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Ethylene Production by Chilled Cucumbers (*Cucumis sativus* L.)[1]

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ABSTRACT

Chilling at 2.5 C accelerated the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) and C_2H_4 production in cucumber fruit. Skin tissue contained higher levels of ACC and was more sensitive to chilling than was cortex tissue. Accumulation of ACC in chilled tissue was detected after 1 day of chilling and remained elevated even after C_2H_4 production started to decline. These data suggest that ACC synthesis is readily stimulated by chilling, whereas the system that converts ACC to C_2H_4 is vulnerable to chilling injury. Chilling-induced C_2H_4 production was inhibited by aminoethoxyvinylglycine, sodium benzoate, propyl gallate, 2,4-dinitrophenol, carbonyl cyanide *m*-chlorophenylhydrazone, and cycloheximide. The utilization of methionine for ACC formation and chilling-induced C_2H_4 biosynthesis was established using L-[3,4-^{14}C]methionine. Chilled tissue had a higher capacity to convert L-[3,4-^{14}C]methionine to ACC and C_2H_4 than did nonchilled tissue.

Chilling temperatures have been observed to stimulate the production of C_2H_4 by tissues of a number of plants, including citrus, avocados, and pears (6, 13, 15). However, very little is known about the mechanism of this chilling-induced C_2H_4 production.

It has recently been recognized that ACC[2] is a precursor for C_2H_4 in many higher plant tissues (1, 4, 9). This unusual amino acid is produced from methionine via SAM. The proposed sequence for the pathway of C_2H_4 biosynthesis thus becomes: methionine → SAM → ACC → C_2H_4. The study presented here was initiated to determine if this pathway accounts for the production of C_2H_4 from chilled tissue and to identify the step(s) that are enhanced by chilling temperature.

The C_2H_4 biosynthetic pathway is inhibited by AVG (7), uncouplers of oxidative phosphorylation (12), and free radical scavengers (2, 3). Therefore, the effect of these inhibitors on C_2H_4 production and ACC levels in chilled cucumbers has been examined.

MATERIALS AND METHODS

Chilling of Samples. Cucumbers (cv. Poinsett 76) grown in Beltsville, Md., were used for all experiments. Freshly harvested samples were divided into two lots and stored at 2.5 C or 13 C. Each day, development of chilling injury symptoms was evaluated in five cucumbers from each treatment group that were transferred to a room maintained at 20 C. Chilling injury was evaluated as previously described (14), and the severity of injury was rated on a scale of 1 to 5 (1, no abnormality; 2, trace; 3, slight; 4, moderate; and 5, severe injury).

C_2H_4 and ACC Determination. C_2H_4 production by intact cucumbers stored at 2.5 C or 13 C was measured daily. Duplicate samples of two cucumbers each from each temperature treatment were transferred to a room maintained at 25 C and were allowed to warm to room temperature. Then they were placed in 1-liter chambers and sealed for 1 h. Gas samples then were taken for C_2H_4 determination by GC (11). After C_2H_4 determination, 3 g skin and 3 g cortex tissue were taken from each cucumber and assayed for ACC content by the method of Lizada and Yang (8).

Incorporation of L-[3,4-^{14}C]Methionine. Three g skin tissue were placed in a 50-ml Erlenmeyer flask with 4 ml 10 mM Mes buffer (pH 6.0) containing 2% sucrose and 33.3 μg/ml chloramphenicol. After addition of 1 ml 2 μCi L[3,4-^{14}C]methionine (52 μCi/μmol), the flasks were sealed with serum caps and incubated at 30 C for 6 h. At the end of the incubation period, gas samples were withdrawn and assayed for radioactive C_2H_4. For this assay, 12-ml samples were injected into evacuated scintillation vials sealed with serum caps that contained 1 ml 100 mM mercuric acetate in glass-distilled methanol as absorbant. The vials were kept at 4 C for 3 h; then, 10 ml Aquasol scintillation fluid were added, and radioactivity then was determined by scintillation counting.

Detection of [^{14}C]ACC. For chromatographic assay of radioactive ACC, the skin tissue was removed from the incubation flasks at the end of the incubation period, washed with H_2O, and homogenized in 70% ethanol. The residue after filtration was re-extracted twice with 70% ethanol, and the combined extracts were concentrated *in vacuo* at 40 C. The residue was taken up in 5 ml H_2O and applied to a Dowex 50 column (H^+ form). The column was washed with H_2O, and the amino acids were eluted with 3 N NH_4OH. An aliquot of the concentrated eluate was chromatographed on Whatman 3MM paper along with 0.1 μmol authentic ACC. Butanol-acetic acid-H_2O (4:1:5, v/v) was used as developing solvent. Authentic ACC on the chromatogram was visualized by spraying with ninhydrin; radioactive ACC was located with a Baird-Atomic radiochromatogram scanner. A second aliquot of the concentrated amino acid fraction was taken for determination of labeled ACC by the method of Lizada and Yang (8). To determine the efficiency of the degradation of ACC to C_2H_4, 100 nmol unlabeled ACC was added to the sample as an internal standard, and the per cent recovery was calculated from the C_2H_4 produced after the reaction. Labeled C_2H_4 formed from [^{14}C]ACC was absorbed in mercuric acetate and counted as described above.

Testing of Possible Inhibitors of C_2H_4 Production. Test solutions of AVG (0.005, 0.05, and 0.1 mM), SB (1 mM), PG (1 mM), DNP (0.05 mM), CCCP (0.05 mM) and cycloheximide (0.05 mM) were each made up in 10 mM Mes buffer (pH 6.0) and 2% sucrose. Each 25-ml Erlenmeyer flask contained 1 g skin tissue and 100

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[2] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine [2-amino-4-(2'-aminoethoxy)-*trans*-3-butenoic acid]; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; SAM, *S*-adenocylmethionine; PG: propyl gallate; SB, sodium benzoate.

μg chloramphenicol in 3 ml test solution. In an additional series of tests, 0.1 mM ACC was included with each of the test solutions. The flasks were sealed with serum caps and incubated at 30 C in a shaking water bath. Gas samples were removed after 2 h for C_2H_4 determination by GC. After incubation, skin tissue samples that did not receive exogenous ACC were extracted and assayed for ACC content.

RESULTS AND DISCUSSION

Chilling Injury. Chilling injury appeared first as numerous tiny pitted areas on the surface of the cucumber skin. The pitted areas gradually enlarged and became more prominent. In severe cases, the skin became loose and decay developed throughout the entire cucumber. Symptoms of chilling injury developed rapidly after samples were transferred to 20 C. The severity of the symptoms of chilling injury observed on day 7 after transfer to 20 C after chilling (2.5 C) for various periods is shown in Figure 1. Samples did not show chilling symptoms until exposed to 2.5 C for 3 days or more. Those exposed for 5 days or more exhibited severe chilling symptoms.

C_2H_4 Production and ACC Levels. A higher level of ACC was detected in chilled (2.5 C) than in the nonchilled (13 C) cucumber skin tissue after only 1 day exposure to chilling temperatures (Fig. 2). Thereafter, ACC continued to accumulate in chilled tissues, especially in the skin, for the first 7 days. However, C_2H_4 production by cucumbers from the two temperatures showed no difference until day 2. The reason for this apparent delay in C_2H_4 production is not clear but may be a result of the fact that even a moderate increase in C_2H_4 production by skin tissue could be difficult to detect because the skin is such a small fraction of the total mass of the cucumber fruit. However, at later times, C_2H_4 production from the skin might increase manyfold, thus giving rise to detectable differences in C_2H_4 production between chilled and nonchilled fruit. Nevertheless, it appears that chilling stimulates ACC synthesis before detectable differences in C_2H_4 production can be observed.

C_2H_4 production remained low and stable in cucumbers stored at 13 C but was stimulated after 2 days exposure to 2.5 C and reached a peak on day 4. Cameron *et al.* (5) showed that C_2H_4 production by cucumbers increased markedly after application of 0.1 mM ACC, suggesting that ACC levels could limit C_2H_4 production in cucumber tissue. This observation and the data presented here suggest that a higher capacity for ACC synthesis could be one factor leading to higher C_2H_4 production by chilled tissue. The results here are similar to those of Yu *et al.* (17, 19) which showed that ACC levels increased dramatically after treatment of mungbean hypocotyls with IAA. They concluded that C_2H_4 production in hypocotyl tissue was limited by the availability of ACC; this conclusion also seems to hold for cucumber tissues.

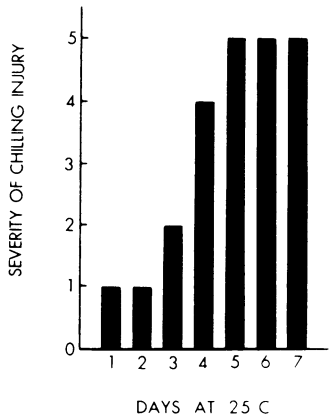

FIG. 1. Development of chilling injury symptoms in cucumbers. Chilling injury was evaluated 7 days after cucumbers were transferred to 20 C. Severity was rated on a scale of 1 to 5: 1, no abnormality; 2, trace; 3, slight; 4, moderate; and 5, severe injury.

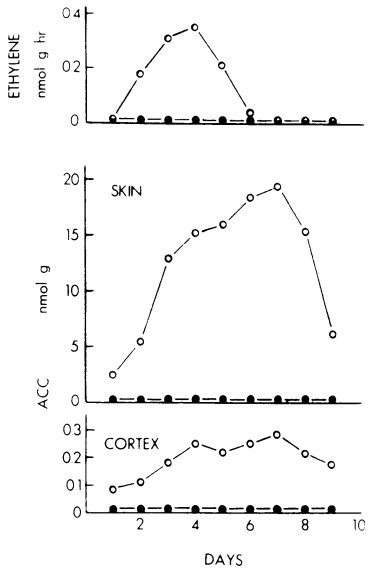

FIG. 2. ACC levels in skin and cortex tissues and C_2H_4 production of whole cucumbers during storage at 2.5 C (○——○) and 13 C (●——●). Cucumbers were removed from storage and warmed to 25 C for C_2H_4 determination.

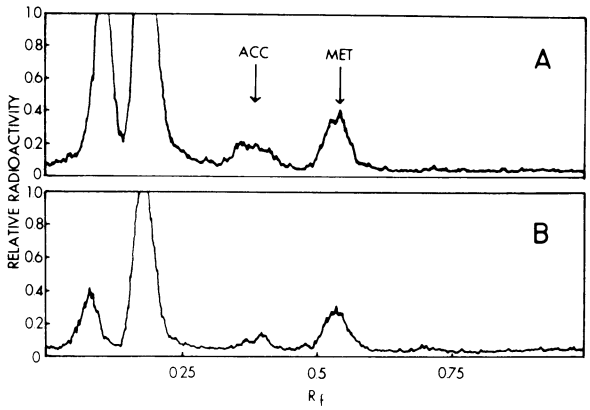

FIG. 3. Radiochromatogram scans of ethanol extracts prepared from chilled (2 days, 2.5 C) (A) or nonchilled (13 C) (B) cucumber skin tissues incubated with 2 μCi L-[3,4-^{14}C]methionine. All tissues were incubated for 6 h at 30 C and the specific radioactivity of methionine was 52 μCi/μmol.

C_2H_4 production by chilled cucumbers declined after 4 days exposure to 2.5 C despite elevated levels of ACC in the tissue. It seems likely that the system that converts ACC to C_2H_4 might have been damaged by the chilling temperature, for cucumbers at this point exhibited severe chilling injury symptoms (Fig. 1). ACC changes in the cortex tissue generally followed the same pattern as in the skin but at a lower level.

Pathway of C_2H_4 Biosynthesis in Cucumber Skin Tissue. Feeding L-[3,4-^{14}C]methionine to chilled as well as unchilled cucumber tissue gave rise to labeled C_2H_4 and a radioactive metabolite that co-chromatographed with authentic ACC (R_F = 0.38) in the solvent system used here (Fig. 3). This metabolite likewise yielded labeled C_2H_4 in the chemical degradation assay of Lizada and Yang (8) and, therefore, is presumed to be labeled ACC formed from the labeled methionine. These experiments indicate that C_2H_4 is produced in cucumber tissue via the same pathway that has been proposed for a number of other plant tissues (1, 4, 9, 19).

Chilled tissue produced more labeled ACC from labeled methionine than did nonchilled tissue. It is likely that the ACC formed from L-[3,4-^{14}C]methionine in control tissue could be a response to wounding, as wounding is well-known to induce C_2H_4 production (16). The data indicate that chilled tissue had a greater capacity for converting methionine to ACC, as would be expected from the ACC levels found in the tissue (Table I; Fig. 2).

Effect of Inhibitors. AVG at 0.005, 0.05, and 0.1 mM inhibited C_2H_4 production in chilled cucumber tissue (Table II). AVG reduced ACC accumulation even at 5 μM. These results are in agreement with previous findings that AVG inhibits the conversion of SAM to ACC by blocking ACC synthase (4, 18). AVG did not inhibit C_2H_4 production when exogenous ACC was added, likewise suggesting that AVG blocked C_2H_4 biosynthesis before the step that converts ACC to C_2H_4.

C_2H_4 production in chilled cucumber tissue was also inhibited by free radical scavengers (PG and SB, Table II). However, unlike AVG, PG and SB increased ACC levels in the tissue and inhibited C_2H_4 production even in the presence of exogenous ACC. This suggests that the step at which these compounds exert their inhibition is between ACC and C_2H_4. Inhibition of C_2H_4 production by free radical scavengers was previously demonstrated by Baker *et al.* (3) and Apelbaum *et al.* (2) in apple, avocado, tomato, bean, and pea tissues. It was proposed that a free radical reaction is involved in one of the final steps in C_2H_4 biosynthesis. The uncouplers DNP and CCCP reduced ACC levels and inhibited C_2H_4 production in cucumber tissue with or without exogenous ACC. It appears that DNP and CCCP at these concentrations inhibit both conversion of methionine to ACC and conversion of ACC to C_2H_4. Since conversion of methionine to SAM requires ATP, these uncouplers may decrease the amount of ACC in the tissue by making less ATP available for SAM synthesis. However, the reason for the inhibitory effect of these compounds in the presence of exogenous ACC is unclear and deserves further attention. In the presence of cycloheximide, both ACC accumulation and C_2H_4 production from exogenous ACC were inhibited. This could suggest that continuous protein synthesis is required for the operation of this pathway; however, this inhibitor is known to have other side effects (10).

Taken together, the data presented here indicate that the pathway for C_2H_4 production in chilled cucumbers is the same as has been proposed for a number of higher plant tissues (1, 4, 9, 19). Likewise, C_2H_4 production in cucumber tissue seems to be limited by the availability of ACC. Increased C_2H_4 production from chilled cucumbers appears to be a result of increased capacity of the tissue to make ACC. Prolonged chilling appears to damage the system that converts ACC to C_2H_4, for ACC levels were still elevated when C_2H_4 production decreased.

Table I. *[^{14}C]ACC and [^{14}C]C_2H_4 Production in Skin Tissues of Chilled (2 Days, 2.5 C) and Nonchilled (13 C) Cucumbers after Incubation with 2 μCi L-[3,4-^{14}C]Methionine for 6 h at 30 C*

Treatment	[^{14}C]ACC	[^{14}C]C_2H_4
	nCi	
Chilled (2.5 C)	4.96	0.285
Nonchilled (13 C)	2.97	0.194

Table II. *Effect of Various Inhibitors on Endogenous ACC Level and C_2H_4 Production in Skin Tissue of Cucumbers Exposed to 2.5 C for 2 Days*

One g skin tissue was taken from each of the chilled cucumbers and incubated in 25-ml flasks containing the inhibitor in 3 ml 10 mM Mes buffer (pH 6.0), 2% sucrose, and 100 μg chloramphenicol. ACC content and C_2H_4 production were analyzed after 2 h incubation at 30 C. Absolute control values for ACC and C_2H_4 production with (+) and without (−) exogenous ACC (0.1 mM) were 3.28 nmol/g, 1.68, and 0.14 nmol/g·h, respectively.

Inhibitor	Concn.	ACC Level	C_2H_4	
			−ACC	+ACC
	mM	*% control*	*% control*	
AVG	0.005	39.1	34.9	96.2
	0.05	28.4	25.2	96.7
	0.1	12.6	7.6	91.6
PG	1.0	120.3	64.9	59.2
SB	1.0	114.7	73.4	70.1
DNP	0.05	88.6	56.3	49.7
CCCP	0.05	52.4	61.8	43.5
Cycloheximide	0.05	46.4	40.7	38.3

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