Glutamic acid and 5-aminolevulinic acid may function as precursors of system II ethylene in ripening tomato fruits

by

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1. GENERAL INTRODUCTION

Ethylene (C_2H_4) triggers changes during all phases of plant growth and development, and it is the causal agent for many dramatic changes during fruit ripening (Abeles et al., 1992). Methionine (MET) is considered the first committed precursor of C_2H_4, and the C_2H_4 biosynthetic pathway has been established as MET → S-adenosylmethionine (AdoMet) → 1-aminocyclopropane-1-carboxylic acid (ACC) → C_2H_4 (Yang and Hoffman, 1984). Previous studies have shown that during ripening tomato fruit tissue switches from MET to an unknown compound as the major precursor of C_2H_4, but it retains its ability to utilize MET as a precursor of C_2H_4 (Baker et al., 1976). It has been suggested that another pathway to C_2H_4 may exist, and this pathway has been labeled System II (McMurchie et al., 1972). Little is known about the compounds that may function as the precursors for System II C_2H_4, and identification of these possible precursors might help scientists better understand and control this pathway to C_2H_4. Subsequently, elimination of the burst of C_2H_4 production during the ripening of some fruits and vegetables may allow them to be harvested, handled, and marketed without the large percentage of waste now experienced. The overall objective of this research project was to evaluate several compounds as possible precursors of System II C_2H_4 in ripening tomato fruit. These possible precursors are: ACC, MET, 5-aminolevulinic acid (ALA), homocysteine (HOM), glutamic acid (GLU), and α-ketoglutarate (KG). The specific objectives of this research were to study the influence of these possible precursors on 1) C_2H_4 production and 2) free and conjugated ACC synthesis in mature green, pink, and red ripe tomato fruits.

Thesis Organization

This thesis is organized by using journal manuscript format. In addition to the included paper, a general review of literature and general summary and conclusions are included. A list of references cited in the introduction, the general review of literature, and

the summary and conclusions follows the summary and conclusions chapter. The senior author, H.P.V. Rupasinghe, solely conducted the research included in the journal manuscript that constitutes the main body of this thesis.

2. GENERAL REVIEW OF LITERATURE

Ethylene (C_2H_4) is a plant hormone that initiates ripening and regulates many aspects of plant growth and development (Abeles et al., 1992). C_2H_4 biosynthesis occurs via the following pathway (Yang and Hoffman,1984): Methionine (MET) → S-adenosylmethionine (AdoMet) → 1-aminocyclopropane-1-carboxylic acid (ACC) → C_2H_4. MET is converted to AdoMet by AdoMet Synthetase, and AdoMet is converted to ACC by ACC Synthase. The final step, ACC → C_2H_4, is catalyzed by ethylene-forming enzyme (EFE), which also is known as ACC Oxidase (ACCO). The methylthio (CH_3S-) group of MET and adenosine is released from AdoMet as 5-methylthioadenosine (MTA) during the formation of ACC, which comes from carbon atoms 1 to 4 of MET. MTA is hydrolyzed rapidly to 5-methylthioribose (MTR), and MTR then is converted into MET via 2-keto-4-methylthiobutyric acid (KMBA) as an intermediate. This recycling pathway for MET is known as the Yang Cycle (Abeles et al., 1992). Several other compounds may act as precursors under certain conditions, and there is evidence that suggests that C_2H_4 production may occur through other pathways (Yang and Hoffman, 1984; Lieberman, 1979; Baker et al., 1976).

The concentration of free MET was two or three times greater in unripe avocado fruits than in those fully ripe, but this concentration of MET could not account for the quantity of C_2H_4 produced during the climacteric rise (Baur et al., 1971). Furthermore, Baur et al. (1971) showed that the endogenous level of MET could sustain this rate of C_2H_4 production for only about 3 h, assuming that MET is the sole or the predominant precursor of C_2H_4 (Yang and Baur, 1969). Similarly, free MET in tomato fruits at the green stage was relatively low (13.5 μmol/100g fresh weight), and it decreased as the fruits ripened (6.5 μmol/100g fresh weight during the climacteric rise and 7.5 μmol/100g fresh weight at the climacteric peak) (Yu et al., 1967).

The rhizobitoxine analog, L-2-amino-4-(2-aminoethoxy)-*trans*-3-butenoic acid (Ro), which blocks the conversion of SAM to ACC, inhibits C_2H_4 production by 50 to 70% in green tomato fruit, but only by about 15% in climacteric (pink) and postclimacteric (red) tomato fruit slices (Baker et al., 1976; Baker et al., 1978). Incorporation of ^{14}C from [^{14}C]methionine into C_2H_4 in green and pink tomato tissues was inhibited by Ro to about the same extent as inhibition of total C_2H_4 production. The same researchers also reported similar results when they used avocado tissues, and these tissues do not behave like apple, certain flowers, and other tissues that produce C_2H_4 from MET via Ro-sensitive pathways. Similar results were found by Kushad et al. (1985) when tomato plugs at the breaker stage were incubated with aminoethoxyvinylglycine (AVG). These results suggest that the ability of tomato fruits to convert MET into C_2H_4 does not parallel their ability to produce C_2H_4 naturally. Baker et al. (1976) suggested that, during ripening, tomato fruit tissue switches from MET to an unknown compound as the major precursor of C_2H_4, but it retains its ability to utilize MET as a precursor of C_2H_4.

Kushad et al. (1985) found that the Yang Cycle activity decreased during ripening of tomato fruit. They found that the greatest MTA nucleosidase and MTR kinase activity was observed in the green and breaker stages, respectively, and the activity declined sharply at the pink and red stages. Similarly, treatment with 1 mM ACC invariably increased the rate of C_2H_4 production to 10 to 1000 times greater than the controls. In work by other researchers, MET at the same concentration was shown to be ineffective (Cameron et al., 1979).

C_2H_4 and polyamine metabolism share a common precursor, AdoMet, during tomato fruit ripening, and the possibility arises that the biosynthesis of both may be linked closely (Casas et al., 1990). Bakanashvili et al. (1987) found a decrease in the concentration of the natural polyamines putrescine (PUT), spermidine, and spermine at fruit maturation of 'Rutgers' tomato. On the other hand, other researchers showed that the PUT concentration

of 'Lorena' tomato increased during the climacteric peak (Casas et al., 1990).

These results allow the interpretation that either MET is turned over rapidly or there are precursors of C_2H_4 other than MET. Thus, two pathways, or systems, may be involved in C_2H_4 production, and this may be the basis of System I and System II C_2H_4 production that has been postulated previously (McMurchie et al., 1972). System I seems to control basal C_2H_4 production and stress-related C_2H_4 production, and the System I receptor seems to respond to C_2H_4 by promoting the activity of EFE and ACC malonylation (Mattoo and White, 1991). In contrast, System II seems to regulate autostimulation (autocatalysis) of C_2H_4 and operates only in climacteric fruit (Mattoo and White, 1991). The System II receptor responds temporally to C_2H_4 and induces ACC synthase activity (Mattoo and White, 1991).

In plant tissue, chlorophyll and heme biosynthesis occur via a common pathway from 5-aminolevulinic acid (ALA) through monopyrrole porphobilinogen (PBG) to the asymmetric, cyclized tetrapyrrole protoporphyrin IX (Smith and Griffiths, 1993). However, there is evidence that ALA can be metabolized to other compounds not in the porphyrin pathways that are in some plant species (Beale, 1978; Duggan et al., 1982). El-Rayes (1987) has shown that application of (2,3-^{3}H) ALA caused the accumulation of radioactivity in both ACC and C_2H_4 but not in MET. These data suggest that during ripening of tomato fruits, C_2H_4 is formed from ALA via ACC. One route by which ALA may be converted into ACC is transamination followed by decarboxylation of carbon atom 5 and then cyclization to ACC (El-Rayes, 1987). It also has been shown that carbon atom 5 of ALA is metabolized to CO_2 during metabolism of ALA in ripening tomato fruit tissue (El-Rayes, 1987).

These data support the idea that there is another separate and independent pathway by which C_2H_4 is synthesized during tomato fruit ripening and System II C_2H_4 synthesis. Tomato fruit may utilize MET as the major precursor of C_2H_4 during the green stage of development (Baker et al., 1976; Baker et al., 1978) while PBG deaminase (PBGD) activity and its concomitant use of ALA is high (McMahon et al., 1990). In addition, ALA

dehydratase (ALAD) activity in tomato fruit tissue declines over time, with the most pronounced decrease occurring between days 10 and 25. At the mature green stage (about day 40), and before carotenoids appear (about day 45), activity of ALAD had declined to a minimum but remained detectable at residual levels through ripening (days 40 to 60) (Kyriacou et al., 1996). Therefore, as the activities of both PBGD and ALAD decline during the later stages of growth and development, ALA may become the precursor of C_2H_4 and the role of MET as a precursor of C_2H_4 may become reduced (Baker et al., 1976; Baker et al., 1978).

Glutamic acid (GLU) and α-ketoglutarate (KG) serve as possible ALA precursors (Castelfranco and Beale, 1983), and it has been shown that they participate in the C_2H_4 biosynthetic pathway in *Penicillium digitatum* (Yang, 1973). In this system, C_2H_4 was derived from carbons 3 and 4 of GLU and KG (Chou and Yang, 1973). *P. digitatum* is known to produce C_2H_4 via pathways that do not utilize MET as a precursor (Jacobsen and Wang, 1968). In this fungus system, rhizobitoxine did not inhibit C_2H_4 production (Chalutz and Lieberman, 1977; Owens et al., 1971), and uniformly labeled MET was not metabolized to labeled C_2H_4 (Ketring et al., 1968).

In senescing flower tissue of morning glory (*Ipomea tricolor* Cav.) and auxin-treated pea (*Pisum sativum* L.), MET had little effect on C_2H_4 production, and they utilized selenoamino acids as more efficient precursors of C_2H_4 than MET (Konze et al., 1978). Hanson and Kende (1976) found that in the presence of homocysteine (HOM) thiolactone, C_2H_4 synthesis was enhanced and occurred prematurely in flower tissue of *I. tricolor*. In apple tissue, it has been shown that HOM serves as a precursor of C_2H_4 through its conversion into MET (Baur and Yang, 1972). Stimulation of C_2H_4 synthesis was consistently greater with HOM thiolactone than with MET. Subsequently, Schilling and Kende (1979) found, in *P. sativum* L. cv. Alaska stem sections, that MET slightly promoted C_2H_4 synthesis (5 to 10%), whereas HOM thiolactone enhanced C_2H_4 production by 20 to 25%. These

authors also found that when unlabelled MET or HOM thiolactone was supplied to incubating, IAA-treated pea stem sections treated with L-[U-^{14}C]MET, the specific radioactivity of C_2H_4 was reduced about equally. Gonzalez (1994) has shown that HOM induced more rapid production of C_2H_4 and caused the accumulation of more ACC than did MET in senescing carnation petals. These findings also reveal the possibility that other compounds may be precursors of System II C_2H_4.

3. GLUTAMIC ACID AND 5-AMINOLEVULINIC ACID MAY FUNCTION AS PRECURSORS OF SYSTEM II ETHYLENE IN RIPENING TOMATO FRUITS

A paper to be submitted to *HortScience*

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Abstract. Methionine (MET) is considered the first committed precursor of ethylene (C_2H_4), and the C_2H_4 biosynthetic pathway has been established as MET → S-adenosylmethionine (AdoMet) → 1-aminocyclopropane-1-carboxylic acid (ACC) → C_2H_4. It has been suggested that another pathway to C_2H_4 may exist during times when the synthesis of massive amounts of C_2H_4 occurs, and this pathway has been labeled System II. The overall objective of this research was to evaluate the efficacy of several compounds as possible precursors of System II C_2H_4. Fruit discs of 'Rutgers' tomato pericarp tissue at the mature green, pink, and red ripe stages were incubated continuously in 10, 25, or 40 mM solutions of ACC, MET, 5-aminolevulinic acid (ALA), homocysteine (HOM), glutamic acid (GLU), and α-ketoglutarate (KG). C_2H_4 production rate at 8-h intervals during the 32 h-incubation period and free and conjugated ACC content at the end of the incubation period were quantified. Fruit discs at the mature green stage treated with ACC and MET exhibited increased C_2H_4 production and free ACC content. These results confirmed the role of ACC and MET as the predominant precursors of C_2H_4 during the early stages of fruit development in tomato (System I). At the pink stage (System II), however, ALA increased C_2H_4 production to 175% and free ACC content to 146% of the control, and MET increased C_2H_4 production to 127% and ACC content to 157% of the control. These results suggest that ALA may function as a more

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efficient precursor of C_2H_4 than MET during ripening. ALA and GLU caused the greatest increase in C_2H_4 production in fruit discs at the red ripe stage. C_2H_4 production in response to exogenous ACC at this stage of fruit development was diminished and was not different from the control. Also at the red ripe stage, the C_2H_4 response to MET was similar to the response to ACC except at 40 mM. These results suggest that GLU and ALA may be metabolized to C_2H_4 via System II during tomato fruit ripening.

Introduction

Ethylene (C_2H_4) triggers dramatic changes during fruit ripening (Liu et al., 1989). C_2H_4 biosynthesis occurs via the following pathway (Yang and Hoffman,1984): Methionine (MET) → S-adenosylmethionine (AdoMet) → 1-aminocyclopropane-1-carboxylic acid (ACC) → C_2H_4. MET is converted to AdoMet by AdoMet synthetase, and AdoMet is converted to ACC by ACC synthase. The final step, ACC → C_2H_4, is catalyzed by ethylene-forming enzyme (EFE), which also is known as ACC oxidase (ACCO). Several other compounds may be precursors under certain conditions, and there is evidence that suggests that C_2H_4 production may occur through other pathways (Yang and Hoffman, 1984; Lieberman, 1979; Baker et al., 1976).

The concentration of free MET in tomato fruits at the green stage was relatively low (13.5 μmol/100g fresh weight), and it decreased as the fruits ripened (7.5 μmol/100g fresh weight at the climacteric peak) (Yu et al., 1967). Similarly, free MET was two to three times greater in unripe avocado fruits than in those fully ripe, but this concentration of MET could not account for the quantity of C_2H_4 produced during the climacteric rise (Baur et al., 1971). In addition, aminoethoxyvinylglycine (AVG), which blocks the conversion of AdoMet to ACC, inhibits C_2H_4 production in green tomato fruit, but sensitivity of C_2H_4 production to AVG declines considerably during ripening (Baker et al., 1978). Furthermore, treatment with 1 mM ACC invariably increased the rate of C_2H_4 production to 10 to 1000 times greater

than the control, whereas MET at the same concentration was ineffective (Cameron et al., 1979). Kushad et al. (1985) found that the Yang Cycle activity decreased during ripening of tomato fruit. These results suggest that the ability of tomato fruits to convert MET into C_2H_4 does not parallel their ability to produce C_2H_4 naturally. Baker et al. (1976) suggested that, during ripening, tomato fruit tissue switches from MET to an unknown compound as the major precursor of C_2H_4, but it retains its ability to utilize MET as a precursor of C_2H_4. Thus, two pathways, or systems, may be involved in C_2H_4 production, and this may be the basis of System I, which controls basal C_2H_4 production, and System II, which controls autostimulation of C_2H_4 production, postulated by McMurchie et al.(1972). Alternatively, the pool of MET may turn over rapidly, but there is no evidence for this phenomenon because most amino acids exist at or near a steady-state concentration.

In ripening tomato fruits, El-Rayes (1987) showed that application of (2,3-^{3}H) 5-aminolevulinic acid (ALA) caused the accumulation of radioactivity in both ACC and C_2H_4 but not in MET. This suggests that during ripening of tomato fruits, C_2H_4 is formed from ALA via ACC. Therefore, these data support the idea that there is another separate and independent pathway by which C_2H_4 is synthesized during tomato fruit ripening. Tomato fruit may utilize MET as the major precursor of C_2H_4 during the green stage of development (Baker et al., 1976; Baker et al., 1978) while ALA dehydratase (ALAD) activity (Kyriacou et al., 1996) and porphobilinogen deaminase (PBGD) activity and its concomitant use of ALA is high (McMahon et al.,1990). However, as the activity of ALAD and PBGD declines during the later stages of growth and development, ALA may become the precursor of C_2H_4, and the role of MET as a precursor of C_2H_4 may become minimal (Baker et al., 1976; Baker et al., 1978).

Glutamic acid (GLU) and α-ketoglutarate (KG) serve as ALA precursors (Castelfranco and Beale, 1983), and it has been shown that they participate in the C_2H_4 biosynthetic pathway in *Penicillium digitatum* (Yang, 1973). In this system, C_2H_4 was

derived from carbon atoms 3 and 4 of GLU and KG (Chou and Yang, 1973). Konze et al. (1978) found that senescing flower tissue of *Ipomea tricolor* and auxin-treated *Pisum sativum* L. stem sections utilized selenoamino acids as precursors of C_2H_4 more efficiently than MET. In apple tissue, it has been shown that homocysteine (HOM) serves as a precursor of C_2H_4 through its conversion into MET (Baur and Yang, 1972). Similarly, Gonzalez (1994) has shown that HOM induced more rapid production of C_2H_4 and caused the accumulation of more ACC than did MET in senescing carnation petals.

These data suggest that there may be one or more compounds that function as the precursor(s) for System II C_2H_4 during ripening of tomato fruit. Identification of these possible precursors might allow researchers to gain control over this process and thereby reduce or eliminate the burst in C_2H_4 production during fruit ripening of several fruits and vegetables. Subsequent fruit ripening then could occur at will, and that could lead to a reduction of the large percentages of postharvest losses now experienced. The purpose of this research was to evaluate whether or not ACC, MET, ALA, HOM, GLU, and KG serve as precursors of System II in ripening tomato fruits. The specific objectives of this research were to study the influence of these possible precursors of System II C_2H_4 on 1) C_2H_4 production, and 2) free and conjugated ACC content of mature green, pink, and red ripe tomato fruits.

Materials and Methods

Plant material

Lycopersicon esculentum Mill. 'Rutgers' were grown in an environmentally controlled (20C night, 24C day) greenhouse under standard cultural practices. Uniform transplants were produced in a rockwool-based hydroponic system. Four weeks after sowing, seedlings were transplanted into pots of 3 soil: 4 peat: 3 perlite (by vol.) for the remainder of one fruiting cycle. Seedlings were maintained with Peters® Excel® Cal-Mag nutrient solution (15-

2.2-12.45; The Scotts Co., Marietta, GA). Plants were trained to a single stem, and the stem apex was removed above the first leaf that followed the first inflorescence. Flowers were pollinated and tagged at anthesis (Lyons and Pratt, 1964), and three fruits were grown to completion per plant. Stages of fruit development that were used were: (i) mature green at 37 days, (ii) pink at 42 days, and (iii) red ripe at 57 days (Martin et al., 1979).

Fruit disc preparation and incubation

Discs of tomato outer pericarp tissue (1 cm diam., 2.5 mm thick, and 320±25 mg fresh weight per disc), with the epidermis intact, were excised from central to blossom end of tomato fruits with a stainless-steel cork borer at the mature green, pink, and red ripe stages just after harvest. Four discs were incubated for 32 h at 25C in a 125 ml erlenmeyer flask. Each flask contained one Whatman no. 1 filter paper wetted with 1.75 ml of either 10, 25, or 40 mM solutions of ACC, MET, ALA, HOM, GLU, or KG (Sigma Chemical Co., St. Louis, MO) or citrate buffer as the control. Citrate buffer at pH 5.4 was used to prepare each of the solutions tested. One-half ml of each corresponding solution was added to each erlenmeyer flasks with fruit discs after withdrawing gas samples at 8, 16, and 24 h during the incubation period.

Analysis of C_2H_4

The erlenmeyer flasks with fruit disks were fitted with rubber septa (catalog no. Z10,145-1, Aldrich®, Milwaukee, WI) 1 h before sampling times that were at 8-h intervals during the incubation period. A 1-ml (for pink) or 2-ml (for mature green and red ripe) sample of the gas in the headspace of the flask was withdrawn. C_2H_4 was analyzed by using a Varian Star 3600 CX gas chromatograph equipped with an activated alumina column and flame ionization detector as described previously (Sinska and Gladon, 1984). The carrier gas was He, and the oven temperature was 90C. The rubber serum stopper was

removed after the analysis of the headspace gases.

Analysis of free ACC and conjugated ACC

At the end of the incubation period, immediately after the final analysis for C_2H_4, the fruit discs were analyzed for free ACC and total ACC content. The fruit discs were ground and homogenized in 10 ml of 80% (v/v) ethanol at 70C by using a Polytron® homogenizer (Brinkmann Instruments, Westbury, NY, model PT 10-35) at a speed setting of 4 for 30s. The homogenate was centrifuged at 12,000 x *g* for 10 min, and the pellet was resuspended in 10 ml of 80% (v/v) ethanol at 70C and the mixture was homogenized and centrifuged again. Twenty ml of combined supernatant was evaporated to dryness under an air stream at 22C. The dry extract was dissolved in 2 ml water, and a 0.5 ml sample of this solution was employed for determination of free ACC or total ACC. Free ACC was quantified by the method of Lizada and Yang (1979), and total ACC was quantified by the method of Hoffman et al. (1983). Conjugated ACC was calculated by subtracting the content of free ACC from the content of total ACC. Efficiency of conversion of ACC to C_2H_4 was determined by using a known amount of ACC as an internal standard, and it was uniformly greater than 95%.

Experimental design and statistical analysis

Three independent experiments were conducted for each stage of physiological maturity. Replicates of fruit of the same stage of physiological maturity were obtained from the entire plant population. A randomized complete block design with 4 blocks was used for each independent experiment. Each block consisted of 21 treatments that were a factorial combination of 7 substrates, each with 3 concentrations. A 125 ml erlenmeyer flask with 4 fruit discs represented an experimental unit. The 3 experiments were conducted independently 2 times, and the data were pooled for statistical analysis. C_2H_4 production

and free ACC content at the end of the incubation period were analyzed statistically. C_2H_4 production was estimated by integrating the areas under the C_2H_4 production rates during the 8, 16, 24, and 32 h incubation periods. The data from ACC treatments were not included in the statistical analysis of free ACC because the amounts of free ACC were extraordinarily large compared with the other treatments. For statistical analysis, software package SAS Release 6.06 (SAS Institute, Cary, NC) was used.

Results

C_2H_4 production in response to 7 substrates in tomato pericarp tissue

When tomato fruit discs at the mature green stage were treated with ACC, they showed the greatest C_2H_4 production rate of all substrates at each of the 3 concentrations (Fig. 1A). At the pink stage, ACC caused the greatest C_2H_4 production both at 10 and 25 mM, but not at 40 mM (Fig. 1B). C_2H_4 production in response to ACC treatment in tomato fruit disks at the red ripe stage did not show statistical differences ($p = 0.05$) when compared with that of the control. Moreover, ALA, GLU, and KG caused a greater amount of C_2H_4 production than did ACC (Fig. 1C). C_2H_4 production in response to treatment of fruit discs with ACC in the green and pink stages of maturity declined as the concentration ACC that was applied increased (Figs. 1A, 1B).

C_2H_4 production in response to MET in mature green fruit discs was 17.4 $nl \cdot g^{-1} \cdot h^{-1}$ whereas it was 9.2 $nl \cdot g^{-1} \cdot h^{-1}$ in response to the citrate buffer control. This proportion was relatively less at the pink (51.6 $nl \cdot g^{-1} \cdot h^{-1}$ *vs* 40.7 $nl \cdot g^{-1} \cdot h^{-1}$) and red ripe (27.5 $nl \cdot g^{-1} \cdot h^{-1}$ *vs* 23.3 $nl \cdot g^{-1} \cdot h^{-1}$) stages of maturity (Table 1).

In fruit discs at the mature green stage, substrates other than ACC and MET did not enhance C_2H_4 production over the control (Fig. 1A). However, it is interesting that fruit discs at the pink stage treated with 40 mM ALA, and fruit discs at the red ripe stage treated with all the 3 concentrations of ALA showed the greatest C_2H_4 production over all

other substrates including ACC and MET (Figs. 1B, 1C). The fruit discs at the red ripe stage treated with GLU also exhibited an increased rate of C_2H_4 production, and this was closely similar to ALA treatment (Fig. 1C).

ACC content in response to 7 substrates in tomato pericarp tissue

Accumulated MACC content measured after the incubation of fruit discs treated with exogenous ACC ranged from 0 to 7.6 nmol/g. The MACC content in control and other treatments was undetectable. Free ACC content of fruit discs that were treated with ACC was extraordinarily greater than that of all other treatments and was not included in the statistical analysis (Table 1). At each of the 3 maturity levels, accumulated free ACC was greater with increasing concentrations of treatment ACC (data not presented). When data for ACC are not included in the analysis, MET caused the greatest free ACC content at each of the maturity levels (Figs. 2A, 2B, 2C, Table 1). Accumulated free ACC content was greater with increasing concentrations of MET in each of the 3 stages of maturity (Figs. 2A, 2B, 2C). Accumulated free ACC content in response to MET in fruit discs at the mature green stage was different ($p = 0.05$) from that of all other substrates (Fig. 2A). Furthermore, it was different ($p = 0.05$) from the control at each stage of maturity and at each concentration, but it was not statistically different ($p = 0.05$) from the ALA treatment at concentrations of 25 and 40 mM in pink fruit discs, and at 10 mM in red ripe fruit discs (Figs. 2B, 2C).

ALA caused an increase in free ACC content, and this was statistically different ($p = 0.05$) from that of the control at each of the 3 concentrations and maturity stages except 10 mM of mature green fruit discs (Fig. 2A, 2B, 2C). None of the other substrates except ALA caused an increase in free ACC content that was greater ($p = 0.05$) than that of the control in fruit discs at mature green stage (Fig. 2A).

Discussion

When ACC was applied to tomato fruit discs at the mature green stage, there was a dramatic increase in C_2H_4 production and accumulated free ACC content (Figs. 1A, 2A). However, high external ACC concentrations reduced the enhancement of C_2H_4 production relative to the stimulation obtained with lower external concentration 8 h after incubation of fruit discs (Figs. 1A, 1B, 1C). Similar types of responses were reported by Apelbaum et al. (1981) in apple discs where concentrations of 10 mM were less stimulatory to C_2H_4 production than lower concentrations (0.1-1.0 mM) after 4 h of incubation. These results suggest an autoinhibitory effect of longterm incubation of fruit discs in elevated concentrations of ACC. ACC might exhibit a negative feedback regulation that leads to a decrease in ACCO activity. The ability of tomato fruit tissue to utilize exogenous ACC for C_2H_4 production was greatest in fruit discs at the pink stage and it was lowest in fruit discs at the mature green stage (Figs. 1A, 1B). The same result was reported previously (Apelbaum et al., 1981; Cameron et al., 1979), and this suggests that the ability to convert ACC to C_2H_4 is greatest at the time normal C_2H_4 production is greatest. Surprisingly, C_2H_4 production in red ripe fruit discs in response to exogenous ACC was very low and was not different ($p = 0.05$) from that of the control (Fig. 1C, Table 1). This reduced metabolism of ACC to C_2H_4 could be due to inactivation of ACCO that is associated with cellular membranes. Therefore, membrane disintegration that accompanies the red ripe stage of fruit development may have affected the conversion of ACC to C_2H_4 (Yang and Hoffman, 1984; Abeles et al., 1992).

In addition to the metabolism of ACC to C_2H_4, ACC has been recognized to be conjugated into the biologically stable 1-(malonylamino)-cyclopropane-1-carboxylic acid (MACC) in many plant tissues (Abeles et al., 1992). However, accumulated MACC content in fruit discs treated with exogenous ACC were not considerable (0 to 7.6 nmol/g). Thus, malonylation of ACC to MACC may result in lower levels of free ACC and consequently a

lower C_2H_4 production could not be caused low C_2H_4 production in response to treatment ACC in the red ripe fruit discs. Unexpectedly, accumulated MACC in control and other treatments were undetectable. This may be due to conversion of MACC to some unknown compound due to the effect of long incubation period. However, it is unlikely that MACC serves as a source of ACC (McKeon and Yang, 1987).

In mature green fruit discs, C_2H_4 production and free ACC content in response to MET, when compared with that of the control, was greatest but that was relatively less in fruit discs at the pink and red ripe stages (Table 1). Similar to our results, Cameron et al. (1979) found that the stimulatory effect of MET on C_2H_4 production declined as the tomato fruit ripened. In addition, the same researchers found that mature green tomatoes possess the enzyme(s) necessary to convert MET to C_2H_4. These results showed that once fruit tissue turns to the pink and red ripe stages, its ability to convert MET to C_2H_4 declined. Two possible reasons for this observation are the decrease in Yang cycle activity during ripening of tomato fruit (Kushad et al, 1985) and the limited ability of ACCO to convert ACC to C_2H_4 during the late fruit development (Rothan and Nicolas, 1988). None of the substrates other than ACC and MET at the mature green stage showed differences ($p = 0.05$) in C_2H_4 production over the control. Hence, our observation at mature green stage demonstrated the typical C_2H_4 biosynthesis pathway that previously was found by Yang and Hoffman (1984).

In contrast, the contribution by ALA to C_2H_4 production and free ACC content was greater in fruit discs at the pink and red ripe stages compared with the mature green stage (Figs. 1B, 1C, 2B, 2C, Table 1.). El-Rayes (1987) observed the same response to ALA with respect to C_2H_4 production. However, contrary to El-Raye (1987) findings, these results showed accumulated free ACC content in response to treated ALA was not greater than that of the treated MET in pink fruit discs. Therefore, our data do not show strong evidence for a pathway that converts ALA to C_2H_4 via ACC as proposed by El-Rayes (1987).

Perhaps ALA may be converted to C_2H_4 either via ACC or directly (without going through ACC) in tomato fruit tissue at the pink and red ripe stages. However, in view of these observations, these data suggest that ALA may function as a precursor of C_2H_4 during late fruit development and may be involved in the control of autostimulation of C_2H_4 (System II).

Fruit discs at the red ripe stage treated with GLU also exhibited greater C_2H_4 production, and the values for C_2H_4 production were similar to those obtained with ALA treatment. However, there is no strong evidence that GLU stimulated greater free ACC content over the control (Fig. 2C). It is interesting that GLU, the precursor of C_2H_4 in *P. digitatum*, has some structural similarity to MET, the precursor of C_2H_4 in higher plants, and that in both systems, C_2H_4 derives from carbons 3 and 4 of their respective substrate (Lieberman, 1979) without going through ACC. Biosynthesis of ALA from GLU has been found in a variety of cell types (Beale and Weinstein, 1991), including higher plant tissues such as greening barley (*Hordeum vulgare* L.) (Beale et al., 1975) and greening maize leaves (Meller et al., 1975). Furthermore, expression of genes that encode glutamate-1-semialdehyde-2,1-aminotransferase (GSAAM), which is involved in the conversion of GLU to ALA, decreased with increasing age of tomato fruit (Polking, 1995). Therefore, our results suggest that GLU also may function as a precursor of C_2H_4 in tomato fruit tissue at the red ripe stage and GLU may be metabolized to C_2H_4 either directly (Lieberman, 1979) or via ALA (Beale et al., 1975; Meller et al., 1975).

All substrates, including HOM and KG, showed a greater C_2H_4 production and free ACC content in fruit discs at the pink stage, as compared with the other 2 stages (Figs. 1B, 2B). This result suggests that these compounds may be metabolized to C_2H_4 while MET could not account for the quantity of C_2H_4 produced during ripening (Baur et al., 1971). HOM can serve as an C_2H_4 precursor in apple tissue (Baur and Yang, 1972) and pea stem sections (Schilling and Kende, 1979). Gonzalez (1994) showed that HOM increased C_2H_4 production and free ACC content before MET did in senescencing carnation petals.

Hence, there may be other independent pathway(s) by which C_2H_4 could be synthesized during System II C_2H_4 production and later stages in fruit ontogeny. The pathway starting from MET seems to operate at all times, predominantly during early fruit development, but at a relatively low rate during the late stages of fruit development. The other pathway(s) may utilize ALA and GLU and metabolize ALA and GLU to C_2H_4 through ACC or directly. This later pathway(s) may be operable when the activities of the enzymes ALAD (Kyriacou et al., 1996) and PBGD (McMahon et al., 1990) decrease and GLU and ALA is no longer utilized for chlorophyll biosynthesis. However, further research must be conducted by using radiolabeled GLU and ALA to confirm their metabolism to C_2H_4 as well as to determine their specific biosynthetic pathway(s).

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Table 1. Ethylene production and free ACC content in response to 7 substrates that may have acted as a precursor of ethylene in tomato pericarp tissue. Ethylene production is expressed as an hourly mean of the ethylene production rates over the period starting at hour 8 and ending at hour 32 of incubation. Free ACC content was measured only at the end of the 32-h incubation period. Pericarp tissue received constant exposure to 10, 25, or 40 mM solutions of each substrate. Data represent means of 3 concentrations of each substrate.

Substrate	Stage of Fruit Development					
	Mature Green		Pink		Red Ripe	
	C_2H_4 ($nl \cdot g^{-1} \cdot h^{-1}$)	ACC ($nmol \cdot g^{-1}$)	C_2H_4 ($nl \cdot g^{-1} \cdot h^{-1}$)	ACC ($nmol \cdot g^{-1}$)	C_2H_4 ($nl \cdot g^{-1} \cdot h^{-1}$)	ACC ($nmol \cdot g^{-1}$)
Control	9.2	25.2	40.7	124.9	23.3	83.9
ACC	39.5	15168.0	75.4	11492.0	24.9	11760.0
MET	17.4	154.9	51.6	196.8	27.5	199.9
ALA	12.8	70.7	71.1	182.5	31.5	130.3
HOM	11.4	31.3	51.8	155.0	22.7	102.8
GLU	9.1	32.8	48.6	143.7	30.6	112.2
KG	11.0	32.9	53.3	143.4	27.1	117.7
$LSD_{(0.05)}$	1.5	20.0	1.6	8.3	2.3	11.8

FIGURE CAPTIONS

Fig. 1. Ethylene production in response to 7 substrates that may have acted as a precursor of System II ethylene: Control (○), ACC (□), MET (●), ALA (■), HOM (▲), GLU (▼), and KG (✖) in tomato pericarp tissue at mature green (A), pink (B), and red ripe (C) stages. Ethylene production is expressed as an hourly mean of the ethylene production rates over the period starting at hour 8 and ending at hour 32 of incubation. Each data point represents the mean of 8 replicates.

Fig. 2. Free ACC content in response to 6 substrates that may have acted as a precursor of System II ethylene: Control (○), MET (●), ALA (■), HOM (▲), GLU (▼), and KG (✖) in tomato pericarp tissue at mature green (A), pink (B), and red ripe (C) stages. Free ACC content was measured at the end of the 32-h incubation period. Each data point represents the mean of 8 replicates.

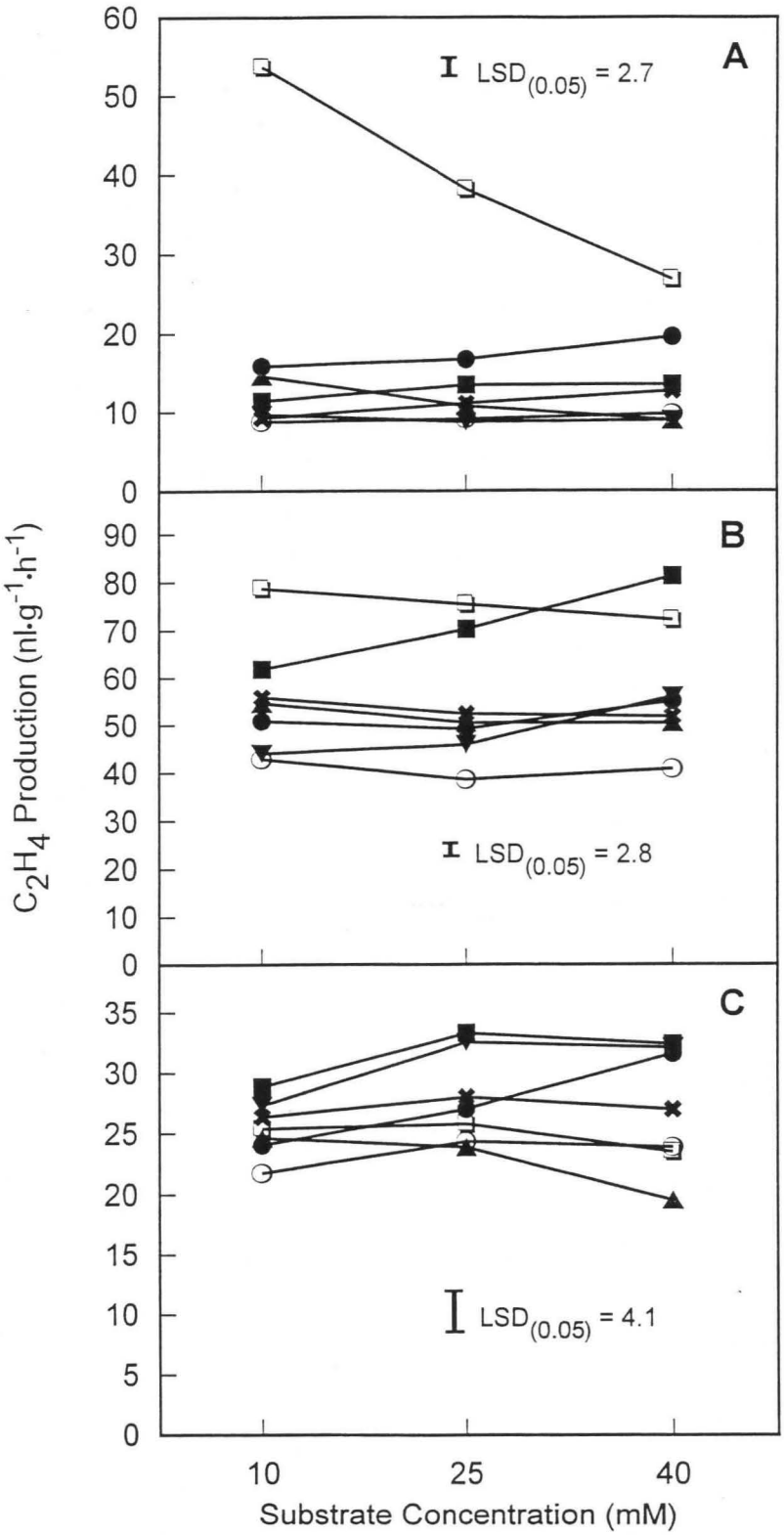
A
B
C
LSD(0.05) = 2.7
LSD(0.05) = 2.8
LSD(0.05) = 4.1
C2H4 Production (nl·g-1·h-1)
Substrate Concentration (mM)
10
25
40

Fig. 1

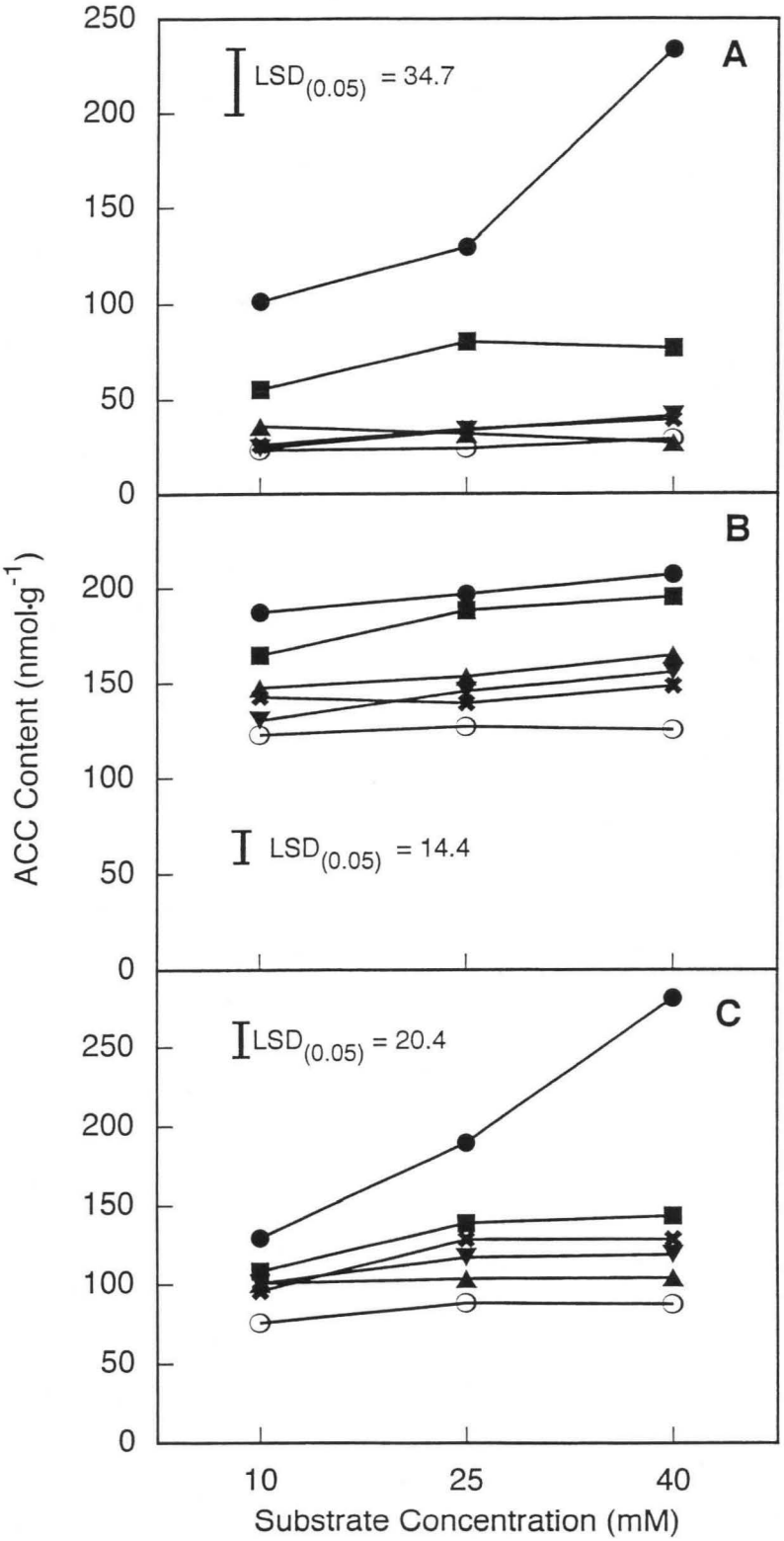
A
B
C
LSD(0.05) = 34.7
LSD(0.05) = 14.4
LSD(0.05) = 20.4
ACC Content (nmol·g-1)
Substrate Concentration (mM)
0
50
100
150
200
250
10
25
40

Fig. 2

4. GENERAL SUMMARY AND CONCLUSIONS

The effect on C_2H_4 production and free and conjugated ACC content in 'Rutgers' tomato fruit by several possible precursors of System II C_2H_4 was evaluated. The possible precursors were 1-aminocyclopropane-1-carboxylic acid (ACC), methionine (MET), 5-aminolevulinic acid (ALA), homocysteine (HOM), glutamic acid (GLU), and alpha-ketoglutarate (KG). Discs of pericarp tissue at the mature green, pink, and red ripe stages of fruit development were incubated continuously in individual treatments of 10, 25, and 40 mM concentrations of substrate in citric acid buffer (pH = 5.4) that functioned as the control. The C_2H_4 production rate was measured at each 8-h interval during a 32 h-incubation period, and free and conjugated ACC content were quantified at the end of the incubation period.

Fruit discs at the mature green stage showed a dramatic increase in C_2H_4 production and free ACC content in response to exogenous ACC. MET showed a similar response that was second only to ACC at this stage of fruit development. These results confirmed the role of ACC and MET as the predominant precursors of C_2H_4 during the early stages of fruit development in tomato fruit.

At the pink stage of development, C_2H_4 production in response to ACC was greatest at lower concentrations (10 and 25 mM), but it was relatively lower at the 40 mM concentration where ALA enhanced the largest C_2H_4 production over all other treatments. ALA increased C_2H_4 production to 175% and ACC content to 146% of the control, whereas MET increased C_2H_4 production only to 127% and ACC content to 157% of the control. These results suggest that ALA may function as a precursor of C_2H_4 more efficiently than MET during ripening, when tomatoes are at the pink stage of development.

ALA and GLU showed the greatest C_2H_4 production in fruit discs at the red ripe stage. C_2H_4 production in response to ACC treatment at this stage of fruit development was diminished and did not show differences over that of the control. The response of C_2H_4

production in response to MET was similar to that of ACC, except at 40 mM where it was greater. However, accumulated free ACC content was greatest with fruit discs treated with ACC and followed by MET. These results lead us to a conclusion that ALA may be metabolized to C_2H_4 during System II C_2H_4 synthesis and late fruit development of tomato. Evidence also was found that GLU could be a potential precursor of C_2H_4 during red ripe stage of tomato fruit.

Further research should be conducted with radioactive ALA and GLU to confirm the results obtained with the present research and to have a better understanding of the role of these possible precursors in C_2H_4 production during ripening of tomato fruit and other climacteric fruits and other plant tissues. Also, further research must be done on pathway(s) by which ALA and GLU is metabolized to C_2H_4.

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