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Chilling-Induced Ethylene Production in Cucumbers (*Cucumis sativus* L.)[1]

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ABSTRACT

1-Aminocyclopropane-1-carboxylic acid (ACC) level, ACC synthase activity, and ethylene production in cucumbers (*Cucumis sativus* L.) remain low while the fruit are held at a temperature which causes chilling injury (2.5°C) and increase rapidly only upon transfer to warmer temperatures. The increase in ACC synthase activity during the warming period is inhibited by cycloheximide but not cordycepin or α-amanitin. Our data indicate that the synthesis of ACC synthase, which results in increased ACC levels and accelerated ethylene production, occurs only upon warming, possibly from a message produced or unmasked during the chilling period. Ethylene production by chilled (2.5°C) cucumbers increased very little upon transfer to 25°C if the fruit were chilled for more than 4 days. The fruit held for 4 days or longer showed a large increase in ACC levels but little ethylene production even in the presence of exogenous ACC. This suggests that the system which converts ACC to ethylene is damaged by prolonged exposure to the chilling temperature. Cucumbers stored at a low but nonchilling temperature (13°C) showed very little change in ACC level, ethylene production, or ACC synthase activity even after transfer to 25°C.

Low-temperature stress induces ethylene production from plant tissues which do not normally produce significant amounts of ethylene (1, 8, 13, 20, 21, 24). Our previous study (21) showed that the pathway for ethylene biosynthesis in chilled cucumbers (*Cucumis sativus* L.) is similar to that in ripening fruits. Furthermore, the increased ethylene production by chilled cucumbers appeared to be a result of an increased capacity of the tissue to make ACC[2]; the synthesis of ACC was identified as the step stimulated by chilling (21) and higher ACC levels were found in tissues after exposure to the chilling temperature. Since the ethylene production and ACC levels in our previous study were not measured until after the fruit were transferred to warmer temperatures, it was not known whether the stimulation of ACC synthesis and ethylene production occurred during the chilling period or took place during warming. Although prolonged chilling caused a large increase in ACC levels, the fruit exhibited reduced ethylene production even after they were allowed to warm (21), suggesting that prolonged chilling damages the system which converts ACC to ethylene.

The synthesis of ACC has been shown to be the rate-limiting step in ethylene biosynthesis in several plant tissues (3, 7, 16). ACC synthase, which catalyzes the conversion of SAM to ACC, has been studied in tomato fruit tissue by Boller *et al.* (4) and Yu *et al.* (25), and the amount of that enzyme has been implicated as the controlling factor for ethylene production in apple cell suspension cultures (2). The present study was undertaken to determine ACC levels and ACC synthase activity during chilling and upon warming in order to characterize the kinetics and timing of chilling-induced ethylene production in cucumber fruit.

MATERIALS AND METHODS

Plant Materials. Cucumbers (*Cucumis sativus* L., cv. Poinsett 76) were harvested from a farm at the Beltsville Agricultural Research Center. Samples were selected for uniformity of size (5.5 × 20 cm) and green color. They were randomly divided into two lots and immediately stored at 2.5 or 13°C. Fruit were transferred daily from both temperatures to a room maintained at 25°C and the change in pulp temperature was monitored. The severity of chilling injury was evaluated as described previously (22).

Determination of ACC and Ethylene. One-g skin samples were periodically taken for ACC analysis (15) after cucumbers were transferred from 2.5 or 13 to 25°C. For ethylene analysis, two intact cucumbers were enclosed in 1-liter chambers for 1 h after which gas samples were taken and ethylene therein determined by gas chromatography. The chambers were flushed with fresh air between samplings.

Evaluation of the Capability of Tissue to Convert ACC to Ethylene. One g of skin tissue was taken from cucumbers stored at 2.5 or 13°C and incubated with or without 0.1 mM ACC in 25-ml Erlenmeyer flasks. The incubation solution in each flask contained 3 ml 10 mM Mes buffer (pH 6.0) with 2% sucrose. The flasks were sealed with serum caps and shaken in a water bath at 30°C. At 2-h intervals, a 3-ml gas sample was withdrawn from each flask with a syringe and ethylene was determined by gas chromatography. The flasks were flushed with air between each sampling period.

Assay of ACC Synthase. Methods for extraction and assay of the enzyme activity were modified from that described by Boller *et al.* (4). Samples were periodically taken for the determination of ACC synthase activity after cucumbers were transferred from 2.5 or 13 to 25°C. Another set of samples were taken after 0 and 5 h warming at 25°C. Two strips of cucumber skin (about 4 g) were chopped into very fine pieces with a razor blade in a cold Petri dish which contained 10 ml of homogenization buffer. The homogenization buffer consisted of 100 mM EPPS (pH 8.5), 2 mM DTT, and 5 μM pyridoxal phosphate. The chopped pieces were further ground in a cold mortar and pestle for approximately 5 min then filtered through one layer of cheesecloth into a cold 16-ml centrifuge tube and centrifuged at 9,000*g* for 15 min. An aliquot of 500 μl of the supernatant was passed through a 0.8 ×

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[2] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; SAM, *S*-adenosylmethionine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EPPS, *N*-2-hydroxyethylpiperazine propane sulfonic acid; AVG, aminoethoxyvinylglycine [2-amino-4-(2′-aminoethoxy)-*trans*-3-butenoic acid].

3.4 cm (2 ml bed volume) Sephadex G25 column which had previously been equilibrated at 1.5°C with 2 mM EPPS (pH 8.5), 0.1 mM DTT, and 0.5 μM pyridoxal phosphate. The fraction of the effluent containing protein was collected and used for the ACC synthase assay.

The assay mixture contained 800 μl enzyme preparation, 100 μl 0.5 M EPPS (pH 8.5), and 100 μl 0.5 mM SAM. The mixture was incubated at 30°C for 2 h. The amount of ACC produced at the end of incubation was assayed according to the method of Lizada and Yang (15).

Additional assay mixtures were incubated with or without SAM (50 μM) and with or without AVG (25 μM), to demonstrate that the enzyme activity was characteristic of ACC synthase. No activity was found in the absence of SAM, and 25 μM AVG completely inhibited the reaction. The enzyme activities were expressed as nmol ACC formed/mg protein · h. The protein content was determined by the Bradford method (6).

Application of Inhibitors. Before removal from 2.5°C, cucumber skins were removed and soaked in 10 mM Mes buffer (pH 6.0) and 2% sucrose, with or without 50 μM cycloheximide, 50 μM cordycepin, or 50 μM α-amanitin. Vacuum infiltration (253 mm Hg for 3 min) was used to facilitate the absorption of the solutions by skin tissues. Following the vacuum infiltration treatment, tissues were incubated for 1 h at 2.5°C then transferred to 30°C and incubated for an additional 3 h.

ACC synthase was assayed as described above and ACC content was analyzed according to the method of Lizada and Yang (15). For ethylene determination, separate 1-g skin tissue samples with or without inhibitors were placed in 25-ml Erlenmeyer flasks containing 3 ml of 10 mM Mes buffer (pH 6.0) and 2% sucrose. The flasks were incubated for 1 h at 30°C, then flushed with fresh air and covered with serum caps. Gas samples were withdrawn after an additional 2 h incubation at 30°C and the amount of ethylene produced was determined by gas chromatography.

RESULTS AND DISCUSSION

Changes of ACC Level and Ethylene Production During Storage at Chilling and Nonchilling Temperatures. Both the ACC content and ethylene production in the cucumber tissues remained low during storage at either chilling (2.5°C) or nonchilling (13°C) temperature (Fig. 1). No accumulation of ACC or increase in ethylene production was detected throughout the storage period at either temperature. Similar results were reported in chilling-susceptible lemon fruit by Eaks (10). He found that the internal ethylene concentration of the fruit remained relatively constant during 12 weeks of storage at 5 or 12.8°C.

Warming Rate of Cucumber Tissues After Transfer from 2.5 to 25°C. The greatest rate of warming occurred within the 1st h after transfer (Fig. 2) and gradually decreased thereafter. The temperature of the cucumber tissue reached 25°C within 5 h.

Stimulation of ACC Synthase, ACC Level, and Ethylene Production in Chilled Tissues During Warming. A rapid increase in ACC level occurred after the cucumbers were transferred from 2.5 to 25°C, and it reached a maximum in 7 h at which time the level had increased about 50 times (Fig. 3). The greatest rate of increase occurred between 1 and 3 h after transfer, and accelerated ethylene production paralleled the increase in ACC content. The time course of ethylene production in relation to ACC formation during warming is shown in Figure 3. ACC synthase activity was very low at 2.5°C before transfer. This accounts for the low levels of ACC and ethylene production before the chilled tissues are warmed. ACC synthase activity increased rapidly upon warming of the chilled tissues and reached a plateau in 3 h. These data suggest that upon warming there is a rapid increase in ACC synthase, giving rise to increased ACC levels which in turn causes increased ethylene production. This is consistent with our previous findings (21) that the synthesis of ACC is the rate-limiting step in chilling-induced ethylene production.

Tissues from the nonchilling temperature (13°C) exhibited no increase in ACC synthase activity, ACC level, or ethylene pro-

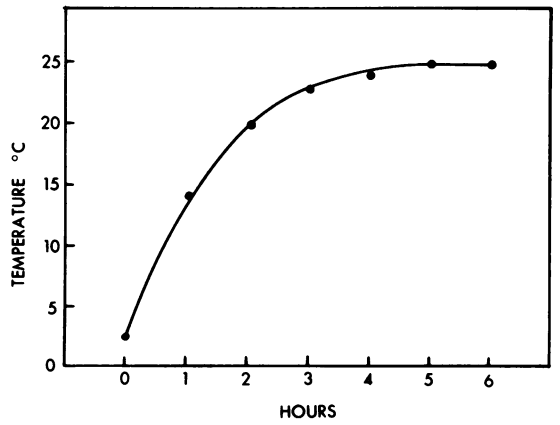

FIG. 2. Rate of warming of cucumber fruit tissues after transfer from 2.5 to 25°C.

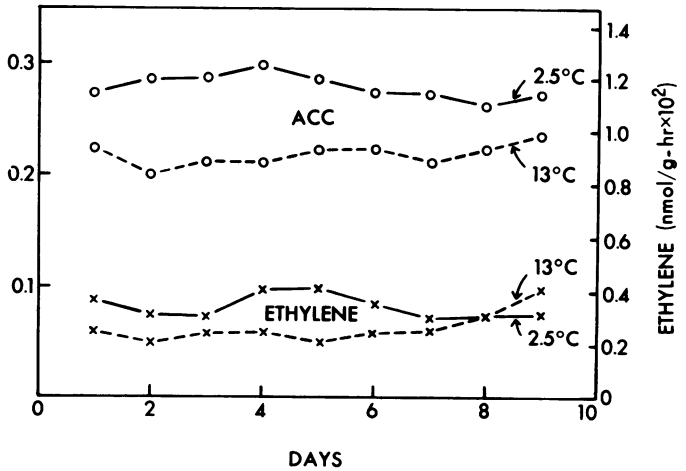

FIG. 1. ACC content (○) and ethylene production (×) of cucumber fruit during storage at 2.5°C (——) and 13°C (– – –). Intact fruit were used for ethylene measurement and skin tissues were used for ACC analysis.

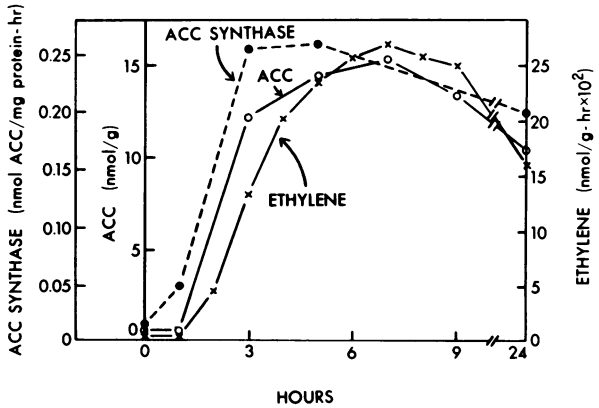

FIG. 3. Kinetic changes of ACC synthase (●), ACC level (○), and ethylene production (×) in cucumbers after transfer from a chilling temperature (2.5°C) to a warmer temperature (25°C). Cucumbers were exposed to chilling temperature for 4 d. ACC synthase and ACC level were measured in the skin tissues but intact fruit were used for the determination of ethylene production.

duction after transfer to 25°C (data not shown). Thus, chilling stress appears to induce changes which result in an increase in ACC synthase activity, ACC level, and higher ethylene production at warmer temperatures.

Wounding also induces a rapid increase in ACC synthase activity (5, 28). However, the "wound effect" is not a factor in our experiments, inasmuch as intact fruit were used for the determination of ethylene production, and extraction of tissue for ACC and ACC synthase was performed immediately after separation of the skin samples.

Effect of Varying Degrees of Chilling on ACC Level and Ethylene Production at 25°C. Seven h after the transfer of cucumbers from 2.5 to 25°C, both ACC level and ethylene production reached a maximum (Fig. 3). A comparison of the ACC levels and ethylene production among tissues with various degrees of chilling revealed that both ACC level and ethylene production increased with increasing exposure to the chilling temperature up to 4 d (Fig. 4). The ethylene production rate rose to a maximum of 0.27 nmol/g·h after 4 d at the chilling temperature and declined thereafter. The ACC content continued to increase even after the decline in ethylene production rate, suggesting that the system that converts ACC to ethylene is damaged by prolonged chilling.

Evaluation of the System Which Converts ACC to Ethylene After Different Degrees of Chilling. Exogenous ACC stimulated ethylene production for at least 4 d in tissues which were exposed to either 2.5 or 13°C (Table I). This suggests that the amount of ACC was the limiting factor for ethylene production in these tissues and that a sound system which converts ACC to ethylene was present. The difference in ethylene production between chilled and nonchilled samples without exogenous ACC (Table I) was not as great as that in Figure 4. In this case (Table I), the ethylene production was not measured until 3 h after cutting, whereas intact cucumbers were used for the data shown in Figure 4. Therefore, wounding might have contributed to the ethylene produced from the nonchilled tissue.

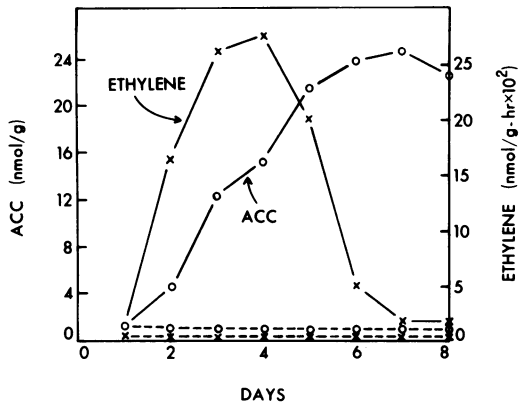

FIG. 4. ACC content (O) and ethylene production (×) of cucumbers 7 h after transfer to 25°C following various periods at 2.5°C (——) and 13 C (– – –). Intact fruit were used for ethylene measurement and skin tissues were used for ACC analysis.

Table I. *Ethylene Production of Cucumber Skin Tissues Incubated with or Without Exogenous ACC After Varying Degrees of Exposure to Chilling and Nonchilling Temperatures*

Samples of 1-g skin tissue were incubated at 30°C in 25-ml flasks containing 3 ml 10 mM Mes buffer (pH 6.0) and 2% sucrose. Ethylene production was determined after 3 h incubation.

Treatment	Ethylene Production				
	2	4	6	8	10
	Days of exposure				
	nmol/g·h × 10²				
2.5°C	20.4	27.9	10.0	3.3	1.2
2.5°C + ACC, 0.1 mM	45.0	46.6	22.1	5.0	1.6
13°C	8.3	9.5	7.5	7.5	8.3
13°C + ACC, 0.1 mM	47.9	45.8	47.9	42.9	50.8

Table II. *Effect of Cycloheximide, Cordycepin, and α-Amanitin on ACC Synthase, ACC Level, and Ethylene Production in Cucumber Skin Tissues After Transfer from 2.5 to 30°C.*

Cucumbers were chilled at 2.5°C for 4 d. Cycloheximide (50 μM), cordycepin (50 μM), and α-amanitin (50 μM) were vacuum infiltrated into the tissues before transfer of the skin samples to 30°C. Development of ACC synthase, formation of ACC, and production of ethylene were measured after 3 h incubation of samples with or without inhibitors in 10 mM Mes buffer (pH 6.0) and 2% sucrose at 30°C.

Treatment	ACC Synthase	ACC Level	Ethylene
	nmol ACC/mg protein·h	*nmol/g*	*nmol/g·h × 10²*
Control	0.32	16.5	25.8
Cycloheximide	0.05	3.8	5.4
Cordycepin	0.34	16.7	25.8
α-Amanitin	0.35	17.1	26.6

Ethylene production from nonchilled samples remained unchanged throughout the 10-d period, whereas the capacity of chilled tissue to convert ACC to ethylene decreased with prolonged chilling exposure (after 4 d) both in the presence and absence of exogenous ACC (Table I). The nonchilled tissues continued to respond to the exogenous ACC even after 10 d storage at 13°C, but stimulation of ethylene production by exogenous ACC in chilled tissue decreased after 4 d exposure to 2.5°C. Failure of chilled tissue to respond to exogenous ACC suggests that it has lost the capacity to convert ACC to ethylene and that depressed ethylene production after prolonged chilling is not due to absence of ACC in the tissue. These results support our previous proposal that the system which converts ACC to ethylene is damaged by prolonged chilling exposure (21). The ACC level continued to increase in chilled tissue even though ethylene production decreased after 4 d of chilling exposure (Fig. 4). An analogous increase in ACC level following the decline of ethylene production in the postclimacteric avocado, banana, and tomato has been reported by Hoffman and Yang (12).

The conversion of ACC to ethylene seems to be a relatively labile system. It is inhibited by high temperature (26), senescence (12), and chilling injury (21).

Effect of the Inhibitors of RNA and Protein Synthesis on ACC Synthase, ACC Level, and Ethylene Production. Very little ACC synthase activity was detected while fruit were held at 2.5°C even after 4 d exposure (Fig. 3). The activity increased markedly only upon transfer to the warmer temperature. This appearance of ACC synthase activity was inhibited by cycloheximide, an inhibitor of protein synthesis (11, 17), but was not affected by cordycepin or α-amanitin (Table II). Cordycepin has been shown to be an inhibitor of post-transcriptional polyadenylation of messenger RNA (9, 19), whereas α-amanitin is thought to inhibit RNA synthesis at the polymerase stage (18). It seems that the steps which are inhibited by cordycepin and α-amanitin have already been completed in the cold. Perhaps the chilling temperature unmasked or stimulated production of mRNA coding for ACC synthase, but translation was not completed until after the tissue was removed from the chilling temperature to 25°C. The increases in ACC level and ethylene production in the chilled tissues were also inhibited by cycloheximide but not by cordycepin or α-amanitin (Table II). Taken together, our data suggest that new protein must be synthesized for the production of ethylene during warming.

Our data indicate that chilling predisposes cucumber tissue to

produce ACC synthase when the fruit are transferred from cold to warm. This in turn leads to formation of ACC and an acceleration of ethylene production. The increase in ACC synthase activity during warming was not inhibited by cordycepin or α-amanitin but was inhibited by cycloheximide, raising the possibility that the message for ACC synthase is made during chilling period, but that translation occurs only during warming. Absence of ACC synthase in the tissues seems to be the main reason for low ethylene production and the low ACC level during the chilling period rather than a general metabolic depression by the chilling temperature. The accelerated rate of ethylene production by the chilled tissues during warming was primarily due to the rapid increase in ACC synthase activity.

While the synthesis of ACC at 25°C is readily promoted by the chilling, the step between ACC and ethylene is vulnerable to damage at chilling temperatures. After 4 d exposure to 2.5°C, ACC levels were still elevated when ethylene production decreased and the tissue gradually lost the ability to respond to exogenous ACC. Since the endogenous ACC level at 25°C changes with different lengths of chilling exposure, it is possible that the activity of ACC synthase or the ACC content itself could serve as an index of chilling exposure or to predict the severity of chilling injury.

ACC synthase activity seems to be a key factor regulating ethylene production in many tissues (2, 4, 23, 25). It controls the amount of ACC available for conversion to ethylene. Various stimuli which increase ethylene production have been shown to enhance ACC synthase activity; these include auxin treatment of etiolated pea epicotyl (14) and mung bean hypocotyl (27), as well as wounding of tomato pericarp (5) and collumella tissues (28). Our data indicate that increased ACC levels and accelerated ethylene production in chilled cucumbers also results from the increased ACC synthase activity induced by the chilling. Chilling stress, thus, provides an ideal system for the study of changes which lead to an increase in ethylene biosynthesis.

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