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Effects of Exogenous Ethylene on Ethylene Production in Citrus Leaf Tissue[1]

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ABSTRACT

Exogenous ethylene stimulated ethylene production in intact citrus (*Citrus sinensis* L. Osbeck cv. "Washington Navel") leaves and leaf discs following a 24-hour exposure. Studies with leaf discs showed that ethylene production decreased when ethylene was removed by aeration. The extent of stimulation was dependent upon the concentration of exogenous ethylene (1–10 microliters per liter). Silver ion blocked the autocatalytic effect of ethylene at concentrations of 0.5 millimolar and lower, but increased ethylene production at higher concentrations. The stimulating effect of ethylene resulted from the enhancement of both 1-aminocyclopropane-1-carboxylic acid (ACC) formation and the conversion of ACC to ethylene. Whereas autocatalysis was evident following 24 hours incubation, autoinhibition of wound- and mannitol-induced ethylene production was observed during the first 24-hour incubation. Ethylene treatment during this period resulted in a marked decrease in ACC levels and ethylene production rates. Furthermore, in leaf discs treated for 24 hours with ethylene, ethylene production rates increased greatly during the first 2 hours after removal of exogenous ethylene by aeration. This increase was eliminated if the discs were transferred to propylene instead of air, indicating that the autocatalytic effect of ethylene is counteracted by its autoinhibitory effect. It is suggested that autocatalysis involves increased synthesis of ACC synthase and the enzyme responsible for the conversion of ACC to ethylene, whereas autoinhibition involves suppression of the activity of these two enzymes.

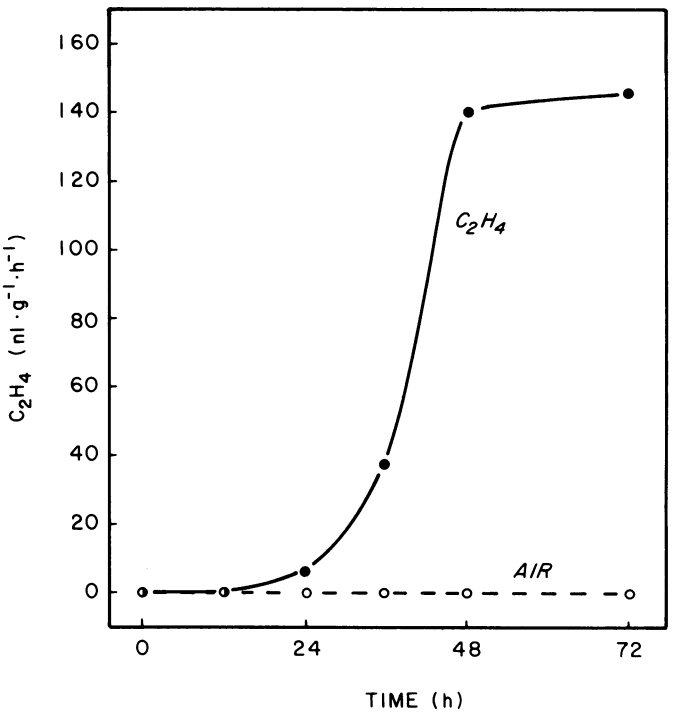

FIG. 1. Effect of exogenous ethylene (12 μl/l) on the time course of ethylene production rates in intact citrus leaves.

In vegetative tissue, the rate of ethylene production is thought to be regulated mainly by the internal levels of IAA, and application of exogenous auxin generally stimulates ethylene production (1, 9). Other plant growth substances, such as cytokinins, also stimulate ethylene production and particularly enhance the IAA-induced ethylene synthesis synergistically (1, 9). Autocatalysis (1, 6, 12) and autoinhibition (13, 18, 21, 22) of ethylene production have been mainly studied in fruit tissues. However, Aharoni and Lieberman (3) reported that tobacco leaf discs treated with ethylene produced somewhat more ethylene than untreated discs. In excised pea segments, applied ethylene or propylene stopped wound ethylene synthesis during the period of application (16). These data suggest that ethylene may also play a role in regulating its own production in vegetative tissues.

During studies of wound ethylene production in citrus leaf discs, we have observed that ethylene inhibits and stimulates ethylene production depending upon the duration of exposure. The present study was undertaken to study at which step in the ethylene biosynthetic sequence (2) ethylene exerts its effects.

MATERIALS AND METHODS

Plant Material and Treatments. Leaves, 3 to 4 months old, were harvested each day of the experiment from mature citrus (*Citrus senensis* [L.] Osbeck cv. "Washington Navel") trees grown in Davis, California. Discs, 8 mm in diameter, were excised with a cork borer and kept in a moistened Petri dish until treatment. Except when otherwise noted, 10 discs, weighing about 140 mg, were placed in a small Petri dish and enclosed in a moistened 1-liter jar into which humidified air, with or without 12 μl/l ethylene, was passed at a rate of 90 ml/min. Incubation was carried out in the dark at 25°C. When discs were treated with various compounds, they were floated on a solution containing 50 mM K-phosphate buffer (pH 5.3) with the adaxial surface down. After treatment, the discs were blotted dry on a filter paper and then placed in either air or ethylene.

In one experiment, whole detached leaves were placed in a moistened 5-liter jar and purged with either humidified air or ethylene (12 μl/l) at a rate of 180 ml/min.

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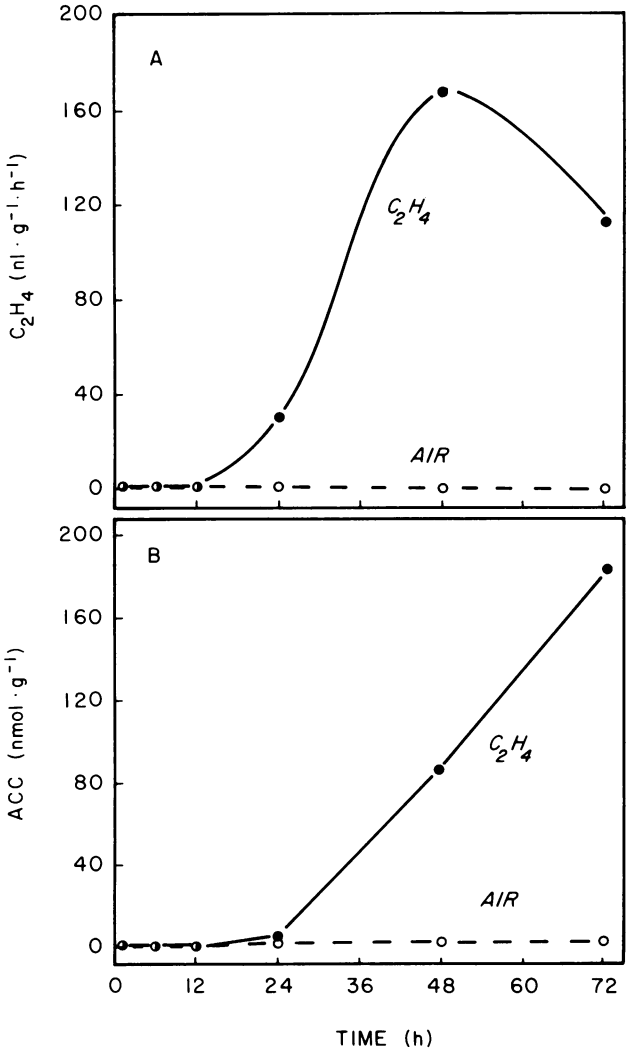

FIG. 2. Effect of exogenous ethylene on promotion of ethylene production rates (A) and ACC accumulation (B) in citrus leaf discs. Discs were incubated in air or ethylene (12 μl/l) for various period as indicated, and their ethylene production rates under ethylene-free air were then determined. The same discs were employed for assay of ACC content after ethylene determination.

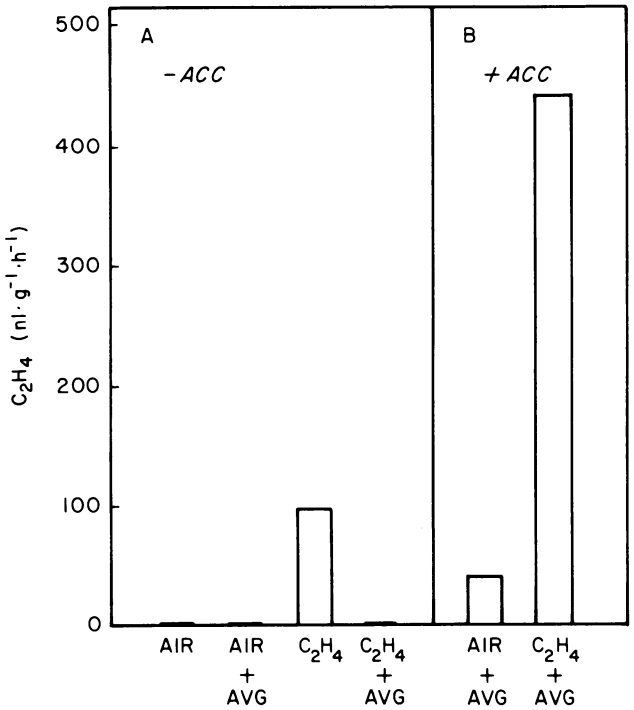

FIG. 3. Effect of ethylene treatment for 30 h on EFE activity in citrus leaf discs. Immediately after excision, discs were treated either with buffer or with 1 mM AVG and incubated for 30 h under air or ethylene (12 μl/l). At the end of incubation, those discs in (B) were further treated with 1 mM ACC. Ethylene production rates under ethylene-free air were determined 15 min after the ACC application.

Chemicals. ACC[3] was purchased from Calbiochem. STS was prepared from $AgNO_3$ and $Na_2S_2O_3$ (1:8, mol/mol) (17). AVG was a gift from J. P. Scannel (Hoffman-LaRoche).

Determination of Ethylene. To monitor ethylene production rates by the discs, they were transferred from the incubation chamber under air or air with ethylene to a 14-ml test tube, which was flushed with ethylene-free air and sealed with a rubber serum stopper. After various periods a 1-ml gas sample was withdrawn with a hypodermic syringe, and ethylene was assayed by gas chromatography, using an activated alumina column and a flame ionization detector. Determination of ethylene production in whole leaves was performed in a similar manner, except that a 32-ml test tube was used.

Various types of evidence, and particularly the data presented in Table III, indicate that the amount of exogenous ethylene absorbed during treatment and diffusing out of the discs during enclosure for ethylene determination is negligible. Measurements of the stimulating effect of exogenous ethylene on ethylene production was performed between 90 and 120 min after terminating ethylene treatment.

Determination of ACC. Discs were ground with a mortar and pestle in 3 ml 80% ethanol. The mortar and pestle were washed with additional 3 ml 80% ethanol and the extract and washing were combined and centrifuged for 10 min at 10,000*g*. The supernatant was concentrated under reduced pressure at 45°C to remove ethanol and the extract was brought to a volume of 2 ml with H_2O and clarified by centrifugation as above. ACC in the extract was assayed by the method of Lizada and Yang (10).

Assay of EFE Activity. *In vivo* activity of EFE was determined by measuring conversion of applied ACC to ethylene. Discs were treated with 1 mM AVG immediately following excision as described above. At the end of incubation, discs were floated for 15 min on a solution containing 1 mM ACC, blotted dry on a filter paper, and then transferred to a test tube for measuring ethylene production.

RESULTS

Autocatalysis of Ethylene Production. Whole deteached citrus leaves produced almost no ethylene following detachment (Fig. 1). Ethylene treatment markedly increased ethylene production in the leaves following a 24-h exposure. Like whole detached leaves, untreated leaf discs produced wound ethylene at a very low rate throughout the experimental period (Fig. 2A). The response of discs to exogenous ethylene resembled that of whole detached leaves. In both cases, ethylene stimulated ethylene production after a lag period of at least 12 h and greatest stimulation occurred at 48 h of exposure. Because of the ease of application of test materials, leaf discs instead of whole leaves were used in subsequent experiments.

[3] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; EFE, ethylene forming enzyme which catalyzes the conversion of ACC to ethylene; SAM, *S*-adenosylmethionine; AVG, aminoethoxyvinylglycine (2-amino-4-[2′-aminoethoxy]-*trans*-3-butenoic acid); STS, silver thiosulfate.

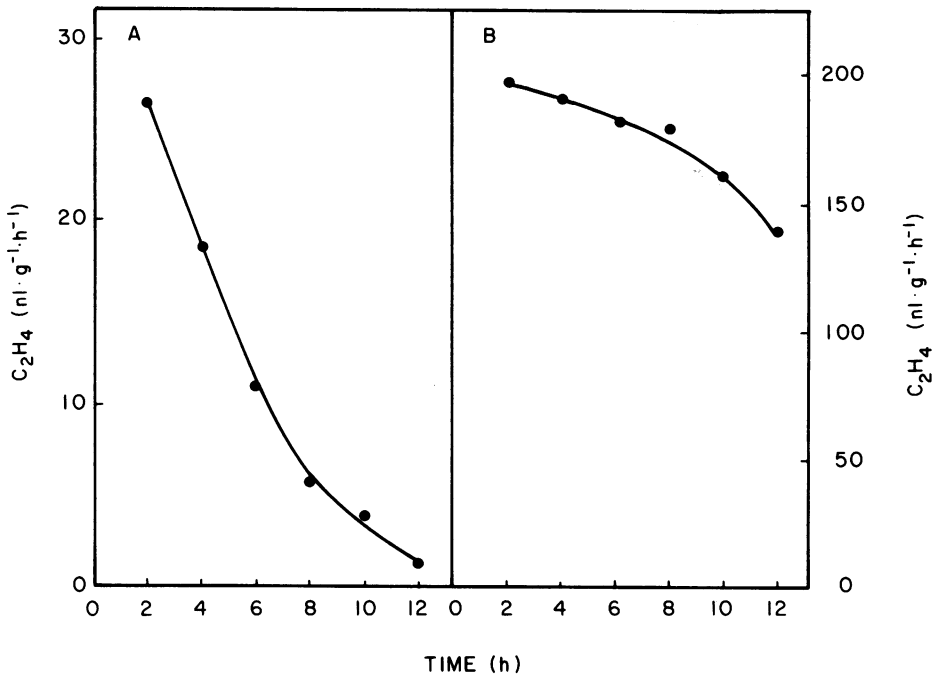

FIG. 4. Ethylene production rates of citrus leaf discs under air following incubation in ethylene (12 μl/l) for 24 h (A) or 48 h (B).

Table I. *Effect of Aeration and Repeated Exposure to Ethylene (12 μl/l) on Ethylene Production Rates in Citrus Leaf Discs*

Ethylene production rates in control discs, which were under air atmosphere continuously, were 1.3, 1.1, 0.5, and 0.2 nl/g·h after 24, 36, 50, and 62 h of incubation, respectively. After each treatment as indicated, ethylene production rates under ethylene-free air were determined.

Treatment	Ethylene Production
	nl/g·h
24 h ethylene	29.1
24 h ethylene + 12 h air	0.9
24 h ethylene + 12 h air + 14 h ethylene	53.8
24 h ethylene + 12 h air + 14 h ethylene + 12 h air	0.4

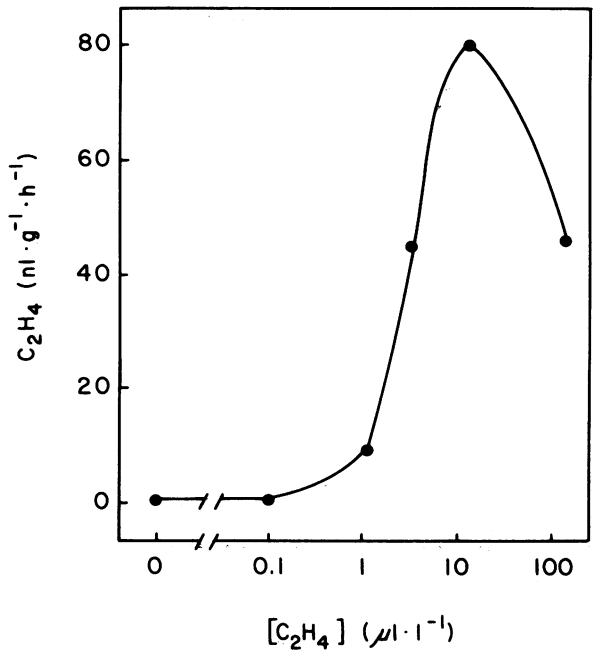

FIG. 5. Effect of exogenous ethylene concentration on ethylene production rates in citrus leaf discs incubated for 30 h. Discs were enclosed in 1-liter jars into which ethylene was injected to give the desired concentration. The actual concentration of ethylene in each jar was determined by GC 1 h after injection.

Inasmuch as the pathway of ethylene biosynthesis is now known (2) it is possible to determine at which steps in the sequence ethylene exerts its stimulating effect. Recent studies on ethylene biosynthesis suggest that one or two of the following steps may become rate-limiting: the conversion of SAM to ACC and the conversion of ACC to ethylene (19). Accordingly, the effect of ethylene on ACC content of leaf discs at various periods after excision was first determined in relation to their ethylene production rates (Fig. 2B). Discs incubated in air contained very little ACC during the course of incubation. Treatment with ethylene caused a linear increase in ACC levels after a lag period of 24 h. Unlike ethylene production rates (Fig. 2A), ACC content did not decrease after 48 h incubation.

Ethylene production rate in a tissue is dependent upon its EFE activity and endogenous ACC concentration. When the endogenous ACC concentrations are about equal, the rates of ethylene production should represent their relative EFE activities. By employing AVG to block the synthesis of ACC (2, 5, 20), followed by an application of exogenous ACC under specified concentration and duration, the resulting endogenous levels in various discs become about equal. Figure 3A shows that AVG blocked almost completely the stimulating effect of ethylene in leaf discs. Ethylene treatment increased by more than 10-fold the *in vivo* conversion of applied ACC to ethylene as compared to control discs 30 h after excision (Fig. 3B), indicating that ethylene treatment also promotes EFE activity.

Ethylene production of discs removed from an ethylene atmosphere gradually declined, the decrease being more rapid in discs incubated for 24 h in ethylene than in discs incubated for 48 h (Fig. 4). A second exposure of the same discs to ethylene after the ethylene production rate had dropped to the control level following removal from the ethylene atmosphere, resulted in renewed ethylene production (Table I). Discs subjected to a second ethylene exposure seemed to be more sensitive to ethylene than during the

Table II. *Effect of Silver Thiosulfate on Autocatalysis of Ethylene Production in Citrus Leaf Discs*

Discs were pretreated for 30 min with two different concentrations of silver thiosulfate and thereafter incubated under air or 12 μl/l ethylene for 30 h.

Treatment	Ethylene Production	
	Air	Ethylene
	nl/g·h	
Control	0.9	86.1
Thiosulfate (16 mM[a])	2.4	92.2
Silver (0.5 mM) as thiosulfate salt	6.2	0.4
Silver (2 mM) as thiosulfate salt	38.2	151.3

[a] This concentration equals that present in 2 mM silver thiosulfate.

Table III. *Effect of Exogenous Ethylene on Ethylene Production Rates and ACC Level in Citrus Leaf Discs*

Discs were incubated for 6 h under air or ethylene (12 μl/l) in experiment A, or preincubated for 30 min with or without 100 mM mannitol and thereafter incubated under air or ethylene (12 μl/l) for 12 h in experiment B. Ethylene production rates during the following hour were determined under ethylene-free air.

Experiment	Treatment	Ethylene	ACC
		nl/g·h	*nmol/g*
A	Air	4.46	0.35
	Ethylene	0.27	0.08
B	Air	1.6	0.32
	Ethylene	0.2	0.15
	Mannitol + air	28.9	1.03
	Mannitol + ethylene	1.8	0.32

first exposure, producing almost twice as much ethylene.

The effect of different concentration of exogenous ethylene on stimulation of ethylene production in leaf discs is illustrated in Figure 5. A slight stimulation was evident at about 1 μl/l ethylene and maximum stimulation occurred at 10 μl/l. A concentration of 100 μl/l ethylene was less effective than 10 μl/l in stimulating ethylene production.

Silver ion is known to antagonize the action of ethylene in many physiological processes (4). We therefore examined whether Ag^+ might inhibit the ethylene action on autocatalysis of ethylene production in the present system. Silver ion was supplied at two different concentrations of STS and ethylene was measured after a 30-h incubation period. Thiosulfate at 16 mM, a concentration present in the 2 mM Ag^+ solution, had a slight stimulatory effect on ethylene production (Table II). Silver ion stimulated ethylene production in discs stored under air, particularly when applied at the highest concentration of 2 mM. In ethylene-treated discs, 0.5 mM Ag^+ inhibited almost completely the stimulating effect of ethylene on ethylene production, whereas 2 mM Ag^+ increased ethylene production rate above that of untreated discs. A lower concentration (0.1 mM) of Ag^+ was also effective in blocking the autocatalysis of ethylene production in the present system (data not presented).

Autoinhibition of Ethylene Production. The data presented above clearly demonstrate autocatalysis of ethylene production in citrus leaf discs. However, various types of evidence indicate that autoinhibition of ethylene production also takes place in the present system during the first 24-h incubation. In the present system, the rate of wound ethylene production peaked at 6 h. Although ethylene production rate in air was low, it was significantly higher than that produced by ethylene-treated discs; ACC content in air control discs was also considerably higher than that in ethylene-treated discs (Table III, Experiment A). Ethylene

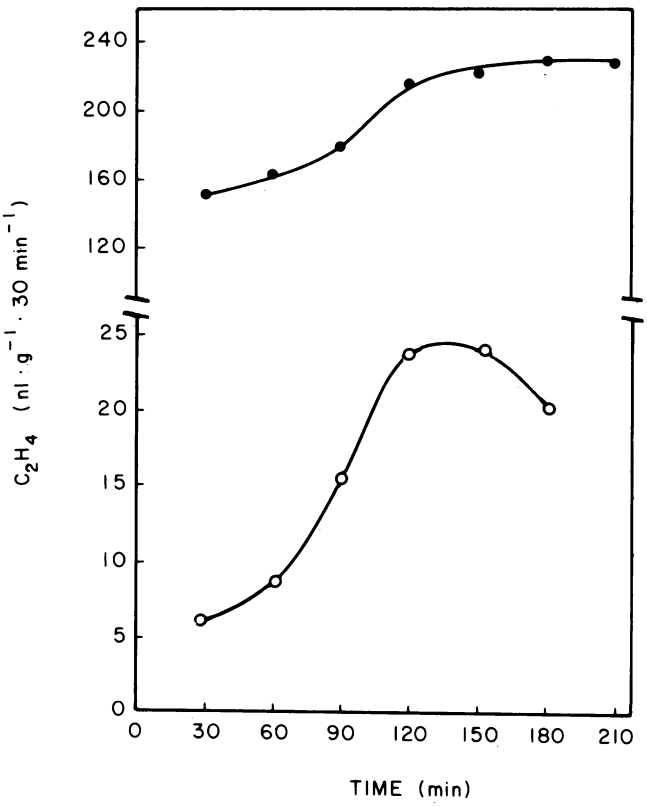

FIG. 6. Ethylene production rates of citrus leaf discs under air during a 3-h period following pretreatment with ethylene (12 μl/l) for 24 h (○) or 48 h (●). Ethylene was measured every 30 min and the test tubes in which the discs were enclosed were flushed with fresh air between each measurement.

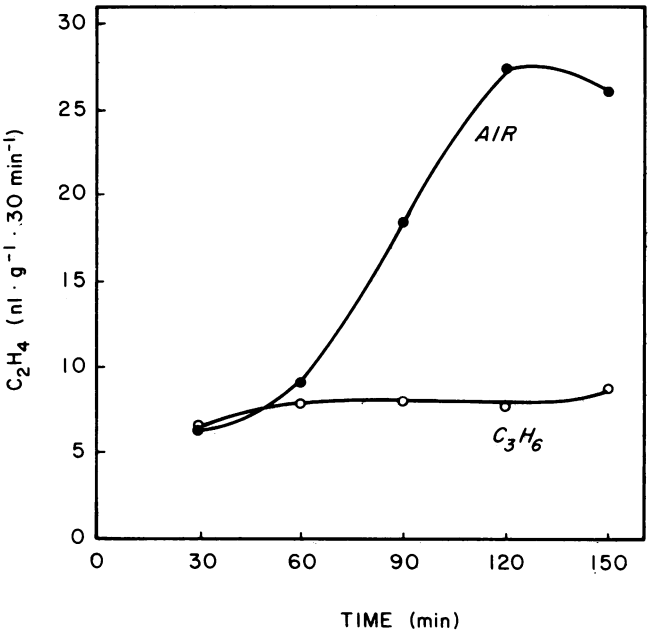

FIG. 7. Effect of propylene treatment on ethylene production rates in citrus leaf discs during a 3-h period following incubation of discs under ethylene (12 μl/l) for 24 h. At the end of ethylene treatment, half of the discs were enclosed in test tubes under air and the other half under 1,400 μl/l propylene. Ethylene production rates under air or propylene atmosphere were determined every 30 min. Fresh air or propylene atmospheres were replaced every 60 min.

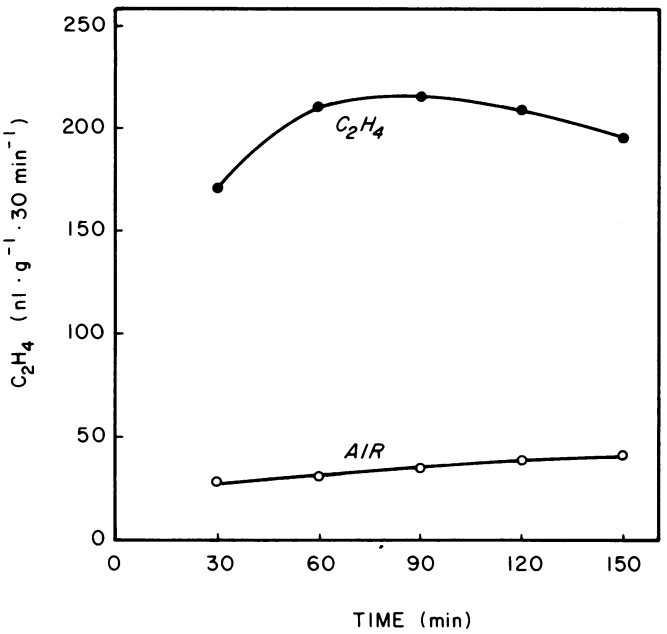

FIG. 8. EFE activity in citrus leaf discs during a 3-h period following incubation in air or ethylene (12 μl/l) for 24 h. Discs were treated immediately after excision with 1 mM AVG. At the end of incubation, 1 mM ACC was applied. Ethylene production was measured every 30 min, starting 15 min after application of ACC. The test tubes in which the discs were enclosed were flushed with fresh air between each measurement.

production rates in leaf discs increased greatly during the first 2 h after removal of exogenous ethylene, particularly in leaf discs incubated for 24 h (Fig. 6). A possible explanation for this phenomenon is that ethylene also inhibits ethylene production, and once ethylene is removed, the autoinhibition is relieved. Occurrence of autoinhibition of ethylene production in the present system was further demonstrated by incubating mannitol-treated discs under ethylene (Table III). Previous studies have shown that mannitol pretreatment stimulates ethylene production in citrus leaf discs (14). Ethylene treatment for 12 h eliminated the stimulating effect of mannitol on ethylene production and decreased the level of ACC in the discs compared to mannitol-treated discs incubated under air (Table III).

Additional experiments were performed to investigate the rapid increase in ethylene production following removal of exogenous ethylene (Fig. 6). To examine whether removal of exogenous ethylene was directly responsible for the observed increase in ethylene production, discs were incubated in ethylene for 24 h and then half of the discs was transferred to air and half to propylene, an ethylene analog. Discs transferred to propylene did not show the characteristic increase in ethylene production (Fig. 7), indicating that the stimulating effect of ethylene was counteracted by its inhibitory effects on ethylene production. We have shown that autocatalysis of ethylene production in the present system results from stimulation of both ACC formation (Fig. 2B) and EFE activity (Fig. 3). Increased ethylene production following removal of exogenous ethylene did not seem to result from enhanced activity of EFE since this activity did not change much during this period (Fig. 8). Consistent with the data of Figure 3, the level of EFE activity was much higher in ethylene-treated discs compared with control discs.

DISCUSSION

The effect of exogenous ethylene on ethylene production in plant tissues can be autocatalytic (1, 6, 12) or autoinhibitory (6, 13, 18, 21, 22). Autocatalysis of ethylene production is a common feature of ripening in climacteric fruits (1, 6, 12) and some other senescing tissues (11), in which an increased synthesis of ethylene is triggered by exposure to ethylene above a threshold level (1, 6, 12). The data presented herein show that citrus leaf tissues respond to ethylene in a similar manner (Figs. 1 and 2A). Autocatalysis of ethylene production in citrus leaf tissues results from the stimulation of both ACC formation (Fig. 2B) and EFE activity (Figs. 3 and 8). This is in variation with the view that in vegetative tissues only the conversion of SAM to ACC is rate-limiting (19). The above view was based on the observations that application of ACC caused a marked increase in ethylene production in various vegetative tissues, which normally produce little ethylene (7). Our results show that although citrus leaf discs produce a high rate of ethylene following application of ACC, ethylene treatment further stimulates EFE activity (Figs. 3 and 8). Similarly, treatment of citrus leaf discs with mannitol greatly enhanced EFE activity (14). These data indicate that conversion of ACC to ethylene in citrus leaf tissues can be regulated by ethylene or other factors, resulting in modulation of their ethylene production rates. It is pertinent to note that excision induced an increase in both ACC synthesis and EFE activities in preclimacteric cantaloupe tissue; ethylene treatment further increased the activity of EFE (8). The observation that the level of ACC increased progressively during ethylene treatment, whereas the rate of ethylene production declined after 72 h of incubation (Fig. 2), also indicates that EFE activity became rate-controlling at that time.

Although exogenous ethylene stimulates ethylene production in both citrus leaves and climacteric fruits, there is a basic difference between the response of these tissues to ethylene. In citrus leaf tissues, a continuous exposure to ethylene is needed to sustain ethylene production, and ethylene production rapidly declines when exogenous ethylene is removed. A repeated exposure to ethylene renews ethylene production (Table I). In contrast, the autocatalytic effect of ethylene in climacteric fruits is irreversible and once the climacteric has been initiated, further exogenous ethylene treatment has no effect in promoting ethylene production and other ripening processes (12).

Silver ion, which has been reported to antagonize ethylene action (4), including the autocatalytic effect of ethylene in climacteric fruits (15) and senescing flowers (4, 17), completely inhibited autocatalysis in citrus leaf tissues when applied as STS at a concentration of 0.5 mM (Table II). It is somewhat surprising that in the presence of 0.5 mM Ag^+, ethylene production rate in ethylene-treated discs was lower than in control discs (0.4 *versus* 6.2 nl/g·h, Table III). Higher concentrations of Ag^+ (2 mM) significantly enhanced ethylene production in discs incubated under air or ethylene, probably as a result of the toxic effects of high Ag^+ concentrations which induce stress ethylene.

Data from several experiments indicate that ethylene has also an inhibitory effect on ethylene production in citrus leaf discs during the first 24-h incubation. First, exogenous ethylene inhibited almost completely wound- and mannitol-induced ethylene production (Table III). Second, a rapid increase in rate of ethylene production occurred in ethylene pretreated discs during the first 2 h after removal of exogenous ethylene (Fig. 6); this increase was eliminated if the discs were enclosed in propylene instead of air during this period (Fig. 7). These observations indicate that a continuous exposure to ethylene decreases ethylene production rates. If the rapid increase in ethylene production following removal of exogenous ethylene is caused by the disappearance of autoinhibition, autoinhibition must have been significantly relieved after prolonged incubation under ethylene, since this increase in ethylene production is smaller after 48 h of incubation than after 24 h (Fig. 6).

The data presented in this paper clearly show that autoinhibition results mainly from suppression of ACC formation. Ethylene treatment eliminates the increase in ACC level following excision

or application of mannitol (Table III). In addition the rapid increase in ethylene production after removal of exogenous ethylene (Fig. 6) does not seem to result from increased EFE activity (Fig. 8). Autoinhibition in citrus peel discs has also been shown to result from suppression of ACC synthesis (13). EFE activity of leaf discs incubated in ethylene for 6 h was somewhat lower than in control discs (data not shown), indicating that autoinhibition in leaf tissues may also result from decreasing EFE activity, at least during the first h of incubation.

The mechanism by which exogenous ethylene both stimulates and inhibits ethylene production in citrus leaf discs is not yet clear. A reasonable explanation is that autocatalysis involves increased synthesis of ACC synthase and EFE, whereas autoinhibition involves suppression of the activity of these two enzymes. This may explain why a rapid increase in ethylene production occurs following removal of exogenous ethylene (Fig. 6). During ethylene treatment, the synthesis of the enzymes responsible for ethylene biosynthesis is increased, but their activities are suppressed. Once ethylene is removed, a rapid increase in ethylene production occurs. This would also explain why autocatalysis occurs after a long lag period (Figs. 1 and 2), whereas autoinhibition occurs several hours after application of ethylene (Table III). The above view is consistent with the reports that induction of enzymes by ethylene often represents *de novo* synthesis (1) and that autoinhibition in citrus peel discs involves inhibition of ACC synthase activity (13).

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