



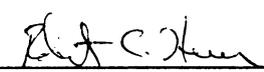
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THESIS



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WOUND ETHYLENE PRODUCTION BY FRUIT PERICARP  
TISSUE OF RIN MUTANT TOMATO  
(LYCOPERSICON ESCULENTUM MILL.)

presented by  
Saichol Ketsa

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of the requirements for  
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WOUND ETHYLENE PRODUCTION BY FRUIT PERICARP  
TISSUE OF RIN MUTANT TOMATO  
(LYCOPERSICON ESCULENTUM MILL.)

By

Saichol Ketsa

A DISSERTATION

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1980

ABSTRACT

WOUND ETHYLENE PRODUCTION BY FRUIT PERICARP  
TISSUE OF RIN MUTANT TOMATO  
(LYCOPERSICON ESCULENTUM MILL.)

By

Saichol Ketsa

Ethylene production by wounded fruit pericarp tissue from rin mutant tomatoes was studied to determine whether the pathway of ethylene biogenesis was similar to that found in ripening fruit. The rin mutant was used to avoid the induction of ethylene as a result of ripening which would occur in normal fruit. The pattern of ethylene production by freshly cut disks of immature- and mature-green fruits was determined. The rate of ethylene production by disks from immature-green fruits was higher than that of disks from mature-green fruits. The rate of wound ethylene production was found to be directly proportional to the cut surface area of disks. Since cycloheximide inhibited ethylene production resulting from rewounding disks it appears that ethylene production is the result of increased enzyme activity rather than the facilitation of gas diffusion.

Disks with intact epidermis produced more wound ethylene than that of disks without epidermis suggesting that the compact cells close to the epidermis may be the site of the ethylene-forming enzyme(s). When 1-aminocyclopropane-1-carboxylic acid (ACC) was applied to disks from

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immature-green fruits much more ethylene was produced than by disks from mature-green fruits. Disks with epidermis produced more ethylene than those without epidermis in response to ACC application.

Rhizobitoxine, cycloleucine and cycloheximide inhibited ethylene production by disks and decreased the amount of ACC in the disks. Cycloheximide prevented ACC application from stimulating ethylene production by tomato disks but rhizobitoxine and cycloleucine did not.

Anaerobiosis completely stopped ethylene production by disks and prevented ACC from stimulating ethylene production. Anaerobiosis also resulted in increased ACC levels in the tomato disks.

Ethylene produced by tomato disks which accumulates in closed containers and propylene both were shown to inhibit ethylene production by the disks. Propylene also caused an accumulation in ACC contained in the disks compared to non-treated controls. This suggests that ethylene and/or propylene inhibit ethylene production through a negative feedback of the conversion of ACC to ethylene.

When carbon dioxide given off by tomato disks was removed by potassium hydroxide ethylene production was reduced. Propylene inhibited ethylene production much more effectively when carbon dioxide was removed by potassium hydroxide. This suggests that endogenous carbon dioxide counteracts the negative feedback of ethylene. On the other hand, applied carbon dioxide was shown to inhibit wound ethylene production. Applied carbon dioxide also inhibited methionine and S-adenosylmethionine stimulation of ethylene production but had no effect on ACC stimulation. This suggests that applied carbon dioxide inhibits ethylene production by disks at step where S-adenosylmethionine is converted to ACC.

The results reported herein strongly suggest that wound ethylene production by tomato disks proceeds through the pathway methionine to S-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid to ethylene.

## DEDICATION

This dissertation is dedicated to the memory of my Uncle, to my Father and to my Mother who have given love and moral patient support during my academic career.

## ACKNOWLEDGMENTS

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## INTRODUCTION

Ethylene is one of the simplest unsaturated carbon compound. It is a gas under physiological conditions of temperature and pressure, and it exerts a major influence on many aspects of plant growth, development and senescence (3,28,85,115). Its role in fruit ripening appears to be particularly important (30,98,126,127). Ethylene is considered to be a plant hormone because it is a natural product of metabolism and acts in trace amounts (29,127).

The general occurrence of ethylene as a plant emanation was recognized from the time it was positively identified (43). One of the earliest observations was that auxin applications can greatly stimulate ethylene production by plants (166). Since ethylene was accepted as a plant hormone more research has been published on ethylene biosynthesis than any other part of ethylene physiology (85,158). Yet, there is much that is not clear and there is considerable disagreement among research workers about the biosynthetic pathway, site of production and mode action of ethylene. However, it has been universally accepted that methionine is a precursor of ethylene in higher plants. Recently, S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) have been found to be intermediates between methionine and ethylene and the chemical reaction(s) that converts ACC to ethylene has been shown to be completely dependent on oxygen (6).



Ethylene production rates are very susceptible to environmental, chemical and physical stress factors (160). Many plant tissues which normally evolve little or no ethylene show a surge in ethylene production two to ten times higher than the basal levels upon physical wounding (23,40,56,58,62,63,64,67,93,96,110,121,132,136,137,165), physical stress without wounding (18,51,84,130,131,140), chilling (39,155), freezing (47), contact with noxious chemicals (1,7,21,22,120,124,150), irradiation (83,105), attack by microorganisms (66,114,124), and water stress (8,44,53).

Ethylene produced by plants under such conditions is usually referred to as "stress ethylene". "Wound ethylene" is another term used for stress ethylene which has widely been used by most researchers dealing with stress ethylene caused particularly by means of cutting, bruising, or dropping. Even though stress ethylene has widely been studied in both vegetative and reproductive tissue of plants since sensitive analytical techniques were developed (35), the mechanism of stress-induced ethylene increase is still obscure. A better understanding of the nature of mechanical stress-induced ethylene biosynthesis could lead to more information of ethylene biosynthesis in both wounded tissue and ripening fruit.

This thesis reports studies on wound-induced ethylene biosynthesis by fruit pericarp tissue of rin mutant tomato fruit. Hopefully, some of these findings will be useful to investigate stress-induced ethylene biosynthesis in the future.

Specifically, the studies were undertaken to investigate the following points:



1. induction of wound ethylene production by fruit pericarp tissue of rin tomato,
2. wound ethylene production in response to applied ACC,
3. mechanism of a negative feedback of wound ethylene production,
4. mode of action of carbon dioxide in wound ethylene production,  
and
5. ACC formation in wounded tissue.



## LITERATURE REVIEW

### WOUND-INDUCED ETHYLENE SYNTHESIS

In many tissues, a burst of ethylene production is evident after cutting, bruising or dropping. In some tissues this burst may last for 2 to 10 h or even up to 40 h (164). In tomato fruit the stimulation of ethylene production by cutting (40,83,110), bruising (96), or dropping (23) may be very large. According to Meigh et al. (110), pericarp and wall tissues from a tomato fruit which was cut into 16 segments were accelerated thirteen-fold in their ethylene production during a 4 to 6 h period, small cubes of tissue were increased twenty-five-fold in their ethylene production during the same time interval and the rate in homogenates was reduced by approximately 50%. McGlasson and Pratt (109) found that muskmelon fruit slices had higher rates of respiration and ethylene production than comparable intact fruit. They suggested that the stimulated production of ethylene may account for at least part of the observed physiological differences between tissue slices and whole fruit. They further pointed out that wounding apparently overcomes whatever factor limits ethylene production in intact (younger) fruits. McGlasson (106) studied ethylene production by slices of green banana fruit which produces a barely detectable amount of ethylene ( $50 \text{ nl kg}^{-1} \text{ h}^{-1}$ ) while intact. The initial rates, measured about 2 h after cutting, were several times higher than the rate in intact fruit.



A second rise in the rate of ethylene production was detected 4 h after cutting, and the maximum rate was reached within 6 to 8 h.

Wounding of figs during the sixteenth to twenty-second day of syconium development induced a fifty-fold increase in the rate of ethylene emanation within the first hour as compared to non-wounded fruit (164). Meigh et al. (110) noted a 20% increase in the ethylene production of apples which had been cut into segments. Loughheed and Franklin (93) showed that bruising substantially increased ethylene production in freshly harvested preclimacteric apples. Dropping grapefruit from heights up to 2 m increased both respiration and ethylene production in fruits that normally produce little ethylene (151). Ethylene production, respiration and rate of ripening were all increased by bruising mature-green tomato fruits (96); the response was proportional to the number of impacts.

Ben-Yehoshua and Eaks (17) observed that the rate of ethylene production by orange fruit was approximately double when incisions were made in the skin. Rivov et al. (129) showed a rapid increase in the rate of ethylene production from disks following excision from grapefruit peel. Hyodo (62) found that when young citrus fruits were cut into small pieces, a large quantity of ethylene was evolved during the course of incubation. The smaller the segments the faster the rise in the rate. Flavedo, albedo and pulp tissue separated from the intact fruit also produced ethylene in large amounts. Bell pepper fruit is non-climacteric. Wounding excised plugs of pepper ovary wall tissue caused an increase in carbon dioxide production within one day, and an increase in ethylene by the second day (136).



Saltveit and Dilley (137) reported that a rapidly induced, transitory increase in the rate of ethylene synthesis occurred in wounded tissue excised from actively growing regions of etiolated barley, cucumber, maize, oat, pea, tomato, and wheat seedlings. Cutting intact stems or excising 9 mm segments of tissue from near the apex of 1-day-old etiolated Pisum sativum L., cv. Alaska seedlings induced a remarkably consistent pattern of ethylene production. Hanson and Kende (56) studied the biosynthesis of wound ethylene in morning-glory flower tissue. The immature segments were cut from buds two days before flower opening, floated overnight on 5 mm KCl solution, and transferred to agar the following morning. These immature segments evolved only a small quantity of ethylene during incubation on agar with most of the ethylene production occurring in the morning. When such segments were wounded mechanically early in the afternoon, the rate of ethylene production rose more than ten-fold within 1 h and returned to a low rate after 3 h.

Cooper (38) observed that mechanical wounding of woody stem tissues of citrus also caused a large increase in ethylene production. When the bark was peeled from the wood of "Robinson" tangerine terminal shoots, five to ten times as much ethylene was produced in the peeled bark and wood as in the intact stems. Slitting the stems into two pieces, or macerating the stems increased the rate of ethylene production more than ten times.

Jackson and Osborne (71) reported that cutting 2 cm petiole segments of bean leaves (Phaseolus vulgaris L., cv. Canadian Wonder) into smaller pieces stimulated ethylene production in proportion to the number of cut surfaces. Imaseki et al. (65) reported that cutting



etiolated rice (Oryza sativa L., cv. Aichi-Asahi) coleoptiles into smaller segments stimulated ethylene production, but that the increase was not proportional to the number of cut surfaces. Increasing the cut surface area of sweet potato roots (Ipomoea batatas L., cv. Norin 1) stimulated ethylene production in proportion to the logarithm of the cut surface area (67).

### BIOSYNTHESIS OF ETHYLENE

Most of the work on the mechanism of ethylene biosynthesis in higher plants has been carried out with ripening fruit tissues which produce large amounts of ethylene. Although cut tissues were used in these studies, there is no indication that the biosynthetic pathway might differ from that of intact fruit since stress-induced ethylene production was of minor significance when compared to the massive ethylene output of the ripening fruit (85,160).

#### Precursor

It is now fairly well established that methionine is the precursor of ethylene in higher plants (5,6). Methionine as a possible precursor of ethylene was suggested first by Lieberman and Mapson (91). In a number of tracer experiments with fruit tissue of apple (5,6,16,33,89, 122,159), avocado (10,16,92), tomato (11,19), cantaloupe (160), citrus (63,64), sweet potato roots (74), pea seedlings (72,141), bean and tobacco leaves (1), mung bean hypocotyls (134,163), flowers of morning-glory (56,57) and Tradescantia (148),  $^{14}\text{C}$ -methionine was converted to  $^{14}\text{C}_2\text{H}_4$ . Labeled ethylene was produced by the tissue only when carbons 3 and 4 of methionine were labeled (33,57,63,89). The L-form of methionine is preferentially converted to ethylene by the tissue (14).



In the conversion of methionine, carbon 1 forms carbon dioxide (33), carbon 2 forms formic acid (143), carbons 3 and 4 form ethylene (56,89), and  $\text{CH}_3\text{S-}$  is retained in the tissue (14,15). The fate of the sulfur atom is of some significance since Yang and Baur (159) determined that the levels of free methionine and protein methionine are too low to provide all the ethylene evolved by apple fruit. Climacteric apple fruit contains about  $60 \text{ mmoles g}^{-1}$  of free methionine plus protein methionine and produces ethylene at an average rate of  $4 \text{ mmoles g}^{-1} \text{ h}^{-1}$ . Therefore, they suggested that at this rate, methionine must be recycled to provide sufficient substrate for continued ethylene production. Recently Adams and Yang (5) confirmed that the sulfur atom, indeed, is recycled to form methionine.

It does appear that most wounded-induced ethylene production is derived from methionine perhaps in the same biochemical pathway that forms basal ethylene during natural physiological synthesis. Abeles and Abeles (1) showed that ethylene production from bean and tobacco leaves increased rapidly following the application of toxic compounds such as  $\text{CuSO}_4$ , Endothal or ozone. The compounds which increased ethylene evolution also increased the conversion of methionine into ethylene. In these experiments, however, the conversion efficiency (specific radioactivity of  $^{14}\text{C-}$  ethylene produced/specific radioactivity of  $^{14}\text{C-}$  methionine administered) was below 1% for unstressed plants, and further decreased (50%) after chemical stress. An extensive dilution of labeled methionine by endogenous methionine must have occurred. If all of the ethylene evolved was derived from methionine, more unlabeled endogenous methionine must have become available as the rate of ethylene production increased following the chemical injury.



Hyodo (64) reported that albedo disks prepared from mandarin fruit produced ethylene at an increasing rate until a maximum (about  $10 \text{ nl g}^{-1} \text{ h}^{-1}$ ) was reached after incubation for 30 h. The ability of albedo disk to convert labeled methionine to ethylene paralleled the rate of endogenous ethylene production. No measurable conversion of labeled methionine into ethylene was found in freshly prepared disks, but aged disks actively converted methionine.

Hanson and Kende (56) presented convincing evidence implicating methionine as the precursor for wound ethylene production from rib segments excised from flower buds of morning-glory flowers (Ipomea tricolor). Such immature segments evolved only a small quantity of ethylene, but upon wounding the rate of ethylene production rose more than ten-fold within 1 h and returned to a low rate after 3 h. A rhizobitoxine analog, aminoethoxyvinylglycine (AVG) completely inhibited ethylene production in both control and wounded tissue, clearly implicating methionine as the precursor of wound-induced ethylene.

Kato and Uritani (74) reported that in diseased tissue of sweet potato, methionine is one of the precursors of ethylene and located at a position closer to ethylene in the biosynthetic pathway than acetate. The methionine system is neither the main pathway of ethylene production in diseased tissue nor involved entirely in the acetate system. They (74) therefore concluded that at least three different ethylene producing systems exist; they are the methionine system, acetate system and pathway "B" in which ethylene originates from substance(s) existing in the tissue which is not easily produced from methionine, glucose or acetate.



### Intermediates in the Pathway from Methionine to Ethylene

Methionine can readily be degraded to ethylene in the in vivo conversion of methionine to ethylene. Burg and Claggett (33) first found that when apple tissue was exposed to L-methionine-<sup>35</sup>S, <sup>35</sup>S was recovered mainly in cationic components such as methionine and S-adenosylmethionine (SAM). Work by Murr and Yang (116) indicated that SAM is an intermediate in the conversion of methionine to ethylene. The evidence for SAM as an intermediate of ethylene biosynthesis was obtained when [methyl-<sup>14</sup>C], or [U-<sup>14</sup>C] methionine was fed to apple plugs, and a radioactive compound was identified as SAM (5). Boller et al. (19) confirmed that SAM is an intermediate of the ethylene biosynthetic pathway. They (19) extracted an enzyme from wild-type tomato fruit which could convert SAM to ACC.

It has long been known that a nitrogen atmosphere reduces and ultimately causes a cessation in ethylene production of pears (55), apples (4), tomatoes (40,97), and bananas (100). Saltveit and Dilley (138) reported that nitrogen atmosphere stopped wound ethylene production. Upon introduction of oxygen to the anaerobic fruit or excised plant tissue, there is an overshoot in ethylene production for a short time period and then a return to basal levels. It was suggested that under the anaerobic condition an intermediate compound was built up which had to react with oxygen to produce ethylene (26). With apple tissue slices, ethylene production and respiration were both inhibited 50% when the partial pressure of oxygen was reduced to 2.5%, whereas at an oxygen tension above 8% there was scarcely any retardation of either process (27). Burg (26) pointed out that if the oxygen requirement is based on the necessity for a supply of high-energy compounds,



anaerobiosis should not totally stop the synthesis of ethylene, but only reduce the rate, since in a tissue such as apple, which shows a well-marked Pasteur effect, some high-energy compounds will be generated even under anaerobic conditions.

Adams and Yang (6) studied the metabolism of  $^{14}\text{C}$ -methionine in apple plugs held in nitrogen and in air. At the end of a 6 h incubation period the plugs were frozen and extracted in 80% ethanol. The plugs in nitrogen atmosphere contained a radioactive compound ("X") which when fed to apple plugs in air produced radioactive ethylene. When [ $^{14}\text{C}$ -methyl] methionine was used in this type of experiment no compound "X" was observed, but when labeled methylthioribose was used, the "X" compound was formed. The "X" compound was identified as ACC. The evidence that an intermediate of ethylene biosynthesis is formed under anaerobiosis is supported by Bradford and Yang (20). They (20) found that ACC levels in xylem sap collected from detopped tomato plants (Lycopersicon esculentum Mill., cv. VFN 8) under the condition of waterlogging was greater than 2  $\mu\text{M}$  but essentially nil in that of controls. The appearance of ACC in the sap preceded the onset of epinasty and was correlated with the elevated rate of ethylene production in flooded plants. They (2) also found similar results obtained with the ethylene-requiring diageotropica (dgt) tomato mutant.

Recently, Boller et al. (19), Suttle (148), Lurssen and Naumann (94), Lurssen et al. (95) and Lizada et al. (92) have confirmed that ACC, indeed, is an intermediate of ethylene biosynthesis in higher plants.

After Adams and Yang (6) elucidated the metabolic role of ACC in ethylene biosynthesis based on the observation that ACC accumulated



under anaerobiosis, they demonstrated the following pathway for ethylene biosynthesis in apple tissue : methionine  $\rightarrow$  SAM  $\rightarrow$  ACC  $\rightarrow$  ethylene.

### Enzymes and Cofactors

Frenkel et al. (49) first showed that ethylene synthesis was blocked by treatment with cycloheximide at the early-climacteric stage of pear fruit without effect on the respiratory climacteric. It was suggested that ethylene synthesis is dependent upon protein synthesis. Lieberman and Kunishi (88) reported that cycloheximide inhibited ethylene production in etiolated pea seedlings and apple tissue. Abeles (2) reported that the stimulation of ethylene production from seedling tissue of Phaseolus vulgaris, Helianthus annuus and Zea mays by auxin was inhibited by protein synthesis inhibitors (actinomycin D and puromycin D). Since the induction of ethylene synthesis by an auxin occurs after a lag of 30 to 60 min, based on protein synthesis inhibitor studies (27) auxin action on enhancement of ethylene production must involve de novo synthesis of requisite enzymes (73,133).

Abeles and Abeles (1) reported that cycloheximide inhibited the production of chemical stress-induced ethylene. Hyodo (62) also found that ethylene production by the excised segments of albedo tissue from the Satsuma mandarin (Citrus unshiu Marc.) fruit was markedly prevented by the addition of cycloheximide. He (64) later showed that incorporation of label into ethylene from L-[3-<sup>14</sup>C] methionine was strongly inhibited by cycloheximide but not actinomycin D. Cycloheximide also inhibited the conversion of labeled methionine into ethylene in freshly cut cantaloupe plugs (160). Saltveit and Dilley (139) reported that D-chloramphenicol, actinomycin D, and cycloheximide inhibited wound ethylene synthesis in excised sections of etiolated pea seedlings.



A number of ethylene-forming model systems were developed and these led to the proposals of enzymes for ethylene biosynthesis in vivo (87, 97). Although enzymatic formation of ethylene from methional was demonstrated with extracts of cauliflower florets (99,101,102) and pea seedlings (80), some investigations failed to show enzymatic formation of ethylene by extracts (86). It was assumed that the ethylene-producing mechanism was so labile that ethylene production by fruit tissue ceased when the cells were disintegrated. Yang showed that ethylene was formed rapidly from methional (156) or from  $\alpha$ -keto- $\gamma$ -methylthiobutyrate (157) by horseradish peroxidase in the presence of  $Mn^{++}$ , sulfite, oxygen and certain phenols. Ku et al. (81) found that cell-free extracts prepared from tomato fruits (Lycopersicon esculentum Mill.) were not capable of catalyzing ethylene production from  $\alpha$ -keto- $\gamma$ -methylthiobutyrate, in the presence of  $Mn^{++}$ , sulfite, and phenol, until an endogenous heat-stable and dializable inhibitor was removed. After its removal, the enzyme catalyzed formation of ethylene was readily demonstrated. However, neither methional or  $\alpha$ -keto- $\gamma$ -methylthiobutyrate are intermediates of methionine conversion to ethylene in vivo in higher plants (87). Therefore, these enzymes are definitely not involved in ethylene biosynthesis of higher plants.

Konze and Kende (77) extracted methionine adenosyltransferase (ATP : methionine S-adenosyltransferase, EC 2.5.1.6) from the rib segments of flower buds of morning-glory plants (Ipomoea tricolor Cav., cv. Heavenly Blue). This enzyme activates methionine in the presence of ATP and forms SAM which is an intermediate of the ethylene biosynthetic pathway (5). Recently, Boller et al. (19) developed an assay for the ACC-forming enzyme from tomato fruit. A soluble enzyme from tomato



fruit converted SAM to ACC. The enzyme has a  $K_m$  of 13  $\mu\text{M}$  for SAM and conversion of SAM to ACC was competitively and reversibly inhibited by the rhizobitoxine analog, aminoethoxyvinylglycine (AVG). The level of the ACC-forming enzyme activity was positively correlated with the content of ACC and the rate of ethylene formation in wild-type tomatoes at different developmental stages.

Inhibitor studies with 2,4-dinitrophenol and other compounds which block the formation of ATP during respiration showed that oxidative phosphorylation metabolism is required for ethylene biosynthesis (34,82, 116,142,146). Murr and Yang (116) reported that when 2, 4-dinitrophenol was fed to apple tissue, it inhibited the conversion of radioactive methionine to ethylene by 50% at a concentration of 60  $\mu\text{M}$  and by 90% at a concentration of 100  $\mu\text{M}$ . Though Yu et al. (164) have recently reported that 2, 4-dinitrophenol at low concentrations (below 50  $\mu\text{M}$ ) exerted its effect primarily on the conversion of ACC to ethylene and the enzymatic formation of ethylene from ACC has been reported by Konze and Kende (78), many of the reported characteristics of the enzyme(s) did not seem to require ATP. However, Adams and Yang (5) confirmed that ATP is indeed required for SAM formation.

Ethylene biosynthesis from methionine appears to be sensitive to rhizobitoxine and its analogs (90,122). It was suggested that a pyridoxal phosphate-linked enzyme is involved in ethylene biosynthesis since rhizobitoxine has been shown to irreversibly inhibit pyridoxal phosphate-linked enzymes (50). Rhizobitoxine and its analog inhibited the conversion of methionine to ethylene in fruit tissue of apple (6,11, 13,90,122), banana (11), tomato (11,19), pear (154), avocado (10,94), snapdragon flower (153), pea seedling (139), sorghum seedling (122),



corolla rib segments of morning-glory flower (56,57), carnation flower (12), broccoli (152), and petals of Tradescantia flower (148).

Canaline ( $\alpha$ -amino- $\gamma$ -aminoxybutyric acid) which is known to inhibit pyridoxal phosphate-dependent enzymes (128) also inhibited the conversion of methionine to ethylene in the fruit tissue of apple (116), citrus (64) and tobacco callus (61). Therefore, it is believed that a pyridoxal phosphate-linked enzyme is involved in ethylene biosynthesis. Furthermore, Konze and Elstner (76) showed that pyridoxal phosphate is indeed involved in ethylene biosynthesis. They (76) found that the rate of ethylene formation from methionine in the presence of illuminated chloroplasts and ferridoxin was greatly enhanced by the addition of pyridoxal phosphate. Ethylene formation from methionine in the presence of pyridoxal phosphate was inhibited by canaline. Recently, Yu et al. (161) confirmed that ACC synthase is a pyridoxal enzyme. The biochemical role of pyridoxal phosphate is catalyzing the formation of ACC by  $\alpha,\gamma$ -elimination of SAM.

Lieberman et al. (89) reported that metal chelates such as ethylenediamine tetraacetic acid (EDTA), sodium diethyl dithiocarbamate and cuprizone inhibited ethylene production in apple fruit tissue. Shimokama and Kasia (142) also reported that the production of ethylene from apple slices was inhibited by EDTA. Konze and Kenda (78) reported that EDTA inhibited the conversion of ACC to ethylene by homogenates of etiolated pea seedling. They, therefore, suggested the involvement of metal cofactors in ethylene biosynthesis.

#### Role of Auxin

The initial discovery that auxin regulated ethylene production was made in 1935 by Crocker, Hitchcock, and Zimmerman (41). They observed



that IAA and NAA (indoleacetic and naphthaleneacetic acids) produced effects on plants similar to those caused by ethylene, including epinasty, inhibition of growth, root induction, tissue swelling, and anesthesia of Mimosa pudica pulvinal tissue. Hansen (54) presented direct chemical evidence that 2,4-D (2,4-dichlorophenoxyacetic acid) accelerated the rate of ethylene production of immature and mature pear fruits. IAA and 2,4-D were also found to stimulate ethylene production in banana fruit.

The apical tissue of seedlings is thought to be an important site of auxin synthesis. The hormone is translocated from the apex of the stem tissue below where it promotes cell elongation. A number of investigators have shown that ethylene production in seedlings is greatest in the apex or immediately below it (31,32). Removal of the apex by excision or by ringing the stem with an inhibitor of auxin transport (TIBA), reduced the rate of ethylene production in subtending tissues. It was possible to restore the original rate of ethylene production by applying IAA to the decapitated stem tissue (4).

Kang et al. (73) reported that IAA caused a rapid increase in ethylene production from apical and subapical portions of pea seedlings followed by a gradual decline and return to pretreatment levels after 24 h. The increase in ethylene production induced by auxin is due to an increase in enzymes associated with the methionine pathway. Studies with inhibitors have shown that the increase in ethylene production induced by auxin can be blocked by the addition of inhibitors of RNA and protein synthesis (1,2,27,133). Burg and Claggett (33) demonstrated that the methionine pathway operates only when pea sections were treated



with auxin. Untreated sections did not convert methionine into ethylene while auxin-treated ones did.

Yu et al. (162) proposed that auxin stimulates ethylene production by inducing the synthesis of an enzyme involved in the conversion of SAM to ACC because the rhizobitoxine analog, AVG, inhibited ethylene production stimulated by IAA. Jones and Kende (72) reported that levels of endogenous ACC paralleled the increase in ethylene production in sub-apical stem sections of etiolated pea (cv. Alaska) seedlings treated with different concentrations of IAA. The IAA-induced formation of both ACC and ethylene was blocked by AVG. Labelling studies with L-{U-<sup>14</sup>C} methionine showed an increase in the labelling of ethylene and ACC after treatment with IAA. IAA had no specific effect on the incorporation of label into SAM or homoserine. The specific radioactivity of carbon atoms 2 and 3 of ACC increased after treatment with IAA, indicating that all of the ethylene was derived from ACC. The activity of the ACC-forming enzyme was higher in sections incubated with IAA than in sections incubated with water alone. Recently Yu et al. (163) obtained similar results from mung bean hypocotyls treated with IAA.

Trauma-induced ethylene production, whether resulting from physical, chemical, or biological stress does not appear to be directly associated with normal auxin-mediated ethylene production exhibited by young developing plant parts (160). Abeles and Abeles (1) reported that nonhormonal stress-inducing chemicals, such as CuSO<sub>4</sub> and Endothol, speed up the conversion of methionine into ethylene. In addition, cyclohexamide inhibited the increased rate of ethylene production induced by these chemicals. The mutant diageotropica of tomato required exogenous ethylene for normal growth and development. High concentration of IAA



application can also normalize the mutant diageotropica (167,168). However, there is no difference in the rate of ethylene production by segments of petiole or stem apex between mutant diageotropica and non-mutant, upright tomato plants (69). Bradford and Yang (20) reported that ACC levels greater than 2  $\mu\text{M}$  were found in the xylem sap of flooded tomato plants (Lycopersicon esculentum Mill., cv. VNF 8) but essentially nil in that of controls. Similar results were obtained with the ethylene-requiring diageotropica (dgt) mutant.

#### LOCALIZATION OF ETHYLENE SYNTHESIS IN HIGHER PLANTS

The site of ethylene synthesis is not yet completely known. A number of investigators experimented with the possibility that subcellular organelles contained enzymes required for ethylene biosynthesis. Subdividing apple fruits into smaller slices decreased ethylene synthesis (34,110). Tomato fruits, on the other hand, produced more ethylene as they were sectioned (40,110). However, ethylene synthesis from both fruits stopped when they were homogenized. It was suggested that the ethylene producing system did not survive during cell destruction. Ethylene synthesis from chloroplasts (45,46,76) and mitochondria (36,37, 110,147) was also described. However, Ku and Pratt (79) failed to observe ethylene synthesis by purified preparations of mitochondria.

It was shown that ethylene production by sections of apples (34) and pears (26) was suppressed by immersing the tissue in water or hypotonic solutions but this effect could be largely prevented by transferring the tissue to a solution of high tonicity; glycerol at 0.3 M and KCl at 0.15 M were equally effective in preventing the reduction of ethylene synthesis. Burg and Thimann (34) concluded that the site of ethylene synthesis could be an organelle other than the mitochondrion.



Sakai and Imaseki (135) reported that proteinaceous inhibitors extracted from etiolated mungbean hypocotyls inhibited ethylene production in mungbean seedling which attaches to the cell surface of the tissue, and could be reversed by washing off the inhibiting protein. They suggested that the ethylene synthesizing system is located on the surface of the plasma membrane. Odawara et al. (119) studied the effect of various substances, including lecithin, Tween 20, Triton-X100 and sodium dodecyl sulfate which change membrane structure, on auxin-induced ethylene production in etiolated mung bean hypocotyl segments. They found that these substances significantly inhibited ethylene production. The inhibition by the first two substances was not reversible, and that of the latter two was partially recovered by removing them from the tissue segments. They concluded that inhibition of ethylene production by these substances resulted from structural changes of cell membranes caused by reversible interaction with the lipophilic substances.

Mattoo and Lieberman (103,104) reported that protoplasts prepared from apple tissue did not exhibit ethylene production, but after culturing for several days, some ethylene production was obtained concomitant with regeneration of some of the cell wall. Production of ethylene by the protoplasts was dependent on the addition of methionine, and the system was inhibited by AVG and n-propyl gallate. They suggested that the ethylene-synthesizing enzyme system is highly structured in apple cells and is localized in a cell wall-cell membrane complex. Recently, Anderson et al. (9) reported that freshly prepared protoplasts from apple could produce ethylene from  $^{14}\text{C}$ -labeled methionine in the complete absence of cell walls. They suggested that the ethylene-forming system is localized on the surface of the plasma membrane. Furthermore, they (9)



also suggested that the freshly prepared protoplasts of Mattoo and Lieberman (103,104) did not produce significant amount of ethylene, because of the inhibitory nature of the pectinase and the Rhozyme HP-150 in which the protoplasts were prepared and because of the lack of  $\text{CaCl}_2$ .

In order to exhibit wound ethylene production the wounding process must not destroy all the cells. Imaseki et al. (66) reported that disease-induced ethylene production in sweet potato tissue was observed only by tissue plugs of sweet potato which consisted of both fungus-invaded and non-invaded parts. The fungus-invaded part alone did not release ethylene. Similarly, plugs which were removed from the fungus-invaded part did not produce an appreciable amount of ethylene. Elstner and Kunze (47) froze various areas of beet leaf disks and found that ethylene production increased with the increase in area of frozen tissue until 50% of the leaf area was damaged. Further increases in damage reduced ethylene production. The cells adjacent to the injured or dead cells appeared to produce the surge in ethylene production.

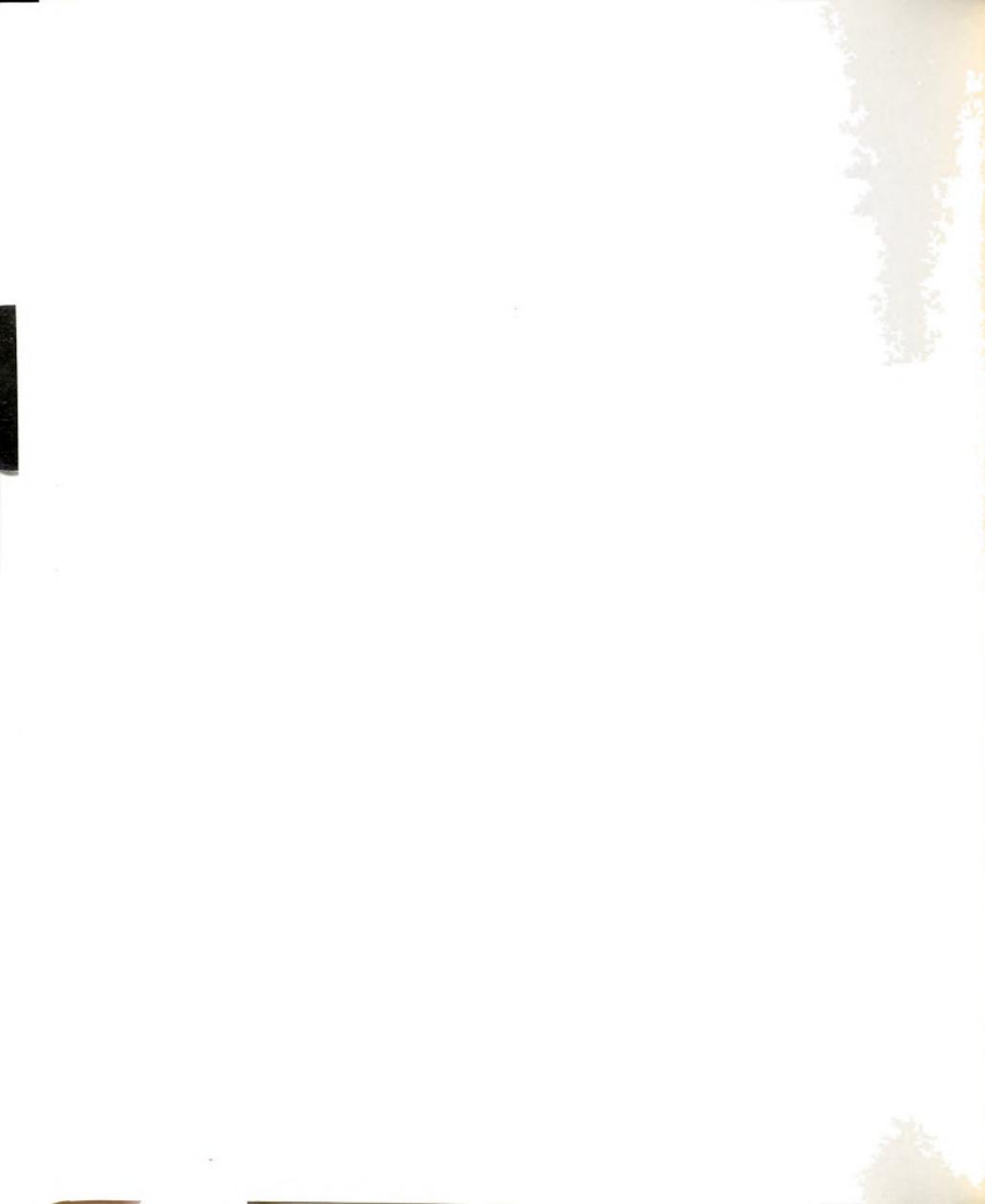
#### CHARACTERISTICS OF RIN MUTANT TOMATO FRUIT

The fruit of rin mutant tomato remains green while wild-type tomato fruit ripens and turns red. Rin fruit eventually turns lemon-yellow color and softens slowly (23,24,48,145). It was shown that rin fruit exhibits a non-climacteric ripening behavior exhibited by (1) a lack of a typical respiratory climacteric during ripening, (2) the stimulation of carbon dioxide production by ethylene or propylene without a concomitant increase in ethylene production and (3) the repeated stimulation of carbon dioxide production which persists only in the presence of exogenous olefins (58,59,107,118).



Buescher and Tigchelaar (25) and Poovaiah and Nukaya (125) found that pectinesterase, polygalacturonase and cellulase activity increased during normal tomato ripening. However, in rin mutant fruit polygalacturonase activity was very low. They, therefore, suggested that the failure of rin mutant tomato fruits to ripen is related to their low polygalacturonase activity during maturity as compared with normal fruits. Suwvan and Poovaiah (149) analyzed calcium and other inorganic ions in the pericarp of rin mutant and wild-type tomato fruits. It was found that during the early stages of fruit development, soluble calcium was higher in wild-type fruit and there were no detectable changes in the accumulation patterns of other inorganic ions. In rin fruits, bound calcium continued to increase with age and it was twice as high as compared to earlier stages of fruit development while in wild-type tomato fruit, bound calcium decreased about three-fold at later stages. They suggested that high levels of bound calcium ions in the rin mutant tomato fruits may be associated with an altered membrane and cell wall and play a role in the non-ripening character of rin mutant tomato fruits.

Davey and Van Staden (42) showed that ripening fruits of wild-type tomato (Rutgers) contained lower levels of endogenous cytokinins than fruits of rin mutant tomato at the same stage of development. The cytokinin content of both strains was high at the green (breaker) stage and decreased as the fruits senesced. This decrease was more pronounced in the wild-type fruits. Fruits of rin tomato not only contained higher levels of the free base cytokinins; zeatin and zeatin riboside, but also high levels of zeatin glucoside, a storage cytokinin. They therefore suggested that the high levels of cytokinin in rin mutant tomato fruits



are involved in delaying the ripening process. However, Mizrahi et al. (111) previously suggested that the endogenous level of cytokinin does not account for the lack of ripening in rin tomato fruits. Nagle (117) found that endogenous levels of free indole acetic acid (IAA) in green-mature fruits of rin tomatoes was only 15% of the average concentration of IAA found in the wild-type fruits (Rutgers) at the same stage of fruit development. In light of the fact that Huang (60) found essentially no difference in the IAA oxidase activity of rin mutant and wild-type tomato fruits, the significantly lower concentration of free IAA in rin mutant tomato fruit may relate to its lack of ripening under normal conditions.

Simpson et al. (144) studied the changes in ultrastructure and pigment content during development and senescence of fruits of wild-type, rin and nor mutant tomatoes. They found that in wild-type fruits, the major change was the transformation of chloroplasts to chromoplasts during ripening; this transformation was complete within five days. A similar, but much slower and less complete, transformation was noted in the mutants. The slow changes in ultrastructural transformation of plastids paralleled the changes in color. It was suggested that there was either suppression of nuclear action or lack of capacity to produce cellular components essential for normal ripening in non-ripening rin and nor mutant tomatoes.

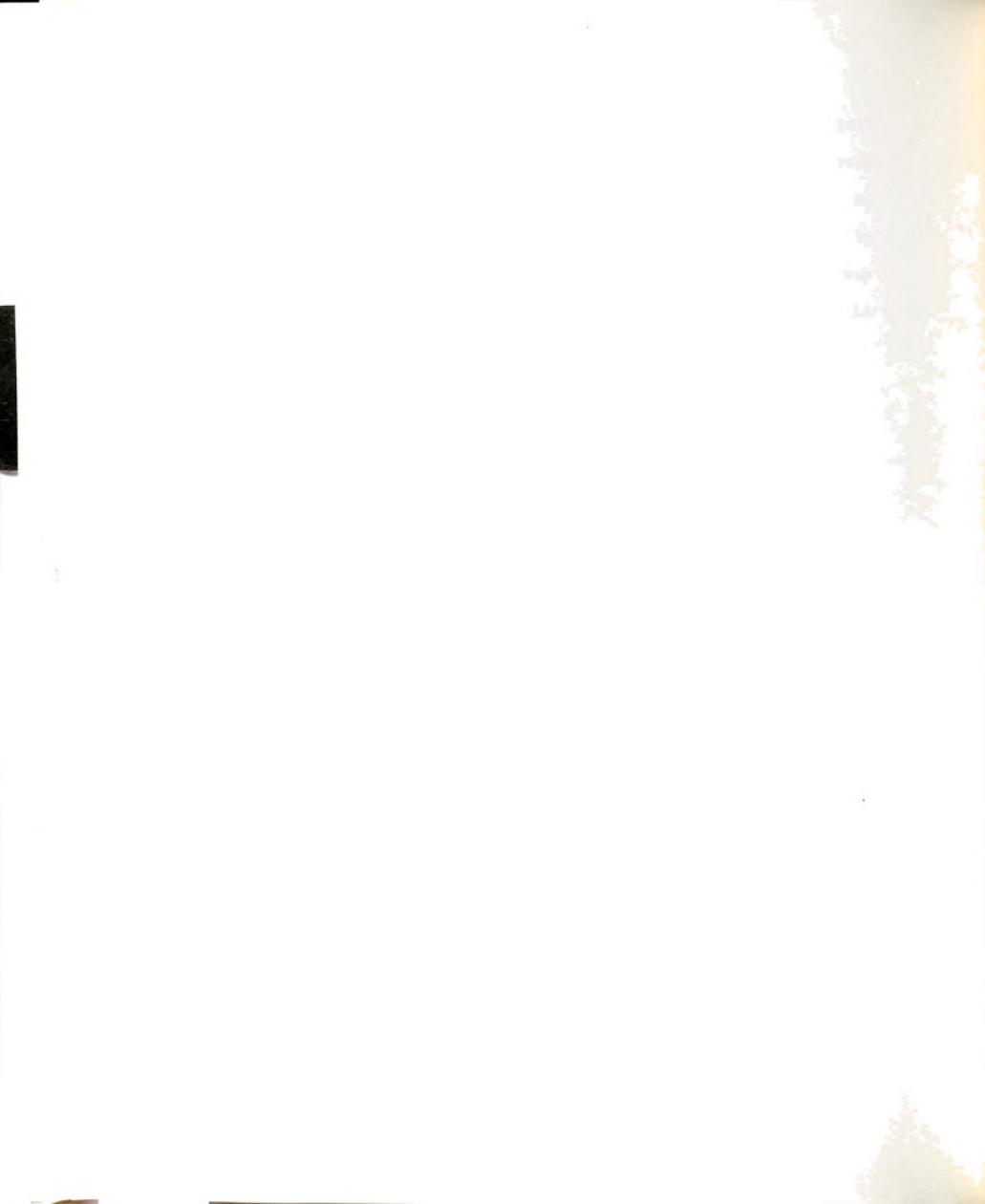
Mizrahi et al. (112) showed that normal ripening was not induced in disks of rin mutant tomato tissue implanted in wild-type fruits although development of yellow or yellow-orange colors associated with senescence of mutant fruits was accelerated. Disks of wild-type fruit tissue implanted in fruits of rin mutant ripened normally and concomitant with the intact wild-type control fruits. It was suggested that the



fruit of rin tomato did not contain translocatable ripening inhibitors. Mizrahi et al. (113) also grafted scions of the non-ripening rin tomato on wild-type understock plants (cv. Rutgers) including reciprocal grafts of Rutgers scions on rin understocks as well as grafted and ungrafted controls. It was found that no alteration in the ethylene, carbon dioxide evolution and color development of either rin mutant fruits on wild-type understock or of wild-type fruits on rin mutant understock occurred. They (113) suggested that the inability of rin mutant fruit to ripen normally stems either from the presence in mutant fruits of a non-translocatable ripening inhibitor or from the absence of a non-translocatable ripening factor.

Gonzalez et al. (52) reported that free levels of methionine are the same for mature-green fruits in rin mutant and wild-type tomatoes and they suggested that the methionine level does not appear to be the limiting factor for ethylene production in rin fruits. Herner and Sink (58) demonstrated that wounding stimulated ethylene production by rin fruit tissue similar to that in wild-type fruit tissue. McGlasson et al. (108) found that there were no substantial differences between the wild-type and rin mutant tomatoes in capacity for the production of ethylene and carbon dioxide from labeled methionine by freshly cut disks of fruit tissue.

Recently, Boller et al. (19) showed that applied ACC, an intermediate of ethylene biosynthesis in higher plants (6), greatly increased ethylene production in fruit tissue of rin mutant tomatoes that otherwise produced little ethylene. They (19) suggested that in rin tomato fruit, the rate-limiting step in ethylene biosynthesis is not the one between ACC and ethylene but between SAM and ACC.



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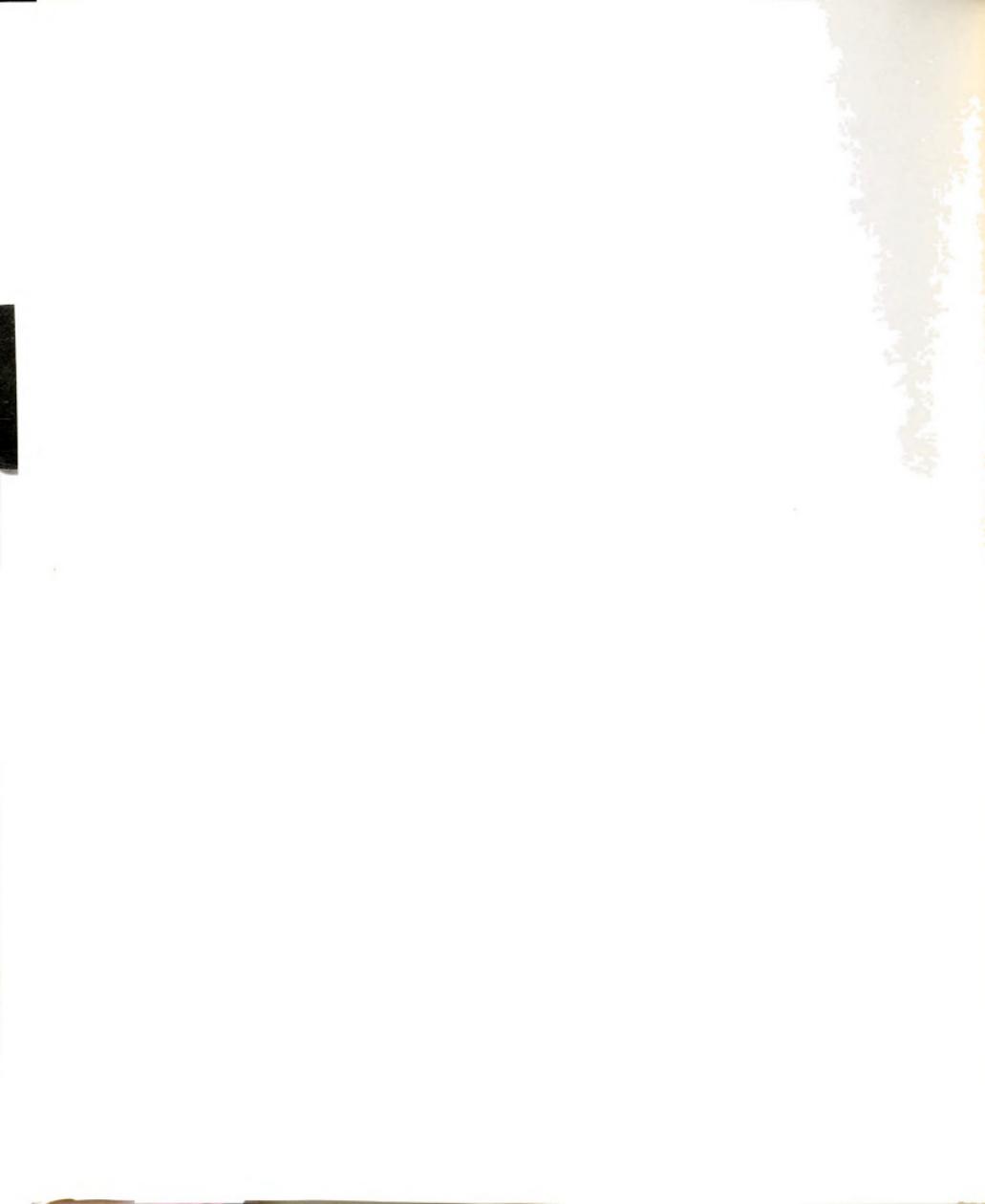
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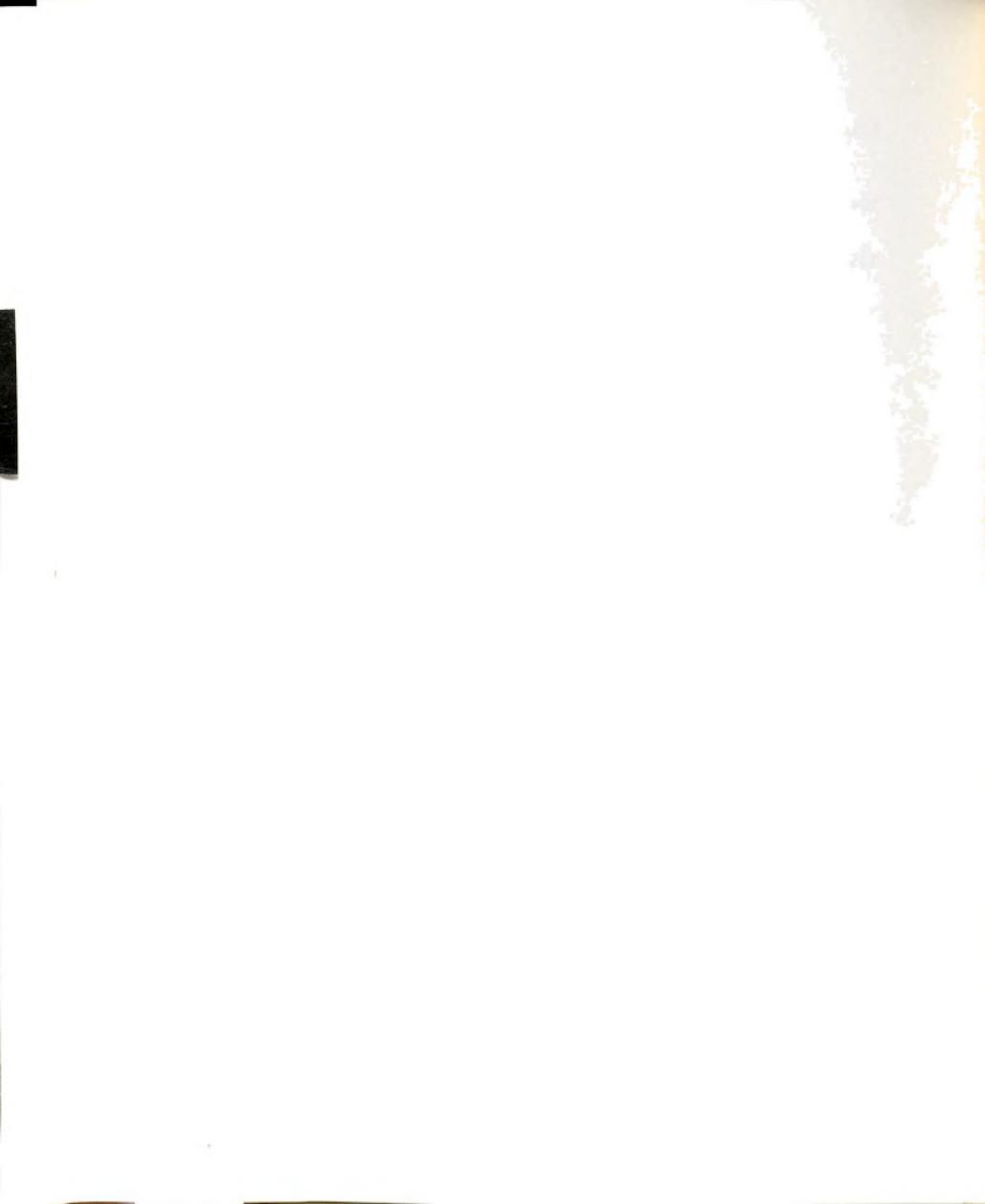
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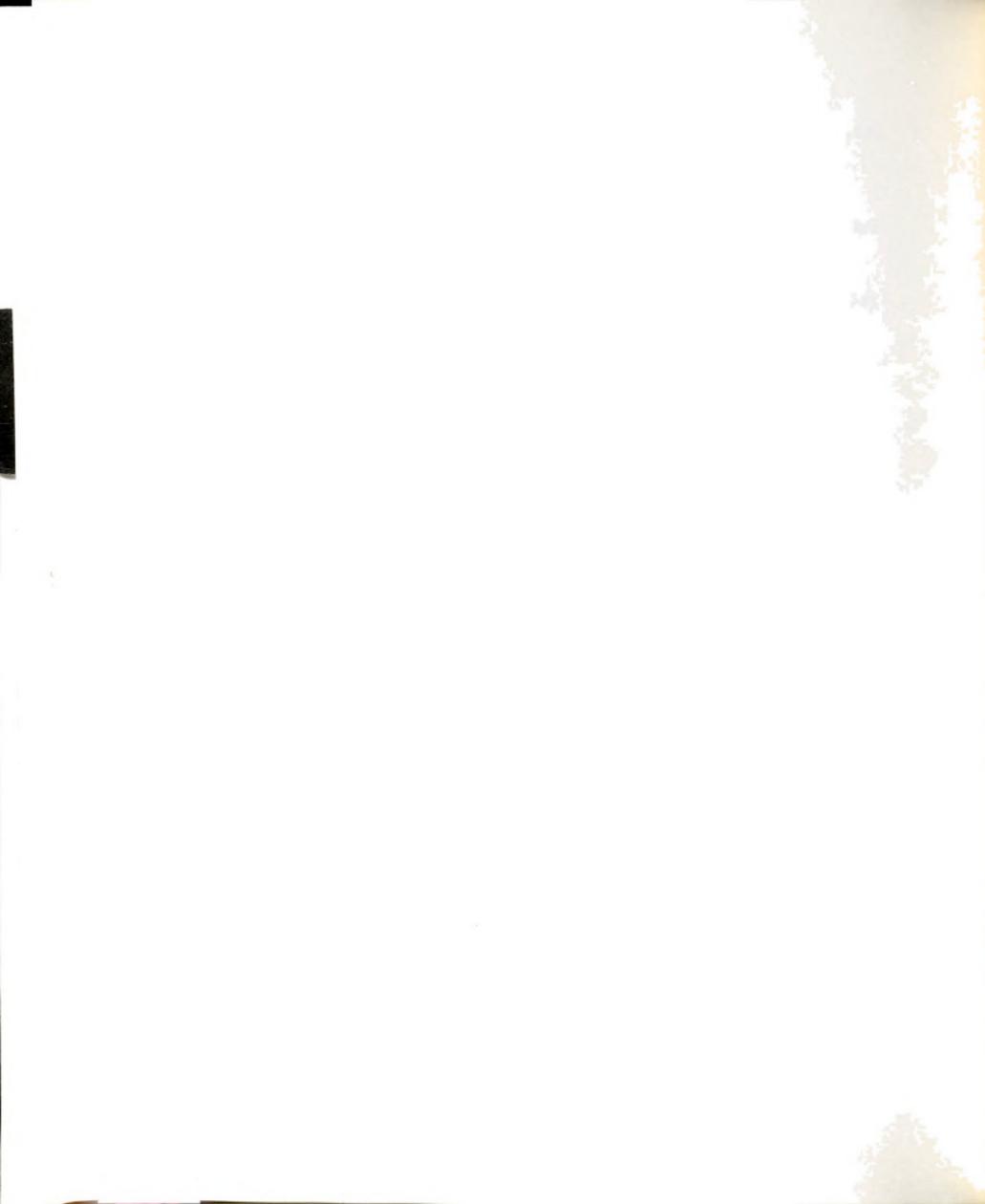
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SECTION ONE

WOUND ETHYLENE PRODUCTION BY FRUIT PERICARP

TISSUE OF RIN MUTANT TOMATO

(LYCOPERSICON ESCULENTUM MILL.)

I. INDUCTION



## I. INDUCTION

### ABSTRACT

The pattern of wound-induced ethylene production by fruit pericarp tissue of rin mutant tomato (Lycopersicon esculentum Mill.) was studied in relation to stage of fruit development, cut surface area, and role of the epidermis. The rate of wound ethylene production by disks from both immature- and mature-green fruits reached the maximum 4 h after cutting, decreased to the minimum 6 to 8 h after cutting and then slowly increased. The rate of wound ethylene production by disks from immature-green fruits was higher than that of disks from mature-green fruits. The rate of wound ethylene production was proportional to the cut surface area of disks and this appeared to be related to the induction of enzyme synthesis, rather than the facilitation of gas diffusion. Disks with intact epidermis produced more wound ethylene than that of disks without epidermis. This suggests that the compact cells close to epidermis may be the location of the ethylene-forming enzyme(s). Anaerobiosis inhibited wound ethylene production by disks. Rhizobitoxine reduced the ACC formation of disks under anaerobic conditions.

### INTRODUCTION

Environmental, chemical, and physical stress factors can alter the rate of ethylene production (1,39). Of these responses to stress,



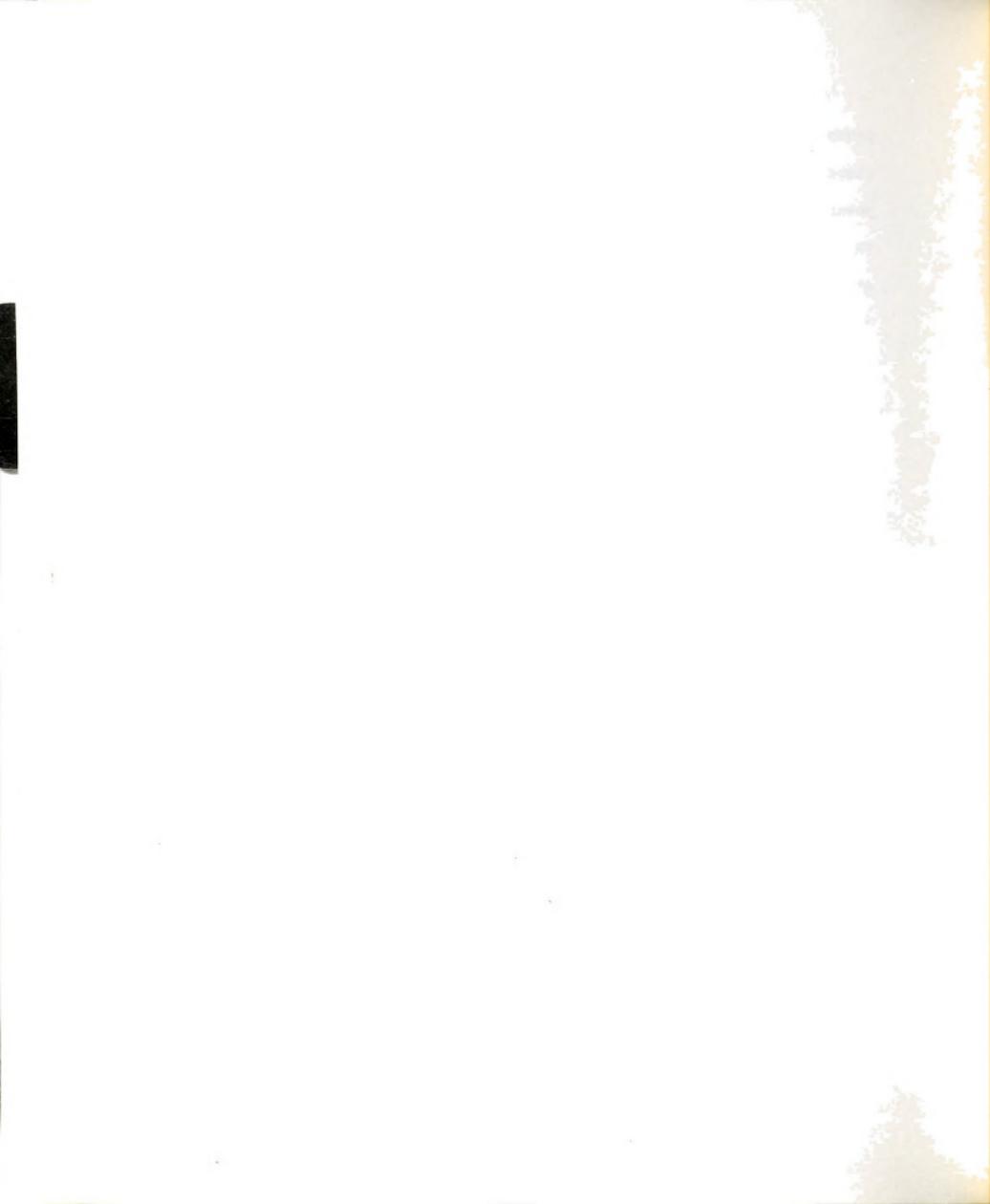
promotion of ethylene production by physical wounding of fruits (7,14), stems (31), leaves (9), and flowers (11) is now a universally recognized phenomenon. Wound ethylene requires unwounded tissue which is adjacent to wounded cells for ethylene synthesis since the severely damaged cells or tissues themselves produce little ethylene (16).

Fruits of the rin mutant tomato are classified as non-climacteric fruit (12), they remain green while wild-type tomato fruits ripen and turn red. The rin fruits eventually turn lemon-yellow color and soften slowly (38). Many researchers have proposed factors which may be involved in the non-ripening rin tomato fruit including non-translocatable inhibitors (25), lack of polygalacturonase (30), calcium ion (37), cytokinin (8), and organelle transformation (36), but none of these has directly been proven to prevent ripening of rin tomato fruit. Herner and Sink (12) demonstrated that wounding stimulated ethylene production by rin tomato fruit tissue at rates similar to that in wild-type fruit tissue. This paper reports more detail of wound-induced ethylene production by fruit pericarp tissue of rin tomato.

#### MATERIALS AND METHODS

##### Plant Materials

Plants of wild-type (Lycopersicon esculentum Mill., 61-37, Fireball x Cornell 54-149) and rin mutant tomatoes were grown in the greenhouse and trained to a single stem. Flowers were tagged at anthesis; only one flower per cluster being pollinated. All others in a cluster were excised. Immature- and mature-green fruits were harvested  $25 \pm 1$  and  $40 \pm 1$  days after anthesis, respectively. Unless noted otherwise, disks of pericarp tissue (diameter : 1.5 cm, thickness : 0.25 cm), with intact



epidermis were prepared from equatorial part of tomato fruits with a cork borer and template. Therefore, all disks were uniformly prepared in size and fresh weight.

#### Experimental Procedure

The rate of wound ethylene production was studied using a static system in which evolved ethylene accumulated. Single disks of pericarp tissue were placed with the epidermis on a layer of glass beads in 20 ml scintillation vials. The vials were flushed with ethylene-free air and closed with a serum cap, the incubated in darkness at 20°C. In order to compare the rate of wound ethylene production by freshly cut disks to the rate of ethylene production by intact fruits, immature- and mature-green fruits of rin tomato were also used to monitor ethylene production by using a static system. Individual fruits were placed in pint canning jars (500 ml).

#### Chemical Treatments

A water-soluble factor necessary for wound ethylene synthesis is apparently lost when tissues of stem sections of pea seedlings (34), tomato fruit (35), and sweet potato root (17) are placed in contact with water. To avoid this problem, in any experiment in which the disks were to be treated with chemical solution(s) or distilled water (control), 20  $\mu$ l of the solution or water were applied to the cut surface of the disks opposite the epidermis. This amount of liquid was found to be totally absorbed by the tissue with no run off.



### Oxygen Dependency

The oxygen requirement for wound-induced ethylene synthesis was studied using a single disk per 20 ml scintillation vial. The vials were purged with N<sub>2</sub> to remove all O<sub>2</sub>.

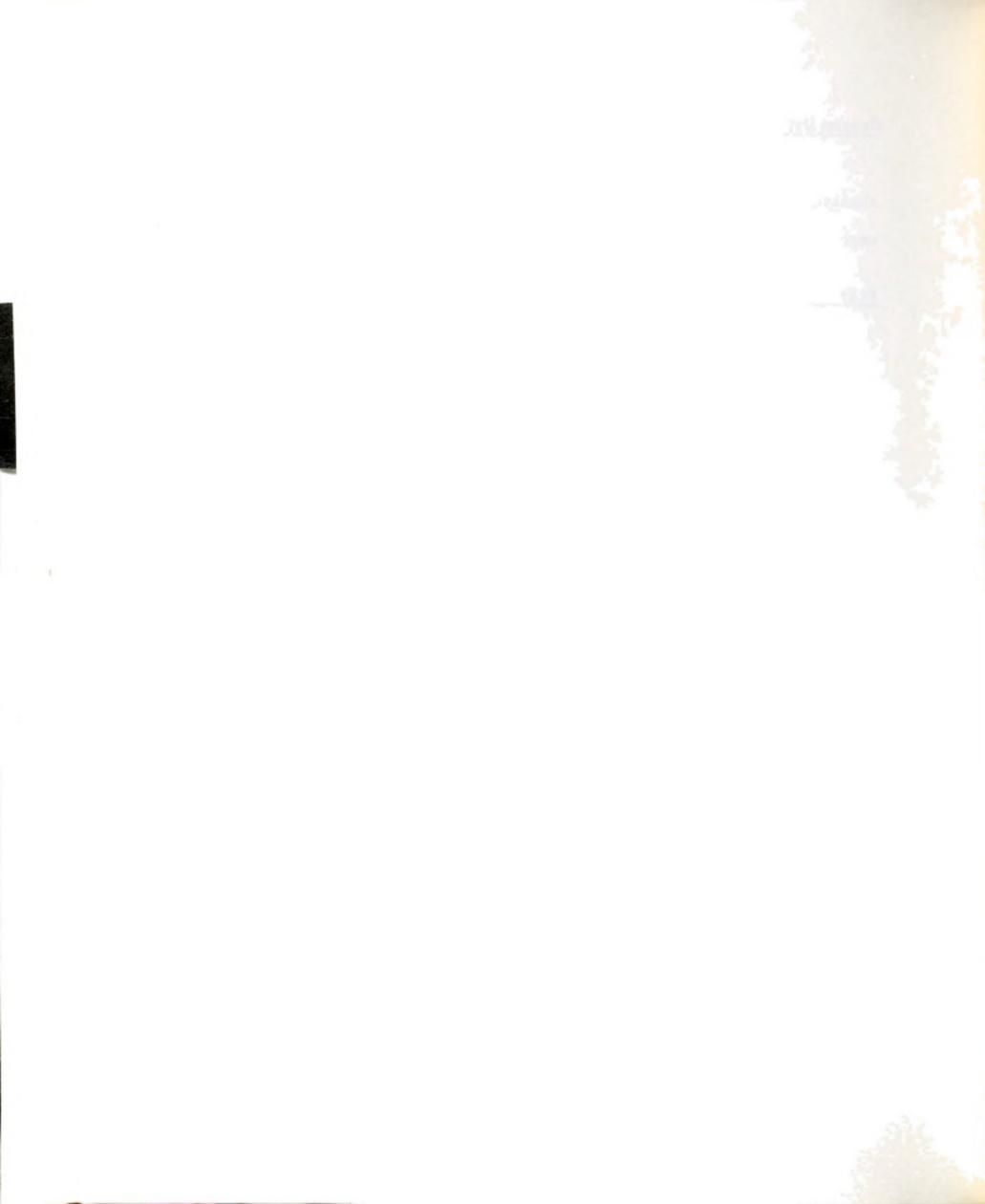
### Ethylene Determination

At the end of every 2 h period, wound ethylene production by freshly cut disks was analyzed by withdrawing a 1 ml gas sample through a serum cap. Vials and jars were flushed with ethylene-free air, sealed and returned to the incubation conditions. Ethylene in the vials and jars was determined by a gas chromatograph (Varian Aerograph Series 1700 or 1400), equipped with a flame ionization detector. A column (45 x 0.32 cm) of 60 to 80 mesh of Al<sub>2</sub>O<sub>3</sub> was used, operating at 60° or 80°C. In calculating the amount of ethylene evolved in an experiment, all data were corrected for any ethylene in blank containers and their replacement with ethylene-free air. Each experiment consisted of five to six disks and was repeated three times. For intact fruits of rin tomato, each stage of fruit development consisted of three fruits and was also repeated three times. Data represent mean values.

## RESULTS

### Stage of Fruit Development

Freshly cut disks from both immature- and mature-green fruits evolved wound ethylene during incubation at 20°C in the dark, reaching a maximum about 4 h after cutting (Figure 1). The rate of wound ethylene production then declined to a minimum 6 or 8 h after cutting for both immature- and mature-green fruits, and slightly increased again



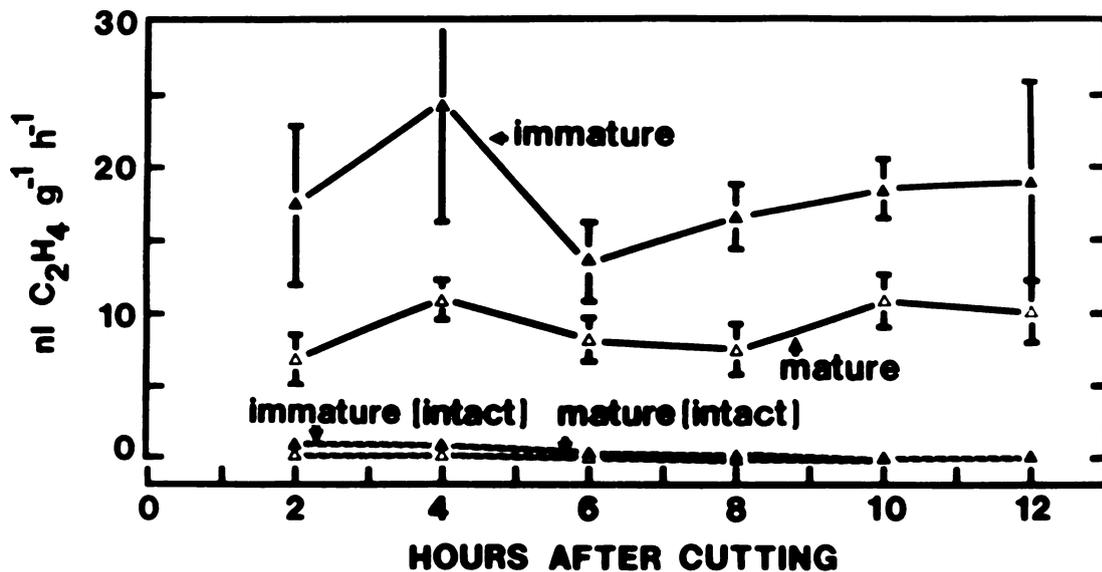


Figure 1. Effect of stage of fruit development on wound ethylene production by freshly cut disks as compared to intact fruits.

thereafter. In contrast, the rates of ethylene production from intact immature- and mature-green fruits were almost the same and very close to nil. The rate of wound ethylene production by disks from immature-green fruits was greater than from disks of mature-green fruits throughout the incubation period. At the maximum, the rate of wound ethylene production by disks from immature-green fruits was  $23.54 \pm 4.49$   $\text{nl g}^{-1}\text{h}^{-1}$  while  $11.67 \pm 0.66$   $\text{nl g}^{-1}\text{h}^{-1}$  was produced from disks of mature-green fruits. The average rate of wound ethylene production throughout the incubation period (12 h) for disks from immature- and mature-green fruits was  $17.26 \pm 3.27$  and  $8.12 \pm 1.93$   $\text{nl g}^{-1}\text{h}^{-1}$ , respectively.

The rate of wound ethylene production by disks from mature-green fruits of both rin and wild-type tomatoes were comparable throughout the incubation period (Figure 2). The average rate of wound ethylene production throughout the incubation period by disks from rin and



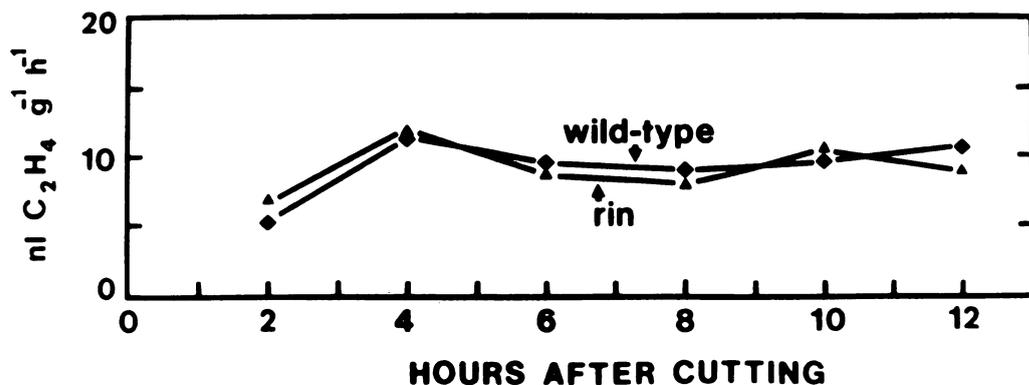


Figure 2. Wound ethylene production by freshly cut disks from mature-green fruits of rin and wild-type tomatoes.

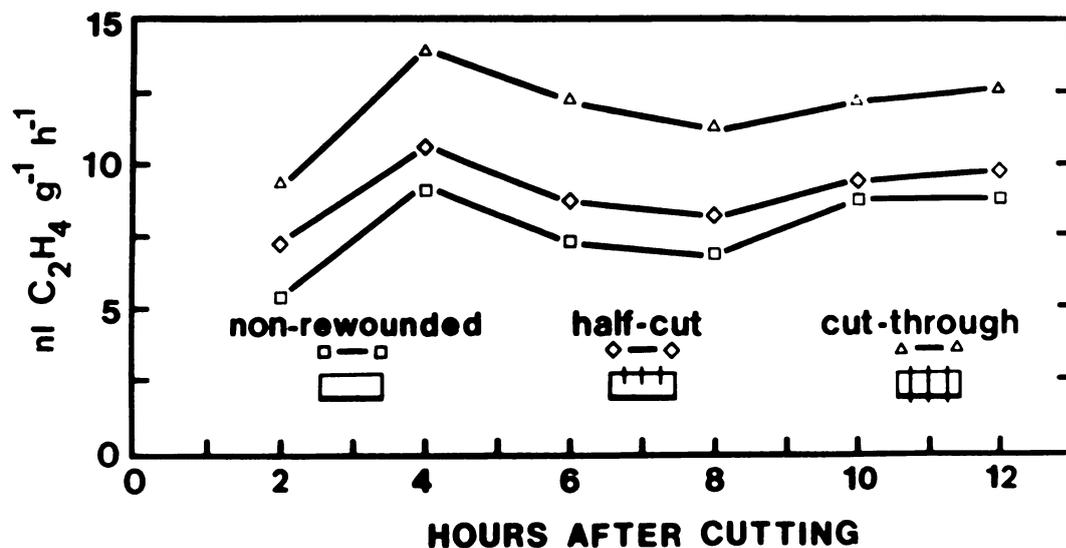
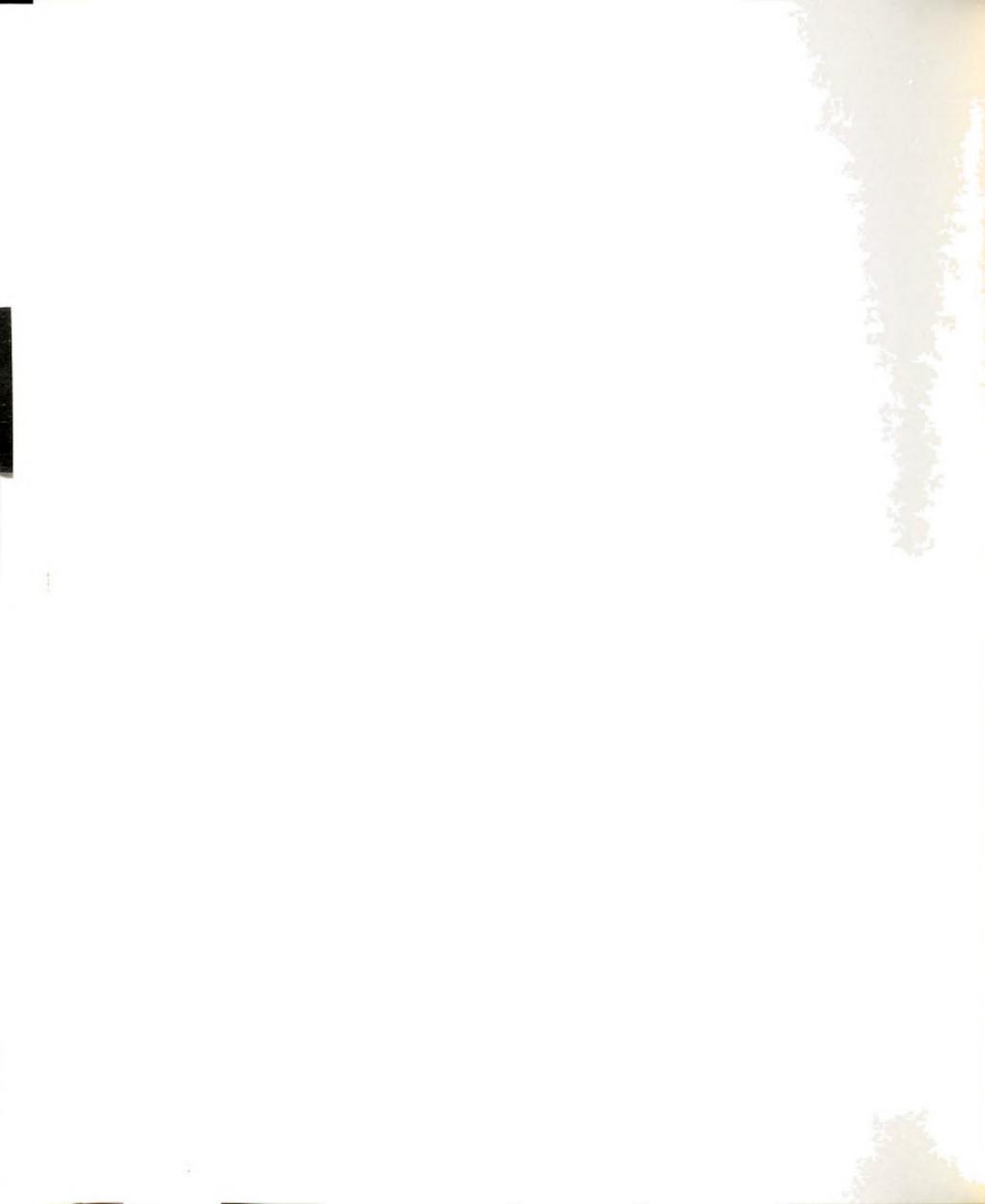


Figure 3. Effect of rewounding on ethylene production by freshly cut disks from mature-green fruits of rin tomato. Freshly cut disks were non-wounded, rewounded by 3 cuts half-way or completely though perpendicular to the cut surface of disks. Wounds were made on largest cut surface opposite the epidermis.



wild-type tomatoes was  $9.45 \pm 1.71$  and  $9.46 \pm 2.04$   $\text{nl g}^{-1}\text{h}^{-1}$ , respectively.

#### Effect of Cut Surface Area of Disks

When freshly cut disks from mature-green fruits were rewounded by three cuts half-way or completely through, perpendicular to the major cut surface, the rates of wound ethylene production were higher than that of non-rewounded disks (Figure 3). Increasing the cut surface area of disks by cutting completely through the disks stimulated more wound ethylene production than by cutting only half-way through the disks. The trend of ethylene production by rewounded and non-rewounded disks was similar, reaching a maximum after 4 h with a minimum 8 h after

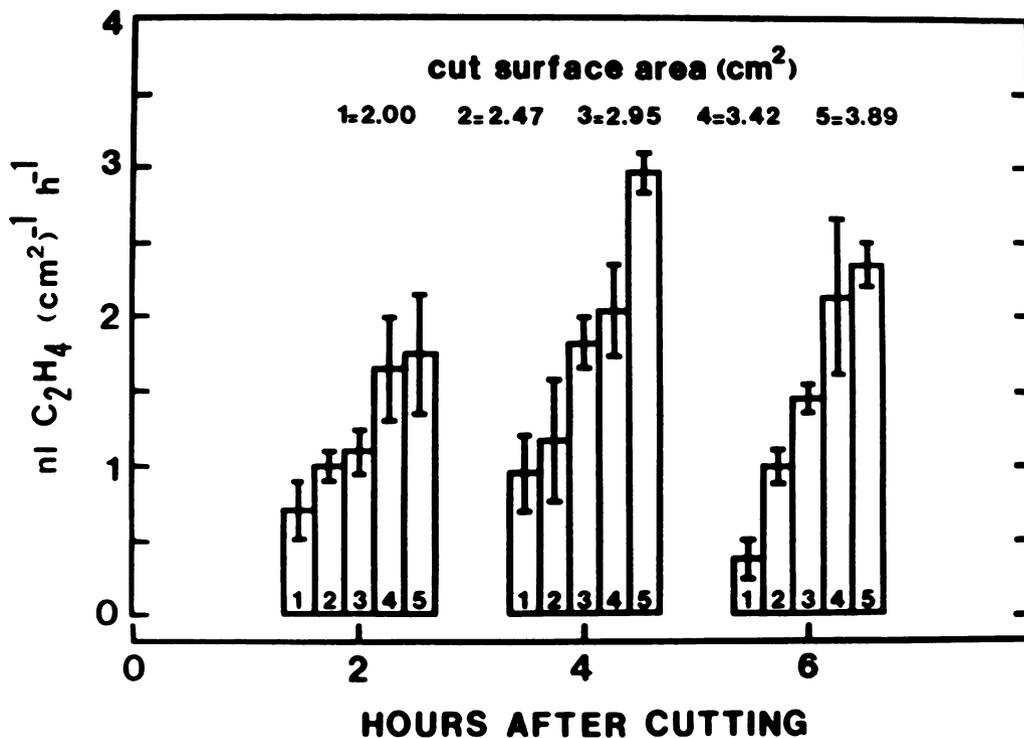


Figure 4. Effect of cut surface area of freshly cut disks on wound ethylene production. Disks with intact epidermis were cut into 0.05, 0.15, 0.25, 0.35, and 0.45 cm thickness resulting in cut surface area of 2.00, 2.47, 2.95, 3.42, and 3.89  $\text{cm}^2$ , respectively.



cutting and then slightly increasing again. The average rates of wound ethylene production throughout the incubation period were  $7.66 \pm 1.26$ ,  $8.79 \pm 1.44$  and  $12.14 \pm 2.43$   $\text{nl g}^{-1}\text{h}^{-1}$  for non-rewounded, three cuts half-way and completely through the disks, respectively.

Instead of rewounding to increase the cut surface area, disks were cut into different thicknesses ranging from 0.05 to 0.45 cm thickness resulting in cut surface areas ranging from 2.00 to 3.89  $\text{cm}^2$ . The rate of wound ethylene production was directly proportional to the cut surface area of disks when expressed on a surface area basis (Figure 4). The rates of wound ethylene production by all disks reached a maximum 4 h after cutting and decreased thereafter. The average rates of wound ethylene production throughout the incubation period (6 h) were 0.78, 1.01, 1.46, 2.10, and 2.33  $\text{nl (cm}^2)^{-1}\text{h}^{-1}$  for disks with cut surface areas of 2.00, 2.47, 2.95, 3.42, and 3.89  $\text{cm}^2$ , respectively. Similarly, when the rate of wound ethylene production of the disks used in Figure 4 was expressed as  $\text{nl disk}^{-1}\text{h}^{-1}$ , the rate of ethylene production was also directly proportional to the thickness of disks (Figure 5).

The correlation between cut surface area and average rate of wound ethylene production throughout the incubation period (6h) was very high;  $r = 0.983$  (Figure 6).

When freshly cut disks were rewounded 6 h later and then treated with 20  $\mu\text{l}$  of  $\text{H}_2\text{O}$ , ethylene production was stimulated as compared to non-rewounded disks (Figure 7). Rewounding the tissue was accomplished by making three cuts with a razor blade half-way through the disks on the surface opposite the epidermis. Ethylene production from the disks rose to a peak 2 h after rewounding and then declined. Non-rewounded tissue produced less ethylene for 2 h and began to increase again.



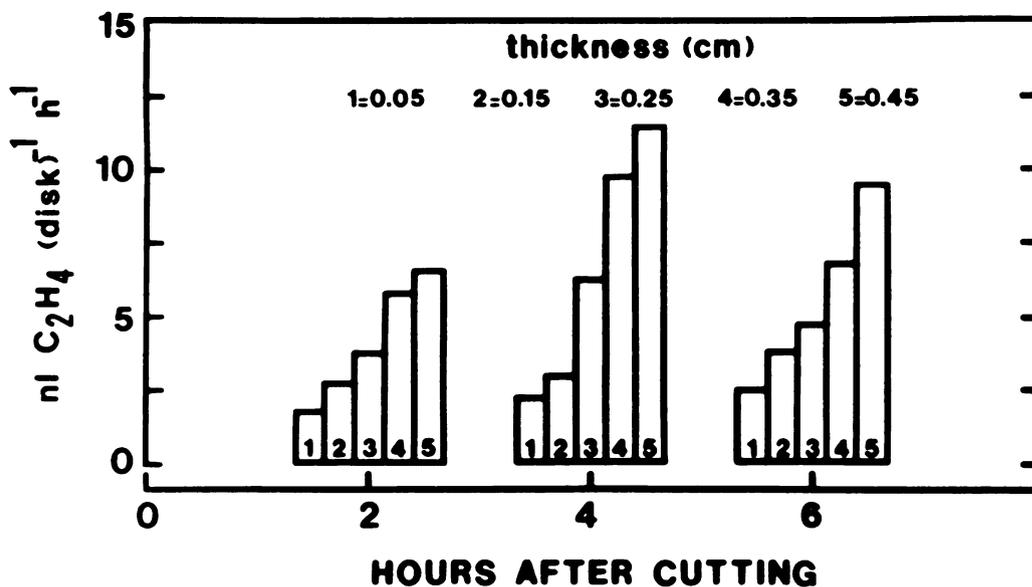


Figure 5. Effect of disk thickness on wound ethylene production. The disks with skin intact at different thicknesses were the same as used in Figure 4.

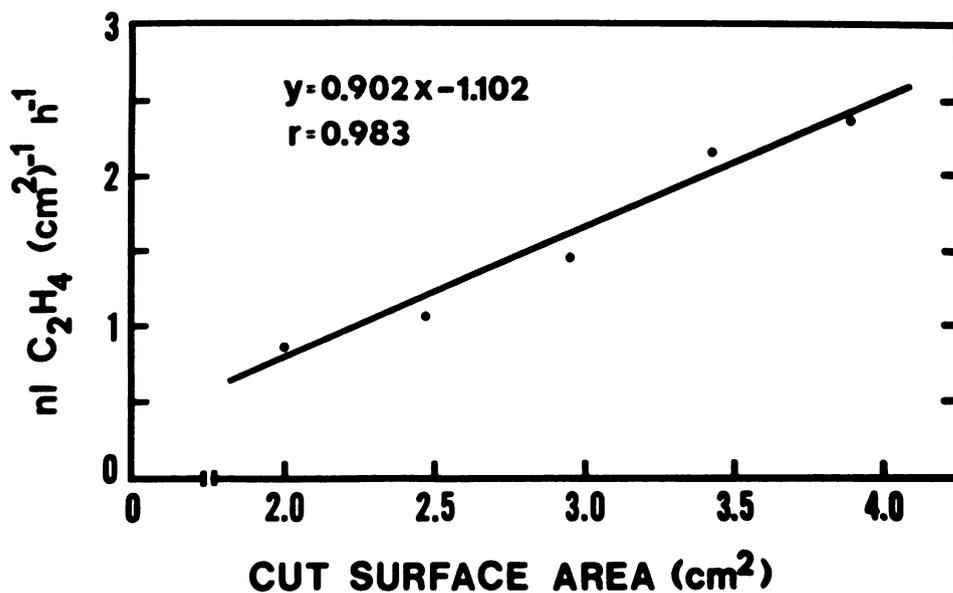


Figure 6. Correlation between the rate of wound ethylene production and cut surface area of freshly cut disks from mature-green fruits of rin tomato. Means of wound ethylene production rates were averaged from three readings during the 6 h incubation period (from Figure 4).



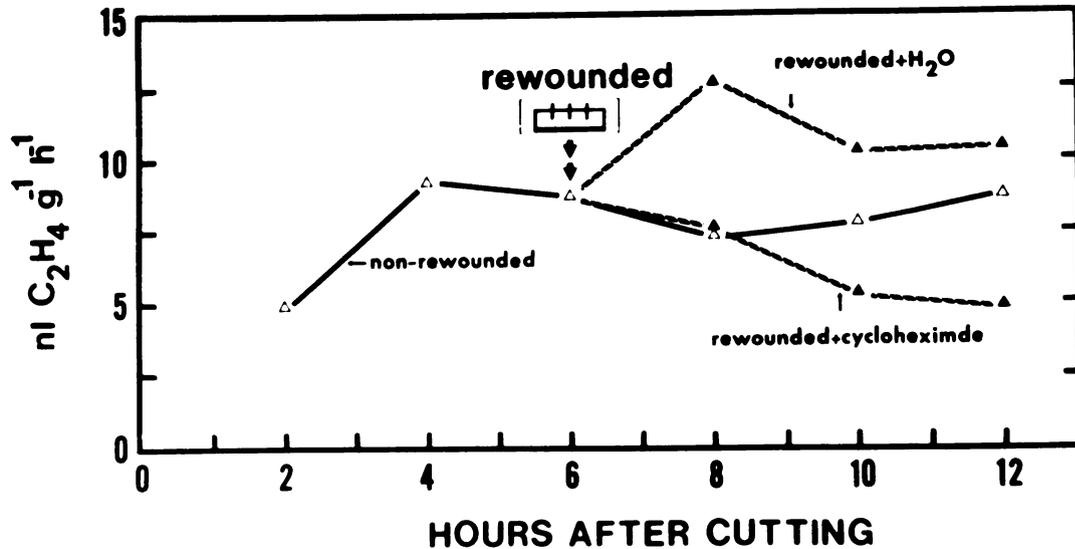


Figure 7. Effect of cycloheximide on ethylene production caused by rewounding of disks cut from mature-green fruits of *rin* tomato. Freshly cut disks were non-rewounded and treated with 20  $\mu$ l of H<sub>2</sub>O or 0.1 mM cycloheximide or rewounded by 3 cuts half-way through the major cut surface of disks 6 h after incubation and treated with 20  $\mu$ l of H<sub>2</sub>O or 0.1 mM cycloheximide as indicated by arrows.

Rewounded disks treated with cycloheximide produced a continually declining amount of ethylene up to 6 h after rewounding. Cycloheximide applied to freshly cut disks greatly reduced ethylene production.

#### Role of Epidermis

The rate of wound ethylene production was increased when the epidermis of disks was closer to the major cut surface (Figure 8). The thinner the disk, the greater the wound ethylene production when expressed on a per unit weight basis. Disks with different thicknesses were the same as those in the experiment concerning the effect of cut surface area of the rate of wound ethylene production was here expressed on a weight basis. The rate of wound ethylene production by all disks reached a maximum 4 h after cutting and then declined. The rate of wound ethylene production among disks with major cut surface 0.15, 0.25



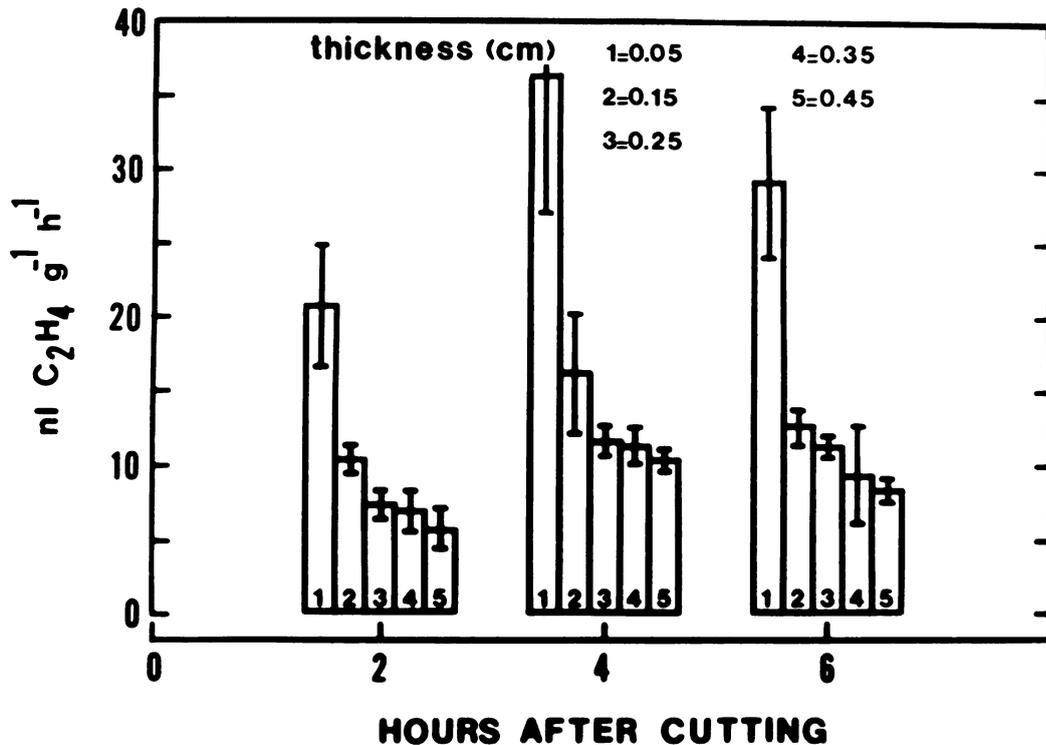
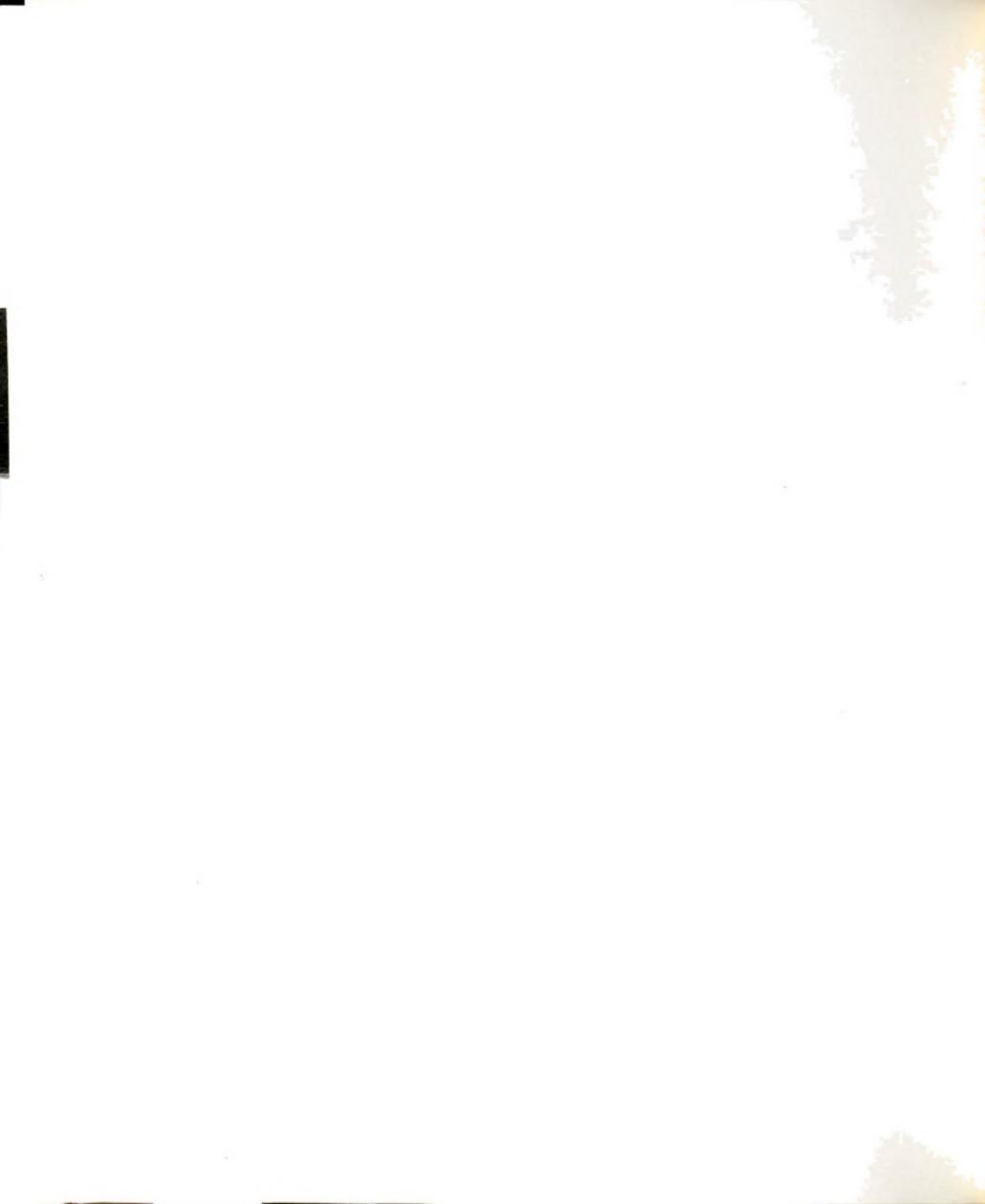


Figure 8. Effect of the distance between the epidermis and the major cut surface on wound ethylene production of freshly cut disks from mature-green fruits of rin tomato. Disks with intact epidermis were cut into 0.05, 0.15, 0.25, 0.35, and 0.45 cm thickness.

0.35, and 0.45 cm away from the epidermis were slightly different while disks with the cut surface only 0.05 cm away from the epidermis produced significantly greater amounts of wound ethylene throughout the incubation period.

When disks with the same thickness were prepared from the plug of pericarp tissue but from different distances from the epidermis (see diagram in Figure 9), it was found that the first disk including the epidermis (intact epidermis) produced the most wound ethylene during the first 4 h following the preparation of the disks. The second, third, and fourth disks had one cut surface covered with vaseline. Six h after preparing the disks, there was little difference in ethylene production between the disks from any of the four positions in the pericarp tissue.



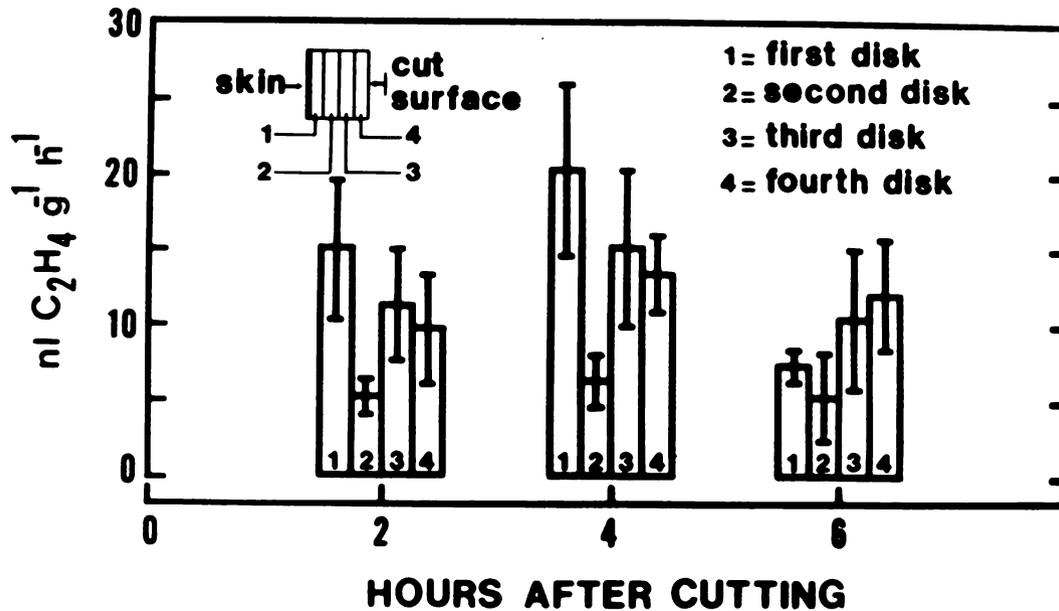


Figure 9. Wound ethylene production by freshly cut disks from different parts of pericarp tissue from mature-green fruits of rin tomato. Plugs of pericarp tissue were cut into consecutive 0.1 cm thick disks beginning with the epidermis. Only the first disk had epidermis and the proximal cut surface of the second, third, and fourth disk was covered with vaseline.

#### Oxygen Dependency

Anaerobiosis almost completely stopped wound ethylene production by freshly cut disks as compared to disks being held in air throughout the incubation period (Figure 10). When anaerobic disks were returned to air their initial rate of wound ethylene production was higher than that of the maximum rate of disks being held throughout in air. The rate of wound ethylene production by disks being held under anaerobic conditions and then transferred to air after 8 h was similar higher than that of disks held in anaerobiosis for 4 h. The rate of wound ethylene production by disks held in anaerobiosis for 4 and 8 h reached a maximum 2 h after return to air, declined thereafter and reached the basal rate of wound ethylene production by disks held throughout in air.



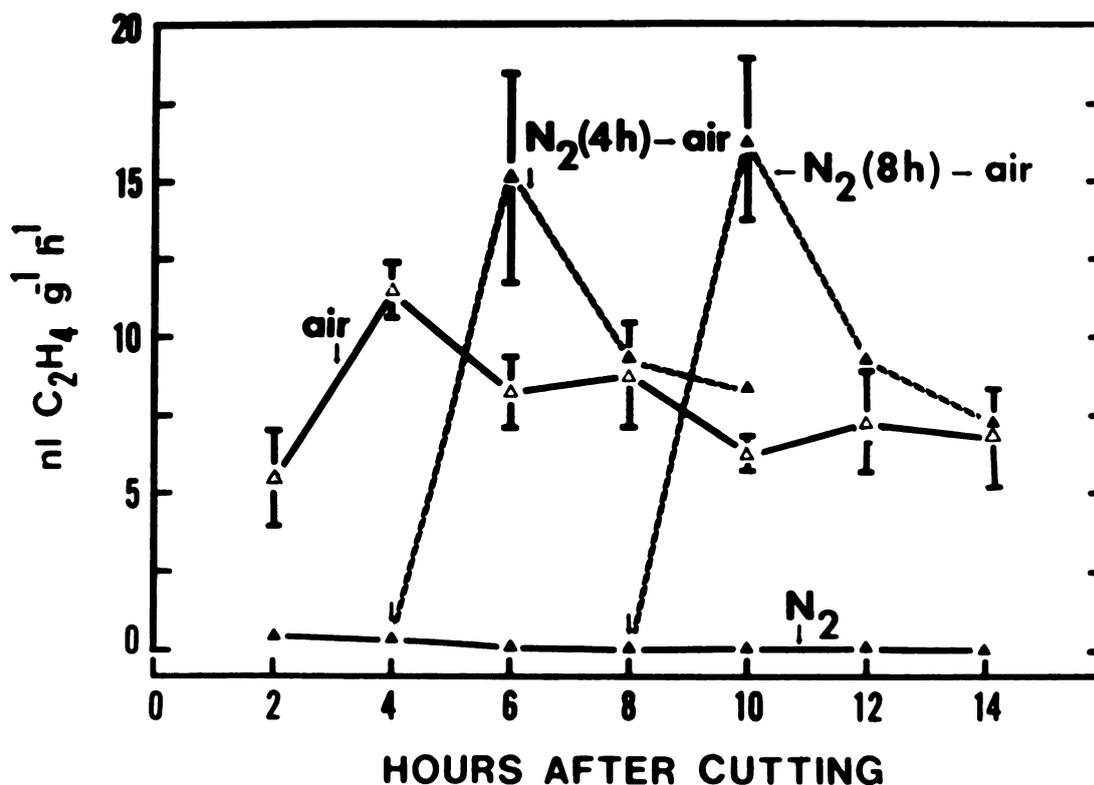


Figure 10. Effect of anaerobiosis on wound ethylene production by freshly cut disks from mature-green fruits of rin tomato. Freshly cut disks were incubated throughout in air, N<sub>2</sub>, or 4 and 8 h in N<sub>2</sub> and returned to air as indicated by arrows.

Figure 11 shows that rhizobitoxine inhibited wound ethylene production when disks were held in air. Holding freshly cut disks in a nitrogen atmosphere decreased wound ethylene to practically nil. Removing the disks from nitrogen to air resulted in a transient increase in ethylene production which then declined. Adding rhizobitoxine to the disks at the time of removal from nitrogen to air had little effect on subsequent ethylene production (compared to water treatment controls) until 4 to 6 h after treatment with rhizobitoxine. Treating disks immediately after cutting with rhizobitoxine and during the nitrogen incubation resulted in a greatly reduced wound ethylene production when these disks were subsequently returned to air.



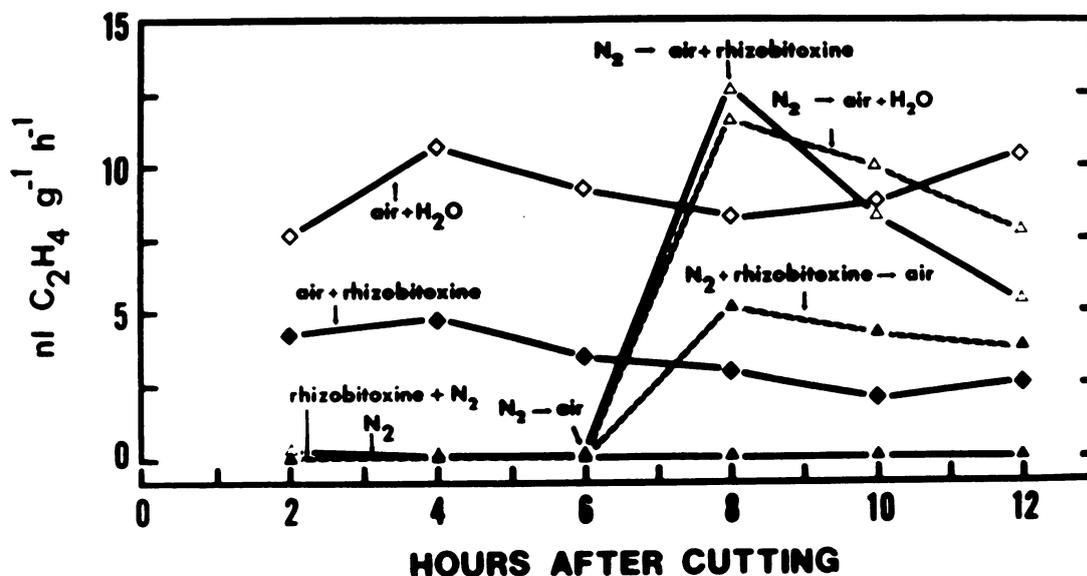


Figure 11. Effect of rhizobitoxine on wound ethylene production by freshly cut disks from mature-green fruits of rin tomato upon return from anaerobiosis to air. Disks were treated with 20  $\mu$ l of  $H_2O$  or 0.1 mM rhizobitoxine and held throughout in air or disks were non-treated or treated with 20  $\mu$ l of 1 mM rhizobitoxine and held in anaerobiosis until the time designated by an arrow (6 h after incubation) then, 20  $\mu$ l of  $H_2O$  or 1 mM rhizobitoxine were applied to non-treated disks.

#### DISCUSSION

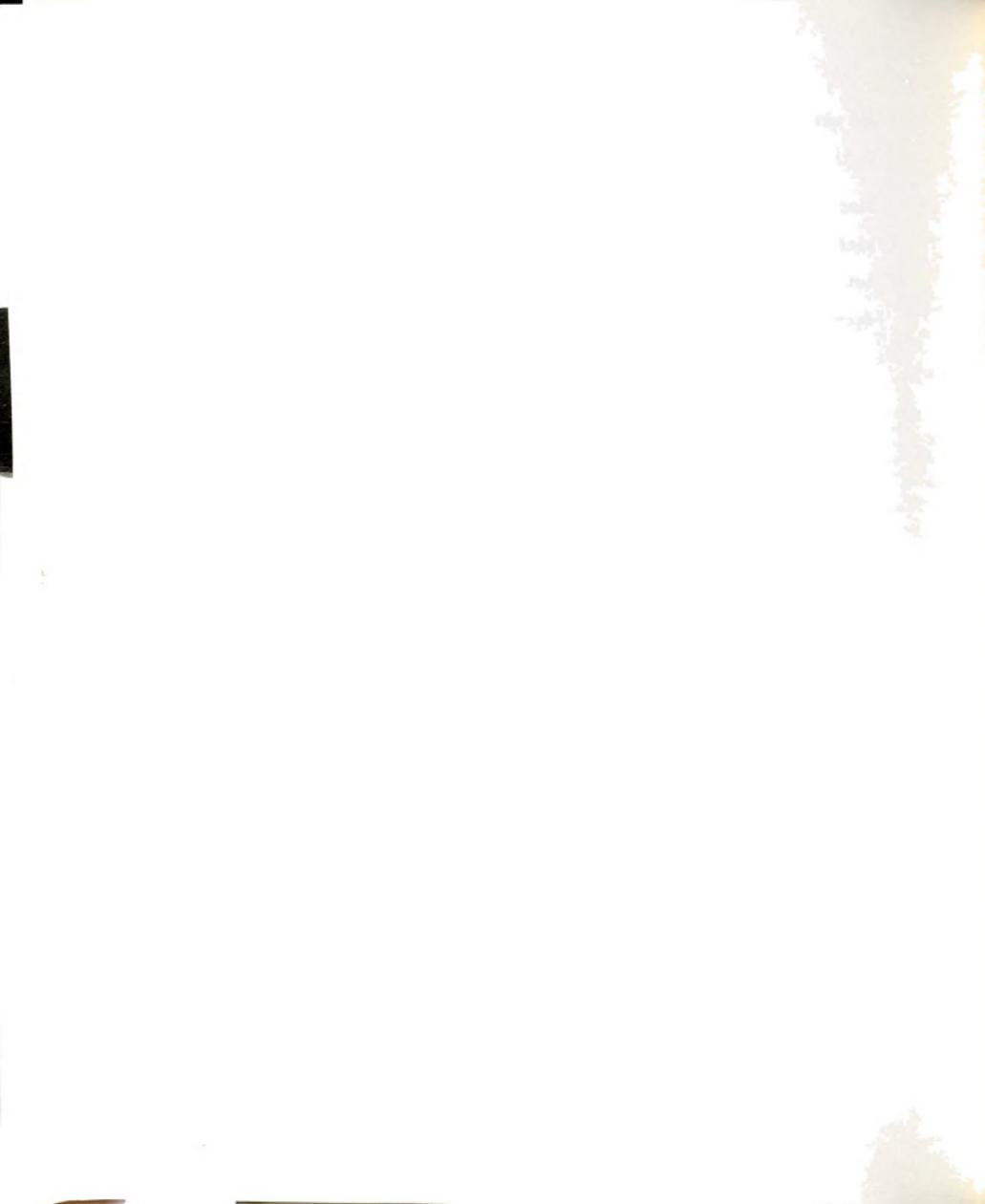
The wound-induced ethylene production was higher by disks from immature-green fruits than from mature-green fruits. Differences in auxin may exist in immature- and mature-green fruits (18) and it is possible that high endogenous levels of auxin may cause high rates of ethylene production by disks from immature-green fruits (2,40). However, since the endogenous levels of auxin in mature-green fruits of rin tomato are only 15% of the auxin content found in wild-type fruits at the same stage of fruit development (27), and disks from mature-green fruits of rin and wild-type tomatoes produced equal amounts of wound-induced ethylene. Therefore, auxin does not directly contribute to the differences in wound ethylene production between disks from immature-

1

and mature-green fruit. This conclusion is supported by other research in the diageotropica mutant tomato (19) and other plant tissues (1).

Since the disks from immature- and mature-green fruits were equal in size and weight, it is likely that the differences in ethylene production between the disks from immature- and mature-green fruits is due to differences in cell number. According to Houghtaling (13), in several cultivars of tomato fruit there is little cell division in the fruit after anthesis. McArthur and Buttler (21) reported that the post-anthesis growth period was characterized chiefly by cell expansion. It would be expected then, that immature tomato fruit disks of equal size would contain more cells than disks from mature tomato fruit. It has been suggested that differences in ethylene production by apical and basal petal disks of carnation flowers was due to cell number differences (28) and McKenzie and Street (22) have reported that ethylene production in sycamore suspension cells was related to cell number.

Increasing the cut surface area of freshly cut disks stimulated more ethylene production and this agrees with other reports with apple (24), citrus (14), sweet potato (17), bean (20), rice (15) and tomato fruit (7). Wounding or increasing cut surface area which causes a temporary increased rate of ethylene production in plant tissues has been thought to be due to increased diffusion of endogenous ethylene through the exposed cut surface (6). However, if the increase in cut surface area of disks increased ethylene by simply increasing diffusion, then cycloheximide should not inhibit wound-induced ethylene production. In these experiments, cycloheximide probably inhibited ethylene production resulting from the increase in the cut surface area of disks by inhibiting the synthesis of enzymes responsible for ethylene synthesis (10).



Perhaps the stimulation of ethylene production by increasing the cut surface area of disks is related to the amount of injured cells located on the wounded surface (9). Other researchers have shown that the capacity to produce wound-induced ethylene is dependent upon the amount of injured tissue, non-injured or injured tissue alone can produce only small amounts of ethylene (16). The increase in cut surface area may increase the production of a wound signal released from the injured tissue (33), and this signal may in turn induce all or parts of the ethylene biosynthetic pathway in the non-injured tissue (41).

The experiments with disks of different thickness, and disks with and without epidermis from the same plug of pericarp tissue demonstrate that the epidermis and/or cells immediately below it play an important role in wound-induced ethylene production. From chemical and morphological studies it has been shown that the tomato epidermis is composed primarily of lipid compounds and there are three to four layers of compact cells close to the epidermis (4,26). Though the step from ACC to ethylene in the pathway of ethylene biosynthesis is totally dependent upon oxygen (3), it is unlikely that when the cut surface was moved closer to the epidermis, as in thinner disks, the compact cells were exposed to more oxygen.

In disks whose thickness was 0.45 cm, cells closest to the cut surface may produce more ethylene than those cells farther from the cut surface (16), therefore, when the rate of ethylene production was expressed on a weight basis, the thickest disks would appear to produce the least and the thinnest disks the most ethylene. When the ethylene production rates of equal sized disks from the same plug of pericarp tissue were compared there were differences in the capacity to produce



ethylene. The first disk containing the epidermis, initially produced the most ethylene, while disks further from the epidermis produced less. This suggests that cells at different distances from the epidermis of tomato fruit pericarp tissue are unequally capable of producing ethylene.

Anaerobiosis stops ethylene biosynthesis in both wounded and senescent tissues (23,32). During the time plant tissues are held under anaerobic conditions, 1-aminocyclopropane-1-carboxylic acid (ACC) builds up and when the tissue is reintroduced into air there is a large initial stimulation of ethylene production. If anaerobiosis stops ethylene synthesis and ACC accumulates; it is logical to assume that the longer the tissue is held under anaerobiosis the more ACC builds up and the more ethylene produced upon introduction to air. Disks of tomatoes held under a nitrogen atmosphere produced very little ethylene and when placed in air, the initial production of ethylene was higher than that by disks held continually in air. However, holding disks for 8 h under anaerobic conditions and returning to air did not result in significantly more ethylene being produced compared to disks held for 4 h.

The ability of rhizobitoxine to inhibit wound-induced ethylene production by disks indicates that methionine is a precursor of wound-induced ethylene (29). However, rhizobitoxine did not inhibit ethylene production if applied to disks after the return from anaerobiosis to air. This is because disks which had been held in nitrogen had a large pool of ACC and rhizobitoxine only inhibits the activity of the ACC-forming enzyme, not the enzyme(s) required for conversion of ACC to ethylene (3,5). Rhizobitoxine inhibited ethylene production of disks



removed from nitrogen to air if it was applied at the time the disks were placed in nitrogen. This suggests that the ACC-forming enzyme separates even though in a nitrogen atmosphere.

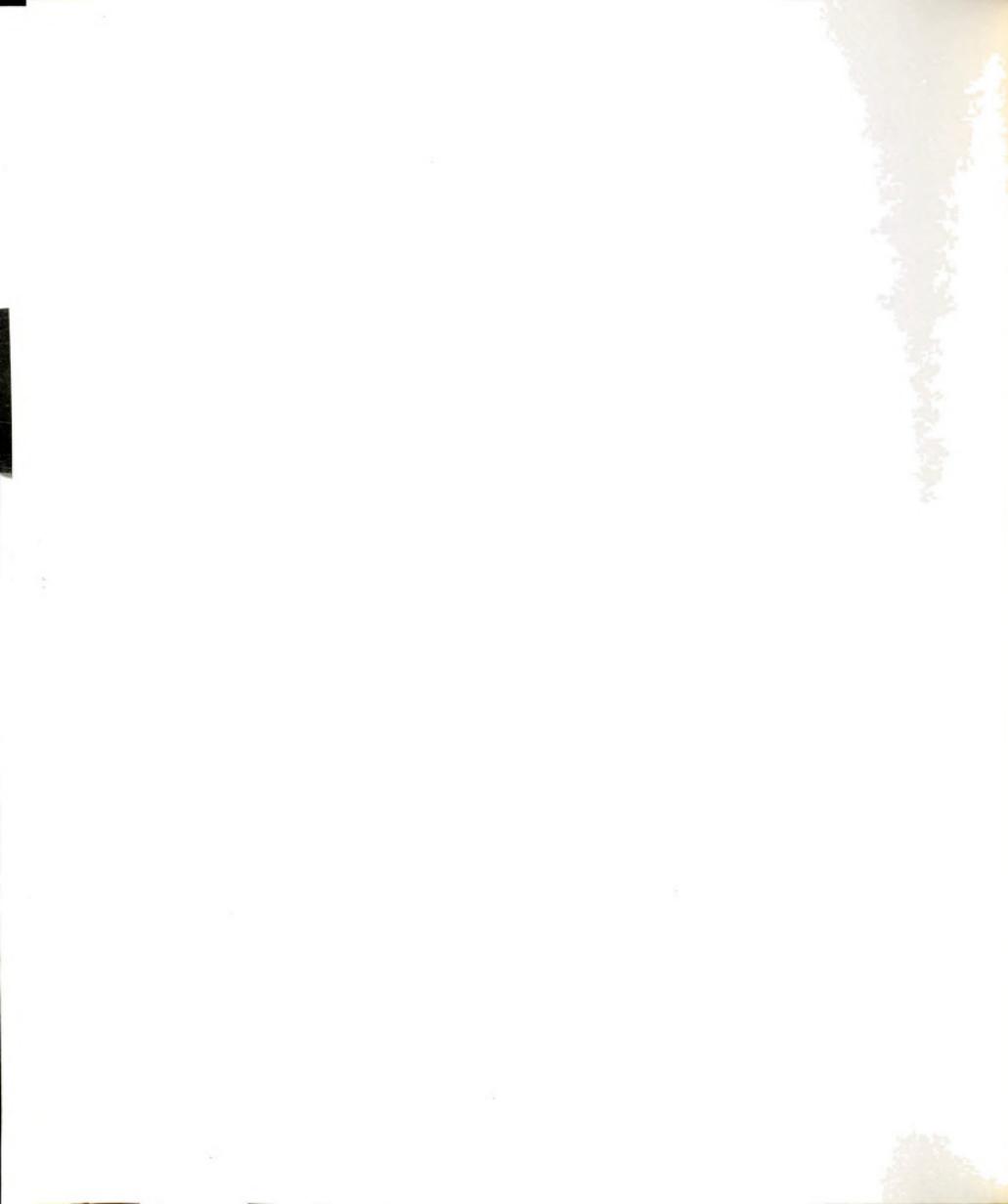


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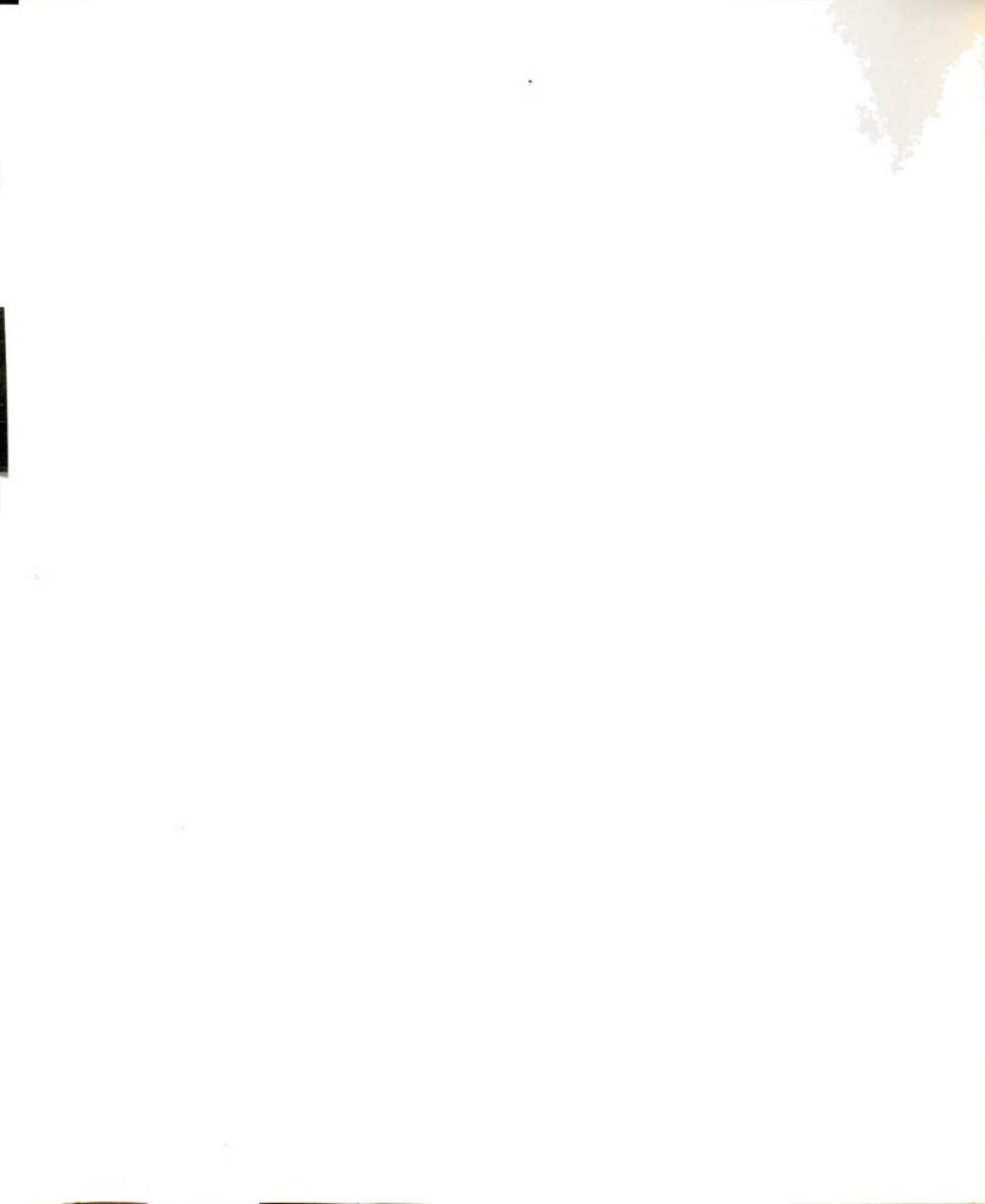
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SECTION TWO

WOUND ETHYLENE PRODUCTION BY FRUIT PERICARP

TISSUE OF RIN MUTANT TOMATO

(LYCOPERSICON ESCULENTUM MILL.)

II. RESPONSE TO APPLIED ACC



## II. RESPONSE TO APPLIED ACC

### ABSTRACT

ACC stimulation of wound ethylene production by fruit pericarp tissue of rin mutant tomato (Lycepersicon esculentum Mill.) was studied in relation to time of ACC application, stage of fruit development, role of the intact epidermis, disks from different parts of the fruit, temperature, inhibitors and anaerobiosis. The later ACC was applied after cutting, the greater the stimulation of wound ethylene production. Severly damaged disks resulting from freezing lost their ability to convert ACC to ethylene. Disks from immature-green fruits gave a greater response to applied ACC than disks from mature-green fruits. Disks from the blossom end of the fruit showed a lag period and gave less response to applied ACC than disks from the stem end and equator of the fruit. Epidermis of pericarp tissue appeared to be the location of enzyme(s) required for the conversion of ACC to ethylene. Temperatures of 10<sup>0</sup> and 40<sup>0</sup>C inhibited ethylene production similarly by disks treated and non-treated with ACC. However, the recovery of ethylene production of disks transferred from 10<sup>0</sup> or 40<sup>0</sup>C to 20<sup>0</sup>C was different. Rhizobitoxine or cycloleucine given alone inhibited wound ethylene production but given along with ACC stimulated more wound ethylene production. Cycloheximide inhibited ethylene production by disks treated and non-treated with ACC. Anaerobiosis blocked the conversion of ACC to ethylene.



INTRODUCTION

Methionine has been shown to be the in vivo precursor of ethylene biosynthesis in both wounded and senescent tissues including flowers, fruits, leaves, stems and roots (1,3,5,8,11,12,14). Wounded tissues convert only C-3 and C-4 of methionine to ethylene (1,11,14) and the conversion of methionine to ethylene is inhibited by rhizobitoxine, its analog, aminovinylglycine (AVG) and cycloheximide (1,2,11,14). Recently, Adams and Yang (2,3) have proposed S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) as intermediates in the conversion of methionine to ethylene. Subsequent work has led to the isolation of the ACC-forming enzyme from tomato fruit tissue (7) and an ACC-dependent ethylene forming system from pea stems (17). The biosynthetic pathway of ethylene is thought to proceed as follows: methionine  $\rightarrow$  SAM  $\rightarrow$  ACC  $\rightarrow$  C<sub>2</sub>H<sub>4</sub>. In this scheme, AVG, a well-known inhibitor of ethylene biosynthesis (19), has been shown to block the formation of ACC (2,7,16). ACC formation from SAM is thought to be the rate-limiting step of ethylene production in plant tissues (3).

Gonzalez et al. (9) reported that levels of free methionine are the same for mature-green fruit in rin mutant and wild-type tomatoes and they suggested that the methionine level does not appear to be the limiting factor for ethylene production in rin fruit. Herner and Sink (13, also see Section One) demonstrated that wounding stimulated ethylene production by rin fruit tissue similar to that in wild-type fruit tissue. McGlasson et al. (22) found that there are no substantial differences between rin and wild-type tomatoes in capacity for the production of ethylene and carbon dioxide from labeled methionine by freshly cut disks of fruit tissue. Recently, Boller et al. (7) showed that fruits of rin



tomato which only produce low levels of ethylene, contained much lower levels of ACC and of the ACC-forming enzyme activity than wild-type tomato fruits of comparable age. Furthermore, they found that applied ACC greatly increased ethylene production in fruit tissue of rin tomato, suggesting that in rin tomato fruit, the rate-limiting step in ethylene biosynthesis is not the one between ACC and ethylene. This paper reports the characteristics of wound ethylene production by fruit pericarp tissue of rin tomato in response to applied ACC.

#### MATERIALS AND METHODS

##### Plant Material and Incubation

Plants of rin and wild-type tomatoes (Lycopersicon esculentum Mill.) were grown as previously described (see Section One). Disks of pericarp tissue (diameter: 1.5 cm; thickness: 0.25 cm) from immature- and mature-green fruits ( $25 \pm 1$  and  $40 \pm 1$  days after anthesis for immature- and mature-green fruits, respectively) were prepared from the equatorial part of tomato fruits using a cork borer and template. The experiments with pericarp tissue from different parts of the fruit were carried out using disks from the stem and blossom end of mature-green fruits. The rate of ACC-stimulated ethylene production was studied using a static system. Single disks of pericarp tissue were placed with epidermis on a layer of glass beads in 20 ml scintillation vials. Vials were flushed with ethylene-free air and sealed with a serum cap then incubated in darkness at 20°C.



### Chemical Treatments

Twenty  $\mu$ l of chemical solution(s) or distilled water (control) were applied to the cut surface of disks opposite the epidermis as previously described (see Section One).

### Oxygen Dependency

The requirement of  $O_2$  for ACC-stimulated ethylene production was studied using a single disk enclosed in a 20 ml scintillation vial. The vials were purged with  $N_2$  to remove all  $O_2$ . At the end of every 2 h period, 1 ml of the gaseous content of the vials was withdrawn by a syringe and analyzed for ethylene.

### Ethylene Determination

At the end of every 2 h period, wound ethylene production by disks was withdrawn with a 1 ml gas tight syringe through the serum cap, was assayed on a gas chromatograph and calculated as previously described (see Section One). The vials were then flushed with ethylene-free air, sealed and returned to the incubation conditions. Each experiment consisted of five to six disks and was repeated three times. Data represent mean values.

## RESULTS

### Time of ACC Application

ACC applied to the cut surface of disks at different times after cutting caused different ethylene production (Figure 12). ACC applied to disks 4 h after cutting gave the least response 2 h after application while ACC applied to disks 12 h after cutting gave the most response 2 h after application. The maximum rates of ethylene production by disks in



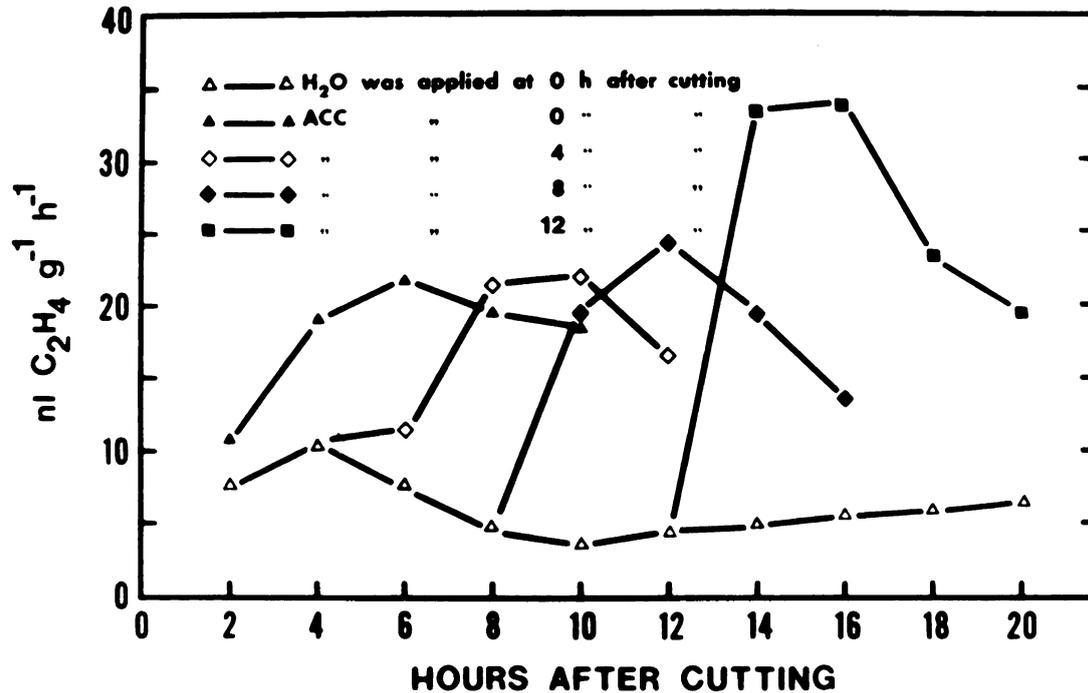


Figure 12. Effect of ACC application time on the stimulation of wound ethylene production by disks from mature-green fruits of rin tomato. Twenty  $\mu$ l of H<sub>2</sub>O at 0 h and 1 mM ACC were applied to the cut surface of disks at 0, 4, 8, or 12 h after cutting.

response to applied ACC at 0, 4, and 8 h after cutting were similar while the maximum rate of ethylene production by disks was in response to applied ACC 12 h after cutting. However, when ACC was applied to severely damaged disks which had been frozen for 1 h at 0 or 12 h after cutting there was little stimulation of ethylene production (Figure 13). Severely damaged disks not only lost the ability to convert ACC to ethylene but also lost their ability to produce wound ethylene as compared to non-frozen disks.

#### Stage of Fruit Development

Disks from immature- and mature-green fruits of rin tomato (Figure 14) gave similar results to those from the wild-type tomato (Figure 15) in response to applied ACC. Ethylene production by disks from

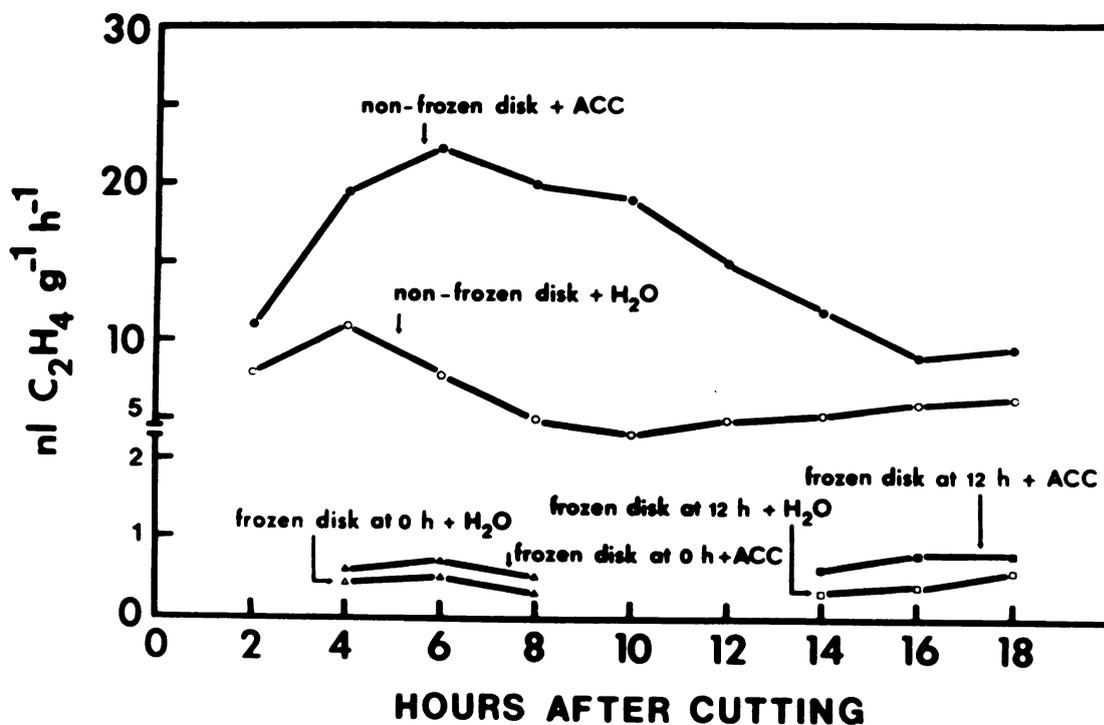


Figure 13. Effect of ACC application time on wound ethylene production by severely damaged disks from mature-green fruits of rin tomato. Disks were frozen for 1 h at 0 h or 12 h after cutting and returned to 20°C. After return to 20°C for 1 h, disks were blotted with paper towels and 20  $\mu$ l of H<sub>2</sub>O or 1 mM ACC were applied to the cut surface.

immature-green fruits was stimulated more than those from mature-green fruits in response to applied ACC.

#### Disks from Different Parts of the Fruit

Figure 16 shows that the rates of ACC-stimulated ethylene production by disks from the stem end and equator of the fruit were comparable throughout the incubation period. In contrast, disks from the blossom end produced ethylene at rates about one half of those from the stem end and equator in response to applied ACC. The ethylene production in response to applied ACC by disks from the blossom end showed a lag period. The stimulatory effect of applied ACC was not shown until 6 h after application and increased until the end of incubation period (12 h). The rates of wound ethylene production by disks without ACC treatment



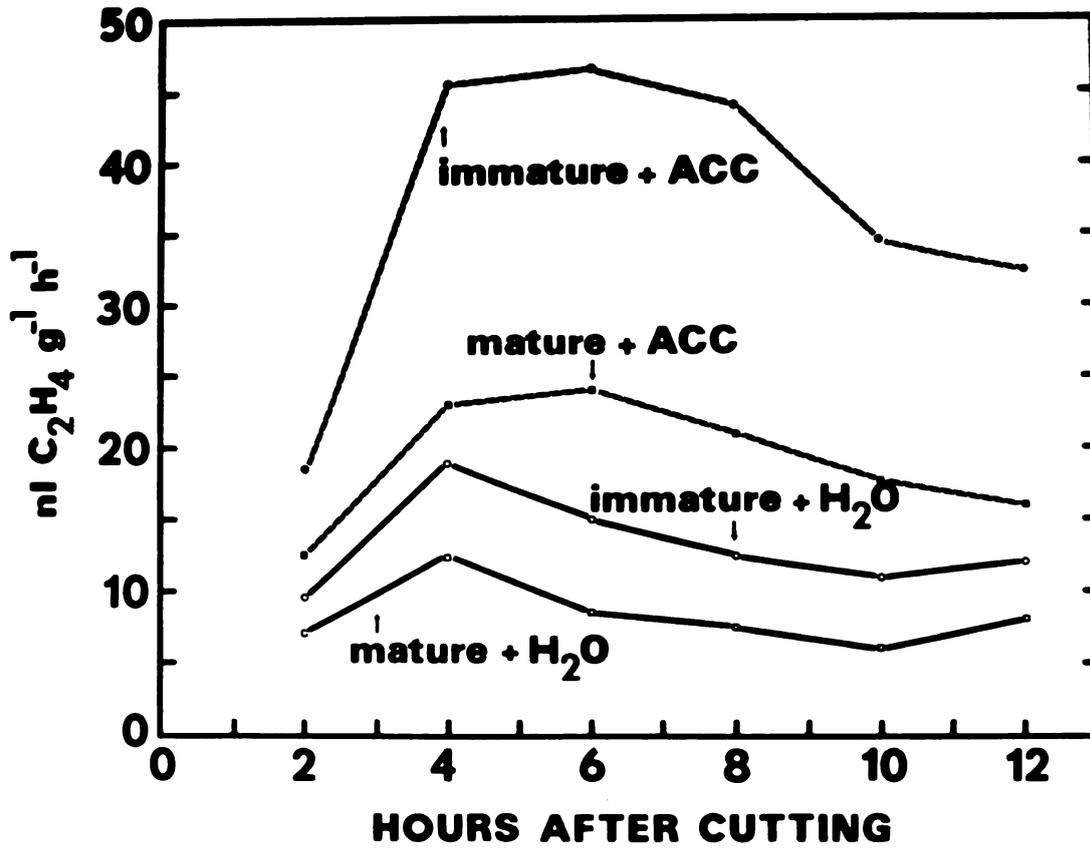
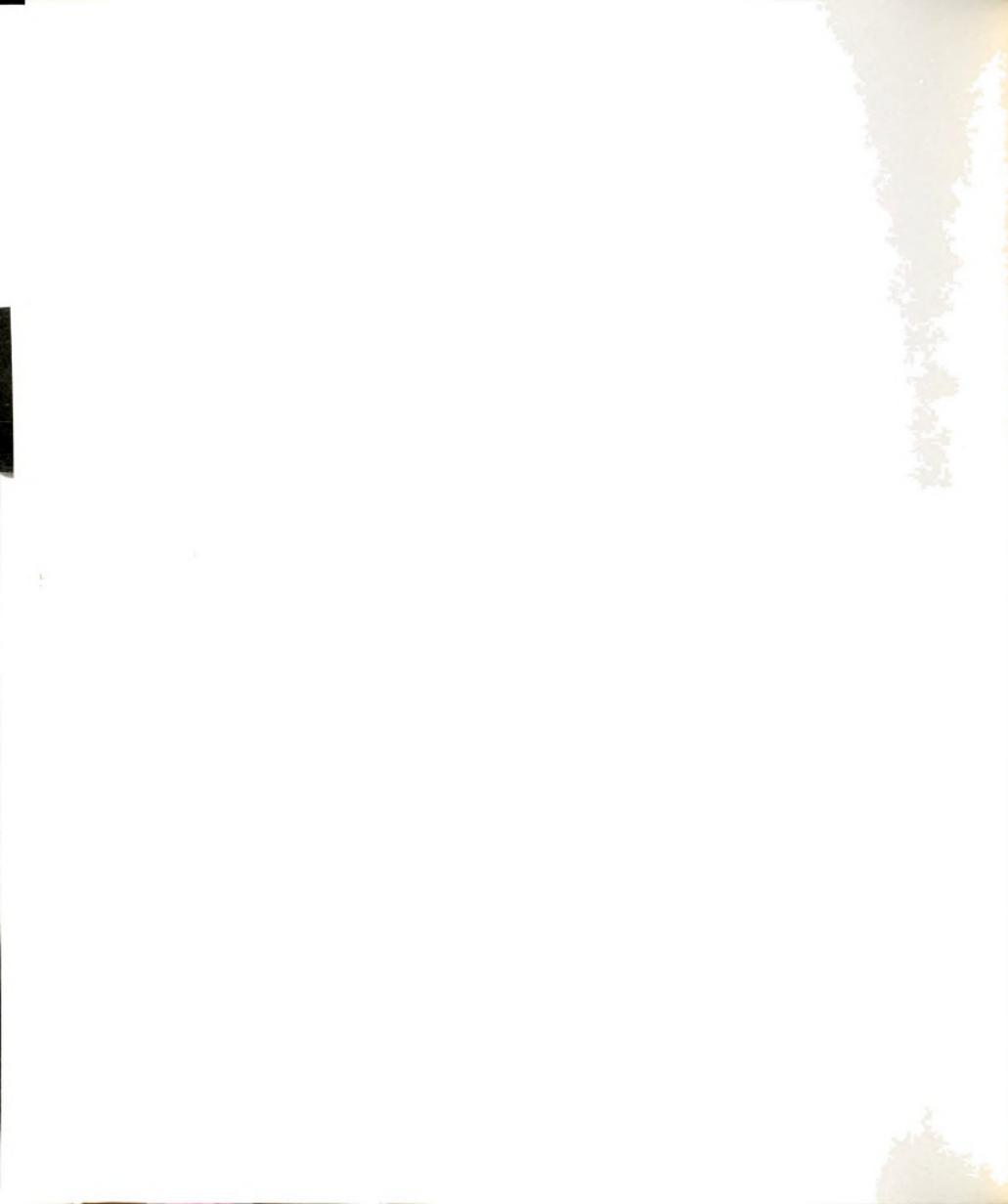


Figure 14. Effect of stage of fruit development of rin tomato on ACC-stimulated ethylene production. Twenty  $\mu$ l of H<sub>2</sub>O or 1 mM ACC were applied to the cut surface of disks from immature- and mature-green fruits at 0 h after cutting.



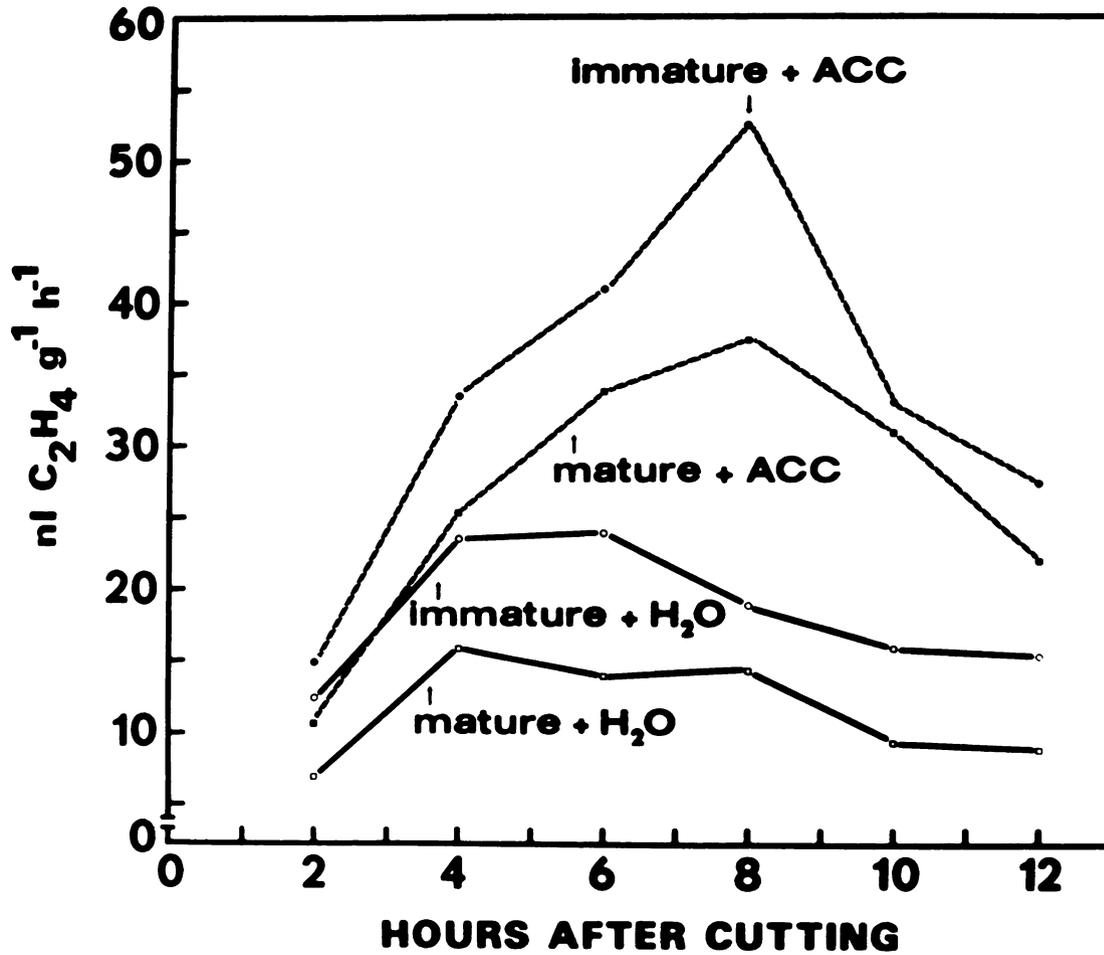


Figure 15. Effect of stage of fruit development of wild-type tomato on ACC-stimulated ethylene production. Twenty  $\mu$ l of H<sub>2</sub>O or 1 mM ACC were applied to the cut surface of disks from immature- and mature-green fruits at 0 h after cutting.



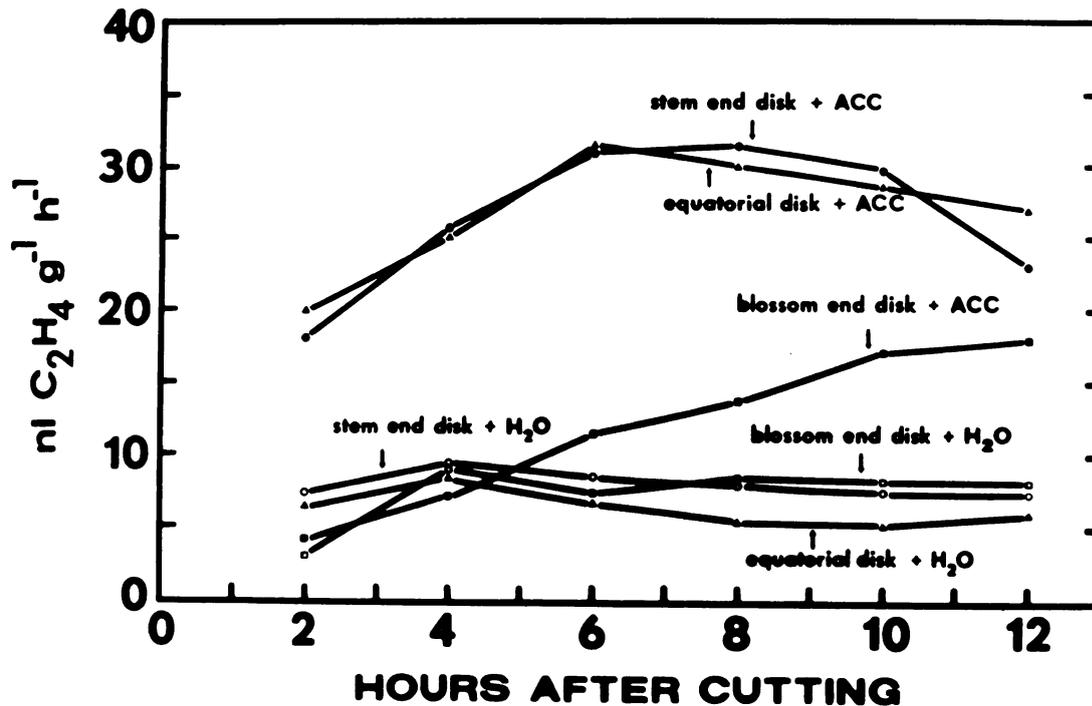
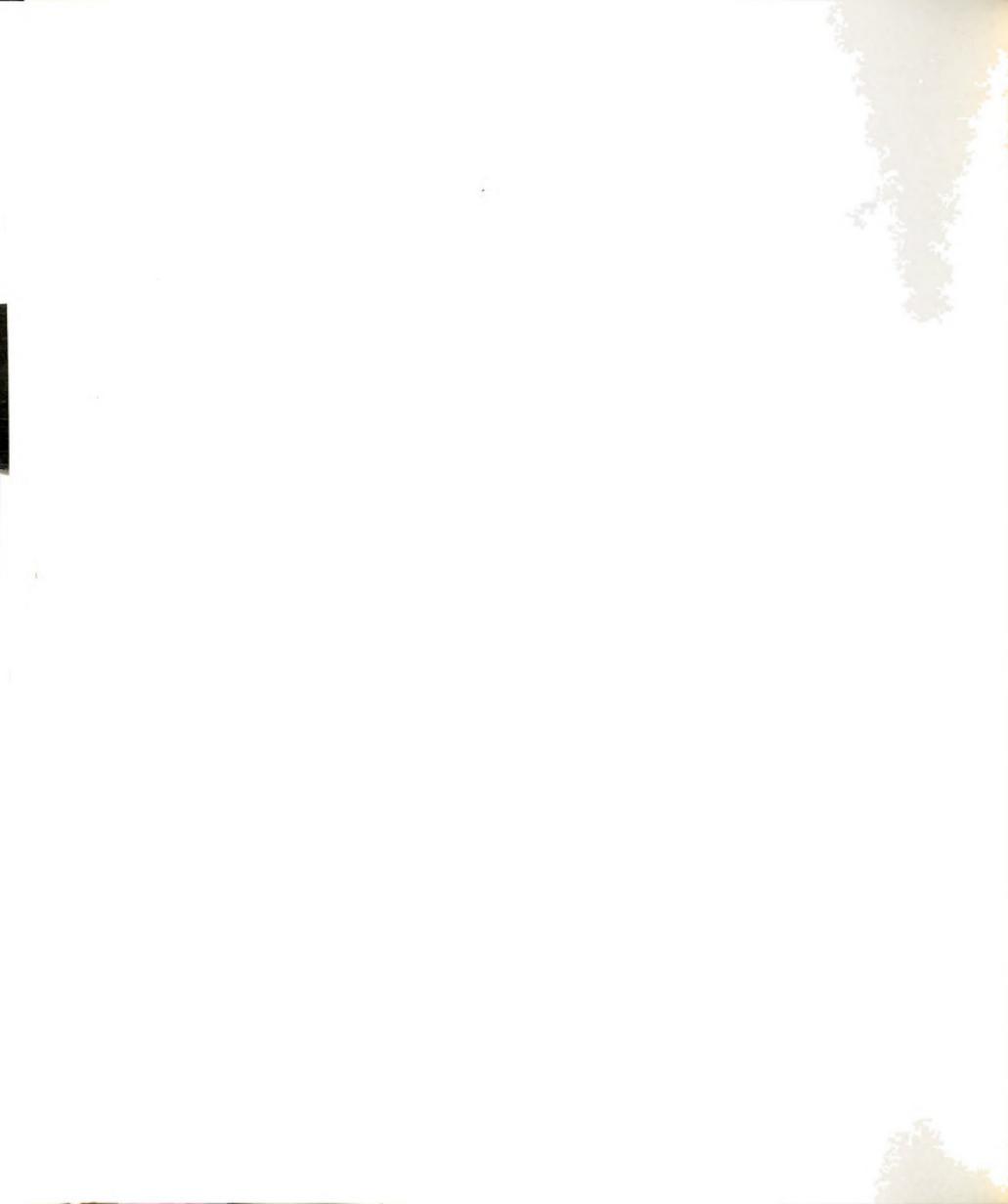


Figure 16. Effect of disks from different parts of mature-green fruits of *rin* tomato on ACC-stimulated ethylene production. Twenty  $\mu$ l of H<sub>2</sub>O or 1 mM ACC were applied to the cut surface of disks originating from the blossom end, equator and stem end of fruits at 0 h after cutting.

were similar except the first 2 h after cutting. When disks from the blossom end had been incubated for 12 h after cutting before ACC treatment showed less response to applied ACC than those disks from the equator, but there was no lag period in response to ACC application like freshly cut disks (Figure 17).

#### Role of Epidermis

Freshly cut disks with intact epidermis were stimulated to produce more ethylene than were disks without epidermis in response to applied ACC (Figure 18). However, disks which had been incubated for 12 h showed the role of intact epidermis more clearly than did freshly cut disks (Figure 19).



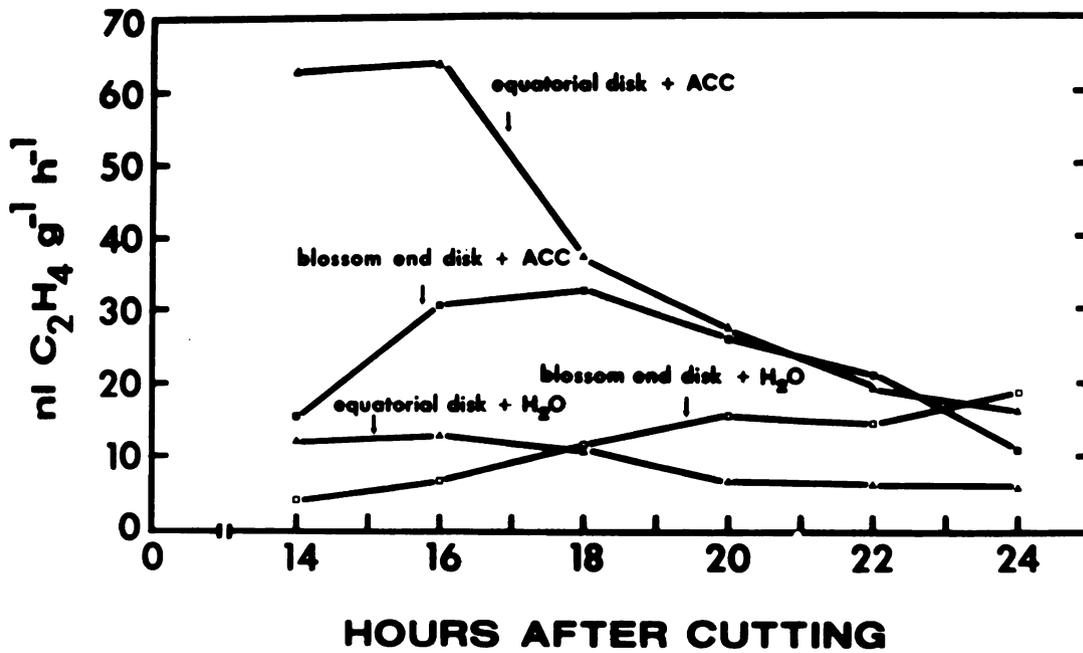


Figure 17. Effect of ACC on stimulation of ethylene production by disks from the blossom end and equator of mature-green fruits of rin tomato. Twenty  $\mu\text{l}$  of  $\text{H}_2\text{O}$  or 1 mM ACC were applied to the cut surface of disks which had been incubated on a layer of glass beads with water underneath in petri dishes for 12 h before being treated.

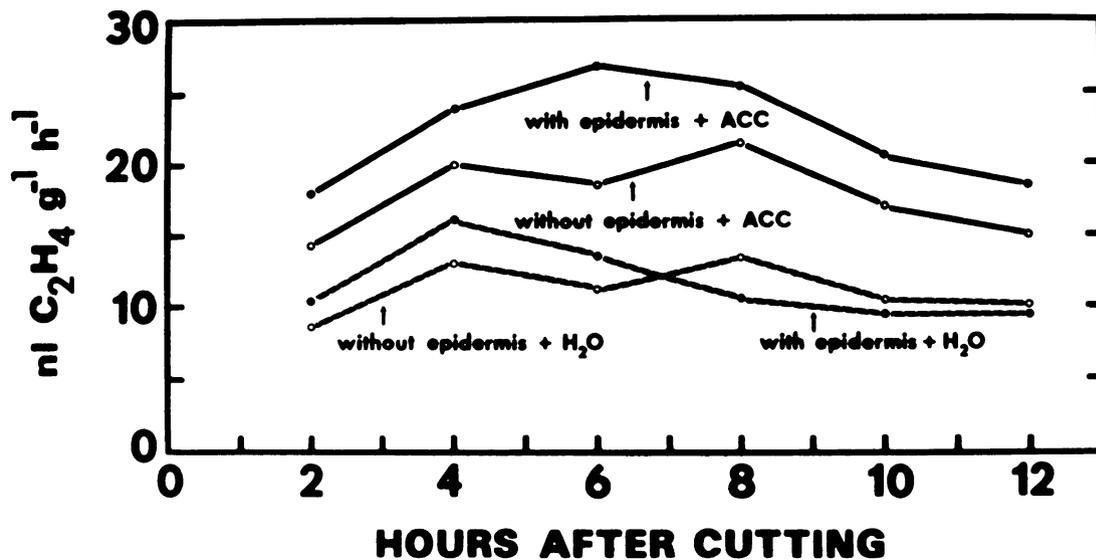


Figure 18. Role of the epidermis of disks from mature-green fruits of rin tomato on ACC-stimulated ethylene production. Disks without epidermis were prepared in the same way as disks with epidermis, then the cut surface of the proximal side was covered with vaseline. Twenty  $\mu\text{l}$  of  $\text{H}_2\text{O}$  or 1 mM ACC were applied to the cut surface of disks.



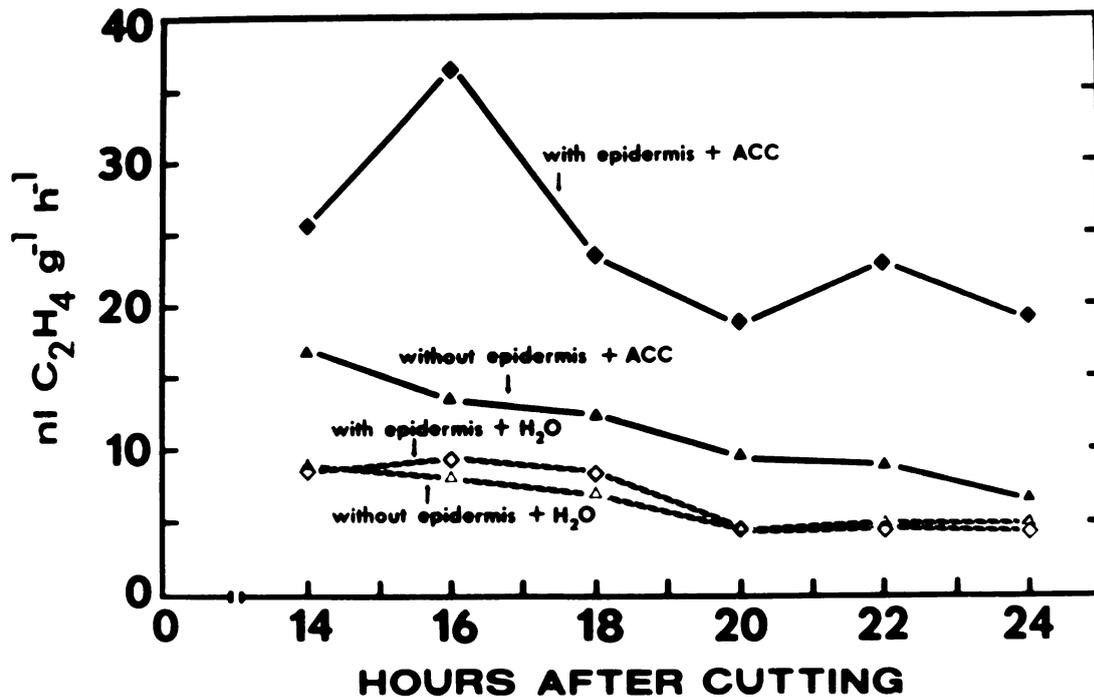
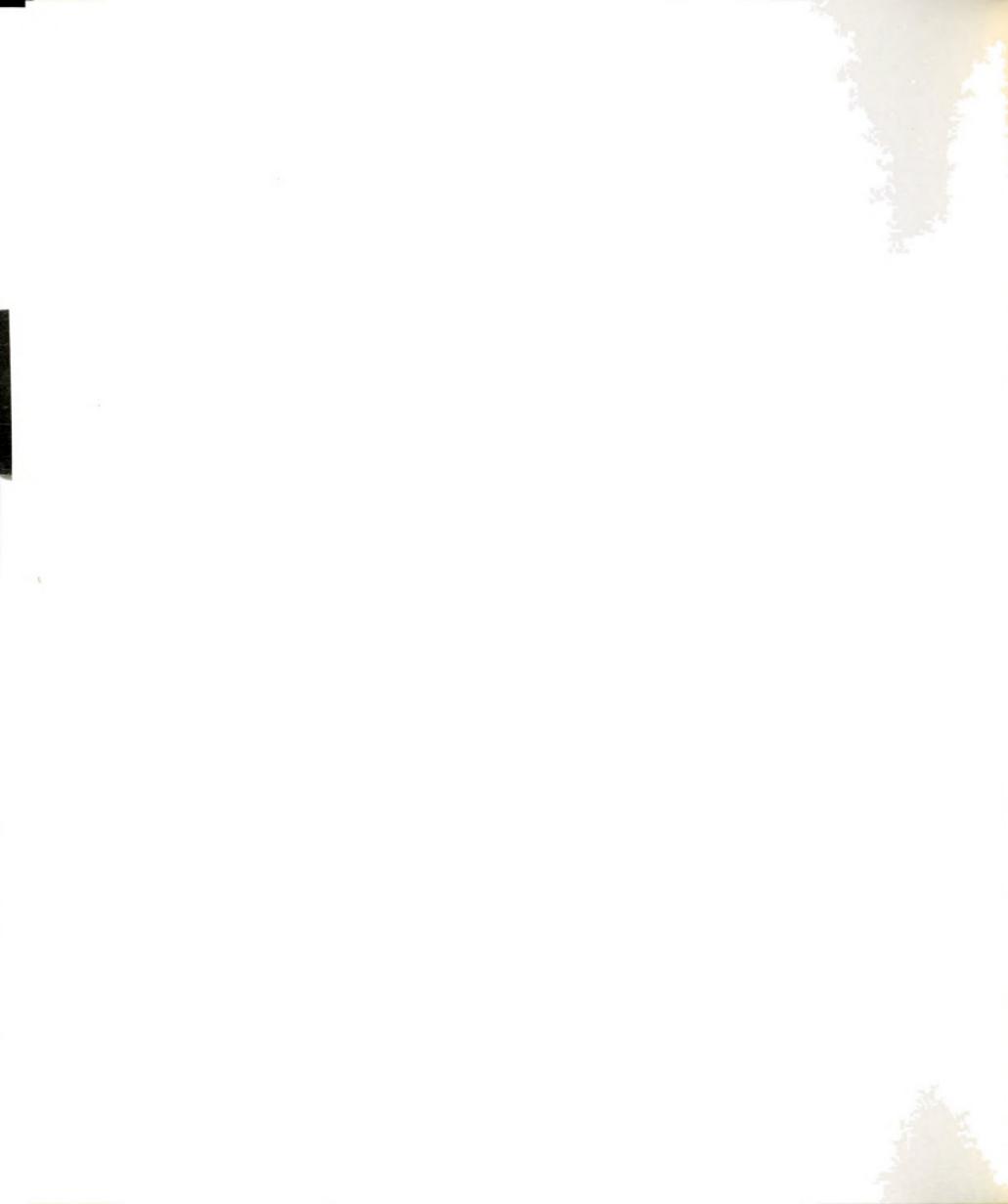


Figure 19. Role of the epidermis of incubated disks from mature-green fruits of *rin* tomato on ACC-stimulated ethylene production. Disks without epidermis were prepared and incubated as previously described in Figures 18 and 17, respectively. At the end of incubation period 20  $\mu$ l of H<sub>2</sub>O or 1 mM ACC were applied to the cut surface of disks.

#### Effect of Temperature

Among temperatures from 10<sup>o</sup> to 40<sup>o</sup>C used (Figure 20), 30<sup>o</sup>C stimulated the most ethylene production in both disks with and without applied ACC. The rates of wound ethylene production by disks with and without applied ACC were similar at temperatures of 10<sup>o</sup> and 40<sup>o</sup>C. The rate of ethylene production began to decrease readily after disks were exposed to 10<sup>o</sup> and 40<sup>o</sup>C.

However, the inhibitory effect of temperatures at 10<sup>o</sup> and 40<sup>o</sup>C seemed to be different for wound ethylene production by disks with and without applied ACC. The longer the disks were held at 10<sup>o</sup>C, the longer the time required for the rate of ethylene production to increase in response to applied ACC (Figure 21). The rate of wound ethylene production by disks with and without applied ACC and held at 40<sup>o</sup>C for 2 h



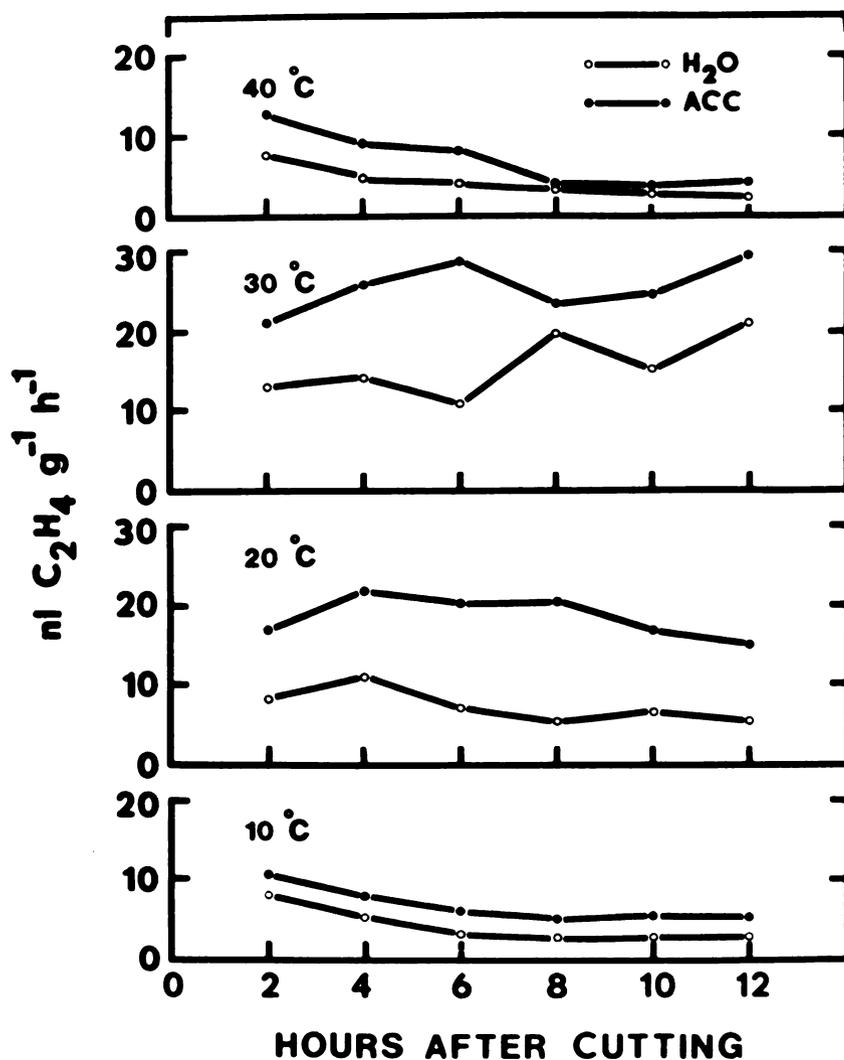


Figure 20. Effect of temperature on ACC-stimulated ethylene production of disks from mature-green fruits of rin tomato. Twenty  $\mu$ l of H<sub>2</sub>O or 1 mM ACC were applied to the cut surface of disks at 0 h after cutting, disks were then incubated at 10<sup>o</sup>, 20<sup>o</sup>, 30<sup>o</sup>, or 40<sup>o</sup>C.



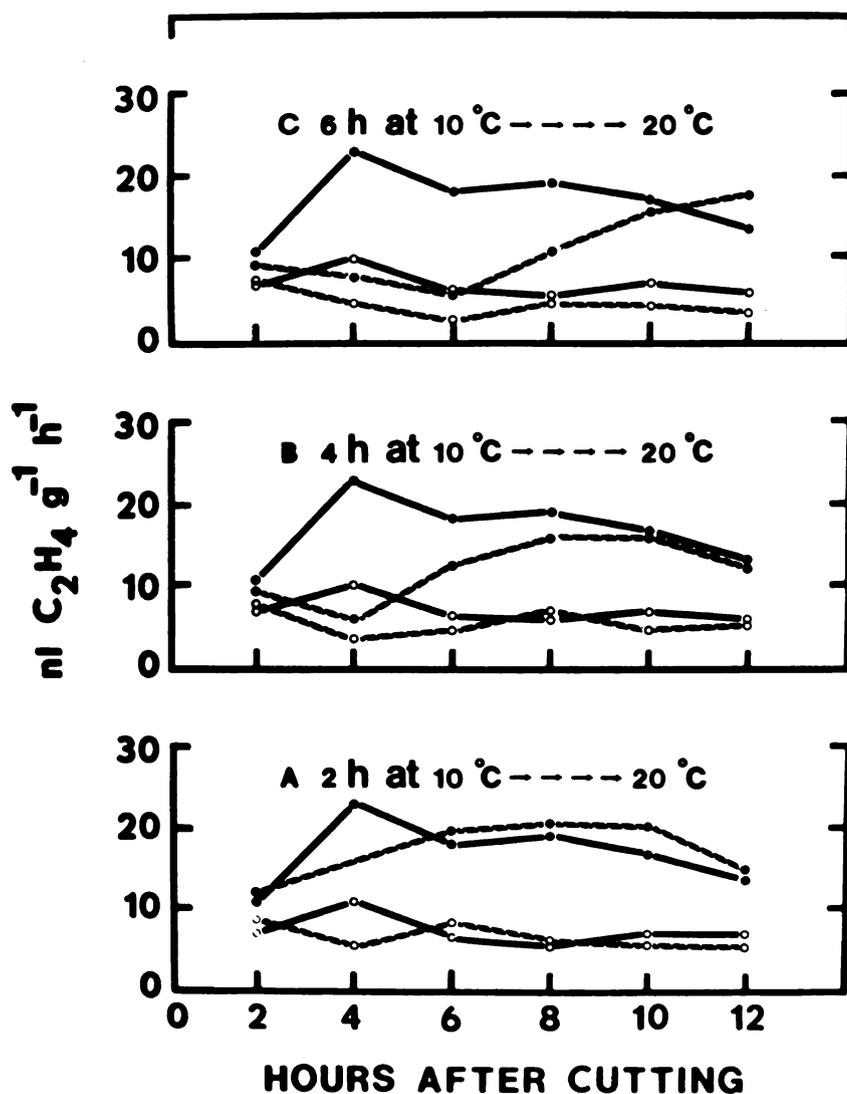


Figure 21. Recovery from the inhibitory effect of 10°C on ACC-stimulated ethylene production by disks from mature-green fruits of rin tomato. Disks were prepared and treated with 20  $\mu\text{l}$  of H<sub>2</sub>O or 1 mM ACC, then held for 2 h (A), 4 h (B), or 6 h (C) at 10°C then removed to 20°C : H<sub>2</sub>O treatment, held at 10°C, then transferred to 20°C (○—○), ACC treatment, held at 10°C, then transferred to 20°C (●—●), H<sub>2</sub>O (○—○) and ACC (●—●) treatment, held at 20°C.



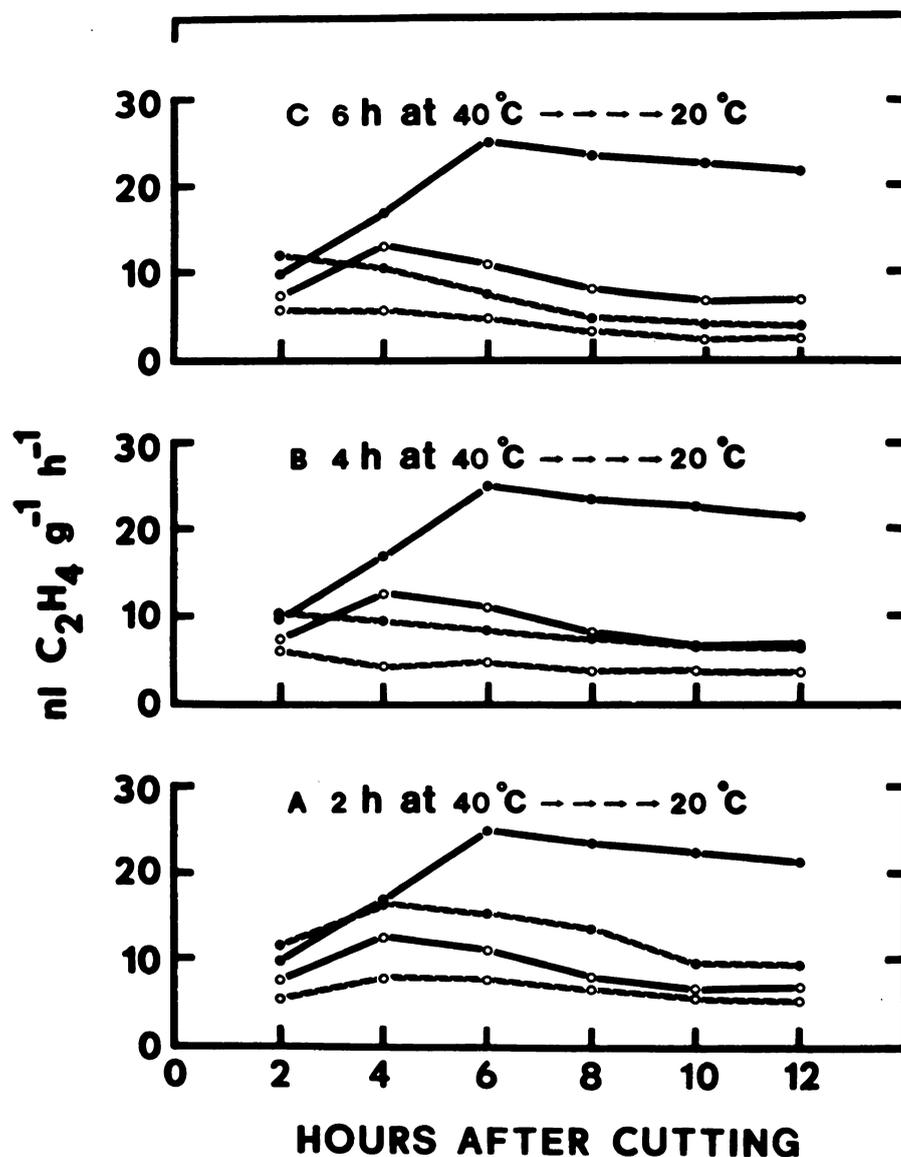


Figure 22. Recovery from the inhibitory effect of 40°C on ACC-stimulated ethylene production by disks from mature-green fruits of *rin* tomato. Disks were prepared and treated with 20  $\mu\text{l}$  of H<sub>2</sub>O or 1 mM ACC, then held for 2 h (A), 4 h (B), or 6 h (C) at 40°C then removed to 20°C : H<sub>2</sub>O treatment, held at 40°C, then transferred to 20°C (○---○), ACC treatment, held at 40°C, then transferred to 20°C (●---●), H<sub>2</sub>O (○—○) and ACC (●—●) treatment, held at 20°C.

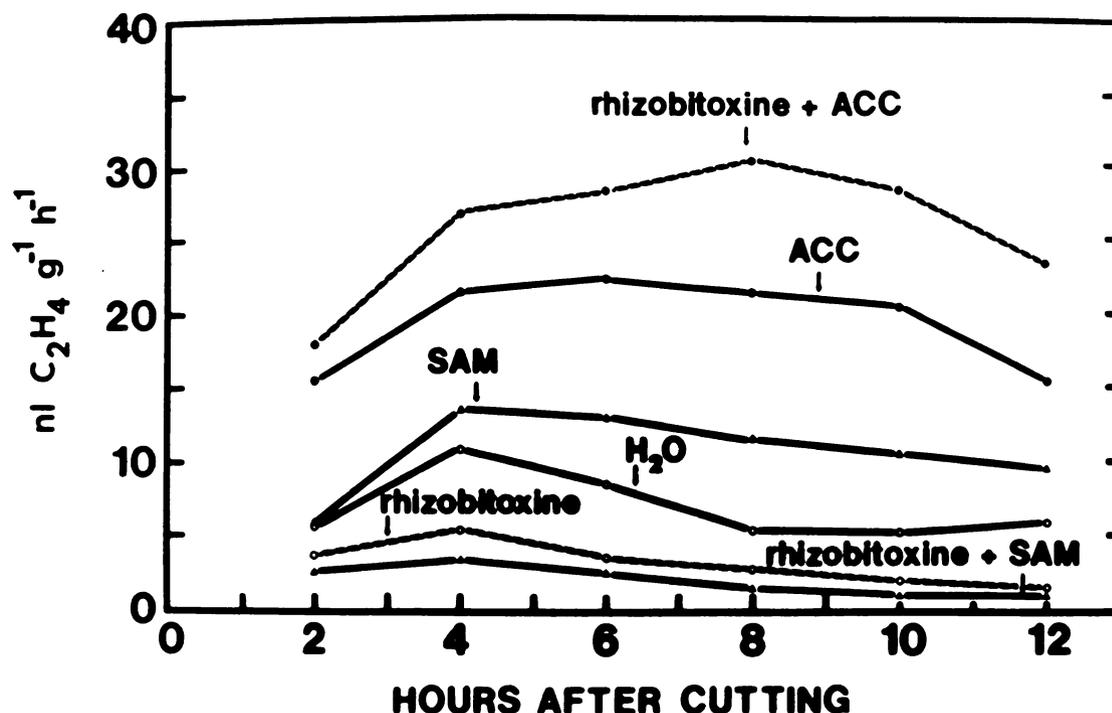


Figure 23. Effect of rhizobitoxine on SAM- and ACC-stimulated ethylene production by disks from mature-green fruits of rin tomato. Twenty  $\mu$ l of H<sub>2</sub>O, 1 mM SAM, 1 mM ACC, 1 mM rhizobitoxine, 1 mM of SAM + rhizobitoxine, or 1 mM of ACC + rhizobitoxine were applied to the cut surface of disks at 0 h after cutting.

showed less recovery than those disks held at 10°C. When the incubation period at 40°C was extended to 4 or 6 h the rates of ethylene production continued to decline after return to 20°C as compared to those which were held throughout at 20°C (Figure 22).

#### Effect of Inhibitors

Figure 23 shows that SAM applied to disks stimulated ethylene production but less than ACC. The response to applied SAM showed a lag period while ACC did not. Rhizobitoxine completely inhibited ethylene production stimulated by applied SAM but it stimulated more ethylene production when it was applied together with ACC. Cycloleucine inhibited wound ethylene production (Figure 24), but it did not inhibit the SAM-stimulated ethylene production. Cycloleucine stimulated more ethylene production when it was applied together with ACC. Cycloheximide



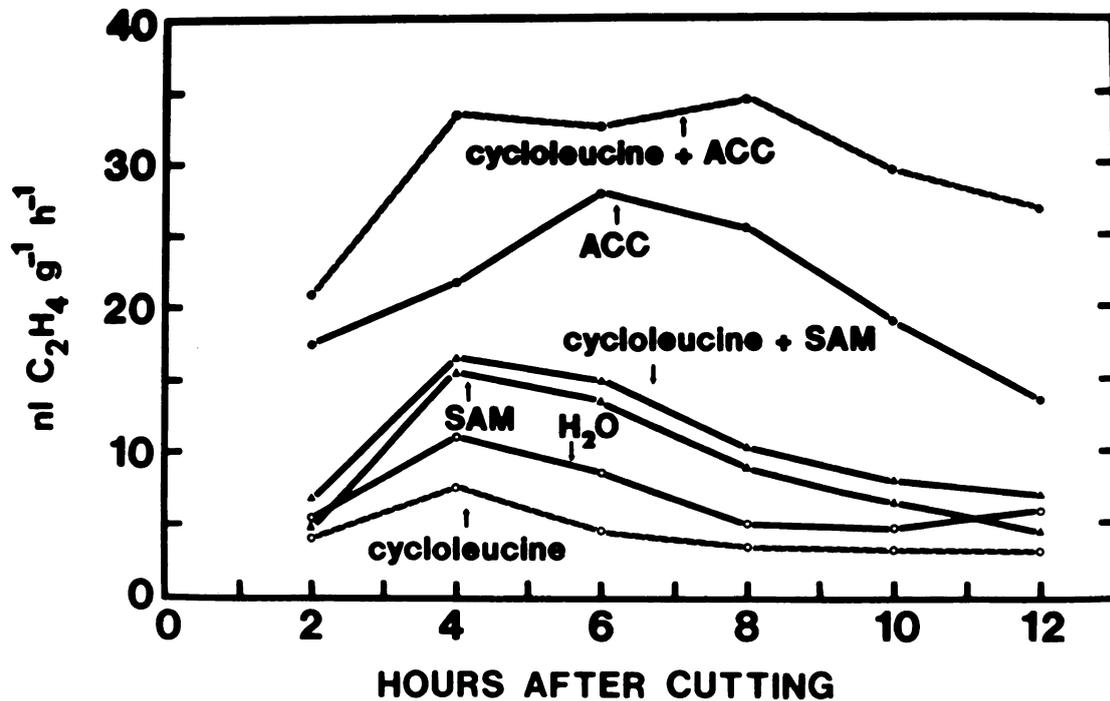


Figure 24. Effect of cycloleucine on SAM- and ACC-stimulated ethylene production by disks from mature-green fruits of *rin* tomato. Twenty  $\mu$ l of H<sub>2</sub>O, 1 mM SAM, 1 mM ACC, 10 mM cycloleucine, 1 mM of SAM + 10 mM cycloleucine, or 1 mM of ACC + 10 mM cycloleucine were applied to the cut surface of disks at 0 h after cutting.

inhibited wound ethylene production similar to the other inhibitors, but instead of increasing the stimulatory effect of ACC, it inhibited the stimulation (Figure 25).

#### Effect of Anaerobiosis

Figure 26 shows that anaerobiosis inhibited almost completely the stimulation of ethylene production by applied ACC. Upon introduction to air the rates of ethylene production by disks with and without applied ACC were about the same as those disks held throughout in air.

#### DISCUSSION

The later ACC was applied to disks after cutting the more ethylene production was stimulated. The effect of differences in absorption of ACC can be ruled out because 2 h after its application, all ACC solution



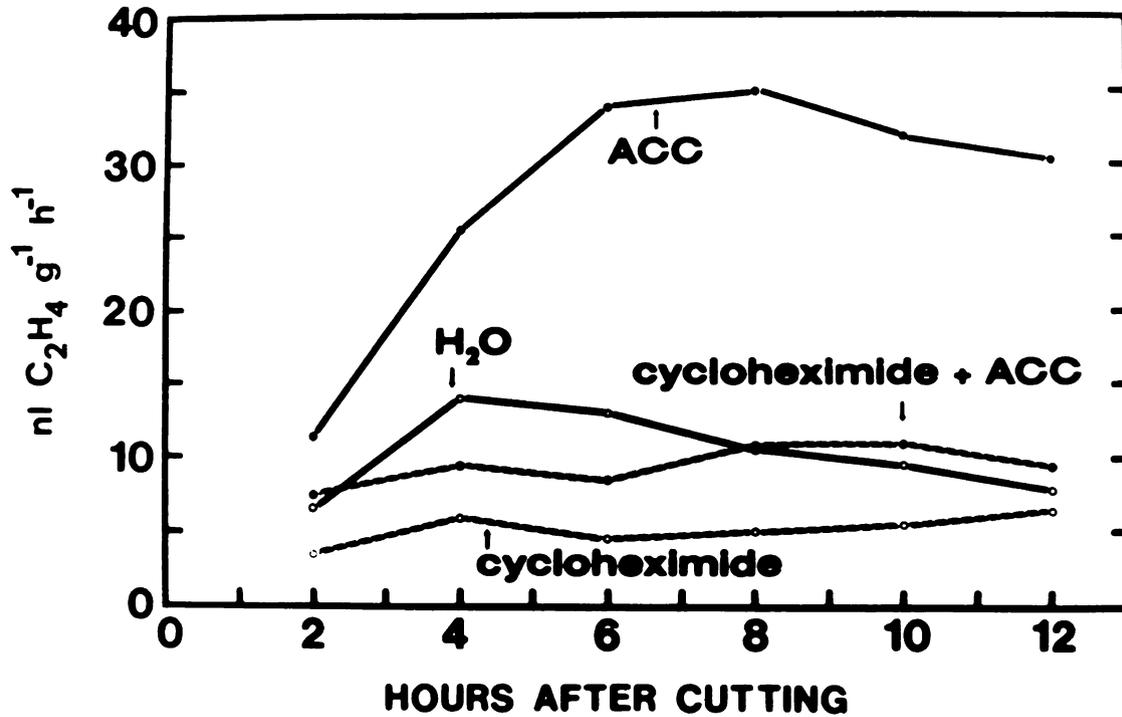


Figure 25. Effect of cycloheximide on ACC-stimulated ethylene production by disks from mature-green fruits of rin tomato. Twenty  $\mu$ l of H<sub>2</sub>O, 0.1 mM cycloheximide, 1 mM ACC, or 1 mM of ACC + 0.1 mM cycloheximide were applied to the cut surface of disks at 0 h after cutting.

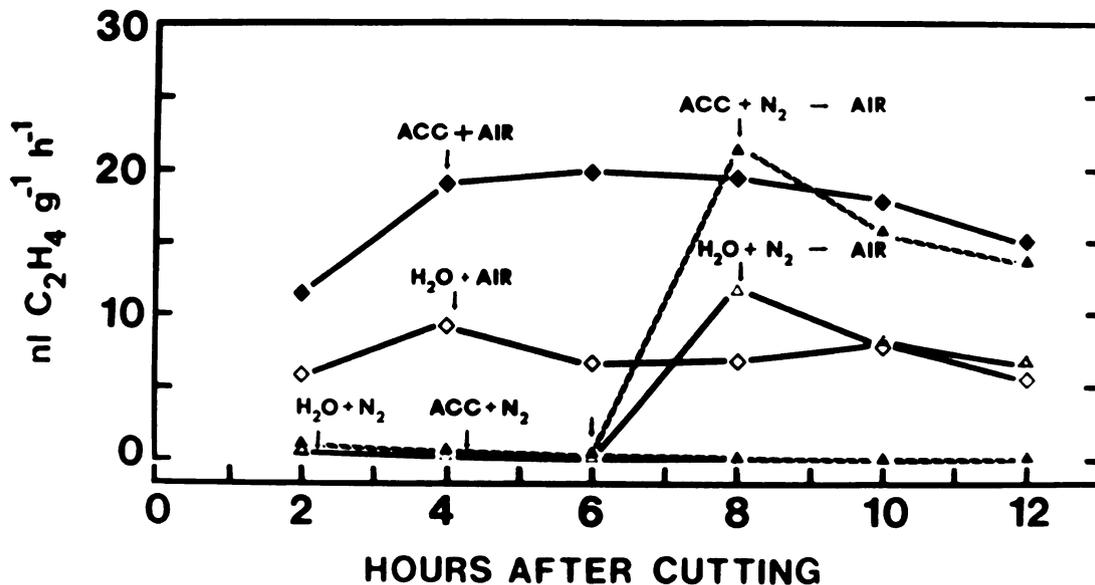


Figure 26. Effect of anaerobiosis on ACC-stimulated ethylene production by disks from mature-green fruits of rin tomato. Twenty  $\mu$ l of H<sub>2</sub>O or 1 mM ACC were applied to the cut surface of disks at 0 h after cutting and incubated throughout in N<sub>2</sub> atmosphere or returned to air at the time designated by the arrow. This is compared to disks with and without ACC treatment and incubating throughout in air.



had completely disappeared from the cut surface of all disks. As the disks passed through the time of maximum wound ethylene production, it would be expected that the endogenous levels of ACC would be decreased (26, also see Section Five). This would lower the ACC pool of disks and when ACC was applied it would be converted more effectively to ethylene than when it was applied earlier. One may argue that since the rates of wound ethylene production by disks without applied ACC at 8 and 12 h after cutting were the same, the difference of ACC levels at 8 and 12 h should not make a great difference in response to applied ACC. This has led to the question as to whether or not wounding can increase the activity of enzyme(s) required for the conversion of ACC to ethylene. If wounding indeed increases the activity of the enzyme(s) required for the conversion of ACC to ethylene, then applying ACC later to disks would be expected to stimulate more ethylene production.

ACC applied to disks which had been frozen and thawed at different times after cutting had little response to ACC and there was no difference because of time of ACC application. This could be due to inactivation of enzyme(s) responsible for conversion of ACC to ethylene or freezing may have caused the breakdown of compartmentation which would possibly bring inhibitors in contact with the enzyme(s) required for the conversion of ACC to ethylene (18). Konze and Kende (17) reported that the homogenates of tomato fruits failed to show the ability to convert ACC to ethylene.

Disks from immature-green fruits not only produced more wound ethylene than did disks from mature-green fruits, but also responded more to applied ACC. Suttle and Kende (25) also obtained similar results when immature sepals of Tradescantia flowers, which normally



produced little ethylene, produced more ethylene than mature sepals in response to applied ACC. Recently, it has been shown that auxin stimulated ethylene production by inducing the ACC-forming enzyme (16) but not the enzyme(s) required for the conversion of ACC to ethylene (17). Although differences in auxin may exist in immature- and mature-green fruits (15), auxin probably is not involved in increasing the ethylene production in response to ACC. Therefore, the difference in stimulation of ethylene production by applied ACC between disks from immature- and mature-green fruits may reflect their difference in cell number (also see Section One). Adams and Yang (4) suggested that the onset of ethylene production in ripening apple tissue results from the induction or activation of the enzyme system that converts SAM to ACC and ACC to ethylene because climacteric apple tissue can produce large amounts of ethylene when given ACC along with AVG but pre-climacteric apple tissue has little ability to produce ethylene even when given ACC. This suggests that the mechanism of induction in ripening apple may be different from ripening tomato, because tissue from both immature- and mature-green fruits of wild-type tomato can be stimulated to produce large amounts of ethylene in response to applied ACC. Although cut (wounded) tissue was used in these studies, there is no indication that the ethylene biosynthetic pathway might be different from intact fruit. The ripening of tomato probably results from the induction of the enzyme system required for the conversion of SAM to ACC but probably not the enzyme(s) for the conversion of ACC to ethylene (7).

Disks from the blossom end of fruits gave less response to applied ACC than those from the stem end and equator of fruits. This can not be explained in terms of their difference in cell number because the



rates of wound ethylene production without ACC application were almost the same from 4 h after cutting until the end of incubation period. The response to applied ACC by disks from the blossom end also showed a lag period. This suggests that tissue from the blossom end of fruits has lower activity of the enzyme(s) required for the conversion of ACC to ethylene than does tissue from the stem and equator of fruits and that the activity of this enzyme(s) may possibly be increased by wounding. This idea is supported by the fact that disks from the blossom end incubated for 12 h showed less response to applied ACC than did disks from the equator. However, further research needs to be done concerning this point.

The results from the experiments of freshly cut and incubated disks with and without epidermis in response to applied ACC clearly showed that three to four layers of compact cells close to the epidermis are more active in the conversion of ACC to ethylene than those cells farther from the epidermis. This strongly supports the idea that the compact cells close to the epidermis play an important role in wound ethylene production by freshly cut disks from rin tomato fruits (see Section One). Similarly, Sakai and Imaseki (24) showed that mechanical destruction or removal of the epidermis from the hypocotyl segments of etiolated mungbean seedlings caused complete loss of ethylene production in response to applied auxin. It is known that auxin stimulates ethylene production in plant tissues by inducing the ACC-forming enzyme (16) which is the rate-limiting step in ethylene biosynthesis (2). This suggests that the epidermis may be the site of the enzyme(s) required for the conversion of ACC to ethylene in hypocotyl segments of etiolated



mungbean seedlings and probably in other plant tissues like tomato pericarp tissue.

Both low and high temperatures have been shown to affect the rate of ethylene production in both wounded and senescent plant tissues (8,10, 21,23). Among tested temperatures, 30°C was optimum for ethylene production by disks with and without ACC treatment. Similarly, Konze and Kende (17) found that the optimum temperature for ethylene formation from ACC in homogenates of etiolated pea seedlings was 30°C. The rates of ethylene production by disks with and without ACC treatment were similar at temperatures of 10° and 40°C. This indicates that the enzyme systems for ethylene biosynthesis appear to be labile. However, the inhibitory effect of ethylene production induced by ACC by temperatures at 10° and 40°C may be different. The rates of ethylene production by disks with ACC treatment showed recovery after they were returned from 10° to 20°C while disks which were returned from 40° to 20°C showed little recovery. Holding the disks at 10°C may have reduced the rate of ACC-stimulated wound ethylene production by inhibiting the activity of the enzyme(s) required for the conversion of ACC to ethylene whereas 40°C may have reduced the rate of ACC-stimulated wound ethylene production by destroying the enzyme(s) required for the conversion of ACC to ethylene (27).

The ability of rhizobitoxine to inhibit wound ethylene production by disks both with and without SAM treatment indicates that the methionine and SAM pathway is operative in this wounded tissue from rin tomato fruits and similar to that found in the majority of wounded and senescent plant tissues (2,5,11,12,25). In tomato disks, applied SAM showed a lag period in ethylene production whereas ACC did not. The inability of



rhizobitoxine to inhibit the conversion of ACC to ethylene by disks is consistent with the hypothesis that rhizobitoxine inhibits the formation rather than the utilization of ACC (2,7). When ACC was applied together with rhizobitoxine more ethylene was formed by disks than when ACC was added alone. Cycloleucine also showed similar results to that of rhizobitoxine when it was applied alone or together with ACC. The mode of action of the inhibitory effect of cycloleucine on ethylene production may be different from that of rhizobitoxine. If cycloleucine inhibited ethylene production by competing with ACC as suggested by Baker et al. (6), then cycloleucine (1-aminocyclopentane-1-carboxylic acid) should reduce the stimulation of ethylene production when it was applied together with ACC. There was in fact a stimulation of ethylene production. It is possible that cycloleucine inhibits the conversion of methionine to SAM. When cycloleucine was applied together with SAM, there was no inhibition of SAM stimulation of ethylene production. Therefore, cycloleucine must inhibit methionine adenosyltransferase which converts methionine to SAM. Lombardini et al. (20) indeed showed that cycloleucine inhibits the activities of methionine adenosyltransferase in E. coli, rat liver and yeast. The ability of cycloheximide to inhibit ethylene production by both treated and non-treated disks with ACC indicates that cycloheximide may interfere at more than one site in the biosynthetic pathway. These results also indicate that sustained protein synthesis is a prerequisite for the ACC-forming enzyme required for wound-induced ethylene biosynthesis (26). It is not known whether or not cycloheximide inhibited the activity of the enzyme(s) required for the conversion of ACC to ethylene and/or inhibited the induction of this enzyme.



Anaerobiosis inhibited almost completely wound ethylene production by disks treated or not treated with ACC and this agrees with many studies with other plant tissues that oxygen is required for the chemical reaction(s) that converts ACC to ethylene (2,8,10). Konze and Kende (17) also demonstrated that the enzyme system from homogenates of etiolated pea seedlings can convert ACC to ethylene and it is totally dependent on oxygen. However, at the present time it is not known whether oxygen serves as a substrate for the conversion or an activator of the enzyme system.



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SECTION THREE

WOUND ETHYLENE PRODUCTION BY FRUIT PERICARP  
TISSUE OF RIN MUTANT TOMATO  
(LYCOPERSICON ESCULENTUM MILL.)

III. MECHANISM OF A NEGATIVE FEEDBACK



### III. MECHANISM OF A NEGATIVE FEEDBACK

#### ABSTRACT

A negative feedback or an autoinhibition of wound ethylene production by fruit pericarp tissue of rin mutant tomato (Lycopersicon esculentum Mill.) was studied in relation to time interval of gas sampling and flushing, number of disks per container, size of containers, treating disks with propylene and action of  $\text{Ag}^+$ . More wound ethylene was produced by disks flushed every 1 h, when only 1 disk per container was present, or when the disks were in a 50 ml container rather than a 20 ml container. Propylene treatment reduced the rate of wound ethylene production by disks and the initial rate of wound ethylene production upon return from propylene to air was similar to that of disks upon return from nitrogen to air but less pronounced. Propylene also inhibited the increased wound ethylene production by applied ACC. These suggest that wound ethylene may inhibit its own production by blocking the conversion of ACC to ethylene.  $\text{Ag}^+$  partially counteracted the inhibitory effect of propylene.  $\text{Ag}^+$  stimulated wound ethylene production by disks with and without applied ACC but rhizobitoxine prevented the stimulatory effect of  $\text{Ag}^+$ .



INTRODUCTION

Ethylene has been shown to have many physiological effects on plants (14). Burg and Burg (8,9) proposed that for ethylene to exert its regulatory effects, it must bind to a metallo-protein site in the cell which can serve in some regulatory manner. This theory is based on two principal lines of evidence: (1) the changes in biological activity with molecular structure of gases similar to ethylene are like the changes in absorption of the gases to heavy metals; and (2) the attachment of ethylene to a heavy metal is inhibited by carbon dioxide in a manner similar to the inhibition of many biological responses to ethylene. Burg and Burg (8,9) also suggested that oxygen is necessary for oxidation of the metal-receptor site to which ethylene becomes attached.

$\text{Ag}^+$  has been reported to counteract ethylene actions on plants such as leaf epinasty (4,7,17), leaf abscission (4,7,17), flower longevity (5,10), fruit ripening (18), chlorophyll loss (3), growth inhibition (5,6), and sex expression (4). It has been suggested that  $\text{Ag}^+$  replaces a metal at the metal-receptor site of ethylene resulting in inhibition of action (6). In fact,  $\text{Ag}^+$  has been reported to inhibit and stimulate ethylene production in many plant tissues (2,22,23,25).

Ethylene producing systems in plants can be categorized into three systems: System I is nonautocatalytic which operates in non-climacteric fruits (15) and exogenously applied ethylene or its analog, propylene, can not induce or trigger increased ethylene production by the fruit (12). System II is autocatalytic which operates in climacteric fruits (15) and other reproductive tissues such as flowers (13). Exogenously applied ethylene or its analog, propylene, can induce or trigger ethylene production by the flower and fruit (16,21). Finally, System III is



stress-induced ethylene which results from any factor including physical, chemical or biological stress (26). This system operates in any plant tissue which possesses either System I or II before they begin to senesce (11,12,19). Saltveit and Dilley (20) reported that the wound-induced ethylene producing system is nonautocatalytic. Vendrell and McGlasson (23) reported that a temporary ethylene treatment, sufficient to stimulate ripening in banana fruit tissue, partly suppressed endogenous ethylene production and the evolution of ethylene from labeled methionine. Zeroni and Galil (27) reported that the autoinhibition of ethylene production in immature fig fruit occurred immediately upon exposure to exogenous ethylene and continued for as long as exogenous ethylene was available. Recently, Aharoni and Lieberman (2) reported that when ethylene was added to mature green leaf disks, IAA and Ag<sup>+</sup> stimulation of the conversion of [<sup>14</sup>C] methionine to [<sup>14</sup>C] ethylene was inhibited. This paper reports evidence of a negative feedback of wound ethylene production in wounded tissue of rin tomato fruit.

#### MATERIALS AND METHODS

##### Plant Material and Incubation

Plants of rin tomato (Lycopersicon esculentum Mill.) were grown as previously described (see Section One). Disks of pericarp tissue (diameter: 1.5 cm; thickness: 0.25 cm) from mature-green fruits (40 ± 1 days after anthesis) were prepared from the equatorial part of tomato fruits using a cork borer and template. Unless noted otherwise, single disks of pericarp tissue were placed with the epidermis on a layer of glass beads in 20 ml scintillation vials. Vials were flushed with



ethylene-free air and sealed with a serum cap, then incubated in darkness at 20°C.

#### Chemical Treatments

Twenty  $\mu\text{l}$  of chemical solution(s) or distilled water (control) were applied to the cut surface of disks opposite the epidermis as previously described (see Section One).

#### Carbon Dioxide Determination

$\text{CO}_2$  production by disks was sampled similar to ethylene and determined using a gas chromatograph (Carle CG 8700) equipped with a thermal conductivity detector.

#### Propylene Treatment

The containers with disks were flushed with ethylene-free air and sealed with serum caps. Pure propylene was added through the serum cap to give a final concentration of 1000, 2000, or 5000  $\mu\text{l l}^{-1}$  propylene. At the end of every 2 h period, the gaseous content of containers was sampled and analyzed for ethylene.

#### Ethylene Determination

At the end of every 2 h period, wound ethylene production by disks was withdrawn with a 1 ml gas tight syringe and assayed using a gas chromatograph. The containers were then flushed with ethylene-free air, sealed and returned to the incubation conditions. Each experiment consisted of five to six disks and was repeated three times. Data represent mean values.



## RESULTS

### Effect of Incubation Methods

When the atmosphere of the 20 ml vial was sampled and flushed every 1 h, more ethylene was produced initially by disks than when the atmosphere was sampled and flushed every 2 h (Figure 27). When the atmosphere was sampled every 2 h but flushed every 1 h, the rate of wound ethylene production was the same as that of disks being sampled and flushed every 1 h. Figure 28 shows that one disk produced more wound ethylene than did three disks when they were placed in the same volume container. The difference between the rates of wound ethylene production could be seen throughout the incubation period of 12 h. When one disk was placed in a 50 ml container more wound ethylene was produced than when one disk was placed in a 20 ml container (Figure 29).

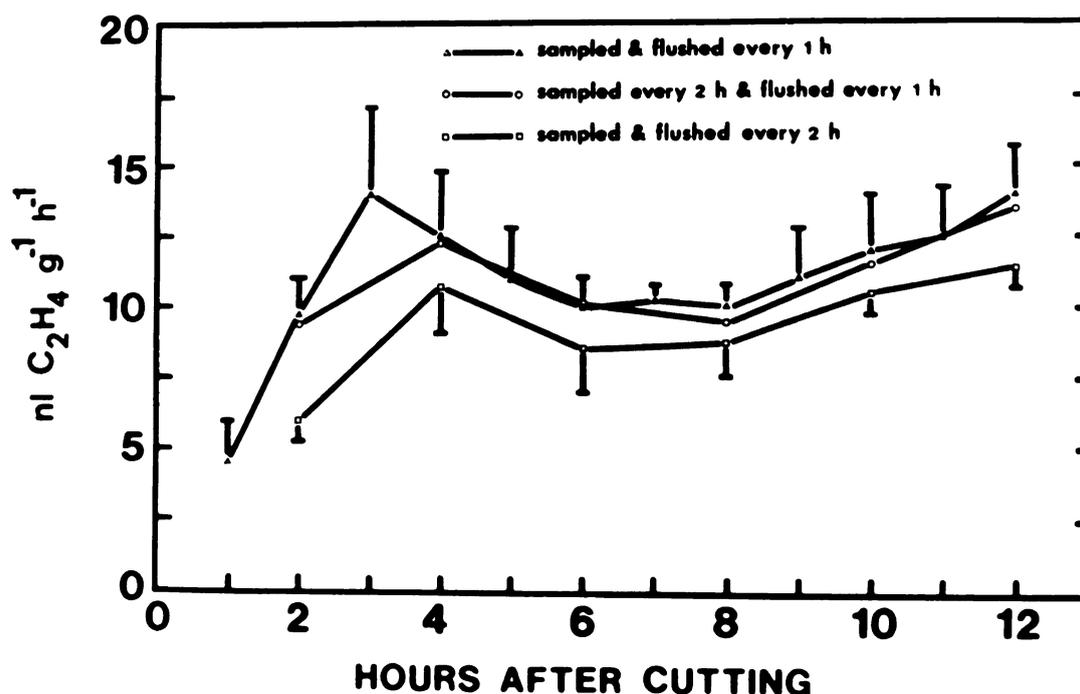


Figure 27. Effect of time interval of gas sampling and flushing on wound ethylene production. Single disks were placed in 20 ml containers and the atmosphere was sampled and flushed every 1 h, sampled every 2 h and flushed every 1 h, or sampled and flushed every 2 h.



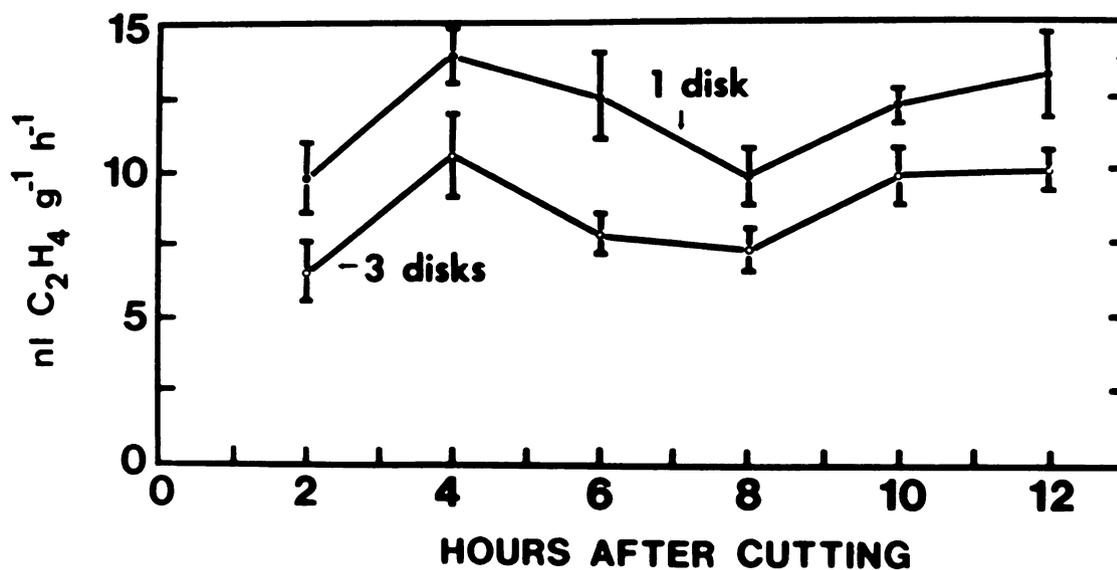


Figure 28. Effect of number of disks per container on wound ethylene production. One disk or three disks were placed in a 50 ml container and the atmosphere was sampled and flushed every 2 h.

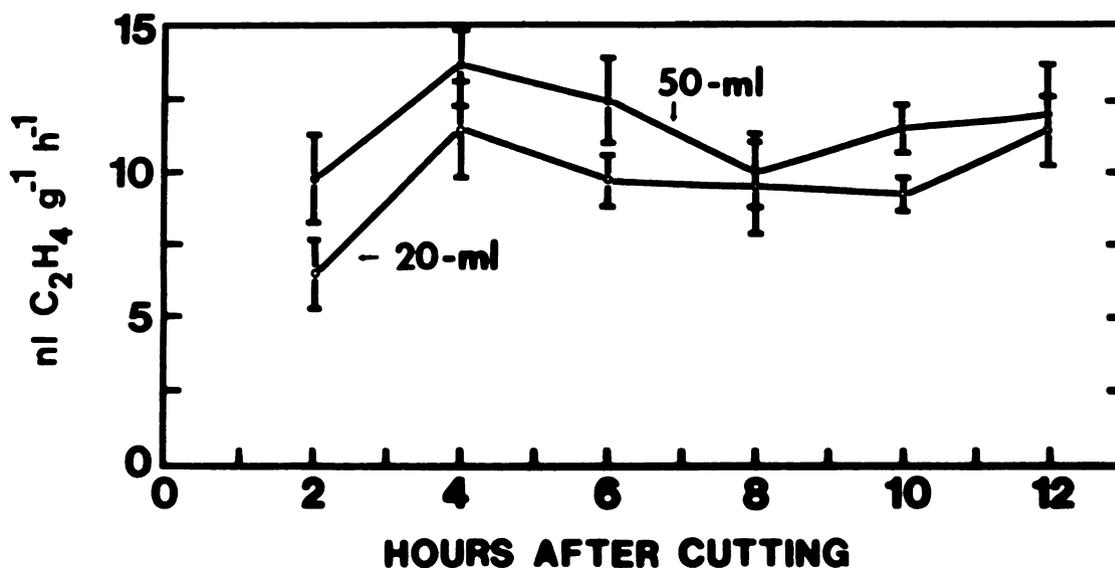


Figure 29. Effect of container size on wound ethylene production. Single disks were placed in 20 or 50 ml containers and the atmosphere was sampled and flushed every 2 h.



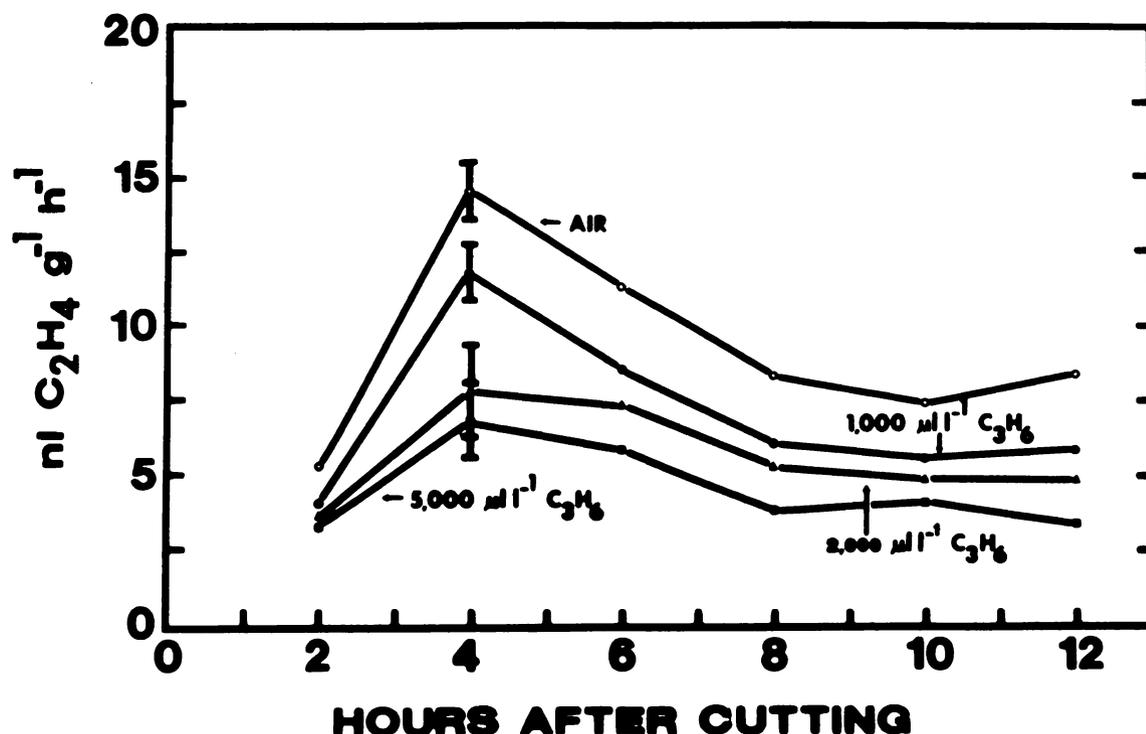


Figure 30. Effect of propylene treatment on wound ethylene production. Disks were held in ethylene-free air or air containing 1000, 2000, and 5000  $\mu\text{l l}^{-1}$  of propylene in air.

#### Effect of Propylene Treatment

Figure 30 shows that 1000, 2000, and 5000  $\mu\text{l l}^{-1}$  propylene inhibited wound ethylene production by disks throughout the incubation period of 12 h. The higher the propylene concentration, the greater the inhibition of wound ethylene. The inhibition by propylene was most pronounced after 4 h. The pattern of wound ethylene production by disks with propylene treatment was similar to that of disks without propylene treatment, however, none of the propylene concentrations brought the maximum rate of wound ethylene production to the basal rate. When disks were gased with 5000  $\mu\text{l l}^{-1}$  propylene for 4 or 8 h after cutting and returned to air, the rate of wound ethylene production by disks increased similar to that of disks returned from nitrogen to air but the increase was less pronounced (Figure 31). Figure 32 shows that



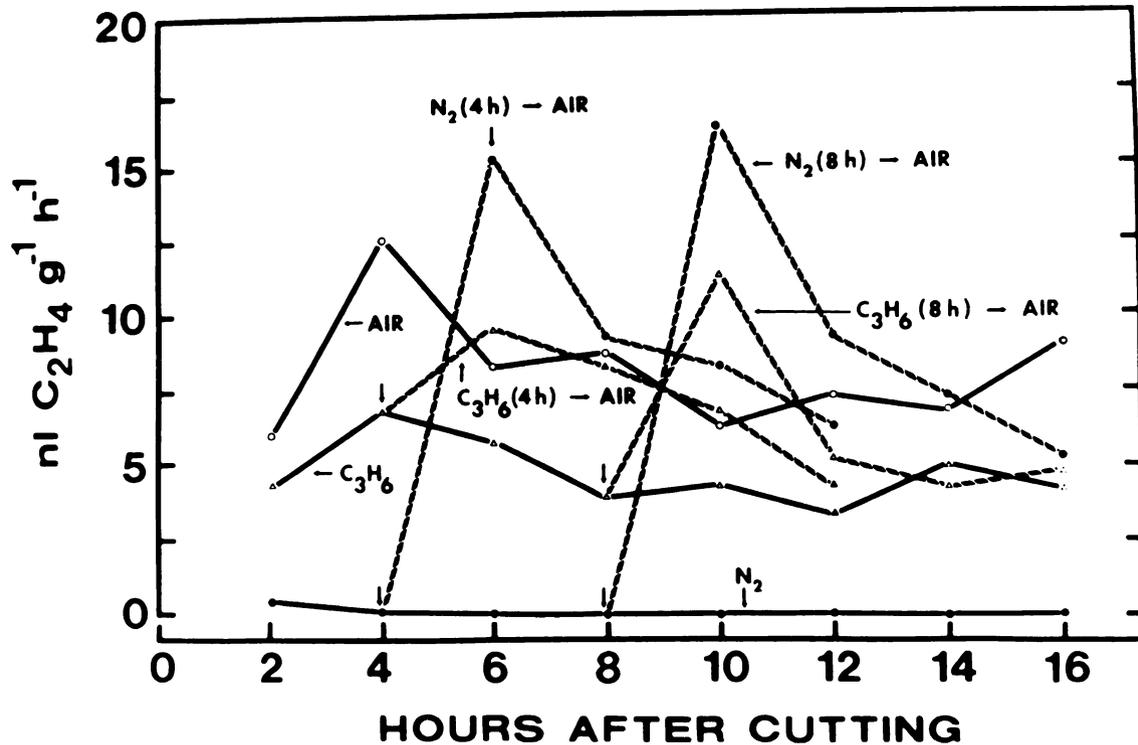


Figure 31. Effect of propylene treatment on wound ethylene production as compared to anaerobiosis. Disks were held in air, 5000  $\mu\text{l l}^{-1}$  propylene, or nitrogen continuously or transferred from propylene or nitrogen to air at 4 or 8 h as shown (arrows).

2000  $\mu\text{l l}^{-1}$  propylene slightly inhibited ACC-stimulated wound ethylene production throughout the incubation period, but the inhibition was not significant.

#### Effect of $\text{Ag}^+$

$\text{Ag}^+$  stimulated wound ethylene production by disks (Table 1) but had no effect on  $\text{CO}_2$  production (Table 2). Figure 33 shows that the inhibitory effect of propylene on wound ethylene production is less in those disks also treated with  $\text{Ag}^+$  compared to disks treated with water. When ACC was added to disks in addition to  $\text{Ag}^+$  there was additional stimulation of ethylene production compared to  $\text{Ag}^+$  or ACC treatment alone (Figure 34). Figure 35 shows that the stimulation of wound ethylene caused by  $\text{Ag}^+$  was completely inhibited by rhizobitoxine.



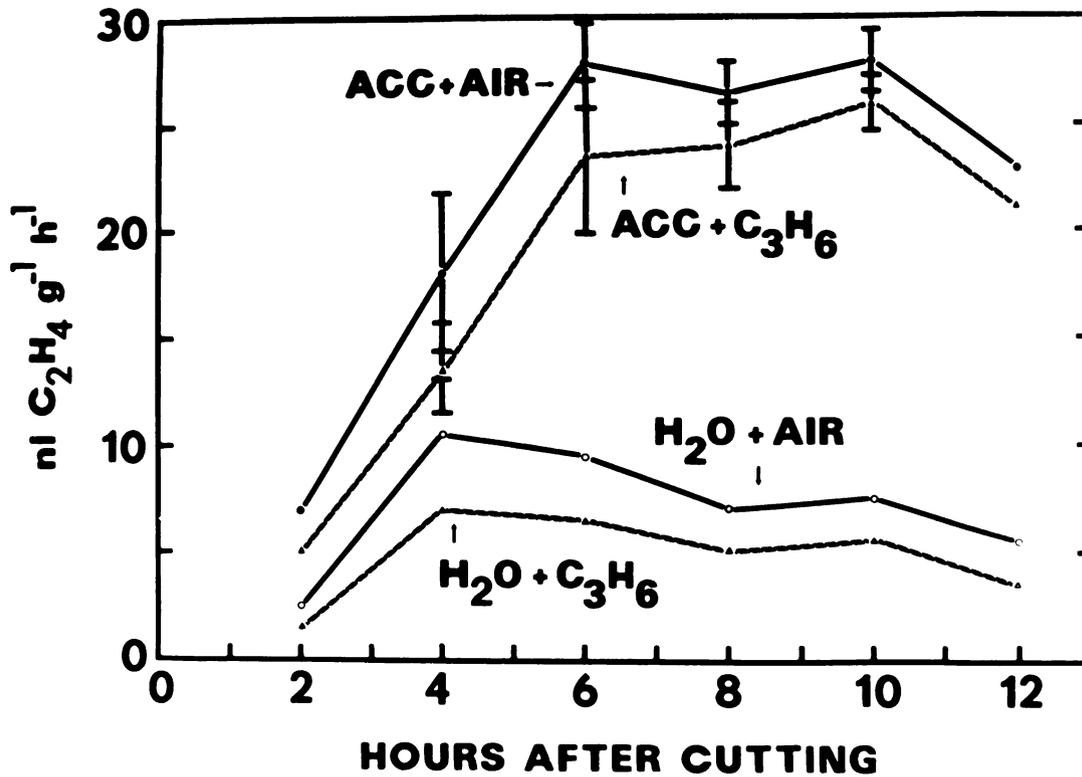


Figure 32. Effect of propylene on ACC-stimulated ethylene production. Twenty  $\mu\text{l}$  of  $\text{H}_2\text{O}$  or 1 mM ACC were applied to the cut surface of disks at 0 h after cutting and disks were held in ethylene-free air or in 2000  $\mu\text{l l}^{-1}$  propylene in air.

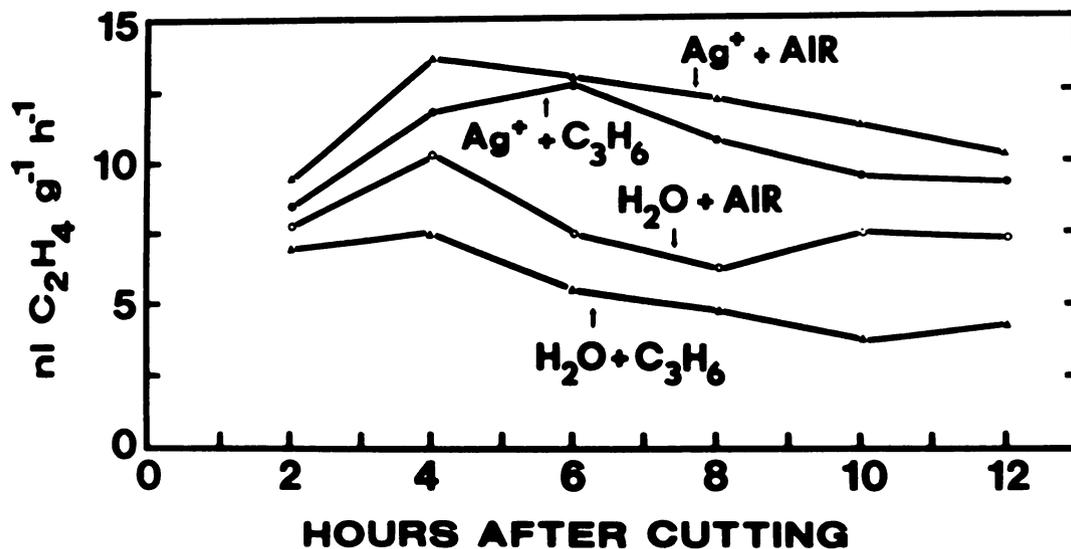


Figure 33. Effect of  $\text{Ag}^+$  and propylene on wound ethylene production. Twenty  $\mu\text{l}$  of  $\text{H}_2\text{O}$  or 1 mM  $\text{Ag}^+$  were applied to the cut surface of disks immediately after cutting and held in ethylene-free air or 2000  $\mu\text{l l}^{-1}$  propylene in air.

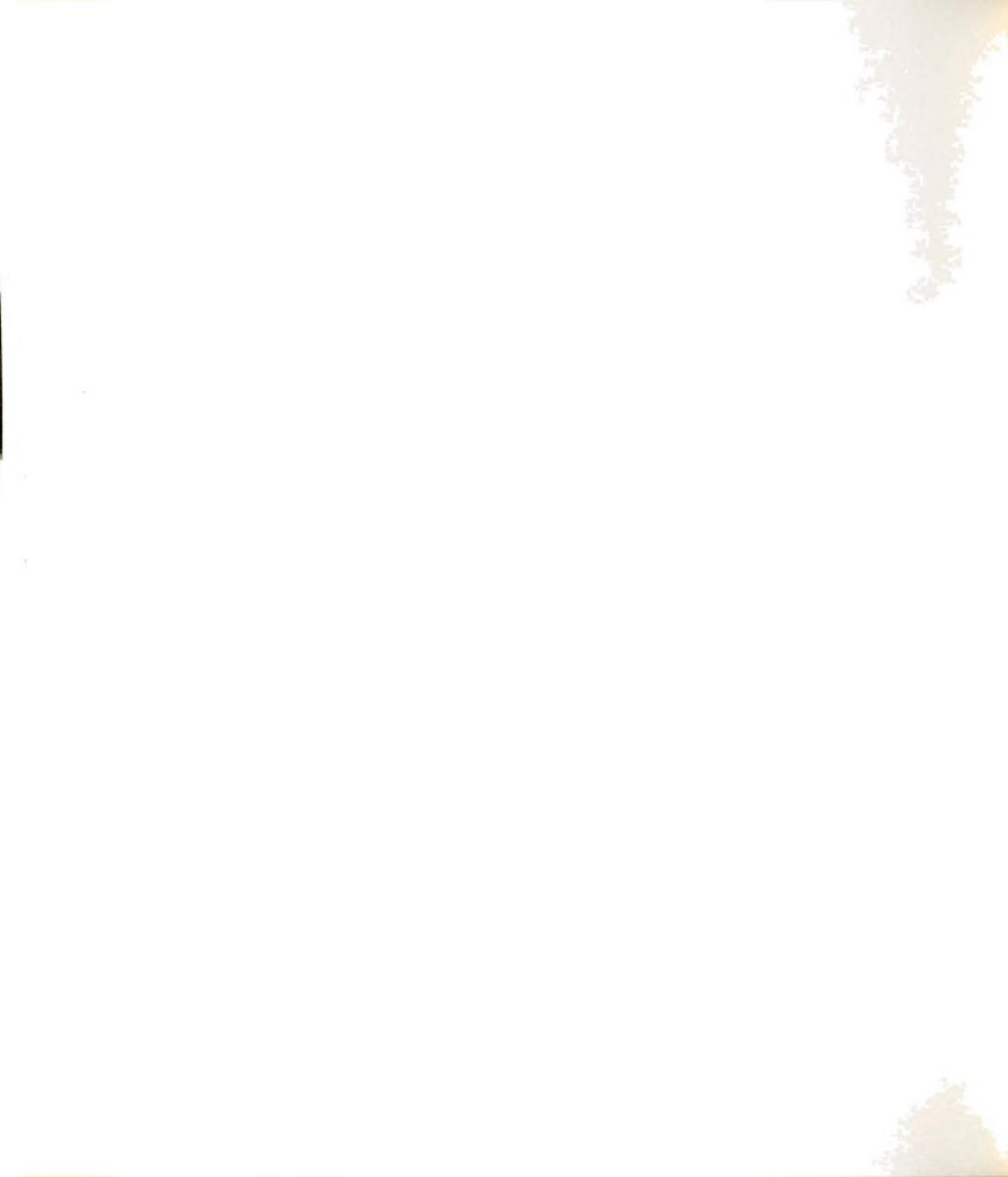


Table 1. Effect of  $\text{Ag}^+$  on wound ethylene production. Twenty  $\mu\text{l}$  of  $\text{H}_2\text{O}$  (control), or solution of  $\text{Ag}^+$  (1, 2, or 4 mM) was applied to the cut surface of disks immediately after cutting

Treatment	Time after cutting (h)		
	0 to 2	2 to 4	4 to 6
	$\text{nl C}_2\text{H}_4 \text{ g}^{-1}\text{h}^{-1}$		
$\text{H}_2\text{O}$	$5.91 \pm 0.73$	$11.39 \pm 1.04$	$9.74 \pm 2.40$
1 mM $\text{Ag}^+$	$9.61 \pm 0.50$	$16.19 \pm 2.23$	$17.89 \pm 1.60$
2 mM $\text{Ag}^+$	$9.14 \pm 0.27$	$16.89 \pm 0.56$	$15.58 \pm 2.59$
4 mM $\text{Ag}^+$	$8.92 \pm 1.58$	$17.92 \pm 1.06$	$21.32 \pm 1.56$

Table 2. Effect of  $\text{Ag}^+$  on carbon dioxide production. Twenty  $\mu\text{l}$  of  $\text{H}_2\text{O}$  or 4 mM  $\text{Ag}^+$  was applied to the cut surface of disks immediately after cutting.

Treatment	Time after cutting (h)		
	0 to 2	2 to 4	4 to 6
	$\mu\text{l CO}_2 \text{ g}^{-1}\text{h}^{-1}$		
$\text{H}_2\text{O}$	$56.99 \pm 2.74$	$57.58 \pm 3.13$	$53.36 \pm 3.53$
$\text{Ag}^+$	$58.34 \pm 3.53$	$55.06 \pm 1.38$	$55.62 \pm 1.80$



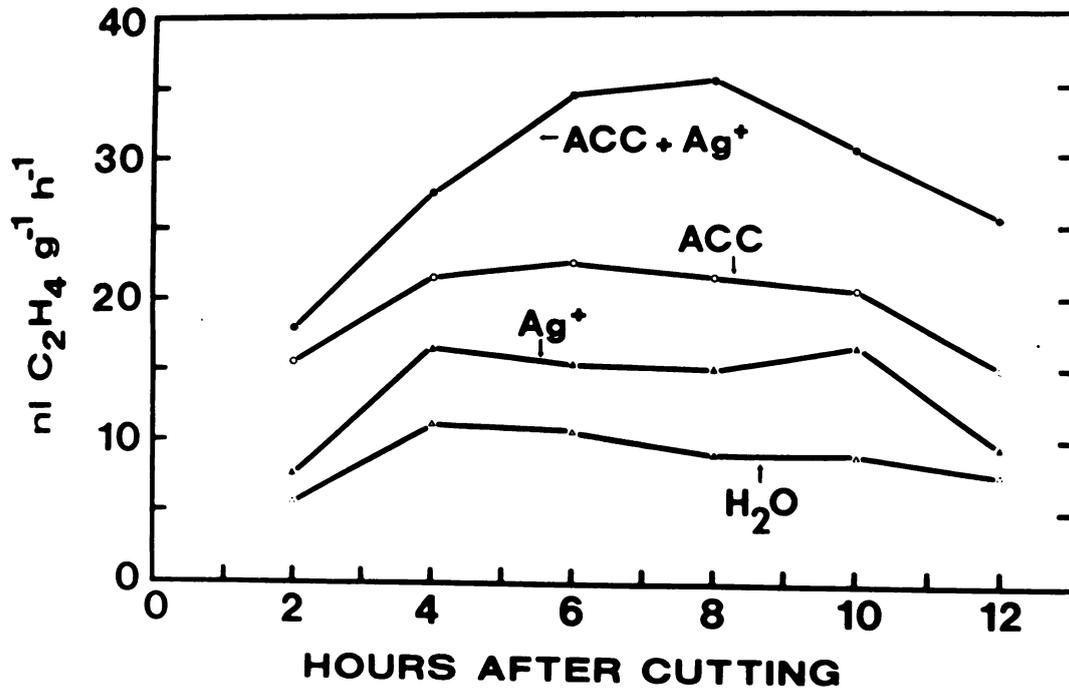


Figure 34. Effect of Ag<sup>+</sup> on ACC-stimulated ethylene production. Twenty  $\mu$ l of H<sub>2</sub>O, 1 mM ACC, 1 mM Ag<sup>+</sup>, or 1 mM of ACC + 1 mM Ag<sup>+</sup> were applied to the cut surface of disks immediately after cutting.

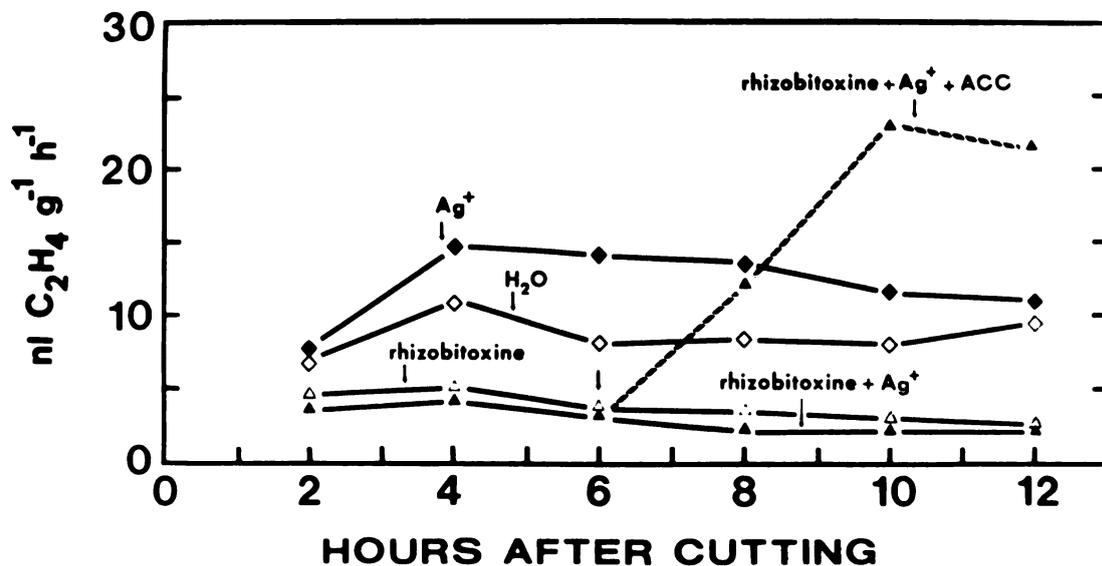


Figure 35. Effect of rhizobitoxine on Ag<sup>+</sup>-stimulated ethylene production. Twenty  $\mu$ l of H<sub>2</sub>O, 1 mM rhizobitoxine, 1 mM Ag<sup>+</sup>, or 1 mM Ag<sup>+</sup> + 1 mM rhizobitoxine were applied to the cut surface of disks immediately after cutting. At 6 h, 20  $\mu$ l of 1 mM ACC were applied to the cut surface of disks which had been treated with Ag<sup>+</sup> + rhizobitoxine.



However, the inhibitory effect of rhizobitoxine on wound ethylene production could be relieved when ACC was applied to disks which had been previously treated with  $\text{Ag}^+$  plus rhizobitoxine.

#### DISCUSSION

When vials containing single disks were flushed every hour rather than every two hours more ethylene was initially produced. One disk per container produced more ethylene per unit weight than three disks per container of the same size. More ethylene was produced per unit weight of disk when one disk was placed in a 50 ml container as opposed to a 20 ml container. This evidence suggests that wound ethylene produced by the tomato disks may have a negative feedback role in regulating ethylene production by the disks themselves (20). In these cases when ethylene concentration built up too rapidly further ethylene production was inhibited by a negative feedback mechanism.

If the above is correct then one would expect that ethylene, or its analog propylene, would inhibit ethylene production at high concentrations. When propylene was added to tomato disks, wound ethylene production was inhibited but not completely stopped. When disks which had been treated with propylene were removed to air, there was a temporary increase in ethylene production above the air treated controls and then ethylene production declined. This response to propylene is similar to but not as great as that of disks transferred from  $\text{N}_2$  (anaerobic conditions) to air. Since anaerobiosis is known to prevent the conversion of ACC to ethylene (1), it is probably that propylene also inhibits to some degree the same step in ethylene biosynthesis.



Propylene should inhibit ACC-stimulated ethylene production but when propylene was added to disks treated with ACC only slight inhibition occurred. Therefore, the step or steps which are inhibited by propylene can not be determined precisely by this experiment.

Silver ion has been shown to interfere with ethylene action in several plant systems (4,5,10,18). In the experiment reported here,  $\text{Ag}^+$  was shown to stimulate ethylene but not  $\text{CO}_2$  production of tomato disks. The stimulation of ethylene production was less than that caused by application of ACC to tomato disks.  $\text{Ag}^+$  was also able to counteract the inhibitory effect of propylene on wound ethylene production. These results indicate that  $\text{Ag}^+$  may prevent a negative feedback mechanism of ethylene.

Rhizobitoxine applied to disks at the same time as  $\text{Ag}^+$  prevented ethylene production. ACC added to disks which had been treated with  $\text{Ag}^+$  and rhizobitoxine stimulated ethylene production of the disks. This would indicate that for  $\text{Ag}^+$  to stimulate ethylene production or counteract a negative feedback, ACC must be present. It is possible that the  $\text{Ag}^+$  stimulation of ethylene production is a reaction to a toxic effect of  $\text{Ag}^+$ , however, no visual symptoms of  $\text{Ag}^+$  toxicity could be seen nor was  $\text{CO}_2$  production stimulated by  $\text{Ag}^+$  application to the disks.

In conclusion, evidence is presented that ethylene or propylene can inhibit ethylene production by tomato disks through a negative feedback mechanism. This feedback mechanism may operate at the ACC to ethylene step of ethylene biosynthesis.  $\text{Ag}^+$  stimulates ethylene production in tomato disks and ACC appears to be necessary for this response to occur.



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SECTION FOUR

WOUND ETHYLENE PRODUCTION BY FRUIT PERICARP

TISSUE OF RIN MUTANT TOMATO

(LYCOPERSICON ESCULENTUM MILL.)

IV. MODE OF ACTION OF CARBON DIOXIDE



#### IV. MODE OF ACTION OF CARBON DIOXIDE

##### ABSTRACT

Wound ethylene production by fruit pericarp tissue of rin mutant tomato (Lycopersicon esculentum Mill.) was studied in relation to the role of endogenous and exogenous carbon dioxide. The removal of ambient CO<sub>2</sub> by KOH reduced wound ethylene production. Propylene was much more effective in inhibiting ethylene production when CO<sub>2</sub> was removed by KOH. These suggest that endogenous CO<sub>2</sub> counteracts a negative feedback of ethylene. On the other hand, exogenously applied CO<sub>2</sub> (1.37 to 7.60%) inhibited wound ethylene production. Ag<sup>+</sup> stimulated ethylene production much more effectively when ambient CO<sub>2</sub> was removed. Exogenously applied 7.60% CO<sub>2</sub> in air reduced the stimulatory effect of Ag<sup>+</sup>. The initial rate of wound ethylene production by disks returned from 7.60% CO<sub>2</sub> to air slowly increased which differed from disks removed from N<sub>2</sub>. Exogenously applied CO<sub>2</sub> inhibited the methionine- and SAM-stimulated wound ethylene production of tomato disks but not ACC stimulation. These results suggest that exogenously applied CO<sub>2</sub> inhibits wound ethylene production by disks in the step from SAM to ACC.

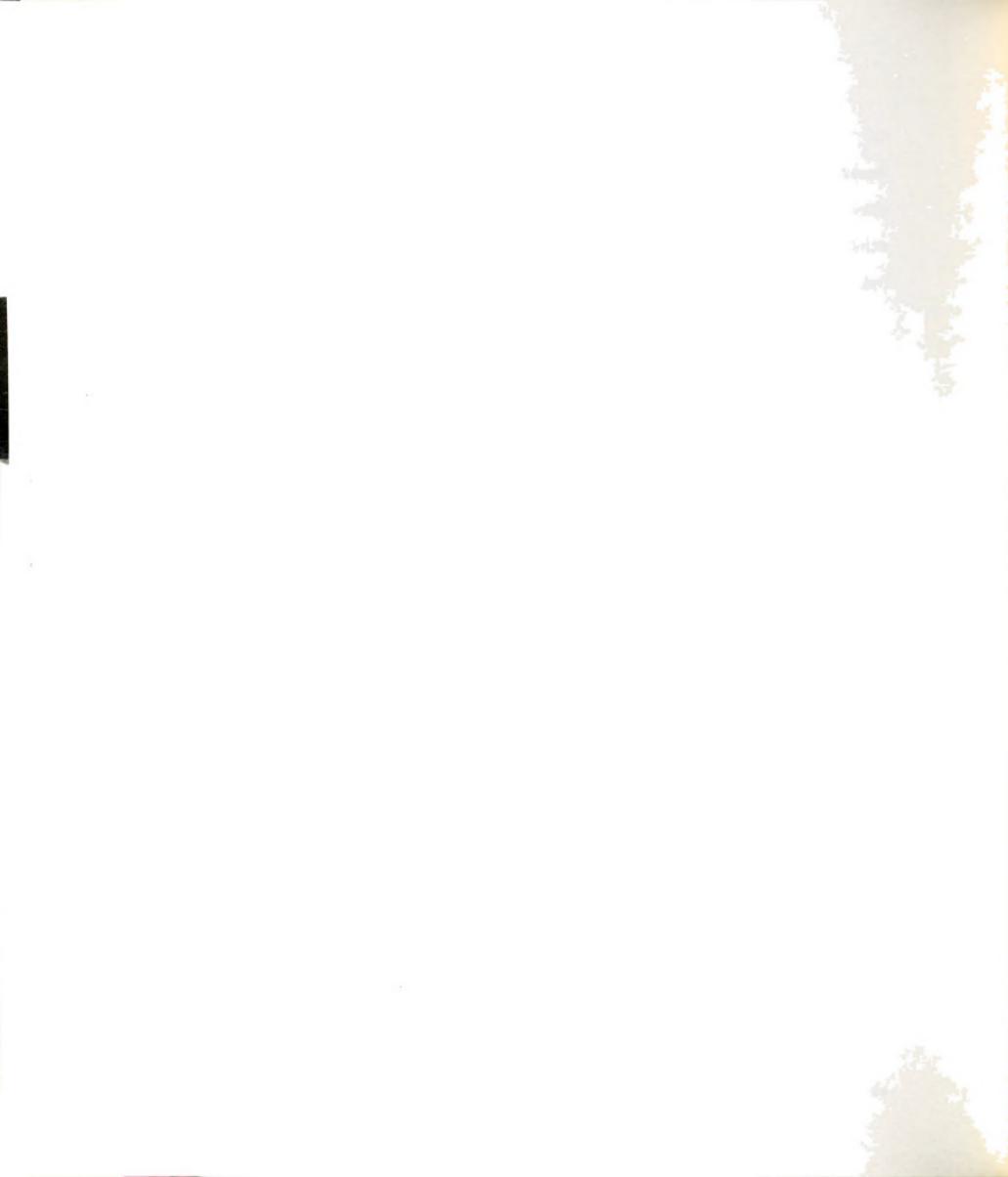
##### INTRODUCTION

CO<sub>2</sub> has long been known to antagonize ethylene action. This was first reported in 1927 by Mack (12) who observed that the addition of



CO<sub>2</sub> to the gas phase reduced the ability of ethylene to blanch celery. Removing CO<sub>2</sub> by absorbing it with KOH enhanced the effectiveness of ethylene. In the same year, Wallace (21) reported that CO<sub>2</sub> decreased ethylene-induced formation of intumescences in apple. Matsushita and Uritami (14) reported that removing CO<sub>2</sub> from the atmosphere increased the stimulatory effect of ethylene on the activity of phenylalanine ammonia lyase and peroxidase in wounded sweet potato root tissue. CO<sub>2</sub> is generally known to block or retard many physiological effects of ethylene action such as abscission (1,8,13), epinasty (9), hook opening (11), sleepiness of carnation flowers (20), chlorophyll loss (2,13), growth inhibition of stems and roots (5,7), and fruit ripening (17). CO<sub>2</sub> has also been shown to inhibit ethylene biosynthesis in both wounded and senescent plant tissues (7,15,16,18). In contrast, removing CO<sub>2</sub> from the atmosphere reduced ethylene production from infected sweet potato root tissue (10).

Although CO<sub>2</sub> has long been recognized as having an effect opposite to ethylene, Burg and Burg (5,6) were the first to clearly state that its action was similar to that observed for competitive inhibitors of enzyme reactions. They pointed out that CO<sub>2</sub> was a close structural analog of allene and CO compounds that mimic ethylene action. Although CO<sub>2</sub> has some structural resemblance to allene and CO could occupy the same site in the cell, when levels of CO<sub>2</sub> were high, it acted as an effector probably because of the partial negative charge on each end of the molecule. This idea was tested by growing pea stem section in different concentrations of CO<sub>2</sub> and ethylene and plotting the resulting data in a Lineweaver-Burk plot. Recently, Beyer (3,4) has suggested that ethylene action is related to its metabolism and CO<sub>2</sub> antagonizes



ethylene action by inhibiting the oxidation of ethylene to  $\text{CO}_2$ . This paper reports the role of endogenous and exogenous  $\text{CO}_2$  on wound ethylene production by fruit pericarp tissue of rin tomato.

## MATERIALS AND METHODS

### Plant Material and Incubation

Plants of rin tomato (Lycopersicon esculentum Mill.) were grown as previously described (see Section One). Disks of pericarp tissue (diameter: 1.5 cm; thickness: 0.25 cm) from mature-green fruits ( $40 \pm 1$  days after anthesis) were prepared from the equatorial part of tomato fruits using a cork borer and template. Disks of pericarp tissue were placed with the epidermis on a layer of glass beads in a 20 ml scintillation vial. The vials were flushed with ethylene-free air and sealed with a serum cap, then incubated in darkness at  $20^\circ\text{C}$ .

### Chemical Treatments

Twenty  $\mu\text{l}$  of chemical solution(s) or distilled water (control) were applied to the cut surface of disks opposite the epidermis as previously described (see Section One).

### Carbon Dioxide Effects

In any experiment which dealt with the effect of endogenous  $\text{CO}_2$ . Three hundred  $\mu\text{l}$  of distilled water (control) or 20% KOH were added to the bottom of containers with a layer of glass beads. The disks were placed with the epidermis on a layer of glass beads, thus, they were not in contact with water or the KOH solution. In any experiment which dealt with the effect of exogenous  $\text{CO}_2$ , the containers were purged with  $\text{N}_2$  to remove all  $\text{CO}_2$  and  $\text{O}_2$ , then an appropriate volume of pure  $\text{O}_2$  and



CO<sub>2</sub> was injected to give specific CO<sub>2</sub> and O<sub>2</sub> concentrations. The containers were flushed with ethylene-free air and sealed with serum caps. At the end of every 2 h period the gaseous content of containers was sampled and analyzed for CO<sub>2</sub>. CO<sub>2</sub> was determined on a gas chromatograph (Carle GC 8700) equipped with a thermal conductivity detector.

#### Propylene Treatment

The containers with disks were flushed with ethylene-free air and sealed with serum caps. Pure propylene was added through the serum cap to give a final concentration of 2000  $\mu\text{l l}^{-1}$  propylene. At the end of every 2 h period, the gaseous content of containers was sampled and analyzed for ethylene.

#### Ethylene Determination

At the end of every 2 h period, wound ethylene production by disks was withdrawn with a 1 ml gas tight syringe and assayed using a gas chromatograph. The containers were then flushed with ethylene-free air, sealed and returned to the incubation conditions. Each experiment consisted of five to six disks and was repeated three times. Data represent mean values.

### RESULTS

#### Effect of Endogenous Carbon Dioxide

When CO<sub>2</sub> given off by disks was removed by KOH, the rate of wound ethylene production was reduced (Figure 36). KOH effectively removed the CO<sub>2</sub> given off by disks throughout the incubation period of 12 h. Not only did the removal of CO<sub>2</sub> result in decreased ethylene production by disks held in air but also resulted in less ethylene production when



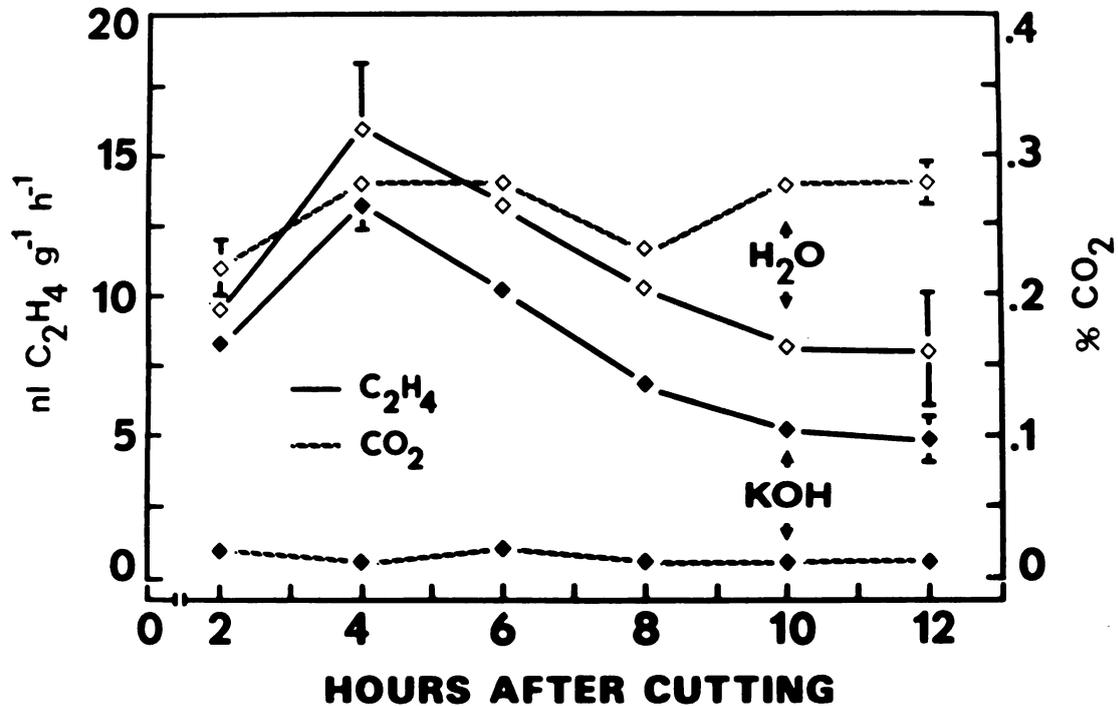


Figure 36. Effect of KOH on wound ethylene production and carbon dioxide in the atmosphere. Disks were placed on a layer of glass beads with 300  $\mu$ l of H<sub>2</sub>O or 20% KOH at the bottom of containers.

the disks were exposed to propylene (Figure 37). In contrast, Ag<sup>+</sup> stimulated more ethylene production by tomato disks in the absence of ambient CO<sub>2</sub> than in the presence of ambient CO<sub>2</sub> levels (Figure 38).

#### Effect of Exogenous Carbon Dioxide

An exogenous application of  $1.37 \pm 0.03$ ,  $3.89 \pm 0.40$ , or  $7.60 \pm 0.40\%$  CO<sub>2</sub> in air inhibited wound ethylene production by tomato disks (Figure 39). The higher the concentration of CO<sub>2</sub>, the greater the reduction of wound ethylene production. The trend of wound ethylene production by disks which were treated with CO<sub>2</sub> was similar to that of control disks throughout the incubation period of 12 h. Figure 40 shows that exogenously applied CO<sub>2</sub> (7.60%) reduced the stimulatory effect of Ag<sup>+</sup> on wound ethylene production. The rate of wound ethylene



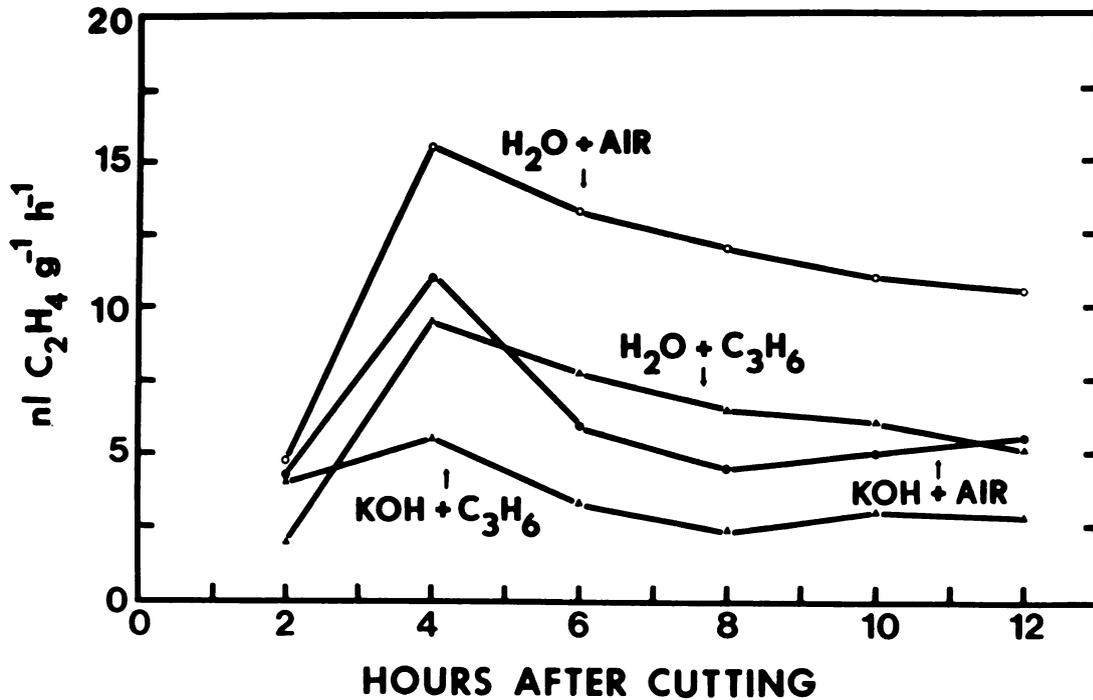


Figure 37. Effect of the removal of carbon dioxide on propylene-inhibited wound ethylene production by disks from rin tomato fruits. Disks were placed on a layer of glass beads with 300  $\mu\text{l}$  of  $\text{H}_2\text{O}$  (open) or 20% KOH (black) at the bottom of containers whose atmosphere were with (triangle) or without (circle) 2000  $\mu\text{l l}^{-1}$  propylene.

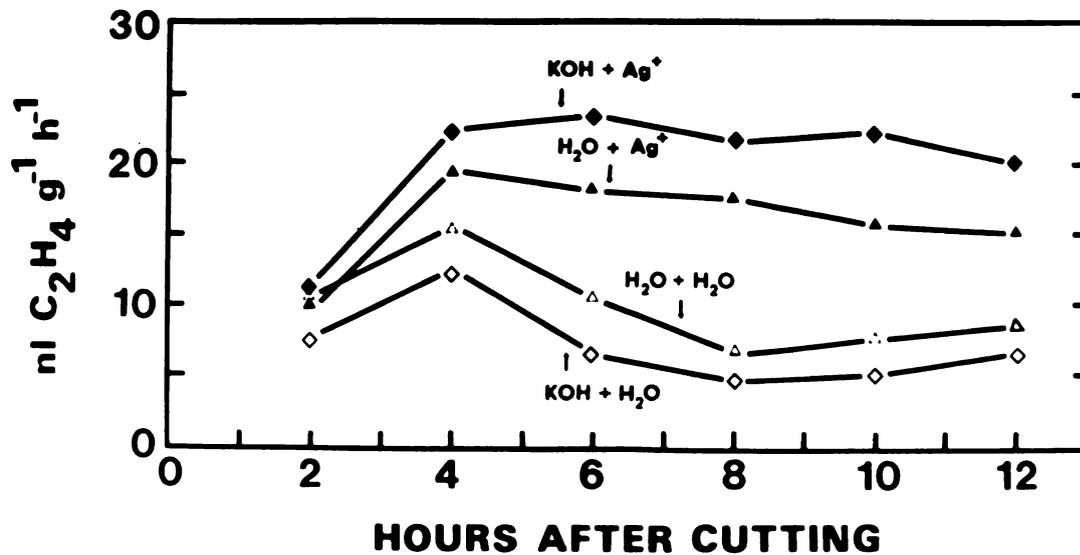


Figure 38. Effect of the removal of carbon dioxide on  $\text{Ag}^+$ -stimulated ethylene production by disks from rin tomato fruits. Disks were placed on a layer of glass beads with 300  $\mu\text{l}$  of  $\text{H}_2\text{O}$  (triangle) or 20% KOH (diamond) at the bottom of containers and 20  $\mu\text{l}$  of  $\text{H}_2\text{O}$  (open) or 1 mM  $\text{Ag}^+$  (black) were applied to the cut surface of disks immediately after cutting.



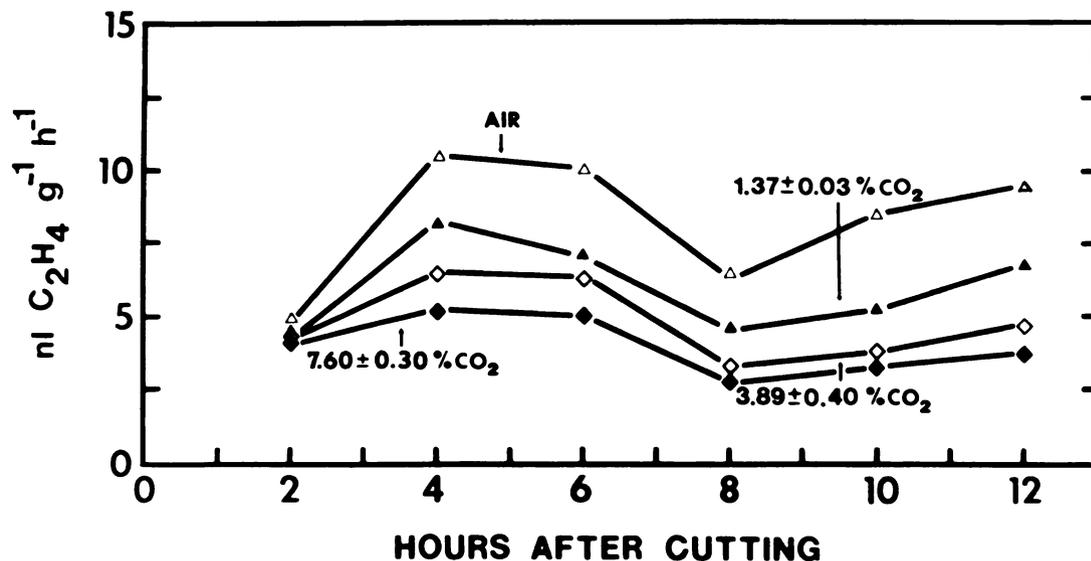


Figure 39. Effect of applied carbon dioxide on wound ethylene production by disks from rin tomato fruits. Disks were held in ethylene-free air,  $1.37 \pm 0.03\%$ ,  $3.89 \pm 0.40\%$ , or  $7.60 \pm 0.30\%$  CO<sub>2</sub> in air.

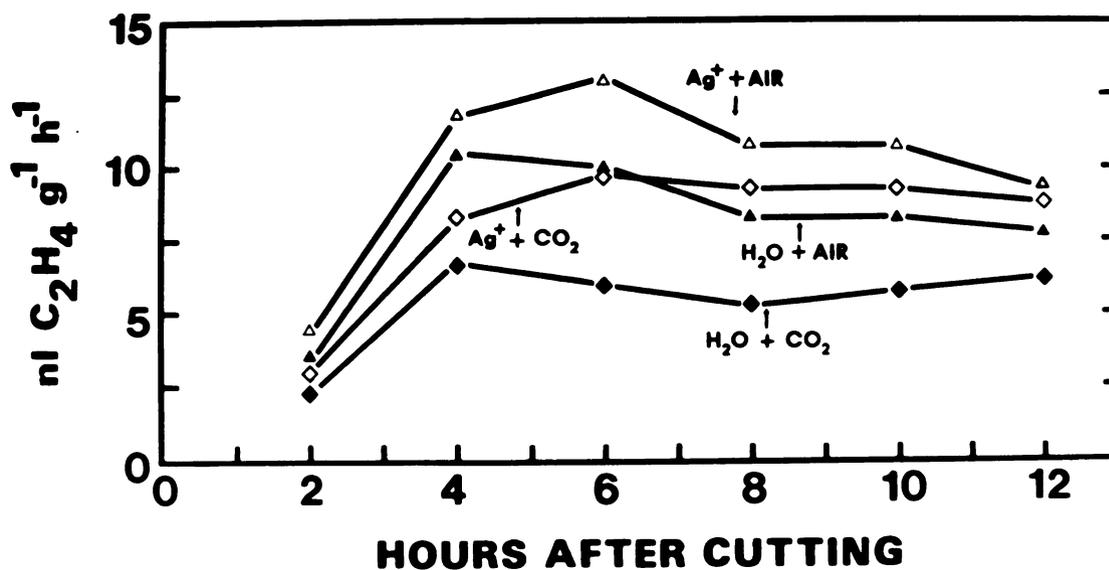


Figure 40. Effect of applied carbon dioxide on Ag<sup>+</sup>-stimulated wound ethylene production by disks from rin tomato fruits. Twenty  $\mu$ l of H<sub>2</sub>O or 1 mM Ag<sup>+</sup> were applied to the cut surface of disks immediately after cutting and held in ethylene-free air with or without  $7.60 \pm 0.20\%$  CO<sub>2</sub>.



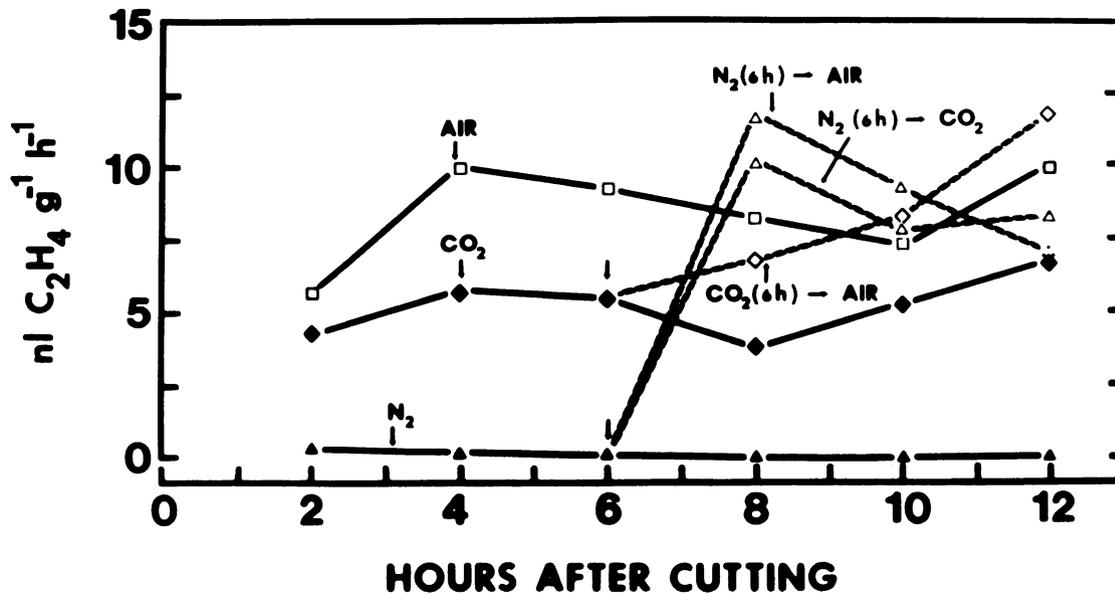


Figure 41. Effect of applied carbon dioxide on wound ethylene production by disks from rin tomato fruits as compared to anaerobiosis. Disks were held in ethylene-free air,  $7.60 \pm 0.20\%$   $\text{CO}_2$ , or  $\text{N}_2$  atmosphere until the designated time shown by arrows at 6 h after cutting, then returned from  $\text{CO}_2$  and  $\text{N}_2$  to air or from  $\text{N}_2$  to  $7.60 \pm 0.20\%$   $\text{CO}_2$ .

production by disks which were treated with  $\text{Ag}^+$  and  $\text{CO}_2$  was lower than that of control disks ( $\text{H}_2\text{O}$  and air) during the first 4 h of incubation but was slightly higher after that.

Figure 41 shows that the rate of wound ethylene production by disks which had been removed from  $7.60\%$   $\text{CO}_2$  and returned to air steadily increased until the end of incubation period. In contrast, disks which had been removed from anaerobiosis to air produced ethylene which reach a peak 2 h after return to air and then steadily declined. Removing disks from anaerobic conditions to high  $\text{CO}_2$  resulted in ethylene production similar to that produced by disks removed from  $\text{N}_2$  to air. Applied  $\text{CO}_2$  (Figure 42) increased ACC-stimulated ethylene production by disks but decreased ethylene production by water treated disks. Figure 43 shows that applied  $\text{CO}_2$  ( $7.60\%$ ) inhibited both methionine- and SAM-stimulated ethylene production by disks.



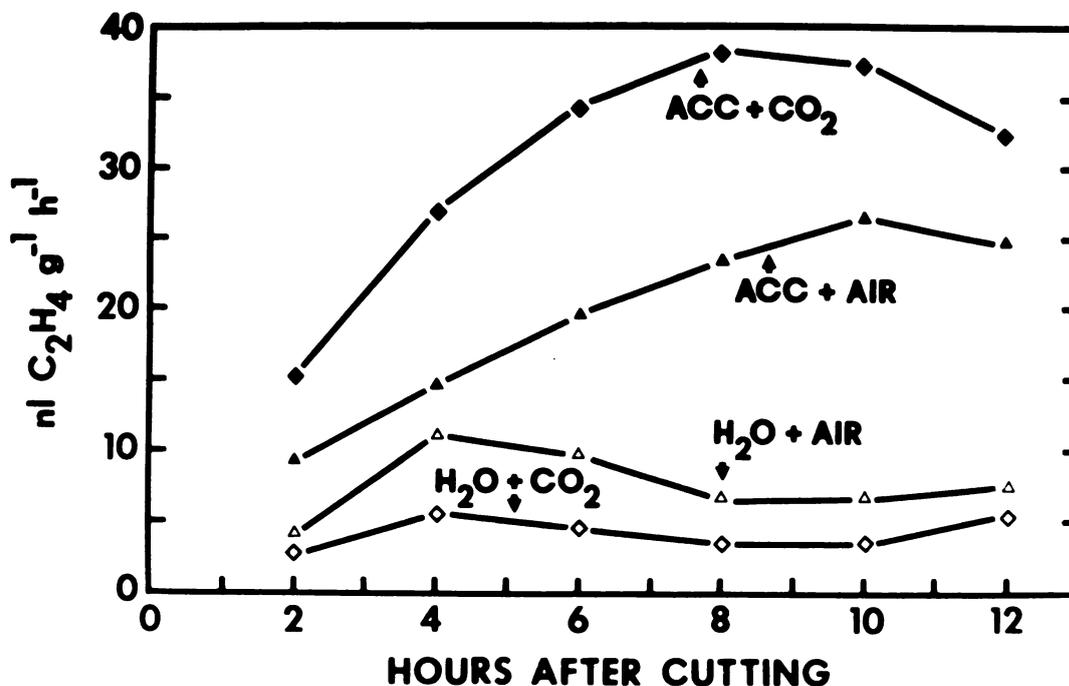


Figure 42. Effect of applied carbon dioxide on ACC-stimulated ethylene production by disks from rin tomato fruits. Twenty  $\mu$ l of H<sub>2</sub>O or 1 mM ACC were applied to the cut surface of disks immediately after cutting and held in ethylene-free air with or without  $7.60 \pm 0.20\%$  CO<sub>2</sub>.

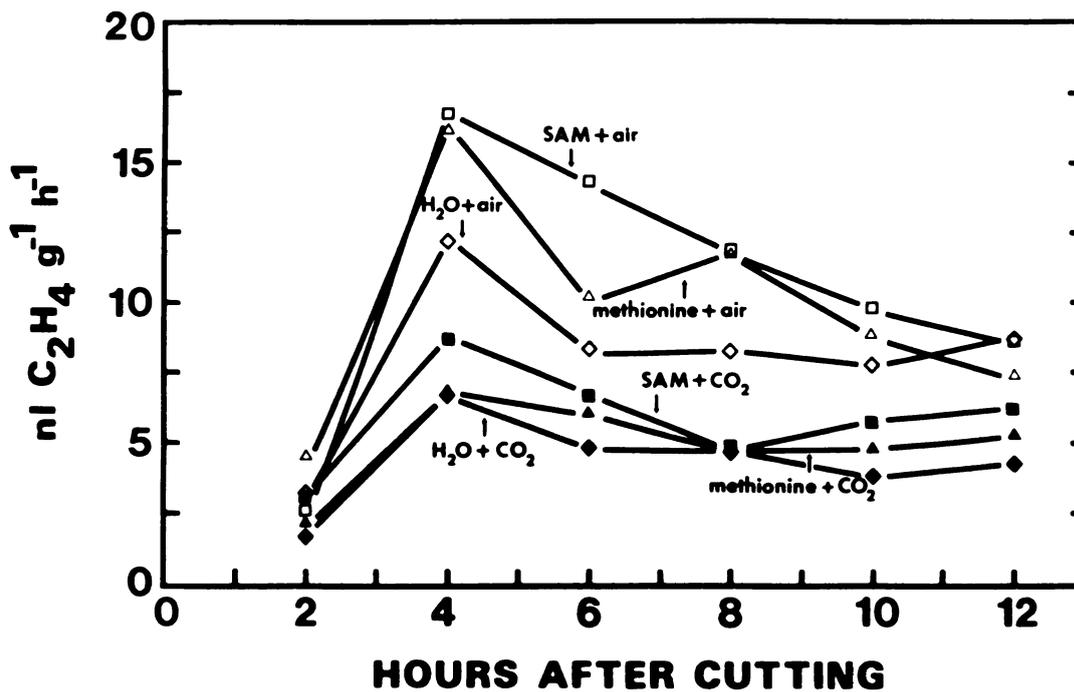


Figure 43. Effect of applied carbon dioxide on methionine- and SAM-stimulated wound ethylene production by disks from rin tomato fruits. Twenty  $\mu$ l of H<sub>2</sub>O, 1 mM methionine, or 1 mM SAM were applied to the cut surface of disks immediately after cutting and held in ethylene-free air or  $7.60 \pm 0.20\%$  CO<sub>2</sub> in air.



DISCUSSION

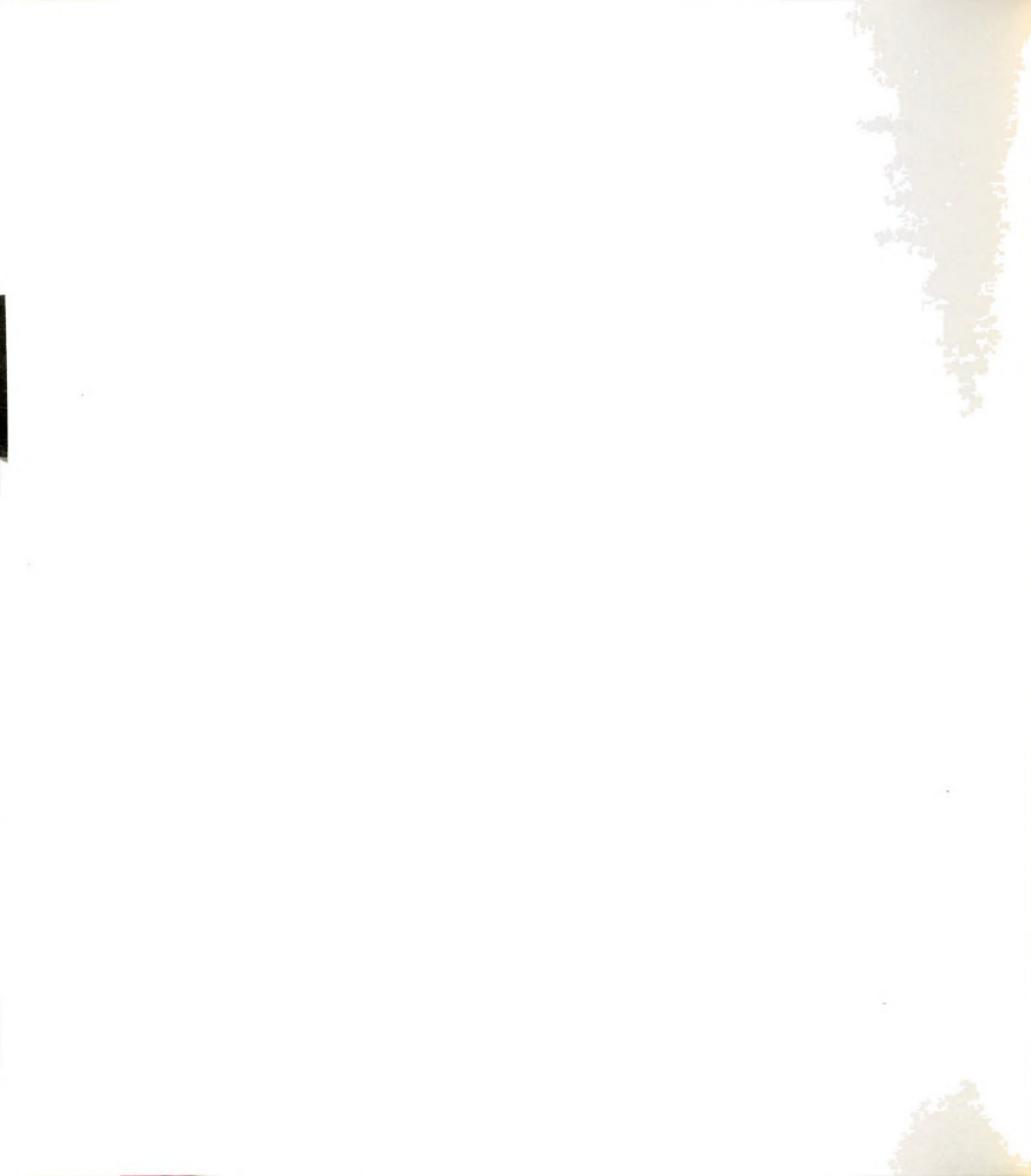
Evidence presented here indicates that endogenous and exogenous  $\text{CO}_2$  may play different roles in the synthesis of wound-induced ethylene. For example, when  $\text{CO}_2$  is absorbed and scrubbed by KOH from the atmosphere surrounding tomato disks, ethylene production is reduced. Also when  $\text{CO}_2$  is removed from the atmosphere,  $\text{Ag}^+$  stimulates more ethylene production by the tomato disks than when  $\text{CO}_2$  is allowed to remain in the atmosphere. In contrast, exogenous  $\text{CO}_2$  causes a decreased ethylene production with increased  $\text{CO}_2$  concentrations and higher  $\text{CO}_2$  tends to decrease  $\text{Ag}^+$  stimulation of ethylene production. These differences may be due to differences of site of action or may be due to differences in concentration of endogenous levels and exogenous levels of  $\text{CO}_2$  used in these experiments.

When endogenous  $\text{CO}_2$  is scrubbed from the atmosphere, propylene inhibits ethylene production by disks more than when  $\text{CO}_2$  is present. This and the fact that removing  $\text{CO}_2$  from disks held in air reduced ethylene synthesis suggests that low endogenous levels of  $\text{CO}_2$  prevents the action of ethylene or propylene from causing the negative feedback of ethylene synthesis.

When  $\text{CO}_2$  is added to the atmosphere, ethylene production by tomato disks is decreased. When the disks are removed from  $\text{CO}_2$  to air the rate of ethylene production steadily increases but there is no immediate surge of ethylene production followed by a decrease as there is following removal of disks from  $\text{N}_2$  or propylene to air. Added  $\text{CO}_2$  also increases ACC-stimulation of ethylene production by tomato disks in a similar way to that of rhizobitoxine and cycloleucine (see Section Two).  $\text{CO}_2$  was shown in these experiments to inhibit the stimulation of ethylene



production caused by methionine and SAM. This suggests that exogenous  $\text{CO}_2$  inhibits ethylene production of tomato disks by inhibiting ethylene biosynthesis prior to the ACC to ethylene step. Perhaps the SAM to ACC step is the one inhibited by exogenous  $\text{CO}_2$ .



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SECTION FIVE

WOUND ETHYLENE PRODUCTION BY FRUIT PERICARP  
TISSUE OF RIN MUTANT TOMATO  
(LYCOPERSICON ESCULENTUM MILL.)

V. ACC FORMATION



## V. ACC FORMATION

### ABSTRACT

Wound ethylene production by fruit pericarp tissue of rin mutant tomato (Lycopersicon esculentum Mill.) was studied in relation to ACC formation. Disks from immature-green fruits formed more ACC and ethylene than disks from mature-green fruits. The increase in cut surface of disks increased both ACC and ethylene. Rhizobitoxine, cycloleucine, and cycloheximide inhibited ACC and ethylene production. Anaerobiosis inhibited wound ethylene production but not the formation of ACC. Air containing  $2000 \mu\text{l l}^{-1}$  propylene caused the accumulation of ACC formed suggesting wound ethylene production inhibits its own production by blocking the conversion of ACC to ethylene. Exogenously applied 7.60% CO<sub>2</sub> inhibited both ACC and ethylene formation.

### INTRODUCTION

The results from previous experiments showed that disks from immature-green fruits produced more wound ethylene than disks from mature-green fruits of rin tomato. The rate of wound ethylene production was proportional to cut surface area of disks and anaerobiosis completely stopped wound ethylene production (see Section One). Disks from immature-green fruits were stimulated to produce more wound ethylene than disks from mature-green fruit in response to applied ACC. Compact



cells close to the epidermis seemed to be the location of the enzyme(s) required for the conversion of ACC to ethylene. Rhizobitoxine, cycloleucine, and cycloheximide inhibited wound ethylene production. Rhizobitoxine and cycloleucine did not inhibit ACC-stimulated wound ethylene production while cycloheximide did (see Section Two). Wound ethylene production by disks from rin tomato was found to be nonautocatalytic and ethylene caused a negative feedback of ethylene production. Wound ethylene inhibited further ethylene production by blocking the conversion of ACC to ethylene (see Section Three). Applied CO<sub>2</sub> was also shown to inhibit ethylene production by tomato disks (see Section Four).

Abeles and Abeles (1) first reported that treatment of toxic compounds, such as CuSO<sub>4</sub>, endothal, and ozone, increased the conversion of [U<sup>14</sup>C] methionine into ethylene from bean and tobacco leaves. Cycloheximide inhibited the production of stress-induced ethylene. Hanson and Kende (9) presented more convincing evidence that wound ethylene production from rib segments excised from immature flower buds of morning-glory flowers was synthesized from carbon atoms 3 and 4 of methionine. Aminoethoxyvinylglycine (AVG) completely inhibited wound ethylene production in wounded tissue. Hyodo (11) reported that the incorporation of label into ethylene from L [3-<sup>14</sup>C] methionine by wounded tissue was strongly inhibited by L-canaline, 2,4-dinitrophenol and cycloheximide. Saltveit and Dilley (16) reported that anaerobiosis completely stopped wound ethylene production and its rate of wound ethylene production was severely reduced by low and high temperatures. After Adams and Yang (2) had found ACC to be the closest intermediate to ethylene in the pathway of ethylene biosynthesis, Bradford and Yang (4) reported that anaerobic stress stimulated production of ACC in the roots of flooded tomato

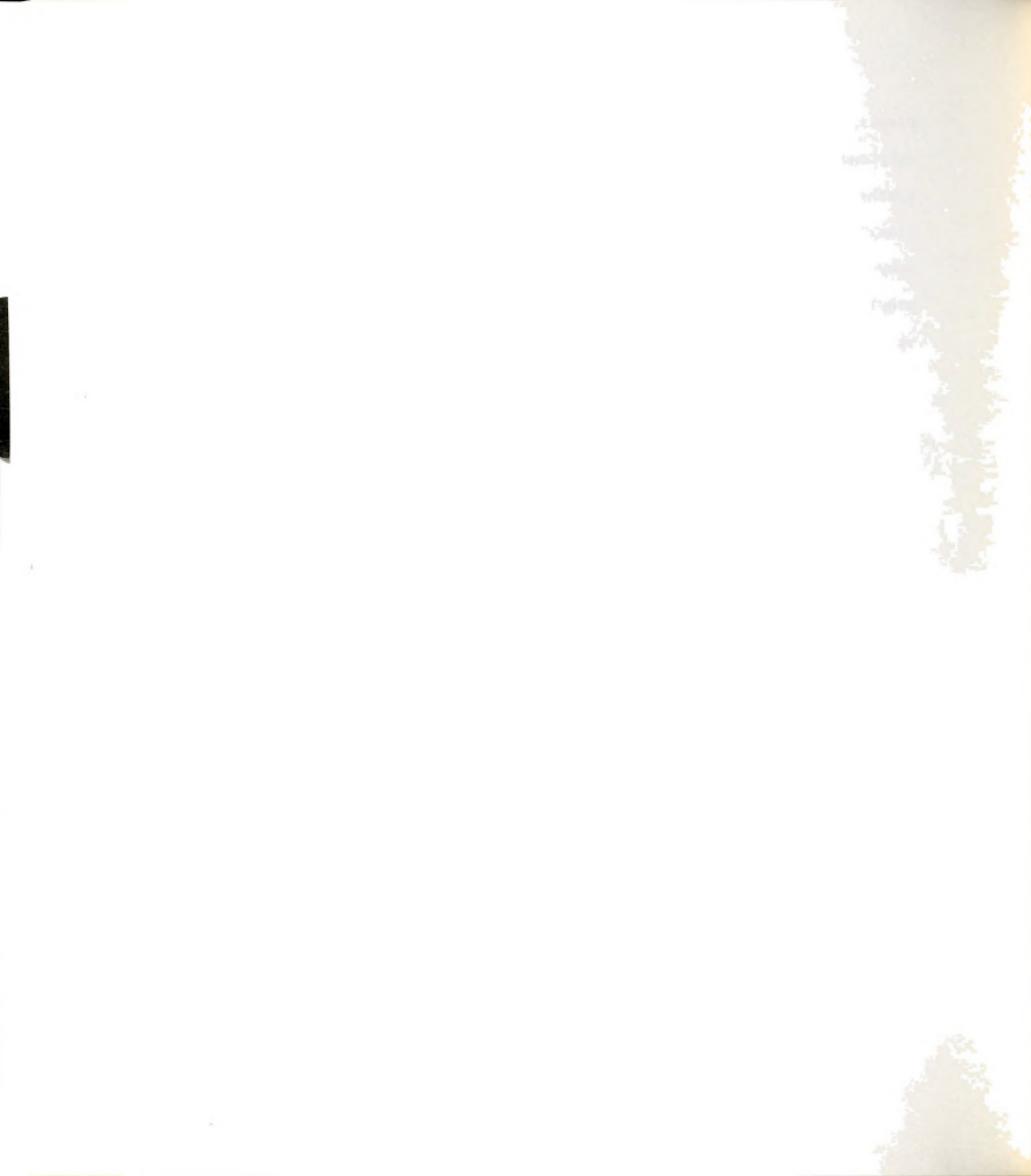


plants. The ACC was transported to the shoot where it was converted to ethylene. Yu and Yang (19) reported that wound or stress induced ACC synthase which is the rate-controlling enzyme in the pathway of ethylene biosynthesis and thereby causes accumulation of ACC and increases ethylene production. Cycloheximide completely blocked wound-induced ethylene production, ACC formation, and development of ACC synthase activity. It does appear that wound- or stress-induced ethylene production has the same biochemical pathway that forms ethylene in natural physiological systems such as ripening fruits of apples (2) and tomatoes (3), floral senescence (10,17) and in auxin-induced ethylene production in vegetative tissues (15,18). This paper reports ACC formation in fruit pericarp tissue of rin tomato in relation to wound-induced ethylene production.

## MATERIALS AND METHODS

### Plant Material and Incubation

Plants of rin tomato (Lycopersicon esculentum Mill.) were grown as previously described (see Section One). Unless noted otherwise, disks of pericarp tissue (diameter: 1.5 cm; thickness: 0.25 cm) from immature- and mature-green fruit ( $25 \pm 1$  and  $40 \pm 1$  days after anthesis for immature- and mature-green fruits, respectively) were prepared from the equatorial part of tomato fruits using a cork borer and template. Disks of pericarp tissue were placed with the epidermis on a layer of glass beads in a 20 ml scintillation vial. The vials were flushed with ethylene-free air every 2 h, sealed with a serum cap, then incubated in darkness at 20°C.



### Chemical Treatments

Twenty  $\mu$ l of chemical solutions or distilled water (control) were applied to the cut surface of disks opposite the epidermis as previously described (see Section One).

### Gas Treatments

The containers with disks were flushed with ethylene-free air and sealed with serum caps. The procedures for the treatment of nitrogen, specific concentration of carbon dioxide and propylene were the same as previously described (see Sections Three and Four).

### Determination of Ethylene Production and ACC Content

At the end of every 2 h incubation period, 1 ml of the atmosphere was withdrawn with a 1 ml gas tight syringe through the serum cap and assayed by a gas chromatograph as previously described (see Section One). After determination of ethylene, ACC content of disks was extracted and assayed by the method of Boller et al. (5). Tomato disks were placed into a glass homogenizer. Five ml of cold 80% ethanol containing 0.05% (v/v) mercaptoethanol were added per two disks, and the disks were thoroughly homogenized. ACC in the ethanol extract was isolated by thin-layer chromatography and was converted to ethylene with pyridoxal phosphate.  $\text{MnCl}_2$  and  $\text{H}_2\text{O}_2$  in a 10 ml test tube. The amount of ethylene liberated was determined by gas chromatography. The efficiency of the conversion of ACC to ethylene was estimated by adding 10 nmol of authentic ACC as internal standard to a separate sample which was then degraded to ethylene by the same method. The amount of ACC was calculated as the quotient of ethylene liberated and the conversion efficiency. Each experiment was repeated two times with three extracts for each treatment.



RESULTSStage of Fruit Development

Disks from immature-green fruits produced more ACC and ethylene than disks from mature-green fruits (Figure 44). The rate of ethylene production was paralleled by ACC levels in both disks from immature- and mature-green fruits throughout the incubation period (6 h).

Increase in the Cut Surface Area

Figure 45 shows that disks rewounded by making three cuts through and perpendicular to the cut surface resulted in more ACC and ethylene being formed than disks which were not rewounded.

Effect of Inhibitors

Rhizobitoxine, cycloleucine, and cycloheximide inhibited both ACC and ethylene production in disks resulting in less ACC and ethylene than that of control disks which were treated with water (Figure 46). Cycloheximide seemed to be the most inhibitory.

Effect of Anaerobiosis, Carbon Dioxide, and Propylene

Figure 47 shows that  $N_2$  atmosphere, air containing 7.60%  $CO_2$ , and  $2000 \mu l l^{-1}$  propylene inhibited ethylene production. However, only the atmosphere containing  $CO_2$  inhibited both ACC and ethylene formation while  $N_2$  atmosphere and air containing propylene increased ACC formation in disks as compared to that of disks incubated in air.

DISCUSSION

The rate of wound ethylene production by disks after cutting was proportional to the endogenous levels of ACC formation. Disks from immature-green fruits formed ACC and produced wound ethylene at a higher rate than disks from mature-green fruits. This is consistent with the



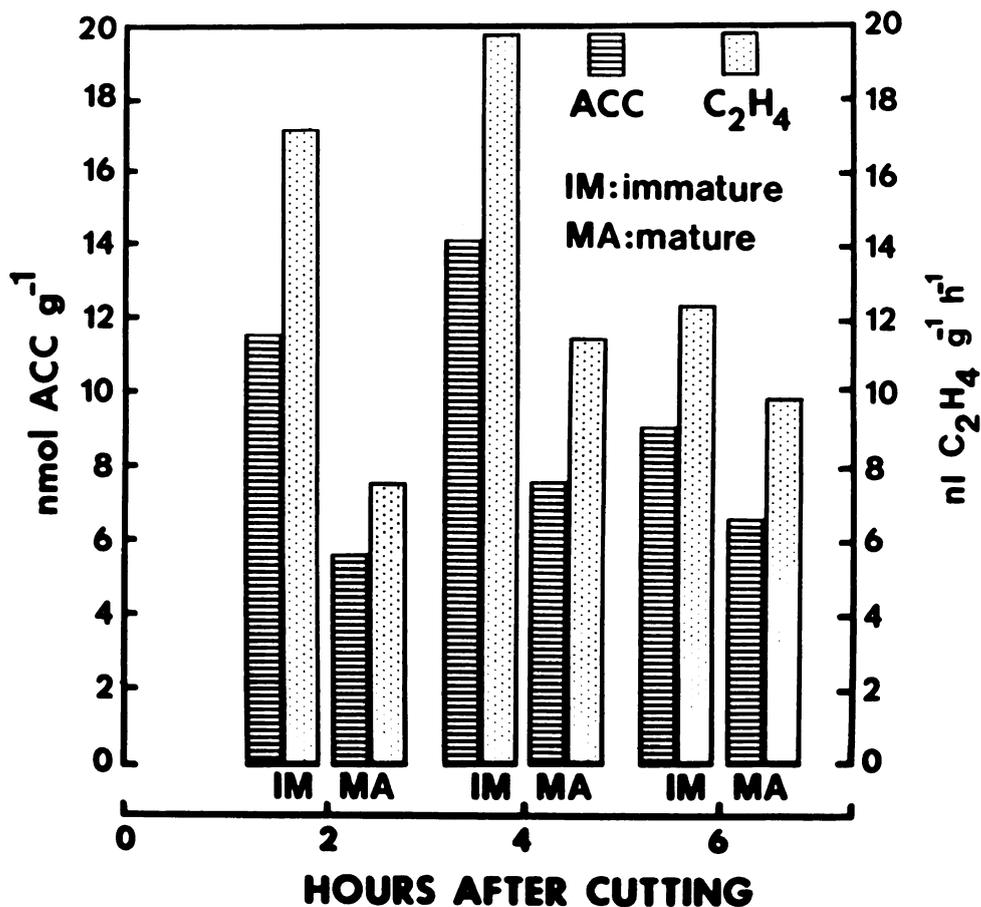
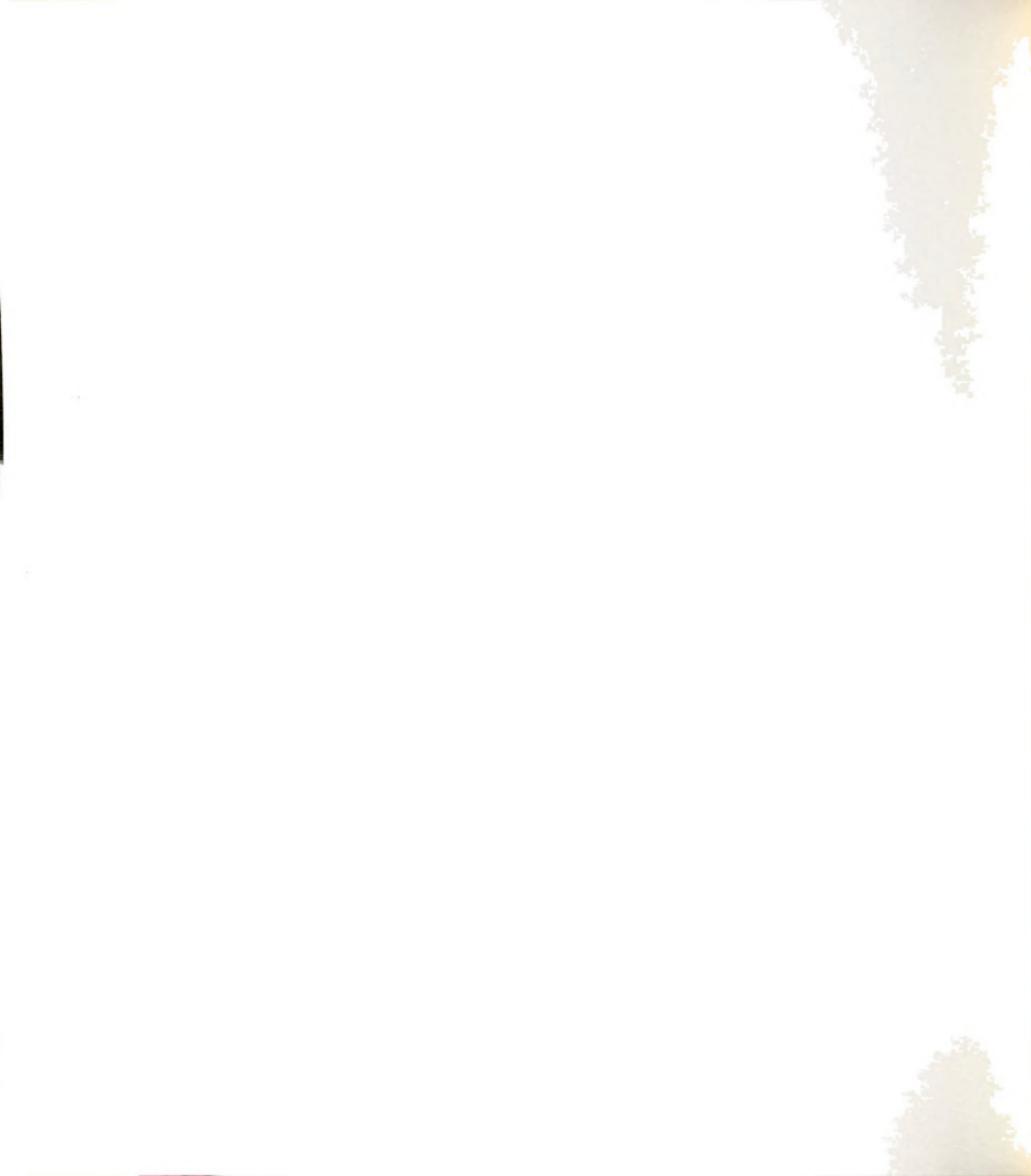


Figure 44. Effect of stage of fruit development on ACC and ethylene formation. Individual disks from immature- and mature-green fruits were incubated in closed containers. Ethylene was determined and then ACC was extracted and assayed at the end of every 2 h period.



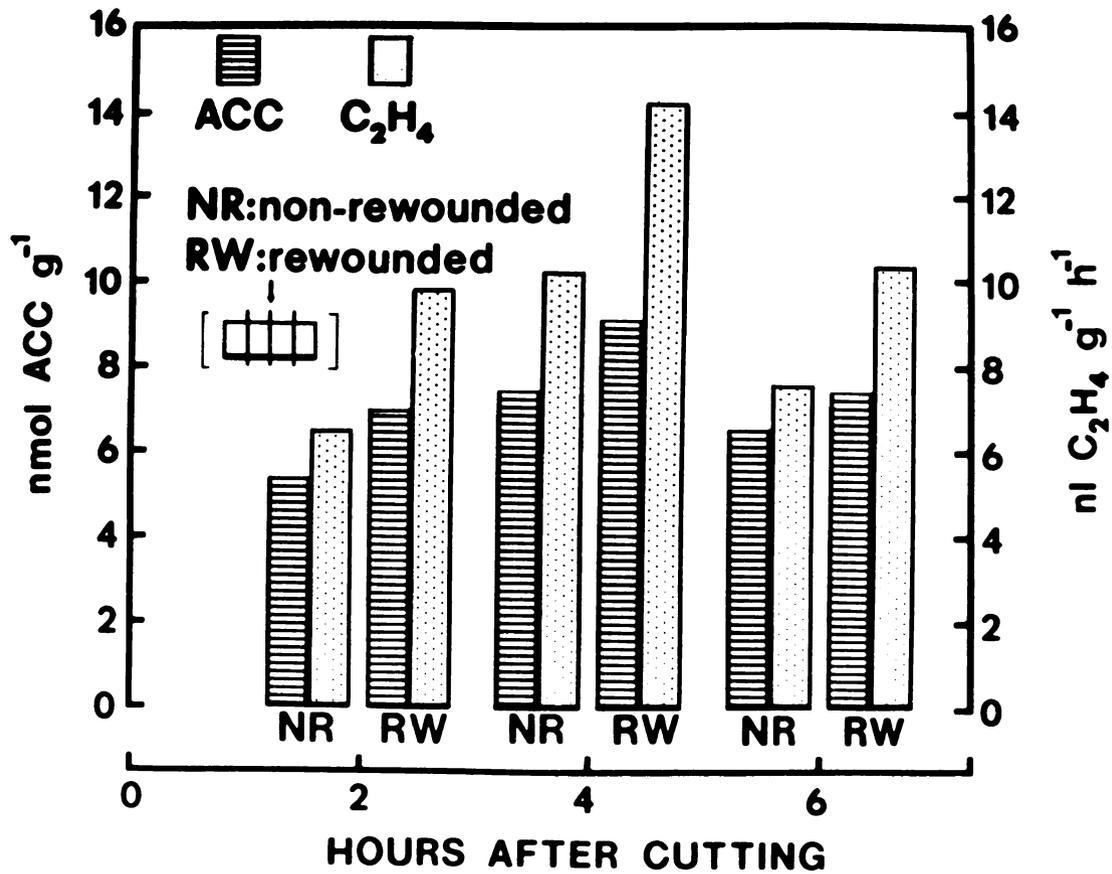


Figure 45. Effect of increase in cut surface area on ACC and ethylene formation of disks from mature-green fruits. Disks were non-rewounded or rewounded by making three cuts through and perpendicular to the cut surface of disks immediately after cutting and incubated in closed containers. Ethylene was determined and then ACC was extracted and assayed at the end of every 2 h period.



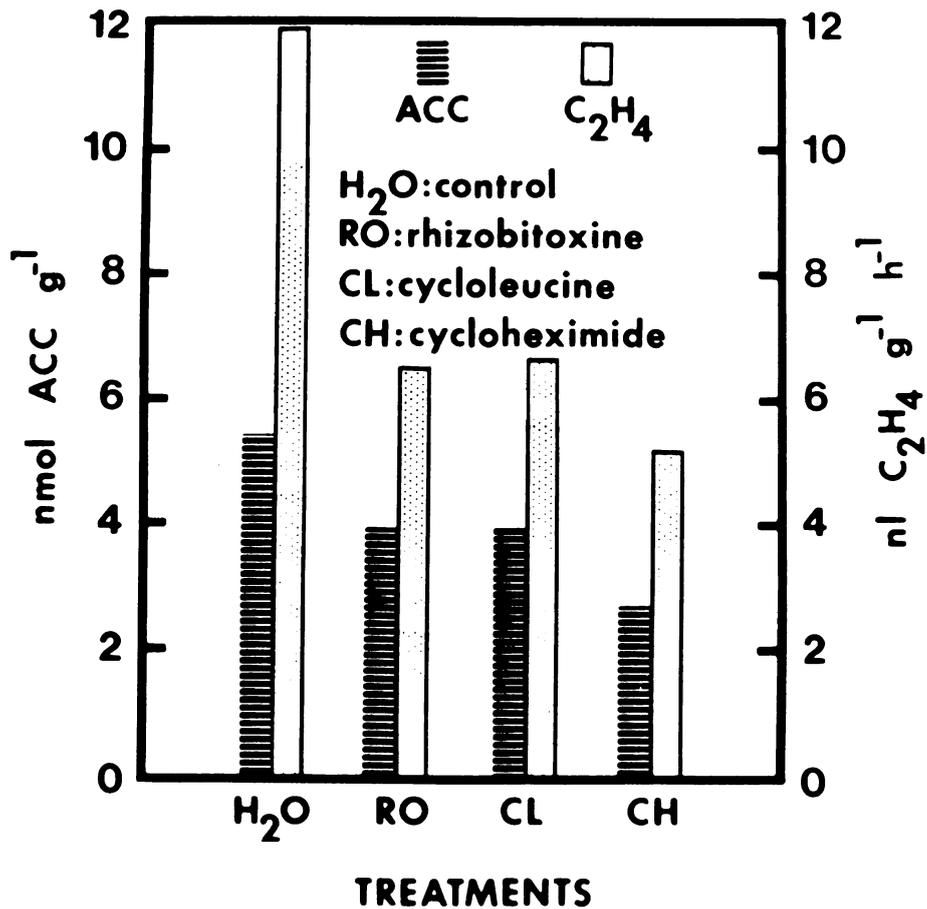


Figure 46. Effect of inhibitors on ACC and ethylene formation of disks from mature-green fruits. Twenty  $\mu$ l of H<sub>2</sub>O, 1 mM rhizobitoxine, 10 mM cycloleucine, and 0.1 mM cycloheximide were applied to the cut surface of disks immediately after cutting and incubated in closed containers for 4 h. Ethylene was determined and then ACC was extracted and assayed.



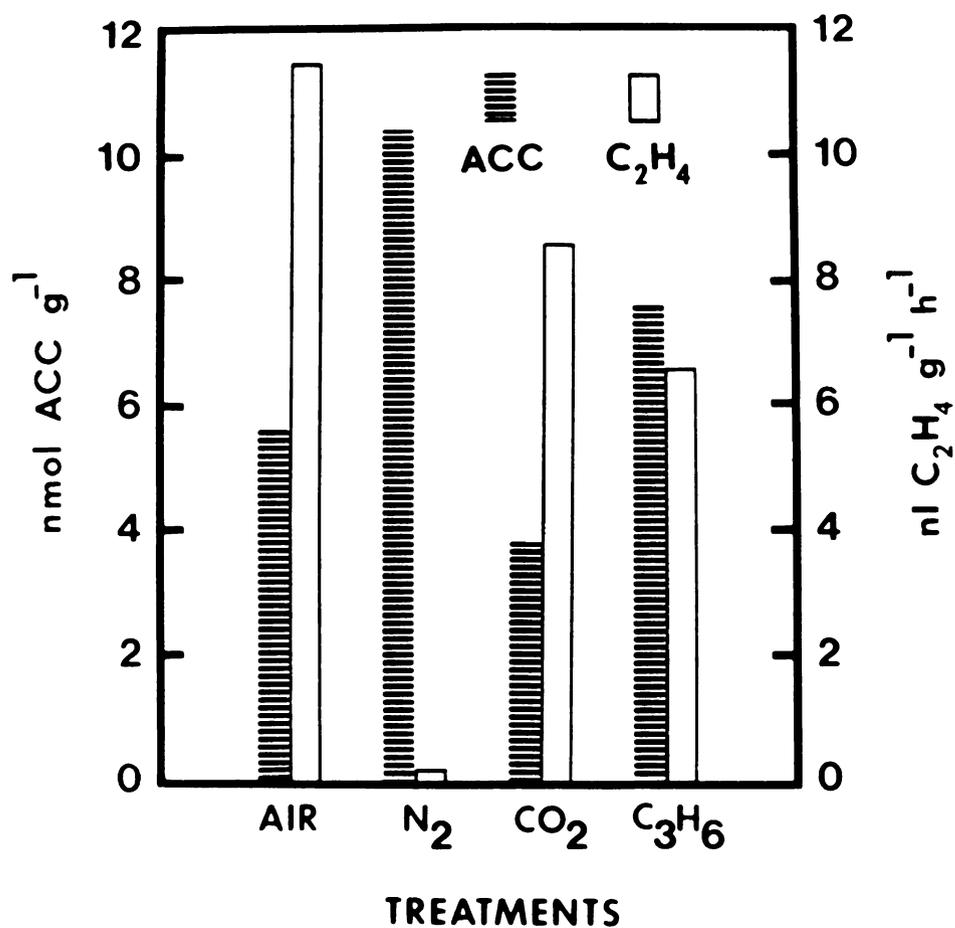
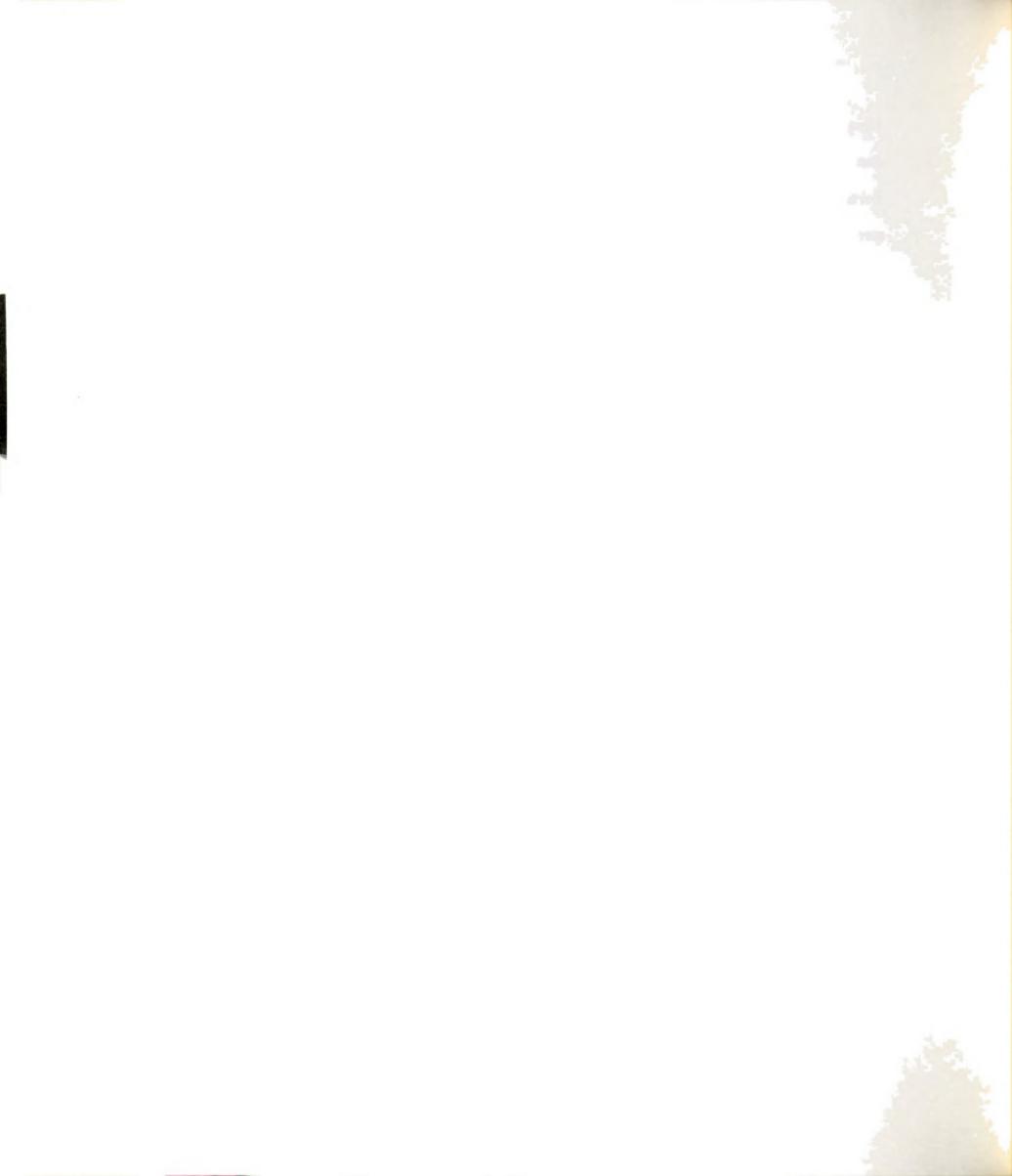


Figure 47. Effect of anaerobiosis, carbon dioxide and propylene on ACC and ethylene formation of disks from mature-green fruits. Disks were incubated in closed containers containing air, N<sub>2</sub>, 7.60% CO<sub>2</sub> or 2000  $\mu\text{l l}^{-1}$  C<sub>3</sub>H<sub>6</sub> for 4 h. At the end of 2 h period, disks were flushed with ethylene-free air and retreated with various gases again. At the end of incubation period, ethylene was determined and then ACC was extracted and assayed.



idea that the rate of ethylene production is proportional to the concentration of ACC (6). Wounding has been shown to induce the ACC-forming enzyme (18). When the disks were rewounded this resulted in more ACC and ethylene formation. This indicates that the increase in cut surface area of disks which subsequently produced more ethylene was not due to the increase in diffusion as has been suggested (5) but is due to increased synthesis of ACC resulting in more ethylene. The relationship between the cut surface area or the degree of injury of tissue and the rate of wound ethylene production has been reported in seedlings of bean (14), rice (12), sweet potato root (13), and wild-type tomato fruits (7). The increase in the degree of injury of tissue may induce the tissue to synthesize more ACC synthase (19).

Rhizobitoxine, cycloleucine and cycloheximide inhibited wound ethylene production because they reduced the formation of ACC. Rhizobitoxine has been shown to inhibit the activity of ACC synthase in ripening tomato fruits (3), auxin-treated mung bean hypocotyls (18) and pea stem sections (15), and wounded tissues of mung bean hypocotyls, orange and tomato fruits (19) but not the enzyme(s) required for the conversion of ACC to ethylene (3, also see Section Two). Though cycloleucine inhibited ACC formation similar to rhizobitoxine, its mode of action in the pathway of wound ethylene biosynthesis in disks of rin tomato fruits seems to differ from that of rhizobitoxine because it inhibited the SAM-stimulated wound ethylene production rather ACC synthase (see Section Two). Cycloheximide may generally inhibit the activity of enzyme systems in the pathway of ethylene biosynthesis because it inhibited both ACC synthase (19) and the enzyme(s) required for the conversion of ACC to ethylene (also see Section Two).



$N_2$  atmosphere has long been known to completely stop ethylene biosynthesis in both wounded and senescent plant tissues (8,16, also see Section One). However,  $N_2$  atmosphere did not stop ACC formation in disks indicating  $N_2$  atmosphere did not inhibit the induction of the ACC synthase in wounded tissue. In fact, the amount of ACC formed by disks incubated in  $N_2$  atmosphere was higher than that of disks incubated in air. This was due to the absence of  $O_2$ ; ACC was formed and accumulated until returned to air, whereby it was converted to ethylene (8). Propylene acted similarly to  $N_2$  in respect to accumulation of ACC in the tissue but was less pronounced. This indicates that ethylene and its analog, propylene, inhibits its own production by blocking the conversion of ACC to ethylene as suggested in the previous experiment (see Section Three) resulting in the accumulation of ACC. Exogenously applied  $CO_2$  seemed to have a different mode of action on ethylene biosynthesis from that of  $N_2$  atmosphere and propylene because it inhibited both ACC and ethylene formation in disks indicating that  $CO_2$  must exert its action on ethylene biosynthesis between methionine and ACC. The results from previous experiments have shown that exogenously applied  $CO_2$  inhibited both methionine- and SAM-stimulated wound ethylene production but not in ACC-stimulated wound ethylene production (see Section Four). It is probable that exogenous  $CO_2$  may inhibit ACC synthase activity.



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## SUMMARY AND CONCLUSIONS

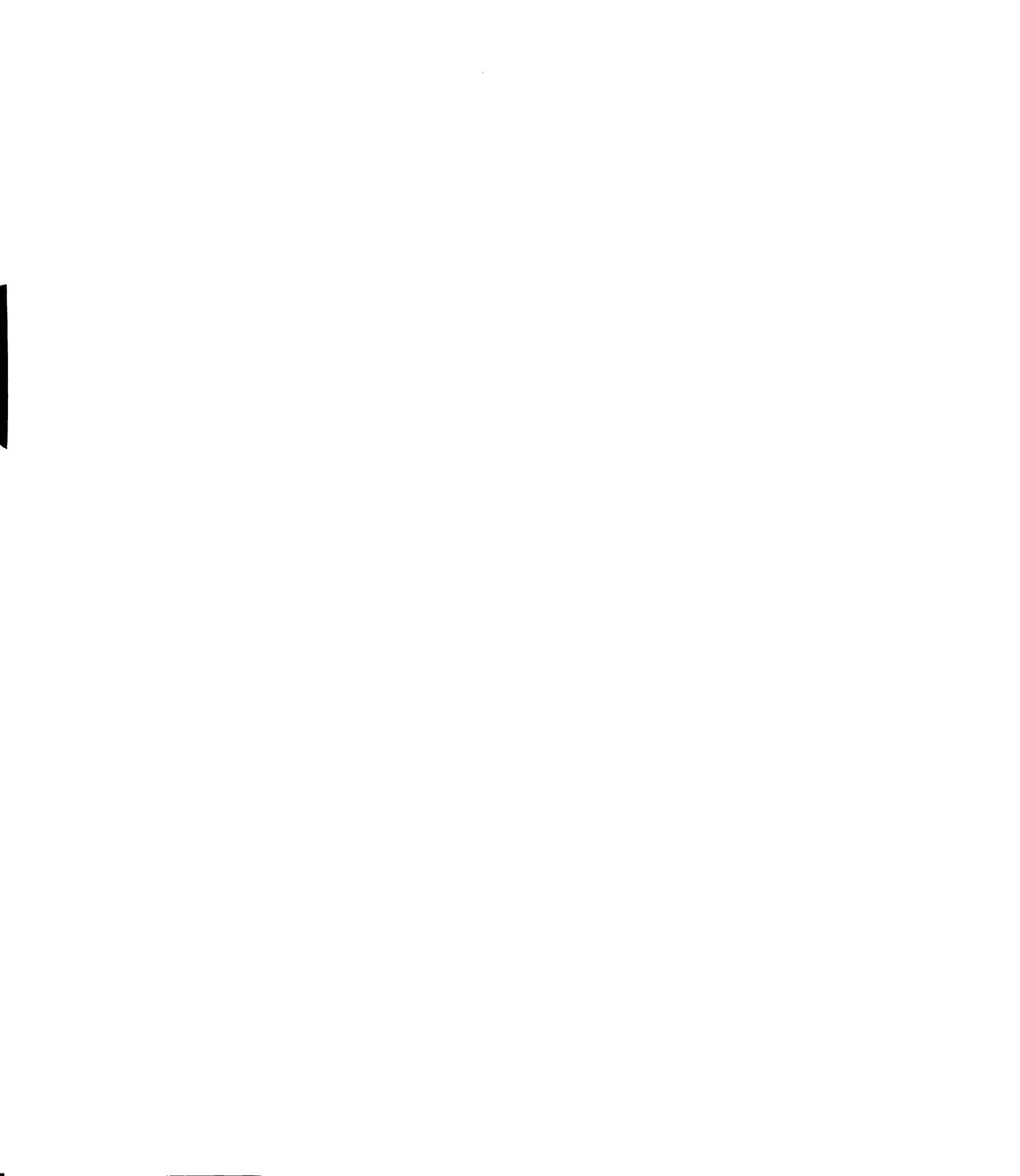
Fruits of rin mutant tomato produce little ethylene, however, upon wounding or cutting disks of pericarp tissue ethylene is produced at a rate higher than intact fruits. Immature-green fruits respond to wounding by producing much higher amounts of ethylene than mature-green fruits. Wounding appears to cause induction of enzyme(s) required for ethylene biosynthesis rather than to the increasing the diffusion of this gas. The intact epidermis of pericarp tissue seems to be the location of enzyme(s) required for ethylene biosynthesis.

Data from applying ACC at different times and the ethylene production from pericarp tissue originating from different parts of the fruit in response to ACC indicate that the enzyme(s) required for the conversion of ACC to ethylene may be induced and/or increased by wounding. Disks from immature-green fruits produce more ethylene in response to ACC application than those from mature-green fruits and this is probably due to the higher number of cells in immature disks. Low (10°C) and high (40°C) temperatures have the different mode of action in the inhibition of ACC-stimulated ethylene production.

Wound-induced ethylene synthesis is autoinhibitory. The rapid increase in ethylene synthesis may cause a negative feedback and this phenomenon appears to occur in the ethylene biosynthesis step from ACC to ethylene. Carbon dioxide released by wounded tissue can antagonize



the negative feedback of ethylene. On the other hand, application of carbon dioxide inhibits the conversion of SAM to ACC.



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