Differential Regulation of Wound-Induced 1-Aminocyclopropane-1-Carboxylate Synthase Activity and Gene Expression in Mesocarp Tissue of Winter Squash Fruit by Carbon Dioxide and Diazocyclopentadiene

Francis M. Mathooko[1], Tetsu Ono[2], Yasutaka Kubo[2], Akitsugu Inaba[2], Reinosuke Nakamura[2]

[1]Department of Food Science and Postharvest Technology, Faculty of Agriculture, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000, Nairobi, KENYA; [2]Laboratory of Postharvest Horticulture, Faculty of Agriculture, Okayama University, Tsushima, JAPAN.

ABSTRACT

Ethylene biosythesis in higher plants induced by various stimuli may be regulated by several factors, among them ethylene action inhibitors. This study investigates the regulation of wound-induced ethylene production, 1-aminocyclopropane-1-carboxylate (ACC) synthase activity and its gene expression and ACC oxidase activity in the mesocarp tissue of winter squash (*Cucurbita maxima* Duch. cv. Ebisu) fruit by elevated $C0_2$ and diazocyclopentadiene (DACP), a new inhibitor of ethylene action and/or synthesis. Northern blot analysis was applied to total RNA using labeled probes prepared by a multiprimer DNA labeling system and α-[^{32}P]dCTP with a *Hind* III fragment from pCMW33, a wound-inducible ACC synthase gene as a template. $C0_2$ suppressed wound-induced ethylene production, ACC synthase and ACC oxidase activities and the expression of ACC synthase gene. The suppression was reversible upon withdrawal of $C0_2$ except that of ACC oxidase activity. On the contrary, DACP stimulated wound-induced ethylene production, ACC synthase activity and accumulation of its mRNA transcripts but suppressed ACC oxidase activity. The DACP data suggest that wound-induced ACC synthase activity and its gene expression, in winter squash fruit are regulated by a negative feedback control mechanism. The $C0_2$ data suggest that $C0_2$ does not regulate wound-induced ACC synthase activity and its gene expression by antagonizing the effects of ethylene produced in response to wounding and may specifically regulate wound-induced ethylene biosynthesis transcriptionally by decreasing ACC synthase mRNA level probably through other yet unknown mechanisms.

KEY WORDS

1-Aminocyclopropane-1-carboxylate , CO_2, *Cucurbita maxima*, diazocyclopentadiene, ethylene

1.0 INTRODUCTION

Ethylene, one of the simplest organic molecules with biological activity, is a plant hormone that controls many aspects of plant growth, development and senescence (Abeles et al. 1992; Yang and Hoffman, 1984), and is synthesized in response to a wide range of stimuli including wounding (Hyodo, 1991; Mathooko, 1995; Mathooko, 1996; Mathooko et al. 1993a). Enhancement of ethylene production serves as a signaling mechanism with profound physiological consequences and large losses of fruits and vegetables are due to ethylene's effects on plant

senescence (Sato et al. 1991). The enzyme whose activity limits ethylene production is presumably 1-aminocyclopropane-1-carboxylate (ACC) synthase which catalyses the formation of ACC, the immediate precursor of ethylene (Abeles et al. 1992; Kende, 1993; Yang and Hoffman, 1984), although accumulated evidence has indicated that ACC oxidase activity may also be limiting (Kende, 1993; Mathooko, 1996). Thus, ACC synthase has been the major subject of investigation concerning the regulation of ethylene biosynthesis at the molecular level.

Studies with several inducers of ethylene production have indicated that the induction of ACC synthase is due to enhanced transcription (Huang et al. 1991; Kende, 1993; Li et al. 1992; Lincoln et al 1993; Nakajima et al. 1990; Rottman et al. 1991) and requires *de novo* protein synthesis. It is well documented that ACC synthase is encoded by more than one gene (Huang et al. 1991; Nakagawa et al. 1991; Olson et al. 1991; Van Der Straeten et al. 1990) and that ACC synthase genes induced by different stimuli are differentially regulated (Huang et al. 1991; Kende, 1993; Nakagawa et al. 1991). Despite all these advances in the regulation of ethylene biosynthesis, a great deal of research is still needed in order to understand the molecular mechanisms by which environmental and endogenous factors regulate ethylene biosynthesis.

The regulation of ethylene biosynthesis at the molecular level by CO_2 has not been well studied (Mathooko et al. 1993a). $C0_2$ inhibits wound-induced ethylene biosynthesis by inhibiting ACC synthase activity and/or synthesis at the transcriptional level (Kubo et al. 1995). Whether the regulation of wound-induced ACC synthase activity and gene expression by $C0_2$ was reversible upon withdrawal of $C0_2$ or not was, however, not studied. Sisler and Blankenship (1993) proposed that diazocyclopentadiene (DACP) is a photoaffinity compound which binds to the ethylene receptor(s) in plants resulting in a strong and continuous inhibition of ethylene action. This compound is, therefore, a useful tool in the characterization of the negative and positive feedback control mechanisms of the regulation of ethylene biosynthesis. We, therefore, compared the effects of $C0_2$ and DACP on wound-induced ACC synthase activity and its gene expression in mesocarp tissue of winter squash fruit and the effects of withdrawing $C0_2$ on these parameters.

2.0 MATERIALS AND METHODS

2.1 Plant material and treatments

Winter squash (*Cucurbita maxima* Duch. cv. Ebisu) fruit were obtained from a commercial supplier in Okayama City, Japan. Cubes (5 mm) were excised from the mesocarp tissue and incubated at 25°C in plastic containers. The containers were ventilated with humidified air (control) or 60% $C0_2$. Since the experiment was designed to investigate the direct effect of elevated $C0_2$ level, the 0_2 level was maintained at 20% with the balance gas being nitrogen. This high level of $C0_2$ was used solely to amplify the tissue response to the treatment. Another set of cubes was sealed in a desiccator and exposed to DACP vapour (10 μl/l) under fluorescent light

(>5000 lux) for 2 h and then treated with humidified air in a continuous flow-through system for additional 10 h. DACP was prepared according to the procedure of Regitz and Liedhegener (1967) and kept in a solution of hexane as previously described (Sisler and Blankenship, 1993). Initially, and 6 and 10 h after excision, cubes were sampled for determination of ethylene production, extraction of total RNA and the activities of ACC synthase and ACC oxidase. In the $C0_2$ treatment, after 6 h incubation, $C0_2$ gas was withdrawn and the cubes incubated in humidified air for a further 4 hours. Samples were also prepared from intact fruit.

2.2 Ethylene determination

A 1-ml gas sample was withdrawn from the headspace gas of the incubating flask using a gas-tight hypodermic syringe. Ethylene concentration in the gas sample was assayed using a Shimadzu gas chromatograph (Model GC-4CM, Shimadzu Corporation, Kyoto, Japan) equipped with an activated alumina column and a flame ionization detector.

2.3 Extraction and assay of ACC synthase activity

The extraction of ACC synthase and assay for its activity was performed according to Boller et al, (1979) with slight modifications as previously described (Mathooko et al. 1993a). Five grams of tissue was homogenized in 10 ml of extraction medium consisting of 0.5 M potassium (K)-phosphate (pH 8.5), 5 μM pyridoxal phosphate (PLP) and 5 mM dithiothreitol (DTT) in the presence of 5% (*w/v*) polyvinylpyrrolidone (PVP).

After centrifugation of the homogenate (10,000 *g*, 30 min, 4°C), the supernatant was passed through a membrane filter and then loaded onto prepacked Sephadex G-25 columns (gel bed dimensions 1.5 x 4.9 cm, NAP-25 columns, Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) previously equilibrated with 0.1 M K-phosphate buffer solution (pH 8.5) containing 5 μM PLP and 5 mM DTT, and the column was eluted with the same buffer solution. The resulting protein fraction, free of low-molecular weight compounds including ACC was collected and used in the enzyme assay. All steps in the enzyme extraction were performed at 0 to 4°C. ACC synthase activity was assayed in a reaction mixture containing 1 ml of 500 μM *S*-adenosylmethionine (AdoMet) and 2 ml of the enzyme preparation. A blank containing no AdoMet was performed concurrently to ensure no contamination of the enzyme preparation with extracted ACC. After incubation of the reaction mixture at 30° C for 30 min., the ACC formed from AdoMet was assayed using the method of Lizada and Yang (1979) based on the chemical degradation of ACC to ethylene. ACC synthase activity was expressed as the amount of ACC (nmol) produced per mg of protein per hour.

2.4 Extraction and assay of ACC oxidase activity

ACC oxidase was extracted as described by Fernandez-Maculet and Yang (1992).

Samples were frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle in the presence of 5% (*w/v*) PVP. The powder was then transferred to centrifuge tubes containing two volumes of an extraction buffer consisting of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.2), 10% (*w/v*) glycerol and 30 mM sodium ascorbate, and the mixture was centrifuged 15,000 *g*, 20 min., 4°C. The resulting supernatant was used as the enzyme source. The supernatant was passed through a membrane filter (Cellulose Nitrate, 0.45 μM: Toyo Roshi Kaisha Ltd., Osaka, Japan) and subjected to chromatography on prepacked Sephadex G-25 columns previously equilibrated with the extraction buffer. The column was eluted with the same buffer. The desalted macromolecular protein fraction was collected and used as the ACC oxidase preparation and for protein estimation. All steps were carried out at 0 - 4°C. ACC oxidase activity was assayed in a reaction mixture containing 1.8 ml of the enzyme preparation, 2 mM ACC (Sigma Chemical Co., St. Louis, MO), and 20 μM $FeSO_4$ in the presence of 5% carbon dioxide as previously described (Mathooko et al. 1993b). The flasks were incubated at 30°C and the ethylene produced in 30 min was determined by withdrawing 1 ml of the headspace gas and the ethylene concentration determined as described above. ACC oxidase activity was expressed as C_2H_4 (nl) produced per mg protein per hour.

2.5 Protein determination

Protein content in the enzyme preparations was determined by the dye-binding method of Bradford (1976) using bovine serum albumin (Sigma Chemical Co.) as a standard.

2.6 Isolation of total RNA

Total RNA was extracted according to the phenol-sodium dodecyl sulphate (SDS) method as described in Sambrook et al. (1989). Samples were either used for extraction immediately after the treatment or were frozen in liquid nitrogen and stored at -80°C until extracted. For samples extracted immediately after the treatment, five grams of tissue was frozen in liquid nitrogen and pulverized to a fine powder in a mortar and pestle. The powder was transferred to sterile polypropylene test tubes and the RNA extracted by adding 7.5 ml of 100 mM Tris-HCl (pH 9.0) containing 100 mM NaCl, 1% (*w/v*) SDS and 9% (*v/v*) 2 - mercaptoethanol as an extraction buffer. To the mixture, an equal volume of 100 mM Tris-HCl (pH 9.0) buffer-saturated phenol, chloroform and isoamyl alcohol (PCIa) (25:24:1 *v/v*) containing 0.5% 8-quinolinol was added and the resulting mixture thoroughly mixed. Phases were separated by centrifugation (2,000 *g*, 20 min., 20°C). The aqueous phase was extracted twice more with PCIa. To the final aqueous phase, one twentieth volume of 5 M NaCl was added and the pH adjusted to 5.0 with acetic acid.

Total nucleic acids were precipitated by adding 2.5 volumes of ice-cold absolute ethanol and allowing the solution to stand at -20°C for at least 20 min. The precipitate was collected by

centrifugation (11,000 *g*, 15 min., 4°C) and the pellet dissolved in 2 ml of 10 mM Tris-HCl and 1mM EDTA (TE) buffer (pH 8.0) containing 0.1 mM DTT and 11 units of human placenta RNAase inhibitor (HPR1). To this solution, 10 M $LiCl_2$, was added to a final concentration of 2 M and the mixture stored at 4°C overnight . After centrifugation (11,000 *g*, 30 min., 4°C), the RNA precipitate was dissolved in 600 μl of TE buffer (pH 8.0) containing 0.1 mM DTT and 11 units of HPR1 and then 30 μl of 5 M NaCl was added to precipitate high molecular weight sugars that might have been extracted with RNA. The mixture was centrifuged (10,000 *g*, 10 min., 4°C). To the supernatant 2.5 volumes of ice-cold absolute ethanol was added and the solution stored at -20°C for at least 30 min. then centrifugation (16,000 *g*, 20 min., 4°C) was carried out. The supernatant was discarded and the RNA precipitate washed once with 300 μl of ice-cold 70% ethanol. After a last centrifugation (16,000 *g*, 5 min., 4°C), the pellet was dried under vacuum and dissolved in 100 μl of sterile distilled water. An aliquot was diluted and scanned with Beckman spectrophotometer (Model DU 640, Beckman Instruments Inc., California, USA) from 200 to 320 nm. The RNA preparation was stored at -80°C until use.

2.7 Preparation of cDNA probe

A *Hind* III fragment from pCMW33, a plasmid clone containing the cDNA (1.8 kb insert) for wound-inducible ACC synthase gene (Nakajima et al. 1990), a generous donation from Prof. H. Imaseki of Nagoya University, Japan was used as probe. The probe DNA (60 μg) in a microcentrifuge tube was denatured by heating for 2 min at 95°C and subsequently cooled on ice. The DNA was labelled using a random primed DNA labeling kit (Boehringer Mannheim GmbH, Germany) which labels DNA using random oligonucleotides as primers (Feiberg and Volgelstein, 1984). The preparations were carried out according to the manufacturer's instructions. To the denatured DNA, the following were added: 3 μl of dATP, dGTP and dTTP mixture (1:1:1), 2 μl of a reaction mixture containing hexanucleotides in 10x concentrated buffer (Boehringer Mannheim GmbH, Germany) and 2 μl of α-[^{32}P]dCTP (Amersham International, England). The volume was made upto 19 μl with sterile distilled water and 1 μl of Klenow enzyme (New England Biolabs, Massachusetts, USA) was added. The solution was mixed and incubated at 37°C for 45 min. To the probe solution, 80 μl of TEN (TEN = 10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 100 mM NaCl) was added and then loaded onto a Sephadex G-50 column previously equilibrated with 100 μl of TEN (pH 8.0) so as to separate radiolabelled DNA from nucleotides. The column in a glass tube was centrifuged (16,000 *g*, 2 min., 25°C). The labelled DNA probe was collected, denatured at 95°C for 7 min and immediately cooled on ice.

2.8 RNA blotting and northern hybridization

Total RNA (20 μg) was denatured at 65°C for 30 min in 10 μl of loading buffer (14% (*v/v*) formaldehyde, 50% (v/v) formamide, 20 mM 3-(N-morpholino)propanesulfonic acid

(MOPS), 1 mM EDTA, 5 mM sodium acetate, pH 7.0). The denatured RNA was separated by electrophoresis on 1.2% agarose gels containing 20x MOPS buffer (1x MOPS buffer is 0.4 M MOPS, 0.1 M sodium acetate, 0.02 M EDTA, pH 7.0) and 0.66 M formaldehyde. The gel was then placed on Whatman 3MM paper supported on Plexglas and both sides dipped into a tank containing 20x SSPE buffer (1x SSPE is 0.15 M sodium chloride, 0.01 M sodium phosphate buffer pH 7.4, and 1 mM EDTA). The gel was then placed in contact with a charged nylon membrane (Hybond N^+, Amersham International, England), and the membrane covered with a Whatmann 3MM paper and a stack of paper towels as described in Sambrook et al. (1989). After 6 - 10 h of blotting, the nylon membrane was peeled from the gel and treated as described in Sambrook et al. (1989).

In prehybridization, the dry membrane was transferred to a heat-sealable bag and then northern hybridization solution containing 5 x Denhardt's reagent [1x Denhardt's reagent is 0.02% (*w/v*) Ficoll-400; 0.02% (*w/v*) Sigma Fraction V BSA], 5x SSPE, 50% deionized formamide, 100 μg/ml denatured fragmented herring sperm DNA and 0.1% SDS and without cDNA probe were added. The bag was sealed and incubated at 42°C for at least 2 h. For hybridization, the prehydridization solution was replaced with fresh hybridization solution. The denatured ^{32}P-labelled cDNA probe coding for a wound-inducible ACC synthase gene was added and the solutions were mixed. The bag was then sealed and hybridized at 42°C overnight. After the hybridization, posthybridization steps were carried out by washing the membranes successively at 60°C for 30 min in 2x, 1x and 0.1x SSPE buffer containing 0.1% (*w/v*) SDS and the amount of radioactivity was checked prior to drying membranes. The dry membranes were exposed to an X-Ray film (Kodak XAR 5) at -80°C for 2 - 5 days to obtain an autoradiographic image. Relative mRNA hybridization for pCMW33 was calculated from computerized images of hybridization signals on imaging plates.

The experiment was repeated several times with similar trends being obtained and only representative data are represented.

3.0 RESULTS AND DISCUSSION

The effects of elevated CO_2 and DACP on the regulation of wound-induced ethylene boisynthesis were compared. It has already been demonstrated that treatment of excised mesocarp tissue of winter squash fruit with elevated CO_2 inhibits wound-induced ethylene production, and the activities of ACC synthase and ACC oxidase (Kubo et al. 1995; Mathooko, 1995; Mathooko et al. 1993a). These previous results were reconfirmed in this study. Elevated $C0_2$ inhibited wound-induced ethylene production (Fig.1), and the activities of ACC synthase and ACC oxidase (Fig. 2). When $C0_2$ was withdrawn, ethylene production and ACC synthase activity increased to almost the same level as in the control. In contrast, withdrawal of $C0_2$ caused a further decrease in ACC oxidase activity.

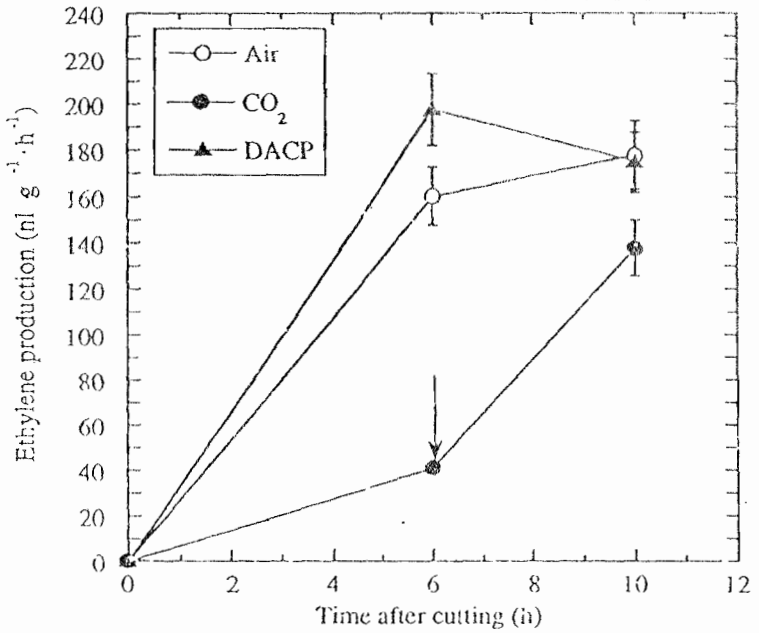

Fig. 1. Time course of changes in ethylene production rates in excised mesocarp tissue of winter squash fruit kept at 25°C. The arrow indicates when samples were transferred from carbon dioxide-enriched atmosphere to air. The vertical bars represent SE of the mean of three replications. When absent, the SE bars fall within the dimensions of the symbols.

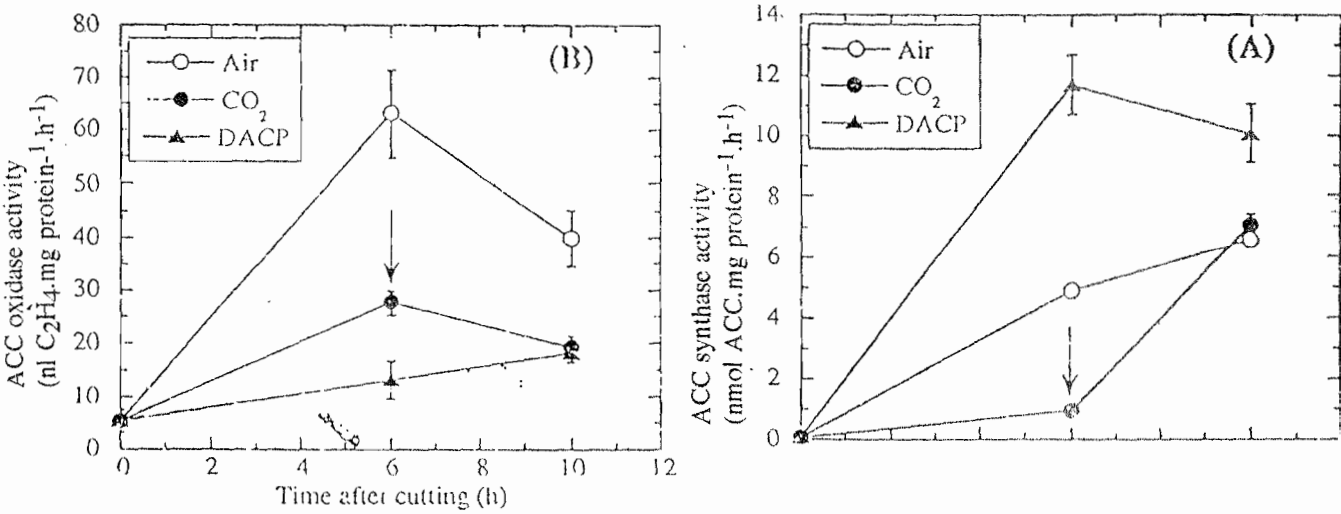

Fig. 2. Time courses of changes in ACC synthase activity (A) and ACC oxidase activity (B) in excised mesocarp tissue of winter squash fruit kept at 25°C. The arrow indicates when samples were transferred from carbon dioxide-enriched atmosphere to air. The vertical bars represent SE of the mean of three replications. When absent, the SE bars fall within the dimensions of the symbols.

Our previous studies indicate that elevated $C0_2$ inhibits ethylene production and the activities of ACC synthase and ACC oxidase (Kubo et al. 1995; Mathooko et al. 1993a) in addition to ACC accumulation (Mathooko, 1995; Mathooko et al. 1993a). DACP, a new inhibitor of ethylene

action (Sisler and Blankenship, 1993) stimulated ethylene production, enhanced and inhibited the activities of ACC synthase and ACC oxidase respectively (Fig. 2). Studies with other inhibitors of ethylene action such as 2,5-norbornadiene (NBD) have indicated that wound-induced ACC synthase is regulated through a negative feedback control mechanism (Hyodo et al. 1993; Mathooko, 1996) since the presence of NBD enhanced wound-induced ACC synthase activity (Hyodo et al. 1993). This hypothesis is well supported by the effects of DACP on wound-induced ACC synthase activity. $C0_2$ is also an inhibitor of ethylene action (Burg and Burg, 1967). Therefore, if $C0_2$ regulates wound-induced ACC synthase by antagonizing the effects of ethylene, it is expected that treatment of wounded tissue with $C0_2$ should enhance ACC synthase activity. Although $C0_2$ and DACP are both inhibitors of ethylene action (Mathooko, 1996) we observed that they had opposite effects on wound-induced ACC synthase activity.

To determine whether the differential effects of $C0_2$ and DACP on ACC synthase activity are at the level of mRNA transcription, total RNA was prepared from the intact fruit, control and treated tissue and subjected to northern blot analysis. A cDNA that encodes the wound-inducible ACC synthase gene, designated pCMW33, which has already been isolated and characterized (Nakajima et al. 1990), was used to probe northern blots of total RNA. Mechanical wounding greatly increased the accumulation of mRNA transcripts for ACC synthase as evidenced by the strong hybridization signal detected by this cDNA probe (Fig. 3) [see figure next page]. Treatment of the excised tissue with DACP further enhanced the accumulation of ACC synthase mRNA transcripts while $C0_2$ treatment inhibited, though not completely the increase in ACC synthase mRNA transcripts. No hybridization signal was detected for RNA from intact fruit. When $C0_2$ was withdrawn, the ACC synthase mRNA transcript accumulation increased and was slightly higher than the control. This increase was mirrored in the increase in the ACC synthase activity.

Complementary DNA clones for wound-inducible ACC synthase in various tissues have been isolated (Kende, 1993). Northern blot analysis of RNAs from wounded winter squash fruit (Li et al. 1992; Lincoln et al. 1993; Mattoo et al. 1993; Olso.. et al. 1991) treated with the respective cDNAs as probes have shown that similar to the ACC synthase activity, the mRNAs are not detected in fresh tissue but increase considerably after wounding. Consistent with these observations no hybridization signal to RNA from intact tissue was detected (Fig. 3). The observation that the accumulation of wound-induced ACC synthase mRNA transcripts was enhanced by DACP treatment agrees well with the physiological evidence of the autoinhibitory effect of ethylene on the accumulation of wound-induced ACC synthase mRNA transcripts. Although we did not examine the effects of exogenous ethylene on the levels of wound-induced ACC synthase mRNA transcripts, Nakajima et al. (1990) reported that exogenous ethylene suppresses the expression of wound-induced ACC synthase gene while NBD promoted its expression.

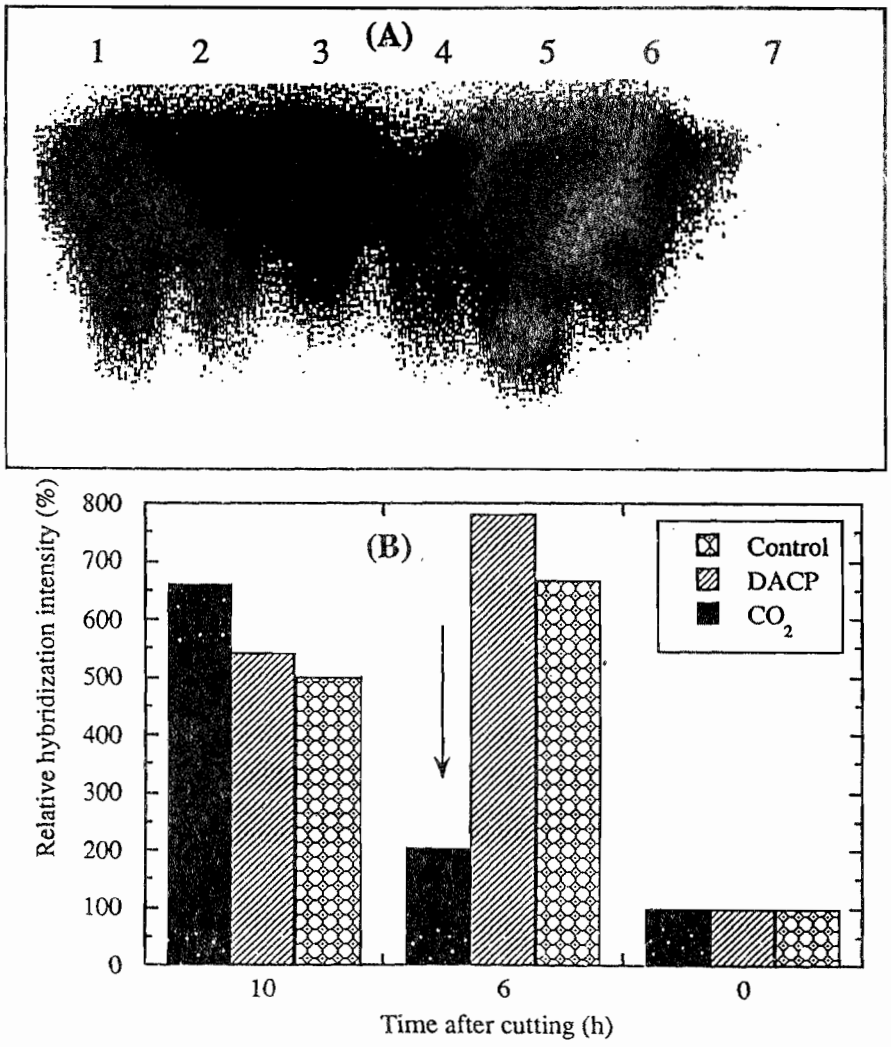

Fig. 3. Time course of appearance of mRNA encoding ACC synthase in excised mesocarp tissue of winter squash fruit after wound induction and storage at 25°C. **A**: Total RNA from intact fruit (lane 7) and from excised tissue treated with air (lane 6), DACP (lane 5) or carbon dioxide (lane 4) for 6 h or treated with air (lane 3), DACP (lane 2) for 10 h or 4 h after transfer from carbon dioxide-enriched atmosphere to air (lane 1; arrow). **B**: Quantification of relative mRNA hybridization for pCWM33 during treatment in **A**.

This observation and that made in this study using DACP suggest that the wound- induced ACC synthase gene expression is regulated by a negative feedback control system and imply that the ethylene produced in response to wounding may inhibit ACC synthase activity and its gene expression.

Elevated levels of $C0_2$ have also been shown to inhibit ethylene action, presumably by interfering with ethylene from binding to its receptor site (Burg and Burg, 1967). Our results show that DACP and $C0_2$ have opposite effects on ACC synthase activity and its gene expression. This suggests that elevated CO_2 effect on the regulation of wound-induced ACC synthase activity and

gene expression could be through another yet unknown mechanism other than inhibition of ethylene binding. Mattoo et al. (1993) reported that the accumulation of a 1.8 kb wound-induced ACC synthase transcript is ethylene inducible. This is because the presence of NBD inhibited wound-inducible ethylene production, ACC synthase activity and accumulation of its transcripts. These workers concluded that NBD might inactivate the ethylene receptor site. Whether $C0_2$ functions in a similar manner remains to be elucidated. In some situations such as cocklebur seed germination (Ishizawa et al. 1988) where ethylene has stimulatory rather than inhibitory effects, $C0_2$ enhances rather than counteracts ethylene action. Indeed, Sisler and Wood (1988) stated that $C0_2$ may act synergistically with ethylene rather than counteract its effects. Moreover, unlike other ethylene action inhibitors, $C0_2$ is a product of the oxidative deamination of ACC to ethylene and $C0_2$ may, therefore, act through a mass action effect in the regulation of wound-induced ACC synthase activity and/or its gene expression (Mathooko, 1996). In the absence of conclusive data showing that $C0_2$ alters ethylene binding, it is possible that $C0_2$ effect on wound-induced ACC synthase activity and/or gene expression is not modulated via ethylene receptor(s).

Both $C0_2$ and DACP inhibited wound-induced ACC oxidase activity consistent with our previous observations (Kubo et al. 1995; Mathooko, 1995; Mathooko et al. 1993a). The effect of elevated $C0_2$ on ACC oxidase activity was contrary to the highly held hypotheses that $C0_2$ stimulates wound-induced ACC oxidase activity (Yang and Hoffman, 1984). It has, however, been indicated that $C0_2$ regulates wound-induced ACC oxidase activity in a commodity specific manner although its mode of action is still not yet known (Mathooko, 1996; Mathooko et al. 1993a). Since there are at least three forms of ACC oxidase including *eth1, eth2 and eth3* from tomato (Bouzayen et al. 1993) which could probably be induced and/or synthesized in different tissues and at different developmental stages, it is quite possible that $C0_2$ can affect each of these forms of ACC oxidase differently (Mathooko, 1996). It has been shown that exogenous ethylene promotes ACC oxidase activity in excised mesocarp tissue of winter squash fruit (Hyodo et al. 1993) among other tissues as well as its mRNA transcripts (Kim and Yang, 1994). This indicates that the conversion of ACC to ethylene is controlled by a positive feedback system. The increase in ACC oxidase activity caused by exogenous ethylene in excised winter squash fruit (Hyodo et al. 1993) was effectively inhibited by NBD. NBD also inhibited ACC oxidase activity and the accumulation of its mRNA transcripts in mung bean hypocotyl (Kim and Yang, 1994) and, therefore, DACP and $C0_2$ may act in similar manner in inhibiting ACC oxidase activity. Hyodo et al. (1993) indicated that the ethylene produced in response to wounding may regulate the rate of ethylene production by suppressing and enhancing the activities of ACC synthase and ACC oxidase respectively. At the moment it is not clear whether or not, unlike ACC synthase, $C0_2$ regulates ACC oxidase by inhibiting ethylene action or through another mechanism. Studies on the expression of wound-induced ACC oxidase gene should further advance our understanding of

the regulation by DACP and $C0_2$ of wound-induced ethylene biosynthesis. The recovery of ethylene production, ACC synthase activity and its mRNA transcripts accumulation upon withdrawal of $C0_2$ indicates that $C0_2$ suppresses the messenger coding for ACC synthase and this messenger is immediately activated upon withdrawal of the gas. This further indicates that the effects of $C0_2$ on wound-induced ethylene biosynthesis is not likely the result of general toxicity but tissue response to the treatment. Since following withdrawal of $C0_2$ ACC oxidase activity declined slightly while ethylene production increased, it is possible, therefore, that ACC oxidase is not a limiting enzyme in wound-induced ethylene biosynthesis.

The DACP results presented in this study support the view that wound-induced ACC synthase and ACC oxidase in winter squash fruit are regulated by negative and positive feedback control systems, respectively. This differs with the situation found in fruit ripening and flower senescence in which endogenous ethylene is known to accelerate the induction of both ACC synthase and ACC oxidase. Results from the $C0_2$ experiment indicate that ACC oxidase is not a limiting enzyme in wound-induced ethylene biosynthesis and $C0_2$ does not regulate wound-induced ACC synthase by antagonizing ethylene action. The results further indicate that $C0_2$ inhibits wound-induced ethylene production by suppressing ACC synthase activity and suggest that this inhibition is due to suppression of ACC synthase gene expression at the transcriptional level. Based on the information from this study and our previous studies (Kubo et al. 1995) that the magnitude and the levels of ACC synthase transcripts were always in parallel with those of the enzyme activity, we may conclude that ACC synthase gene after wounding and treatment with $C0_2$ and DACP is solely subject to transcriptional control and not other factors such as posttranscriptional/translational control and/or enzyme turnover. The modulation of wound-induced ethylene biosynthesis and ACC synthase mRNA transcripts, however, requires continuous presence of $C0_2$.

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