_2H_4$ from ACC or indirectly for regulation of enzyme activities. Unfortunately, protoplasts did not survive treatment with the uncouplers 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone, thereby leaving the question of the role of ATP in the conversion of ACC to $C_2H_4$ unresolved.

Unlabeled ACC reduced the incorporation of radioactivity from [$^{14}$C]methionine into [$^{14}$C]$C_2H_4$ (Table II), indicating that ACC diluted the $^{14}$C-labeled ACC pool and confirming that ACC was converted to $C_2H_4$ by a saturable system. Since application of ACC resulted in the immediate production of $C_2H_4$, formation of $C_2H_4$ must be limited by the availability of ACC and, therefore, must be controlled by either of two enzyme systems, the one converting methionine to SAM (methionine adenosyltransferase) or the other converting SAM to ACC. Methionine adenosyltransferase can be isolated from tissue producing low amounts of $C_2H_4$ (15), indicating that conversion of SAM to ACC is the limiting step in $C_2H_4$ biosynthesis. A comparison of the activity of methionine adenosyltransferase with $C_2H_4$ production in rib segments incubated on methionine or selenomethionine showed that the formation of SAM might become limiting when the tissue was induced to form $C_2H_4$ (15, 16). AVG did not inhibit ACC-enhanced $C_2H_4$ synthesis in rib segments of *I. tricolor* (Fig. 2). This is consistent with the fact that AVG inhibits formation of ACC *in vivo* and the activity of the ACC-forming enzyme *in vitro* (4) and that it does not inhibit ACC-dependent $C_2H_4$ synthesis in mungbeans (21) and in pea homogenates (16). AAA inhibited the natural production of $C_2H_4$ (Fig. 3) but not ACC-enhanced $C_2H_4$ production in rib segments, just as AVG did. Since AAA, like AVG, also inhibits pyridoxal phosphate-requiring enzymes (11), it is assumed that AAA blocks $C_2H_4$ synthesis in *I. tricolor* by inhibiting the ACC-forming enzyme. Yu *et al.* (22) found that AAA acted as an inhibitor of the ACC-forming enzyme obtained from tomato fruit.

Table IV. *Inhibitors of ACC-enhanced $C_2H_4$ Production in Protoplasts Isolated from Flower Bases of I. tricolor*

Protoplasts ($3 \times 10^5$) were incubated with 1, 5, or 6.5 mM ACC in a total volume of 300 μl in 10-ml test tubes closed with serum-vial caps. Different inhibitors were included at the given concentrations. The amount of $C_2H_4$ accumulated in the test tubes after 90 min of incubation at 28 C was determined. The appearance of the protoplasts was not affected by the presence of any of these inhibitors.

| Concentration of ACC | Concentration of Inhibitor | $C_2H_4$ Formed after 90 min | Inhibition |
|---|---|---|---|
| *mM* | | *pmol/3 × $10^6$ protoplasts* | *%* |
| 5 | None | 55 | 0 |
| 5 | 0.01 mM AgCl | 41 | 25 |
| 5 | 0.10 mM AgCl | 4 | 93 |
| 5 | 0.01 mM $CoCl_2$ | 53 | 4 |
| 5 | 0.10 mM $CoCl_2$ | 33 | 40 |
| 5 | 0.01 mM *n*-PG[a] | 50 | 9 |
| 5 | 0.10 mM *n*-PG[a] | 21 | 62 |
| 5 | 1.00 mM *n*-PG[a] | 1 | 98 |
| 6.5 | None | 99 | 0 |
| 6.5 | 1.00 mM $Na_2S$ | 53 | 47 |
| 1 | None | 96 | 0 |
| 1 | 1.00 mM $NaN_3$ | 44 | 54 |
| 1 | 1.00 mM KCN | 9 | 91 |

[a] *n*-PG: *n*-propyl gallate.

Protoplasts are advantageous to study the effect of inhibitors on the formation of $C_2H_4$ from ACC since the incubation medium is in close contact with the cells and uptake problems are reduced. *n*-Propyl gallate inhibits $C_2H_4$ production in several fruit tissues (3) and in a cell-free system from peas (16). However, *n*-propyl gallate has no effect on $C_2H_4$ production in rib segments (unpublished data), although it is an effective inhibitor when tested with protoplasts prepared from the same tissue (Table IV). *n*-Propyl gallate is referred to as a scavenger of oxygen radicals, but it reacts also with peroxide and is a very good substrate of horseradish peroxidase. Peroxidation of *o*-dianisidine, a substrate of horseradish peroxidase (20), was found to be competitively inhibited by *n*-propyl gallate (J. R. Konze, unpublished results). The inhibition

of $C_2H_4$ production from ACC by KCN, $NaN_3$, *n*-propyl gallate, and catalase *in vitro* (16) and by *n*-propyl gallate, KCN, $Na_2S$, and $NaN_3$ in protoplasts (Table IV) indicates that ACC is converted to $C_2H_4$ in a peroxidative reaction. However, no natural peroxidative system for the oxidation of ACC has, as yet, been identified

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