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Communication

Interference of Phenolic Compounds with the 1-Aminocyclopropane-1-Carboxylic Acid Assay[1]

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ABSTRACT

The yields of ethylene from endogenous and exogenous 1-aminocyclopropane-1-carboxylic acid (ACC) in avocado (*Persea Americana* Mill.) fruit pedicel extracts were very low when assayed by the method of Lizada and Yang (1979 Anal Biochem 100: 140–145). Addition of phenolic compounds, which are present in avocado tissues, to the assay mixture significantly reduced the conversion efficiency of ACC to ethylene. A negative correlation was found between the amount of the plant material in the assay mixture and the conversion efficiency of ACC to ethylene. Removal of phenolic compounds from pedicel extracts by polyvinylpolypyrrolidone, Amberlite XAD-7, and Dowex-50 column chromatography or lead acetate precipitation greatly increased the yields of thylene from ACC in these extracts. The use of polyvinylpolypyrrolidone column chromatography also enabled us to obtain more accurate estimations of endogenous ACC levels in carnation (*Dianthus caryophyllus* L.) petal extracts. The conversion efficiency of ACC to ethylene could be improved by increasing the concentrations of mercuric chloride and NaOCl in the assay mixture.

Since Adams and Yang (1) have elucidated ACC[2] as the immediate precursor of ethylene, numerous studies have been reported on the concentration of ACC in various plant tissues (6, 16). ACC is determined by a simple and sensitive method developed by Lizada and Yang (9). This assay is based on oxidation of ACC to ethylene by NaOCl in the presence of mercuric salt. Nieder *et al.* (11) summarized several problems which might give rise to inaccurate measurements of ACC in plant extracts, including a low efficiency of ACC conversion to ethylene. They reported that various amines cause poor yields of ethylene from ACC (11).

In preliminary studies, we have observed that avocado fruit pedicels produced significant amounts of ethylene after excision, but almost no ACC could be detected in this tissue. The yield of ethylene from ACC added to extracts of avocado fruit pedicels was very low. A similar phenomenon has been observed in apple juice (8) and in extracts of carnation petals (S Mayak, personal communication). Since avocado tissues are rich in phenolic substances (2, 5), we examined the possible interference of these compounds with the ACC assay.

MATERIALS AND METHODS

Plant Material. Avocado (*Persea americana* Mill. cv "Nabal") fruits were harvested from mature trees grown in the orchard of the Agriculture Research Organization. Ethylene production of each fruit, with its pedicel attached, was determined. Only fruits that did not produce ethylene were used. Pedicels of these fruits contained less than 0.05 nmol·g^{-1} of ACC.

Ethylene Determination. Carnation flowers (*Dianthus caryophyllus* L. cv "Scania") cut below the calyx or avocado fruits were enclosed in 750-ml jars for 30 min. Two-ml gas samples were withdrawn with a hypodermic syringe for assaying ethylene by GC, equipped with an alumina column and a flame ionizing detector.

ACC Extraction and Determination. Plant tissue, about 1 g fresh weight, was homogenized by means of an Ultra Turrax homogenizer in 8 ml 80% ethanol. The shaft was washed with additional 2 ml 80% ethanol. The extract and washing were combined, filtered through a glass wool, and centrifuged for 10 min at 10,000*g*. The pellet was discarded and the supernatant was evaporated to dryness under reduced pressure at 50°C. The dry residue was dissolved in 2 ml of water and an equal volume of chloroform was added. The tubes were vigorously shaken, and centrifuged as above. ACC in the water phase was assayed by the method of Lizada and Yang (9). One μmol of $HgCl_2$ was added to 0.6 ml extract in a 15 × 125 mm test tube and the volume was brought to 0.9 ml with water. The tube was sealed with a rubber serum cap and kept in ice. Approximately 0.1 ml of a cold mixture of 5.5% NaOCl and saturated NaOH (2:1,v/v) was injected into the test tube through the rubber cap. The mixture was agitated for 20 s on a Vortex. After incubation for 2.5 min in ice, the tube was again agitated and a 1-ml gas sample was withdrawn by a hypodermic syringe for ethylene determination. The conversion efficiency of ACC to ethylene in each sample was determined separately with a replicate sample containing 2 or 8 nmol of ACC as an internal standard. In each experiment the efficiency of ACC conversion to ethylene was determined in an assay mixture without plant extract or phenolic compounds. The conversion efficiency ranged between 70 to 80%. For recovery determination of ACC, a known amount of [2,3-^{14}C]ACC (Centre d'Etudes Nucleaires de Saclay, France) was added after homogenization of the tissue.

Extract Purification. Water extracts, equivalent to 1 g fresh weight of pedicel tissue and containing 8 nmol ACC and [2,3-^{14}C]ACC, were passed through small columns (1 × 5 cm) of

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[2] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; PVPP, polyvinylpolypyrrolidone.

PVPP or Amberlite XAD-7. The columns were eluted with water. Two-ml fractions were collected and those containing radioactivity were pooled and evaporated to dryness *in vacuo* at 50°C. The residue was dissolved in water and ACC was determined. When Dowex-50×8 (H^+ form, 200–400 mesh) was employed, the extract was passed through the column (1 × 2.5 cm) and the column was washed with water. ACC was then eluted by 2 N NH_4OH. Fractions containing radioactivity were pooled and treated as above.

Precipitation of Phenolic Compounds with Lead Acetate. Two hundred mg lead acetate were added to 2 ml water extract equivalent to 1 g fresh weight of pedicel tissue. After gently shaking, the sample was left for 10 min and then centrifuged. The supernatant was collected and the pellet was resuspended in 5 ml of water. The suspension was centrifuged, and the combined supernatants were evaporated. The residue was dissolved in water and ACC was determined.

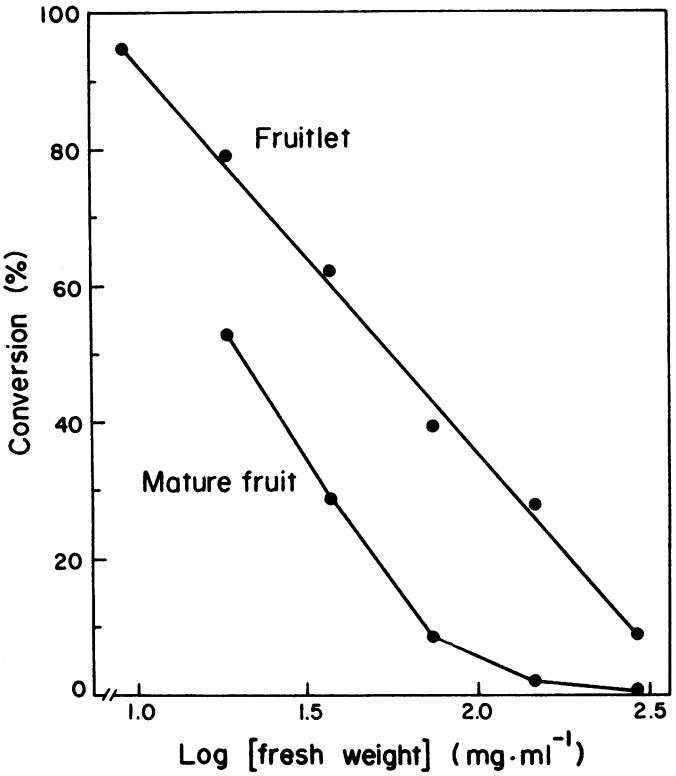

FIG. 1. Effect of avocado pedicel extract concentrations on the conversion of ACC to ethylene. The standard assay mixture contained 8 nmol ACC. The conversion of 8 nmol ACC in water served as 100% of ACC conversion.

Table I. *Effect of Various Phenolic Compounds on the Conversion of ACC to Ethylene*

The standard assay mixture contained 2 nmol ACC, and 5 μmol phenolic compound. The conversion of ACC to ethylene in the same mixture without phenolic compounds served as 100% of ACC conversion.

Phenolic Compound	Conversion
	%
p-Hydroxybenzoic acid	96
Caffeic acid	91
Chlorogenic acid	92
p-Coumaric acid	95
Ferulic acid	95
D-Catechin	60
Epicatechin	56
D-Catechin + epicatechin	22

Table II. *Procedures for Improving the Efficiency of ACC Conversion to Ethylene*

Extracts of avocado pedicels (1 g) containing 8 nmol ACC and [2,3-^{14}C]ACC were partialy purified and the conversion of ACC to ethylene was assayed. The conversion of 8 nmol ACC in a reaction mixture without a plant extract served as 100% of ACC conversion

Treatment	Conversion	Recovery
	%	
Crude extract	5	100
Amberlite XAD-7	63	90
Dowex-50W×8	75	84
PVPP	83	88
Lead acetate	73	88

Table III. *Effect of $HgCl_2$ and NaOCl Concentrations on the Conversion of ACC to Ethylene in Extract of Avocado Fruit Pedicels*

The assay mixture contained crude pedicel extract equivalent to 300 mg fresh wt, 2 nmol ACC, and $HgCl_2$ at the specified concentrations. The reaction was initiated by injecting 0.1 ml of a proper mixture of saturated NaOH and NaOCl to give final concentrations of 50 and 75 mM NaOCl. The total volume of the reaction mixture was 1 ml. The conversion of 2 nmol ACC in an assay mixture containing 1 mM $HgCl_2$ and 50 mM NaOCl, as in the standard method, served as 100% of ACC conversion to ethylene.

$HgCl_2$	NaOCl	
	50 mM	75 mM
mM	*% conversion*	
1	12	21
2	11	28
4	11	38
8	26	61
16	46	87

RESULTS AND DISCUSSION

Inhibition of ACC Conversion to Ethylene by Avocado Pedicel Extracts. A negative correlation was found between the conversion efficiency of ACC to ethylene and the concentration of the pedicel extract in the assay mixture (Fig. 1). The concentration of fruitlet tissue required for 50% conversion was estimated to be 61 mg fresh weight·ml^{-1}. The inhibition of ACC conversion to ethylene in pedicel extracts of mature avocado fruits was higher than in fruitlets (Fig. 1). The higher inhibition in extracts of mature fruits was probably due to accumulation of phenolic compounds in the tissue during fruit development.

Inhibiton of ACC Conversion to Ethylene by Phenolic Compounds. Phenolic substances are widely distributed in fruits and leaves of avocado (2, 13, 15). We have examined the effect of several phenolic compounds, which are present in avocado tissues, on the conversion of ACC to ethylene. D-Catechin and epicatechin reduced the conversion of ACC to ethylene to 60 and 56%, respectively (Table I). When both phenolics were applied together, the conversion was only 22%. Although each of the other phenolic compounds caused only a slight reduction in the yields of ethylene from ACC, the presence of several of these phenolics in pedicel extract may result in a cumulative effect and therefore in more significant reduction in the conversion of ACC to ethylene. The concentrations of the phenolic compounds employed were similar to those occurring naturally in avocado tissues (15).

Effect of Extract Purification on the Conversion of ACC to

Ethylene. To reduce the inhibition of ACC conversion to ethylene, various treatments for removing phenolic compounds from plant extracts were employed (10). All the procedures used significantly increased the yields of ethylene from ACC, with PVPP column chromatography being the most efficient one (Table II). The ability of lead salts to precipitate phenolic compounds is well documented (12) and indeed lead acetate also improved the conversion efficiency of ACC to ethylene (Table II).

The yields of ethylene from ACC in extracts of carnation petals varied greatly between different experiments, ranging from 11 to 77% (data not presented). Purification of the extracts by PVPP resulted in higher and less variable yields (76–93%).

Effect of Mercuric Chloride and NaOCl Concentrations on the Conversion of ACC to Ethylene. Ethylene yields from ACC in juice of apple fruits were reported to increase from 10 to 50% by increasing the final concentration of mercuric chloride from 1 to 10 mM (8). In extracts of avocado fruit pedicels, increasing the concentration of mercuric chloride from 1 to 16 mM increased the conversion of ACC to ethylene from 12 to 46% in the presence of 50 mM NaOCl (Table III). Increasing the concentration of NaOCl from 50 to 75 mM further increased the conversion of ACC to ethylene and with 16 mM $HgCl_2$ the conversion was about 87% (Table III). However, if the concentration of mercuric chloride was increased from 8 to 16 mM in an assay mixture without plant extract, a reduction of 11% in ethylene yield was observed (data not shown). Therefore, the concentration of mercuric chloride in the ACC assay mixture should not exceed 8 mM.

The ability of phenolic compounds to form complexes and insoluble precipitates with metals under alkaline conditions is well documented (7, 12). Crude extracts of avocado pedicels formed a dark brown precipitate in the presence of mercuric chloride under the alkaline conditions of the ACC assay. No formation of such precipitate was observed when the level of the phenolic compounds was reduced by the treatments indicated in Table II. These observations suggest that phenolic compounds interfere with the ACC assay by forming a phenol-mercuric complex and hence the mercuric ions are removed. Another mechanism by which phenolic compounds may interfere with the assay, is by competing with ACC on the NaOCl available for oxidation of ACC to ethylene. Plant phenolics, both monophenols and diphenols, are readily attacked by oxidizing agents such as NaOCl. The oxidizing agent can remove a hydrogen atom from the phenolic compound to form a free radical (14). The phenolic radical so formed can dimerise or react with another radical, forming C—C and C—O bonds (3, 4, 14). Further oxidation can lead to dark brown polymeric products (12). The improvement of the conversion of ACC to ethylene by increasing the concentration of both mercuric chloride and NaOCl (Table III) suggests that both mechanisms are responsible for the poor yields of ethylene from ACC in some plant extracts.

The data presented here may indicate that determination of ACC level in plant tissues that are rich in phenolic compounds requires a step of partial purification. Further improvement of the conversion of ACC to ethylene can be obtained by increasing the concentrations of mercuric chloride and NaOCl in the assay mixture.

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