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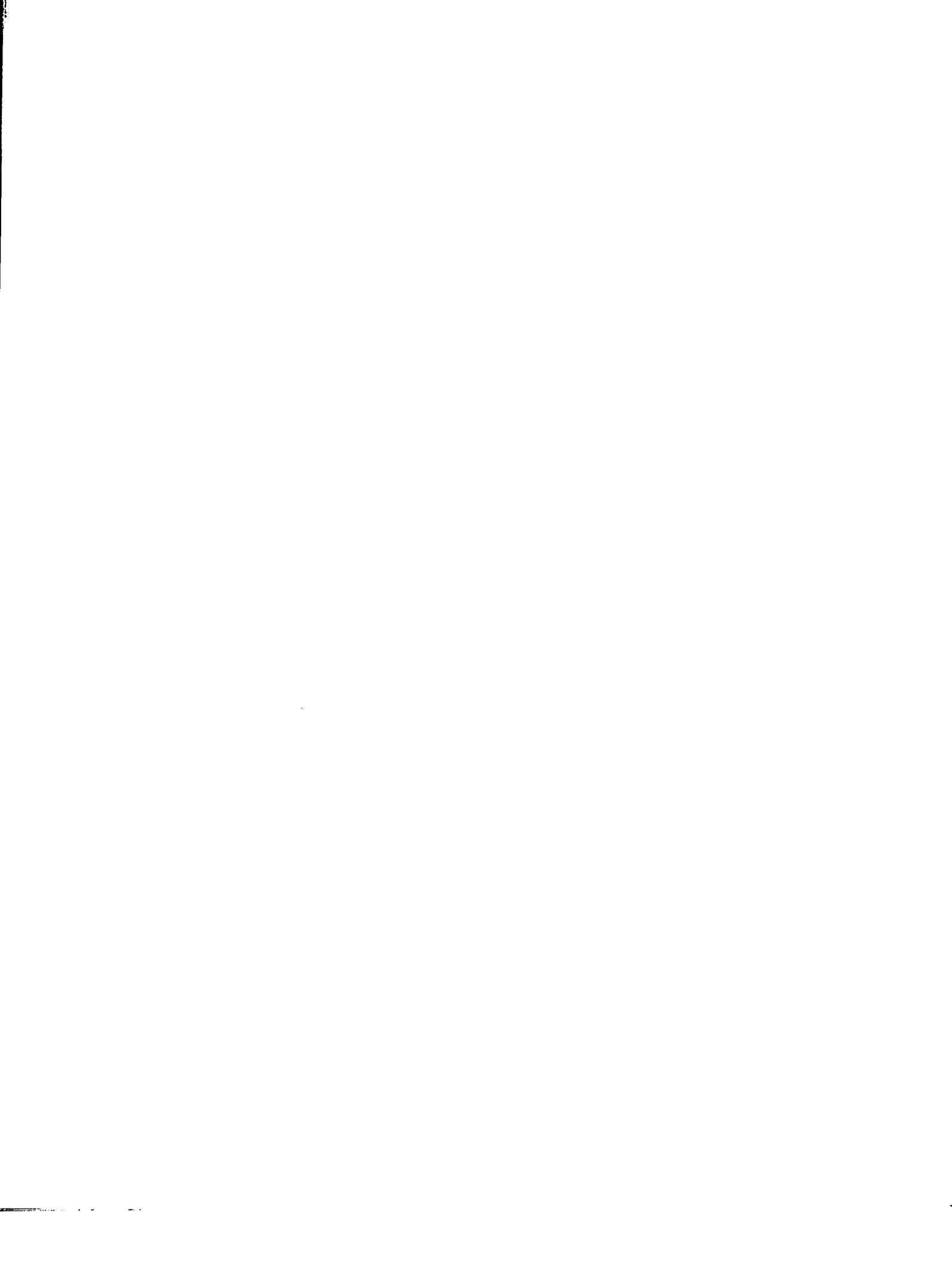
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Relationships between ethylene biosynthetic pathway
metabolites during banana fruit ripening

Honorio, Sylvio Luis, Ph.D.

Iowa State University, 1991

Relationships between ethylene biosynthetic pathway

metabolites during banana fruit ripening

by

Sylvio Luis Honorio

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GENERAL INTRODUCTION

The banana is one of a few fruits available to consumers year-round. It is grown in the tropics and picked in a mature, but unripe, state. It then is shipped to a point close to that of retail sale, and ripening is initiated artificially under controlled conditions. The ripening of the banana can also be initiated artificially in the laboratory under conditions identical to those used in commercial practice, and this makes it ideal for fundamental studies of its metabolic processes.

After harvest, bananas continue ontogeny in a manner similar to that of unharvested fruit. They absorb and metabolize oxygen, produce carbon dioxide, and generate heat. Changes also take place in the composition and structure of the cell wall, and these changes cause fruit softening. The green color of unripe fruit gradually gives way to yellow as chlorophyll is destroyed and the yellow pigment of the skin and flesh is unmasked. Many volatile compounds are produced by the fruit after harvest, and some of these give the fruit its typical aroma and flavor.

Ethylene (C_2H_4) is a volatile compound that is found in fruit at nearly all stages of development. C_2H_4 is the easiest growth substance to monitor because emanated C_2H_4 can be measured directly by gas chromatography and destruction of the tissue is not necessary. On the

other hand, the immediate precursor of C_2H_4, 1-aminocyclopropane-1-carboxylic acid (ACC) and its malonic acid conjugate, 1-(malonylamino)-cyclopropane-1-carboxylic acid (MACC), require more elaborate procedures. At times, indirect and costly direct procedures are used for the quantitation of ACC, and a straight-forward system for the direct identification and accurate quantitation of ACC would aid research on ACC metabolism.

This research evaluated the relationships between concentrations of C_2H_4, ACC, and MACC during maturation, ripening, and senescence of banana fruit. Within this overall objective, the specific objectives were: 1) to quantify the ACC content during fruit ontogeny and correlate it to C_2H_4 production; 2) to quantify the MACC content and determine its relationship to the processes associated with C_2H_4 biosynthesis in banana fruit; and 3) to determine whether or not ACC conjugates with amino acids or organic acids other than malonic acid occur during the postharvest phases of fruit development.

Explanation of Dissertation Format

This dissertation is arranged in the alternate format consisting of one paper that will be submitted to a scientific journal. The format and style of the paper was based on the Journal of the American Society for Horticultural Science. This dissertation also includes a general introduction, general review of literature, general summary and

conclusions, and literature cited for the general review of literature. Sylvio L. Honorio was the principal investigator on all research reported herein, and he is the first author on this paper. Dr. Richard J. Gladon served as Co-major Professor for Mr. Honorio in his research and is listed as an author on the paper.

GENERAL REVIEW OF LITERATURE

Overview of Ethylene Biosynthesis

Ethylene (C_2H_4) is a gaseous, unsaturated hydrocarbon, that is the simplest plant hormone and growth regulator. Ethylene modulates/regulates many aspects of plant growth and development, and its effects are observable from seed germination to organ senescence (Abeles, 1973). Elucidation of the C_2H_4 biosynthetic pathway came from studies that used model systems such as peroxidized linolate (Lieberman and Mapson, 1964), ascorbate (Lieberman and Kunishi, 1971), flavin mononucleotide (Yang et al., 1966), and the horseradish peroxidase system (Lieberman et al., 1965). The most important conclusion drawn from those studies was that, at least in apple tissue, L-methionine (L-MET) was the main physiological precursor of C_2H_4 (Lieberman et al., 1966).

Burg (1973) hypothesized that L-MET was converted to S-adenosylmethionine (SAM) and that SAM was an intermediate of C_2H_4 biosynthesis. Subsequently, Adams and Yang (1977) observed that apple tissue treated with labeled MET generated two radioactive compounds, 5-methylthioadenosine (MTA) and 5-methylthioribose (MTR), and this provided further evidence that SAM might be an intermediate in the conversion of L-MET to C_2H_4. It also was found that tomato

tissue converted SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) and MTR, and it was thought that MTA was hydrolyzed rapidly to MTR by MTA nucleosidase (Yu et al., 1979). Also in tomato tissue, it was shown that the ribose moiety of MTA was metabolized to a four-carbon derivative (2-aminobutyrate) of MET and that the conversion of MTA into MET (completion of the cycle) was oxygen-dependent (Wang et al., 1982).

Cell-free extracts from several fruit tissues have MTR kinase activity (Kushad et al., 1982), and this enzyme catalyzes ATP-dependent phosphorylation of MTR to 5-methylthioribose-1-phosphate (MTR-1-P) (Ferro et al., 1978). In 1983, Giovanelli et al. showed that *Lemna* generated adenine when MTA was hydrolized to MTR. MTR-1-P metabolism in avocado and tomato pericarp tissues produced α-keto-γ-methylthiobutyric acid (α-KMB) and α-hydroxy-γ-methylthiobutyric acid (α-HMB), but only α-KMB served as a substrate for C_2H_4 synthesis, and this indicated that α-KMB was a precursor of MET (Kushad et al., 1983).

The overall result of this methionine-sulfur cycle is that the ribose moiety of ATP furnishes the four-carbon moiety of MET from which C_2H_4 and other products are derived. Also, the CH_3S group of MET is conserved for continued regeneration of MET (Yang and Hoffman, 1984). Finally, *de novo* synthesis of MET from the four-carbon moiety

occurs via the established transulfuration route from ϱ-phosphohomoserine (Giovanelli et al., 1980).

Metabolism of 1-aminocyclopropane-1-carboxylic acid (ACC)

In apple tissue, MET was converted to C_2H_4 in the presence of air, but in nitrogen, it was metabolized to MTR and a compound identified as ACC (Adams and Yang, 1979; Lürssen et al., 1979). In tomato extracts, the conversion of SAM to ACC is catalyzed by the enzyme ACC synthase (Boller et al., 1979; Yu et al., 1979). *In vivo* studies have established that the synthesis of ACC is the rate-limiting step in C_2H_4 biosynthesis (Boller et al., 1979; Cameron et al., 1979; Yu et al., 1979; Yu and Yang, 1980). The precise mechanism of conversion of ACC to C_2H_4 *in vivo* is not clear. In apple, C_2H_4 formation takes place primarily on the surface of the plasma membrane (Mattoo et al., 1979). However, in pea and broadbean, ACC and C_2H_4 synthesis are compartmentalized differently in the protoplast (Guy and Kende, 1984a). Vacuoles from pea and broadbean leaves accounted for 80% of the protoplast C_2H_4 production, and it was suggested that ACC is synthesized mainly in the cytoplasm and transported to the vacuole where it is converted to C_2H_4 (Guy and Kende, 1984b; Bouzayen et al., 1990). This suggests that the vacuole might be the major site of C_2H_4 synthesis and that ethylene-forming-enzyme (EFE) which is known to be membrane-associated

(Apelbaum et al., 1981), is localized in the tonoplast (Yang and Hoffman, 1984).

Conversion of ACC to C_2H_4 *in vivo* is catalyzed by a specific enzyme and not by general peroxidase (Satoh and Esashi, 1980a). It has been proposed that ACC hydroxylase catalyzes the oxidation of ACC to N-hydroxy-ACC, which then is fragmented into C_2H_4 and cyanoformic acid (Yang and Hoffman, 1984). Spontaneous decarboxylation of cyanoformic acid then yields CO_2 and hydrogen cyanide (HCN) (Pirrung, 1983; Peiser et al., 1984; Pirrung, 1985).

An hypothesis for C_2H_4 action and regulation during fruit ripening and seed germination is that both C_2H_4 and HCN act as enzyme inhibitors when cytochrome c oxidase is the prime enzymatic target that affects gene expression (Pirrung and Brauman, 1987). It has been speculated that the difference between climacteric and nonclimacteric fruits may be a difference in the ability to express the genes for ACC synthase in response to alternative pathway activity (Pirrung and Brauman, 1987).

Metabolism of 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC)

It has been suggested that the physiological significance of malonylation of amino acids is to detoxify D-amino acids in plant tissues (Rosa and Neish, 1968). In higher plants, ACC is converted to C_2H_4, but it also is metabolized to N-malonyl-ACC via conjugation with

malonate (Amrhein et al., 1981; Hoffman et al., 1982a). Conjugation between D-amino acids and organic acids (succinic, malonic, acetic) or D-amino acids (D-alanine, D-glutamine, etc.) with themselves occurs naturally in pea, barley, corn, wheat, oat, cauliflower, and *Nicotiana rustica L.* (Amrhein et al., 1981; Amrhein et al., 1982; Good and Andreae, 1957; Ladesic et al., 1971; Rosa and Neish, 1968). In higher plants, conjugates are formed primarily with nonessential D-amino acids (Ladesic et al., 1971; Rosa and Neish, 1968). Ryegrass and maize seedlings also are able to absorb and metabolize D-configuration amino acids such as D-valine, D-leucine, D-alanine, D-methionine, and D-lysine (Aldag and Young, 1970).

In general, D-amino acids, but not L-amino acids, stimulate C_2H_4 production (Hoffman et al., 1982b; Satoh and Esashi, 1980b; Satoh and Esashi, 1982). The efficacy of stimulating C_2H_4 production by D-amino acids varies according to their side-chain structure (Satoh and Esashi, 1981; Satoh and Esashi, 1982). D-amino acids with a polar side-chain, such as D-serine, D-aspartic acid, and D-arginine, were ineffective in stimulating C_2H_4 production, but they raised the concentration of ACC in cotyledonary segments of cocklebur seeds (Satoh and Esashi, 1981; Satoh and Esashi, 1982). D-amino acids, but not L-amino acids, suppressed conjugation of ACC to MACC (Amrhein et al., 1982; Liu et

al., 1983). However, polar D-amino acids were also ineffective in inhibiting malonylation of ACC (Liu et al., 1983).

It has been postulated that EFE interacts with ACC in a configuration that corresponds to an L-amino acid (Amrhein et al., 1981), whereas malonyltransferase (MACC-forming enzyme) (Kionka and Amrhein, 1984) seemed to interact with ACC in a configuration that corresponds to a D-amino acid (Hoffman et al., 1982b; Liu et al., 1983). Because ACC has no asymmetric carbon atom, it can be recognized as either a D- or L-amino acid (Hoffman et al., 1982b; Liu et al., 1983). It has been hypothesized that ACC and D-amino acids might be malonylated by the same (Su and Yang, 1985) or separate enzymes that have cross-substrate specificity (Amrhein et al., 1982; Liu et al., 1983). In cocklebur, peanut seeds, and buckwheat hypocotyls, MACC does not seem to be an active storage form of ACC (Amrhein et al., 1982; Hoffman et al., 1983a; Satoh and Esashi, 1984), but its formation certainly reduces the level of free ACC and might regulate C_2H_4 production partially (Hoffman et al., 1983b; Kionka and Amrhein, 1984; Liu et al., 1983).

The sites of synthesis and storage of MACC in *Acer pseudoplatanus* cells indicate that MACC is synthesized in the cytosol, transported through the tonoplast, and accumulated in the vacuole (Bouzayen et al., 1988).

Quantification of ACC

Identification and accurate quantitation of ACC are critical needs for understanding regulation of C_2H_4 biosynthesis. ACC content in various plant organs can be determined by several methods. Indirect methods that are based on oxidative ring cleavage of ACC to C_2H_4 (Lizada and Yang, 1979; Boller et al., 1979) underestimate or sometimes overestimate ACC concentrations (McGaw et al., 1985; Lanneluc-Sanson et al., 1986). Underestimation is caused by low conversion efficiency in the reaction that generates C_2H_4 (Nieder et al., 1986; Satoh and Esashi, 1982), and overestimation has not been explained (Lanneluc-Sanson et al., 1986). Another indirect method, enzymatic deamination of ACC (Honma, 1983), also contains steps that depend upon determination of the conversion efficiency of the reactions. On the other hand, direct methods for ACC quantitation, such as stable dilution mass spectrometry (McGaw et al., 1985) and gas chromatography-mass spectroscopy (Savidge et al., 1983), are costly and frequently the needed instrumentation is not available routinely.

Preparative high-performance liquid chromatography (HPLC) has been used for purification of ACC (McGaw et al., 1985; Savidge et al., 1983), but only one analytical HPLC method has been used for direct quantitation of ACC (Lanneluc-Sanson et al., 1986). ACC is a nonchiral, α-amino acid, and as such, it should be quantifiable when

HPLC is used as an amino acid analyzer. HPLC analysis, especially with reverse-phase columns, offers greater efficiency, ease of use, and higher flow rates than the conventional ion-exchange techniques (Hill et al., 1982; Lanneluc-Sanson et al., 1986).

SECTION I: RELATIONSHIPS BETWEEN ETHYLENE
BIOSYNTHETIC PATHWAY METABOLITES
DURING BANANA FRUIT RIPENING

ABSTRACT

This report describes a method for extraction, separation, and quantification of 1-aminocyclopropane-1-carboxylic acid (ACC) by reverse-phase, high-performance liquid chromatography (HPLC). A 20 to 21 minute run time (ACC at 19.65 min) in a linear gradient mode yielded a baseline separation of ACC from other α-amino acids. The rates of ethylene (C_2H_4) production and respiration showed two bursts during fruit ripening. The first burst in C_2H_4 production preceded the respiratory climacteric peak, whereas the second burst in C_2H_4 production occurred concurrently with the second burst in respiration. The increase as well as the decrease in C_2H_4 production was related to ACC content, but an unknown mechanism might have also been involved. The content of 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) fluctuated during all phases of banana ripening. MACC content was not related to the rate of C_2H_4 production or respiration.

INTRODUCTION

1-aminocyclopropane-1-carboxylic acid (ACC) is an intermediate in the ethylene (C_2H_4) biosynthetic pathway (Yang and Hoffman, 1984). Identification and accurate quantitation of ACC are critical needs for understanding regulation of C_2H_4 biosynthesis. ACC content in various plant organs can be determined by several methods. Indirect methods that are based on oxidative ring cleavage of ACC to C_2H_4 (Lizada and Yang, 1979; Boller et al., 1979) underestimate ACC concentration (McGaw et al., 1985; Lanneluc-Sanson et al., 1986) due to low conversion efficiency of the reaction that generates C_2H_4 (Nieder et al., 1986). Another indirect method, enzymatic deamination of ACC (Honma, 1983), also contains steps that depend upon determination of the conversion efficiency of the reactions. On the other hand, direct methods for ACC quantitation, such as stable isotope dilution mass spectrometry (McGaw et al., 1985) and gas chromatography-mass spectroscopy (Savidge et al., 1983) are costly and frequently the needed instrumentation is not available routinely.

In most higher plants, oxidative ring cleavage of ACC yields cyanoformic acid, carbon dioxide, and C_2H_4 (Peiser et al., 1984; Pirrung, 1985). C_2H_4 produced by this reaction is biologically active, and it modulates or regulates many aspects of plant growth and development (Abeles, 1973). C_2H_4 production also has been linked

with changes in respiratory activity and alteration of the onset of ripening in bananas (Burg and Burg, 1965).

Many higher plants "detoxify themselves" from D-amino acids by conjugation of the D-amino acids with various organic acids (Ladesic et al., 1971; Zenk and Scherf, 1964). Because ACC is nonchiral, it can be recognized as either a D- or L-amino acid (Hoffman et al., 1982a; Liu et al., 1983). Thus, ethylene-forming-enzyme (EFE) interacts with ACC in a configuration in which ACC is perceived as an L-amino acid (Amrhein et al., 1981), and C_2H_4 then is formed from ACC. On the other hand, the enzyme malonyl transferase seems to perceive ACC as a D-amino acid (Hoffman et al., 1982a; Liu et al., 1983), and malonyl transferase conjugates ACC with malonic acid to form 1-(malonylamino)-cyclopropane-1-carboxylic acid (MACC).

The content of ACC and MACC varies with fruit type and stage of development (Hoffman et al., 1982b; Hoffman et al., 1983; Matern et al., 1984), and they also are present in vegetative tissues (Kionka and Amrhein, 1984; Satoh and Esashi, 1984). In ripening tomato fruits, C_2H_4 production increases MACC content (Liu et al., 1985; Rothan and Nicolas, 1988). The relationships of C_2H_4 production, respiration, and ACC and MACC content during banana ripening have not been studied. The objectives of this research were: 1) to develop an HPLC-based procedure for direct quantitation of ACC; 2) to determine

whether or not ACC from banana fruit conjugates with any organic acid other than malonic acid; and 3) to determine the relationship of C_2H_4 to ACC and MACC content and the respiratory pattern during banana fruit ripening.

MATERIALS AND METHODS

Postharvest treatment of fruit

Hands of ungassed, physiologically mature Honduran 'Grande Naine' bananas [*Musa acuminata* L. (AAA group, Cavendish subgroup)] were obtained locally. Immediately after transport, hands were treated with 500 $mg \cdot l^{-1}$ thiabendazole [2-(4-thiazolyl)benzimidazole] for 3 min and allowed to dry for about 30 min. Fingers (individual fruits) were sampled randomly from different hands and were distributed into 6 experimental groups that each contained 12 fingers. Each group was sealed in a glass desiccator and treated with 100 $\mu l \cdot l^{-1}$ C_2H_4 for 14 hours at $22 \pm 2C$. After the C_2H_4 treatment period, desiccators were evacuated slightly to remove as much C_2H_4 as possible from the intercellular spaces of the fruit. Desiccators then were connected to a compressed air cylinder, and air was passed through the desiccators at 117.5 $ml \cdot min^{-1}$. Color changes in fruit peels were designated by the color index of von Loesecke (1950): 1 = green; 2 = green-trace of yellow; 3 = more green than yellow; 4 = more yellow than green; 5 = green tip; 6 = all yellow; 7 = yellow lightly flecked with brown; and 8 = yellow with increasing brown areas.

Ethylene and carbon dioxide production

C_2H_4 and carbon dioxide (CO_2) production from ripening banana fruits were quantified every 6 h for 138 h after C_2H_4 treatment. Inlet and outlet gas samples from each desiccator were collected and analyzed by gas chromatography. The chromatograph system for C_2H_4 has been described previously (Sinska and Gladon, 1984). CO_2 was quantitated with a thermal conductivity detector after separation on a 183x0.3 cm column of Chromosorb 106. Mean production of C_2H_4 and CO_2 from three experiments each with six replications were plotted as a function of time and color index.

Tissue selection, extraction, and preparation

Nine banana fruits of the same color index (3 replicates of 3 fruits) were removed from six desiccators. Fruits were peeled and the pulps were diced and mixed. A 25-g subsample was extracted with 50 ml of cold 5% (w/v) sulfosalicylic acid. The extract was centrifuged at 10,000xg for 15 min, and the supernatant was decanted to a separate container for subsequent use. The pellet (1 volume) was reextracted for 10 min with 4 volumes of 80% (v/v) hot (70C) ethanol. This extract was centrifuged at 20,000xg for 15 min, and the supernatant from this centrifugation was combined with the previous supernatant. The combined supernatants were concentrated to about 3 ml *in vacuo*,

and the concentrate was passed through a 1x10 cm column of washed ion-exchange resin (Dowex 50x8, H^+-form, 50-100 mesh). Amino acids were eluted with 60 ml of 2 N NH4OH, the eluant was evaporated to dryness *in vacuo*, and it was resuspended in 2 ml deionized water. ACC was identified by thin layer chromatography and verified by cochromatography with authentic ACC (Sigma). Thin layer chromatography was conducted on 20 cmx20 cmx250 μm cellulose plates in n-butanol:acetic acid:water (4:1:5, v/v/v), and ninhydrin was used for visualization. The ACC-conjugate sample was prepared by passing banana tissue extracts through an H^+-form column of ion-exchange resin (to remove amino acids) and then was loaded onto a 1x10 cm column of washed ion-exchange resin (Dowex-1, OH^--form). Conjugates were eluted from this column with 60 ml of 6 N formic acid, the eluant was evaporated to dryness *in vacuo*, and it was resuspended in 2 ml deionized water. Conjugates were hydrolyzed to ACC and organic acids with 2 N HCl at 100C for 2 h. ACC was not degraded during this procedure (data not presented). After hydrolysis, conjugates were quantified as ACC. Conjugates were identified by thin layer chromatography conducted on 20 cmx20 cmx250 μm cellulose plates in 1-propanol: concentrated NH4OH (7:3, v/v). Ehrlich's reagent was used for visualization. Conjugates extracted from banana fruit were

cochromatographed with MACC prepared according to Satoh and Esashi (1984).

HPLC separation and quantitation of ACC

A sample from the resuspended eluant was prepared for HPLC analysis by the method of Hill et al. (1982). ACC was separated from other amino acids on a Waters Associates ALC-244 HPLC with a reversed-phase, μBondapak-C18 analytical column (3.9 mm i.d.x300 mm). Flow rate of the eluting solvent was 2 ml·min^{-1}. An eluting solvent gradient [A/(A+B)] from 85 to 15 percent was used. Solvent A was 15 mM Na2HPO4 and solvent B was CH3CN/H2O (55/45, v/v). ACC was detected by using a Schoeffel FS-970 fluorometer with excitation and emission wavelengths set at 226 and 470 nm, respectively. Preliminary experiments on separation, quantitation, and determination of retention time of ACC were conducted with 10 nmol of reagent grade ACC (Sigma). Positive identification and quantitation were insured by using an internal standard of 5 nmol of ACC for a control in each banana fruit extract. The entire quantification procedure for ACC in banana fruit at various stages of ripeness was conducted three times, and there were three replicates of fruit at each stage of ripeness. ACC content was plotted as a function of time and color index.

Treatment of fruit discs with [2,3-^{14}C]-ACC

Banana fruit discs of each color index (0.6 cm thick, 2.0 cm diameter, and 7.0±2g fresh weight per disc) were cut with a corkborer from a central portion of the fruit. Discs were placed in a petri dish that contained one Whatman No. 1 filter paper wetted with 2.0 ml of solution that contained 8 μCi [2,3-^{14}C]-ACC (sp. act. 80 mcuries/mmol). Labeled ACC was vacuum infiltrated in a desiccator for 1.0 min at 110 mmHg. After infiltration, discs were transferred to 100 ml jars that contained 2.0 ml of a solution of sucrose (2%, w/v) in 0.1 M phosphate (K$_2$HPO$_4$/KH$_2$PO$_4$) buffer at pH 6.8. Fruit discs were incubated for 5 h at 22±2C. Labeled C$_2$H$_4$ produced during this period was trapped in 5.0 ml of 0.1 M mercuric acetate in methanol by passing nitrogen through the jars and into the traps. C$_2$H$_4$ was quantified in a liquid scintillation counter. Fruit discs were washed, blotted dry, frozen in liquid nitrogen, and ground. About 8.5 g of ground tissue was used to extract MACC by the same procedure described under tissue selection, extraction, and preparation. Labeled MACC was quantified in a liquid scintillation counter. Mean content of labeled MACC and production of labeled C$_2$H$_4$ from two complete runs each with three replications were plotted as a function of color index.

Malonic acid identification

Malonic acid was identified by thin layer chromatography and verified by cochromatography with authentic malonic acid (Sigma). Thin layer chromatography was conducted on 20 cmx20 cmx250 μm cellulose plates in tert-amyl alcohol:chloroform:water:formic acid (8:8:8:3, v/v). Bromocresol green (Sigma) was used for visualization.

RESULTS

Separation and quantification of ACC

ACC was extracted from ripening banana fruits, and it was separated and quantitated by using HPLC in a gradient system. ACC was eluted and detected 19.65 min after injection, and baseline separation from other components was achieved (Fig. 1). An internal standard of ACC, in replicate assay vials, confirmed the identity, retention time, and quantity of ACC (data not presented). We did not attempt to identify peaks adjacent to the ACC peak.

Relationship of C_2H_4 production to rate of respiration

A typical burst in C_2H_4 production occurred from 6 to 24 h, during the respiratory climacteric rise (Fig. 2A and 2B). The C_2H_4 peak preceded the climacteric by about 24 h. C_2H_4 production began to decrease during the latter part of the respiratory climacteric rise, and it held a steady-state rate from hours 48 to about 90. The first respiratory climacteric burst peaked at about 48 h, and then decreased to a steady state rate from hours 72 to 120 h. A second burst of C_2H_4 production began at about 96 h, and a second burst in the rate of respiration commenced about 24 h after this increase in C_2H_4 production. Both of these second bursts peaked at about 132 h.
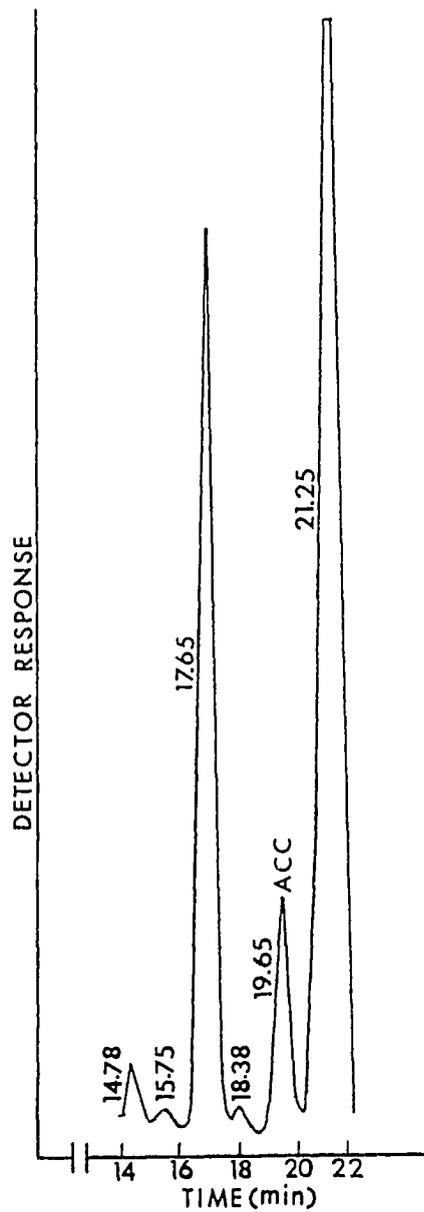
Fig. 1. HPLC chromatogram of color index 7 banana fruit showing baseline separation of ACC (19.65 min). The ACC peak corresponded to 3.13 μmol of ACC. A gradient solvent system of 85% solvent A (15mM Na2HPO4) and 15% solvent B (CH3CN/H20, 55/45, v/v) was used.

Fig. 2. Physiological changes during 'Grande Naine' banana fruit ripening. (A) C_2H_4 production rate measured as $\mu l \cdot kg^{-1} \cdot h^{-1}$. (B) Respiration rate measured as mg $CO_2 \cdot kg^{-1} \cdot h^{-1}$. (C) ACC content measured as $nmol \cdot g^{-1}$. (D) MACC content measured as $nmol \cdot g^{-1}$. LSD 0.05%.
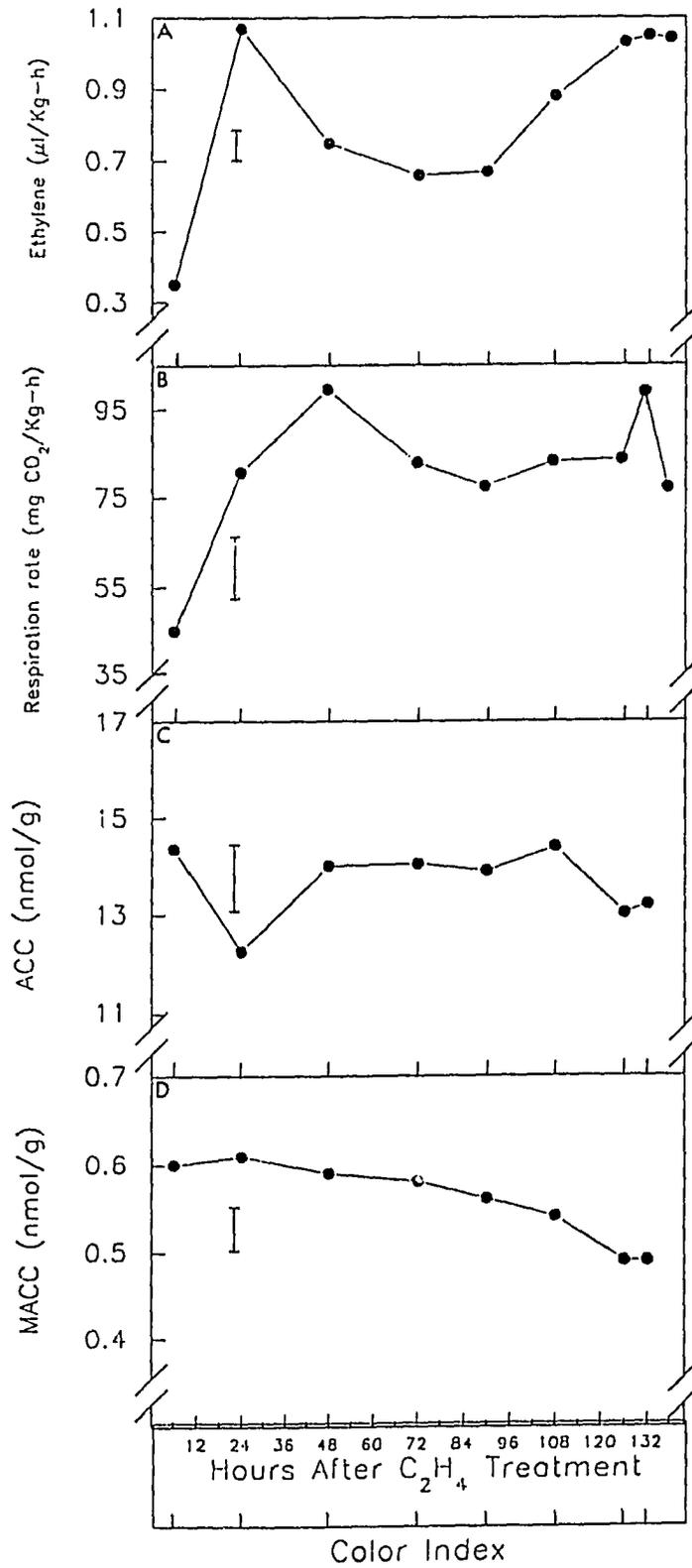
Relationship of C_2H_4 production to ACC content

The climacteric-like burst in C_2H_4 production at about 24 h was associated with a concurrent decrease in ACC content (Fig. 2A and 2C). Similarly, the subsequent decrease in C_2H_4 production was associated with an accumulation of ACC. From 48 to about 90 h, the concentration of ACC and the production rate of C_2H_4 were relatively constant. A second increase in the rate of C_2H_4 production occurred concurrently with an increase in ACC content. However, the increased rate of C_2H_4 production was sustained, but the ACC content began to decrease at hour 108.

Relationship of C_2H_4 production to MACC content

Over the first 24 h period after C_2H_4 treatment, MACC content was relatively constant, but the C_2H_4 production rate increased (Fig. 2A and 2D). At 24 h, both the C_2H_4 production rate and the MACC content began to decrease. Although the C_2H_4 production rate decreased to a lower level by hour 48, the MACC content did not decrease to a significantly lower level until 84 h. MACC content continued to decrease until hour 126; however, C_2H_4 production began its second burst at about 84 h.

Incorporation of $[2-3-^{14}C]$-ACC into C_2H_4 and ACC-conjugate

Thin layer chromatography from extracts of banana fruit discs treated with labeled ACC showed that MACC (Rf of 0.25) was the

only conjugated form of ACC (data not presented). Therefore, malonic acid (Rf of 0.57) was the only organic acid that conjugated with ACC. Fruit discs of all color indices were able to metabolize labeled ACC into labeled C_2H_4 and labeled MACC (Fig. 3). Fruit discs of color indices 1 and 2 incorporated the greatest amount of labeled ACC into both labeled C_2H_4 and labeled MACC. Subsequently, all color indices showed a decreased capability to metabolize labeled ACC. Fruit discs of color indices 5 to 8 incorporated similar amounts of labeled ACC into labeled C_2H_4.
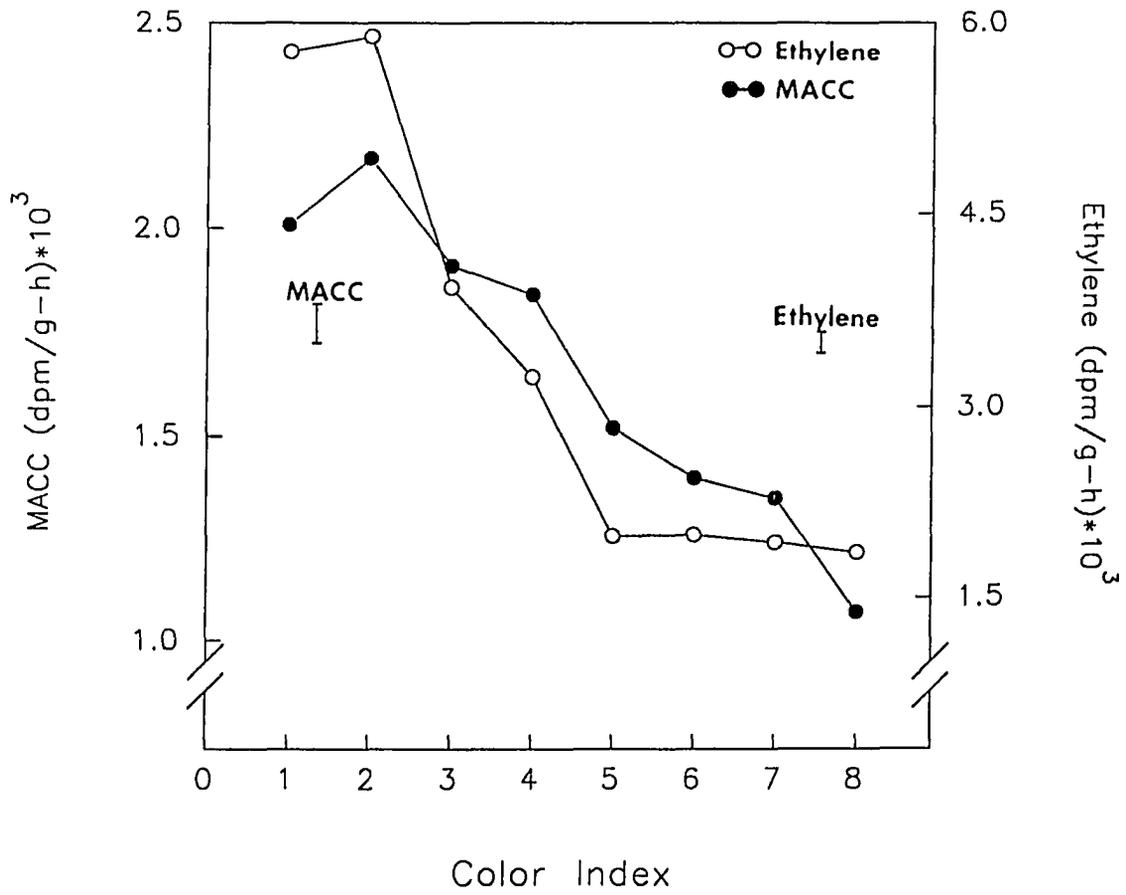
Fig. 3. ^{14}C-MACC and ^{14}C-C2H4 production from 'Grande Naine' fruit discs treated with ^{14}C-ACC.   LSD 0.05%.

DISCUSSION

Our procedure for direct ACC quantitation in banana fruit tissue overcame several difficulties present in other procedures. Problems associated with low conversion efficiency of ACC to C_2H_4 (Lizada and Yang, 1979; Boller at al., 1979) and interferences (Nieder et al., 1986) in indirect procedures are relieved by this direct quantitation procedure. The problem of instrumentation cost (McGaw et al., 1985; Savidge et al., 1983) also is alleviated because most laboratories now contain at least one less-expensive HPLC. An HPLC-based procedure developed by Lanneluc-Sanson et al. (1986) at the same time as ours uses different derivatizing reagents, but the process for quantification of ACC is similar in both procedures. Our method for extraction of ACC from banana fruit tissue is straight forward, and it should be readily adaptable to other types of fruits and plant organs.

The onset of natural ripening events in banana is indicated by increased C_2H_4 production followed by increased respiration. The respiratory climacteric peak in our experiments occurred after the C_2H_4 production peak, and our pattern is in agreement with other reports on banana fruit ripening (Palmer and McGlasson, 1969; Burg and Burg, 1965; Mapson and Robinson, 1966; Hoffman and Yang, 1980; Beaudry et al., 1987). Under various sets of experimental conditions, C_2H_4 production and subsequent ripening of banana fruit may be inhibited

without inhibition of the respiratory climacteric (McGlasson et al., 1971). On the other hand, compounds that inhibit the respiratory climacteric invariably inhibit ripening (McGlasson et al., 1971). This indicates that, as long as the respiratory mechanism is functional, and thus providing energy to the tissue, EFE can generate C_2H_4 and ripening occurs. This seems to explain the second peak of C_2H_4 in 'Grande Naine' banana fruit where an increase in the rate of respiration also was observed.

For most fruits, organic acid content declines during the ripening process, but in banana fruit, the greatest amounts of organic acids are attained at the full-ripe stage (Palmer, 1971). Banana fruit also show a massive conversion of starch to sucrose coincident with the onset of the climacteric rise. These syntheses of organic acids and sucrose constitute a significant sink for respiratory ATP (Beaudry et al., 1989; Hubbard et al., 1990; Kanellis et al., 1989).

Under normal circumstances, C_2H_4 production by plant tissues is limited by the availability of ACC (Apelbaum et al., 1981; Cameron et al., 1979), and this is apparent during the onset of ripening at 6 to 12 h (Fig. 2A and 2C). Increased C_2H_4 production probably caused the concomitant decrease in ACC content during hours 6 to 24. However, the decreased C_2H_4 production that commenced at 36 h may have been caused by decreased ACC content, an impairment of the EFE

system as the tissue ages (Hoffman and Yang, 1980), or some unknown mechanism.

The banana fruit used in this study showed a second increase in C_2H_4 production that started at 96 h. The maximum rate of C_2H_4 production during both of these peaks was the same. Thus, impairment of the EFE system probably is not a valid reason for the decrease in C_2H_4 production that was observed during hours 30 to 60, and decreased ACC content or an unknown mechanism remain as plausible explanations. Vendrell and McGlasson (1971) found a similar pattern of C_2H_4 production in banana fruit treated with 5 to 10 $\mu l \cdot l^{-1} C_2H_4$ for 12 h. They suggested that the cause of the two peaks was that the short C_2H_4 treatment was sufficient to induce partially the ripening process. Future research that employs radiolabeled ACC should resolve this question and explain the relationship of these events to the ripening process.

In several higher plants, ACC conjugates with malonic acid to form MACC (Amrhein et al., 1981; Hoffman et al., 1982a). In our research with 'Grande Naine' bananas, MACC was the only conjugated form of ACC. The greatest amount of MACC occurred coincidentally with the greatest rates of C_2H_4 production. This may reflect production of C_2H_4 that enhances the capability of the fruit tissue to malonate ACC. A similar pattern was found in tomato fruit (Liu et al., 1985; Rothan

and Nicolas, 1988). MACC content seems to be governed by a defined minimum concentration of C_2H_4, because as C_2H_4 production decreased, the MACC content also declined. This was further confirmed when the MACC content stopped decreasing as C_2H_4 production peaked the second time at hour 132 (Fig. 2A and 2D). Conjugation of ACC into MACC may decrease the content of ACC, which in turn, decreases the amount of ACC available for C_2H_4 production. This observation warrants more study because the amount of MACC found in the fruit was small and a delicate balance between ACC, MACC, and C_2H_4 production may exist.

In contrast with tomato fruit (Rothan and Nicolas, 1988), banana fruit does not accumulate a significant amount of MACC even though fruit of all ages were able to metabolize ACC into MACC (Fig. 3). The decreased content of MACC as fruit aged suggests that MACC is hydrolyzed partially to ACC and malonic acid. The occurrence and reason for this hydrolysis might be similar to the hydrolysis that occurred in extracts of senescent peanut plants. In peanut, an aminoacylase catalyzed the hydrolysis of MACC to ACC (Matern et al., 1984). Our findings agree with the postulate that MACC is only a secondary product of ACC, and the role of MACC in C_2H_4 synthesis is minimal (Hoffman et al., 1983; Satoh and Esashi, 1984).

From this research, we conclude that the HPLC assay for ACC is suitable for banana fruit tissues of all ages, and it overcomes conversion efficiency, cost, and equipment availability difficulties. This work also suggests that, as long as the respiratory mechanism is functional, impairment of EFE is retarded and C_2H_4 production can increase even in the latter stages of fruit ripening. Decreases and increases of C_2H_4 production seem to be related to ACC content, but ACC content by itself does not explain these events fully and an unknown mechanism awaits resolution. Finally, conjugation of ACC and malonic acid to yield MACC persists beyond the initiation of rapid C_2H_4 production, and MACC can be hydrolyzed, but it does not contribute significantly to C_2H_4 biosynthesis.

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GENERAL SUMMARY AND CONCLUSIONS

An HPLC procedure was developed for ACC quantitation in banana fruit. This alternative method overcame difficulties encountered in previously published, nonHPLC procedures, and it is comparable to another HPLC procedure published recently. HPLC in a linear gradient mode yielded baseline separation of ACC from other amino acids. It was found that ACC content decreased concomitantly with an increase in C_2H_4 production during the early stages of banana ripening. Conversely, a subsequent decrease in C_2H_4 production may have been caused by decreased ACC content or some unknown mechanism.

Formation of MACC does not seem to contribute to decreased ACC content, because MACC content, relative to ACC content, is small. MACC content is greatest when C_2H_4 production is greatest, and therefore, C_2H_4 seems to induce MACC formation. Malonic acid was the only conjugate of ACC that we found in banana fruit, and conjugation with malonic acid occurred in fruit of all ages. MACC content decreased as fruit aged, and this indicated that hydrolysis of MACC to ACC and malonic acid occurred. However, the small amount that was hydrolyzed to ACC did not seem to contribute to an increase in ACC content.

Respiration increased after the first peak of C_2H_4 production, and it increased concomitantly with the second peak of C_2H_4 production. This indicated that as long as respiration was not impaired, biological activities persisted and impairment of ethylene-forming-enzyme was retarded. Therefore, C_2H_4 production could increase during the later stages of ripening.

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