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**EFFECTS OF CARBON DIOXIDE ON THE ETHYLENE-FORMING
ENZYME IN JAPANESE PEAR AND APPLE FRUITS**

A thesis presented in partial fulfilment
of the requirements for the degree of
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ABSTRACT

The aim of this thesis was to investigate the effects of carbon dioxide on ethylene-forming enzyme (EFE) and its regulation in both Hosui and Granny Smith fruit discs.

1. From measurement of respiration rate and ethylene production after harvest and response of them to propylene treatment, it has been shown that Japanese pear ^{ed} Hosui grown in New Zealand is a nonclimacteric fruit. Granny Smith apple showed different respiration and ethylene production patterns in different seasons. Because fruit produced typical climacteric respiration and ethylene peaks, Granny Smith apple is a climacteric fruit.

2. The rapid rise of ethylene production and respiration measured at 21°C showed no time lag in Granny Smith fruit after storage for 20 days at low temperature ($1^{\circ}\pm 1^{\circ}\text{C}$) compared to those immediately measured after harvest at 21°C. Ethylene production and respiration rate also increased faster and to a greater extent in fruit exposure ^{ed} to low temperature than in fruit measured immediately after harvest. This ripening behavior is similar to that of European pears and Golden Delicious apple. In contrast to Granny Smith apple, ethylene production and respiration patterns of Hosui were not changed by low temperature storage.

3. A reliable method for testing ethylene forming enzyme (EFE) activity in fruit discs of Hosui and Granny Smith was developed.

4. At harvest time EFE activity was present in Hosui fruit, but not in Granny Smith apple. EFE development showed a similar pattern in both fruits, increasing steadily in fruit stored at low temperature.

5. Carbon dioxide stimulated EFE synthesis in fruit discs of preclimacteric Granny Smith, but not in those of Hosui. Carbon dioxide stimulated EFE activity in Hosui fruit discs during the short term storage at $1^{\circ}\pm 1^{\circ}\text{C}$, after ~~that~~ ^{which} CO_2 lost its stimulatory effect. In contrast to Hosui, CO_2 stimulated EFE activity in Granny Smith fruit discs through the measuring period tested.

out

6. Carbon dioxide was not able to reverse Co^{++} (an EFE activity inhibitor) inhibitory effect on EFE activity in ^{2) Hosui} both types of fruit discs. This result showed that CO_2 could directly stimulate EFE activity.

7. The stimulatory effect of CO_2 on EFE activity was dependent on exogenous ACC. EFE activity in both types of fruit discs was lower in 0.4 M mannitol solution than in 0.8 M solution, but EFE lost the capability to respond to CO_2 in 0.8 M mannitol solution. This suggests that the EFE located in the plasma membrane is the main form to respond to CO_2 in both types of fruit discs.

8. Results from kinetic studies indicated that EFE in discs of both Hosui and Granny Smith fruits was not allosteric. The apparent K_m values of EFE for ACC were 0.166 mM for Hosui, and 0.193 mM for Granny Smith apple. Carbon dioxide increased the maximum reaction rate of conversion of ACC to ethylene without changing apparent K_m values of EFE for ACC in discs of both Hosui and Granny Smith fruits. It suggests that the mechanism of the direct stimulatory effect of CO_2 on EFE activity was due to the formation of a CO_2 -EFE-ACC complex and/or EFE-ACC- CO_2 complex which increased the maximum rate of the reaction which resulted in the conversion of ACC to ethylene.

9. Silver ions inhibited ethylene production in Granny Smith fruit discs, but not in Hosui fruit discs which did not produce detectable ethylene. Low concentrations (<0.25 mM) Ag^+ stimulated, but high concentrations (>0.5 mM) inhibited, EFE activity in both types of fruit discs. Carbon dioxide did not reverse the inhibitory effect of Ag^+ . Because only System I ethylene receptor is thought to occur in Hosui, a nonclimacteric fruit, and the inhibitory effect of Ag^+ on EFE activity in discs of both types of fruit were similar, results suggest that EFE activity was regulated by the System I ethylene receptor.

10. Norbornadiene (NDE) is a competitive ethylene action inhibitor, which inhibits ethylene synthesis by binding to the ethylene binding site of ethylene receptors. At 0.5% (v/v), NDE inhibited EFE activity in both types of fruit discs, and this inhibition was partially reversed by CO_2 in discs from unripe Hosui and preclimacteric Granny Smith fruit. These results suggest that CO_2 might indirectly stimulate EFE activity by binding competitively to the System I ethylene receptor at the ethylene binding site. When Granny Smith was at

the climacteric stage CO_2 did not reverse the NDE inhibitory effect on EFE activity.

11. According to the response of EFE to CO_2 and the mechanisms of the response, cells in Hosui and Granny Smith fruits were distinguished into four different types. During ripening, cells in fruits changed their type, thereafter the responsiveness of EFE to CO_2 changed. A model is presented to explain the mechanism of CO_2 on EFE synthesis and its activity through interactions with EFE directly and the ethylene receptors indirectly.

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CHAPTER 1

GENERAL INTRODUCTION

Pome fruit, such as apples, pears and Asian pears are among the most important and popular fruits consumed in temperate countries. Much research work has been done on controlled atmosphere (CA) and modified atmosphere (MA) storage for delaying fruit senescence and reducing postharvest losses. Most studies have involved the technology of CA or MA storage or in developing the optimum CA conditions for each commodity (Brecht, 1980; Kader, 1980; Kader, 1985; Kader and Morris, 1981; Lipton, 1975; Meheriuk and Richardson, 1982). Only a very few reports have reported investigations of the biochemical and physiological effects of modifications in the CO₂ and O₂ contents of the storage atmosphere (Burton, 1974, 1978; Kader, 1986, 1989; Knee, 1973, 1984). Ethylene is a plant hormone which regulates many aspects of growth, development, and senescence (Abeles, 1973). Thus it is important to investigate the role of ethylene in fruit ripening, its biosynthesis and regulation.

1.1 TWO FRUIT RIPENING TYPES

Fruit have been divided into nonclimacteric and climacteric classes on the basis of respiration and ethylene production patterns during maturation and ripening (McMurchie *et al.*, 1972; Biale and Young, 1981). Climacteric fruit undergo a distinct ripening phase which is characterized by a rise in respiration and ethylene production, nonclimacteric fruit do not show this peak; rather respiration and ethylene continue to decline steadily after harvest. The two classes of fruit are distinguished by their responses to treatment with ethylene or its analogues, for example, propylene. In immature (preclimacteric) fruit of the climacteric type, ethylene treatment can accelerate the onset of climacteric of ripening, especially autocatalytic ethylene synthesis and the respiration climacteric rise. Ethylene treatment does not change the climacteric patterns and magnitude of respiration and ethylene evolution, thus responses of respiration and ethylene biosynthesis to ethylene are concentration independent. In a climacteric fruit if a sufficient concentration over a long enough period is applied to cause a respiratory rise, no return to a pre-climacteric stage will occur upon removal of the gas.

In nonclimacteric fruit, the magnitude of the respiratory response increases as a function of ethylene concentration, but this increase in respiratory activity is not accompanied by an increase in ethylene production (Biale and Young, 1981). In nonclimacteric fruits respiration rate will return to the level of untreated control fruit if ethylene is removed at any time during treatment. The internal ethylene levels in climacteric fruit can range from low to high, but in nonclimacteric fruit levels are low.

The main pome fruits are apples and pears which include European and Asian pears. Apples and European pears are climacteric fruits, they show different ripening characteristics. For Asian pears, there are only a few of reports on their post-harvest behaviour. Some Chinese pears, such as Ya-li, Tsu-li, and some Japanese pears, such as Hosui, Shinsui, Kosui, Yakumo and Kikusui are classified as climacteric fruits (Kitamura *et al.*, 1981; Kajiura *et al.*, 1981), whereas others, such as Nijisseiki and Niitaka are nonclimacteric fruits in Japan (Kitamura *et al.*, 1981). In New Zealand, Japanese pear is a new crop. New Zealand reports on fruit maturity indices and storage disorders have been published (Lallu, 1985a,b), but there is little knowledge about their ripening patterns. In previous work in 1986, I found that neither Hosui nor Nijisseiki fruit showed postharvest respiration and ethylene production climacteric peaks. In contrast with Nijisseiki, Hosui fruit did not develop the serious physiological disorders, flesh spot decay and core browning. In this thesis Hosui was selected as experimental material which represented a nonclimacteric type of fruit. 'Granny Smith' is an important apple cultivar grown in New Zealand for local and export markets. Granny Smith has a long storage life compared to other apple cultivars and it was selected as being a representative of climacteric fruit for this study. The ripening patterns and maturity characteristics of both Hosui and Granny Smith fruits grown in New Zealand have been investigated.

1.2 REGULATION OF ETHYLENE BIOSYNTHESIS AND ACTION

1.2.1 Ethylene biosynthesis

1.2.1.1 1-aminocyclopropane-1-carboxylic acid (ACC) pathway

Ethylene synthesis in higher plants occurs predominantly *via* the ACC pathway (Fig.1-1) (Miyazaki and Yang, 1987). Lieberman *et al.* (1966)

showed that the 2 carbons of ethylene were derived from the number 3 and 4 carbons of the amino acid methionine. Adams and Yang (1977, 1979) and Lüssen *et al.* (1979) demonstrated that S-adenosyl methionine (SAM) and ACC were intermediates in the biosynthesis pathway from methionine to ethylene (Fig.1-1). The enzyme which converts SAM to ACC was named ACC synthase (Yu *et al.*, 1979; Boller *et al.*, 1979). ACC conversion to ethylene is mediated by a membrane-associated complex termed ethylene-forming enzyme (EFE) (Yang and Hoffman, 1984). In addition to its conversion to ethylene, ACC can be metabolized to N-malonyl-ACC (MACC) catalyzed by ACC N-malonyltransferase (Amrhein *et al.*, 1981, 1982; Hoffman *et al.*, 1982a).

Here it is worth mentioning that a cycle of reactions in ethylene biosynthesis leads to ACC through SAM (S-adenosylmethionine) - decarboxylated SAM - MTA (5'-methylthioadenosine) - ACC, releasing one of the carbon atoms of methyl thioribose as CO₂ (Miyazaki and Yang, 1987). Tan and Thimann (1989) indicated that CO₂ was needed for the conversion of exogenous ACC to ethylene, but not for the endogenous formation of ethylene and suggested that this CO₂ might remain associated with endogenous ACC, so that satisfied the CO₂ requirement.

1.2.1.2 A non-ACC/EFE pathway

Not all plants possess the methionine or intermediates to produce ethylene through this ACC/EFE pathway (Osborne, 1989). It is possible that this pathway is an evolutionary development which has arisen in higher plants.

In the fern *Regnellidium diphyllum*, a non-ACC ethylene biosynthetic pathway has been found although this plant contains low levels of both ACC and malonyl-ACC (Cookson and Osborne, 1978; Walters and Osborne, 1979). The characteristics of ethylene biosynthesis in this plant are: (1) no conversion of methionine to C₂H₄; (2) no conversion of ACC to C₂H₄; (3) no IAA-induced C₂H₄ formation (Walters and Osborne, 1979); (4) no wounded-induced C₂H₄ formation; (5) C₂H₄ production not blocked by AVG; (6) ACC does not substitute for C₂H₄ in cell growth responses (Osborne, 1989).

Osborne (1989) has shown that although ethylene was produced in a range of lower plants (*Bryophytes*, *Pteridophytes* and certain plants classed

in the *Gymnosperms*), they could not convert ACC to ethylene. Therefore it is likely that an alternative, and as yet unknown pathway for ethylene biosynthesis exists in such plants, where EFE is not an essential intermediate enzyme. She has suggested that ethylene biosynthesis *via* methionine, SAM and ACC evolved as a modern adaptation to the land.

1.2.2 Enzymes of ethylene biosynthesis and their regulation

The pathway and regulation of ethylene biosynthesis are summarized in Fig.1-2 (Yang, 1985). Ethylene production is known to be regulated at the reactions involving in ACC synthesis, conversion of ACC to ethylene and ACC conjugation by a variety of developmental and environmental factors (Yang and Hoffman, 1984).

1.2.2.1 ACC synthase

ACC synthase is the key enzyme which catalyzes the reaction from SAM to ACC. This is the main site of control of ethylene production (Yang and Hoffman, 1984). The molecular mass of ACC synthase is 55-57 KD in tomato pericarp tissue (Kende, 1989) and the K_m of ACC synthase for SAM (S-adenosylmethionine) is 13 μM (Boller *et al.*, 1979). This enzyme requires pyridoxal phosphate for activity and is sensitive to pyridoxal phosphate inhibitors, such as aminoethoxyvinylglycine (AVG) ($K_i=0.2 \mu\text{M}$, K_i is the dissociation constant for the enzyme-inhibitor complex) and aminoxyacetic acid (AOA) ($K_i=0.8 \mu\text{M}$). The substrate for the ACC synthase is (-)-SAM, which has the S-configuration at the sulfonium position and is the naturally occurring isomer of SAM, with a K_m of 20 μM , whereas (+)-SAM is an effective inhibitor (Khani-Oskouee *et al.*, 1984). ACC synthase can be inactivated when incubated with SAM and this process may be responsible for the short half-life (25-30 min) of the ACC synthase *in vivo*. It is proposed that the SAM, when activated by the ACC synthase, can irreversibly modify the enzyme in a 'suicide-inactivation' (Satoh and Esashi, 1986). ACC synthase can be induced by auxin. This shows the interaction of phytohormones, and represents a mechanism for fine endogenous regulation of ACC production levels in the plant. This 'rate control reaction' is regulated by phytochrome, wounding and by almost any kind of environmental stress, e.g. chilling injury, drought stress, flooding (Osborne, 1989; Thomas and Yang, 1987; Yang, 1985; Yang and Hoffman, 1984).

Because the main work in this thesis is involved in EFE and its regulation, the regulation of the conversion of SAM to ACC will not be reviewed in detail.

1.2.2.2 ACC N-malonyltransferase

This transferase is present in a wide range of plant tissues and has been isolated and partially purified from mungbean hypocotyls (Kionka and Amrhein, 1984). The malonyl donor is malonyl-CoA, with a K_m of 0.25 mM; at concentrations greater than 0.75 mM, malonyl CoA inhibits the transferase (Kionka and Amrhein, 1984). The K_m for ACC is 0.15 mM; the (1R,2S)- and (1R,2R)-aminoethyl cyclopropane carboxylic acid (AEC) isomers, which have a D-amino acid configuration, are more effective substrates and inhibitors of malonyltransferase than the (1S,2R)- and (1S,2S)-AEC isomers, which have an L-configuration (Liu *et al.*, 1984a,b). When ACC levels increase during development or when plants are under stress, ACC is malonylated by malonyltransferase which prevents overproduction of ethylene. Thus ethylene production *in vivo* is reduced by malonylation of ACC and promoted by blocking malonylation (Liu *et al.*, 1983). The malonyltransferase activity in preclimacteric tomato fruit is markedly stimulated by ethylene treatment (Liu *et al.*, 1985b), thereby proving an autoregulatory mechanism for limiting ethylene production. In this work EFE activity was measured after fruit tissues were saturated with a high concentration of exogenous ACC (3-5 mM), thus the reaction of ACC malonylation did not affect results of measurement of EFE activity.

1.2.2.3 Ethylene-forming enzyme (EFE)

Another important reaction of ethylene biosynthesis is the conversion of ACC to ethylene which is catalyzed by the ethylene-forming enzyme (EFE). The activity of EFE was first described by Adams and Yang (1979), who demonstrated that O₂ was essential for the conversion of ACC to ethylene. EFE is present in most tissues of higher plants with the exception of unripe fruit (Yang and Hoffman, 1984). Because EFE has not been isolated independent of intact cellular material (vacuoles, protoplasts or tissue), most characterization of the EFE has been done *in vivo*. This situation necessarily complicates interpretation of many experiments because observed effects on EFE can be attributed to direct or indirect

action. Despite this problem, important information about EFE has still been obtained.

Generally the activity of EFE is measured by ethylene production in the presence of a saturating concentration of ACC (Yang and Hoffman, 1984). It is possible that different types of plant material or different tissues have optimum conditions which are specific for that tissue. However only very few papers have reported comparative evaluations of the different methods for measuring EFE activity.

(a) Different buffer or sugar solutions with a wide range of pH values have been used for measuring EFE activity (Table 1-1).

(b) Several methods for saturating ACC have been used (Table 1-2), but no comparisons have been made between these techniques.

(c) EFE activity has been measured:

(1) In medium, such as in buffer for Granny Smith apple (Chaves and Tomas, 1984), in water containing ACC for oat leaves (Preger and Gepstein, 1984), Golden Delicious apple and pinto beans (*Phaseolus vulgaris* L) (Apelbaum *et al.*, 1981), and leaves of *Xanthium strumarium* L. and *Zea mays* L. (Grodzinski *et al.*, 1982); or after discs have been preloaded in water containing ACC for 3 hours, then transferred into water for etiolated wheat seedling (Jiao *et al.*, 1987);

(2) On filter paper wetted with buffer for Granny Smith apple (Cheverry *et al.*, 1988);

(3) In air with no buffer for peel of Golden Delicious apple (Bufler, 1986), tobacco leaf discs (Philosoph-Hadas *et al.*, 1986), wheat leaves (McKeon *et al.*, 1982), rice leaf segments and tobacco leaf discs (Kao and Yang, 1982), citrus leaf discs (Riov and Yang, 1982b).

The wide range of apparently successful methods used for measuring EFE, indicate the robustness of EFE in different systems. Unfortunately none of these techniques indicate the efficiency of the methods. Therefore it is necessary to establish a technique which is simple, reliable and efficient for measuring EFE activity, and which gives consistent results for particular tissue systems.

Table 1-1. Different buffers and solutions for measuring EFE activity.

Plants and solutions	References
<u>Fruits:</u>	
Avocado and tomato 0.028 M citrate, 0.047 M phosphate, 0.4 M sucrose (pH 4.6) 2% sucrose,	Lieberman <i>et al.</i> , 1977
Cantaloupe melons 2% sucrose, 50 mM phosphate buffer (pH 6.8)	Hoffman <i>et al.</i> , 1982b
Granny Smith apples 10 mM phosphate buffer (pH 6.8) 0.6 M sorbitol (pH 7)	Chaves and Tomas, 1984 Cheverry <i>et al.</i> , 1988
Golden Delicious apples 10 mM Mes buffer (pH 6.0) 2% sucrose, 50 mM phosphate buffer (pH 6.8) 0.4 M sucrose	Bufler, 1986 Hoffman <i>et al.</i> , 1982b Lieberman <i>et al.</i> , 1977
<u>Vegetative organs or tissues:</u>	
Hypocotyls of mung bean 2% sucrose, 50 mM phosphate buffer (pH 6.8)	Hoffman <i>et al.</i> , 1982b
Rose petals Distilled H ₂ O	Faragher <i>et al.</i> , 1987
Leaves	
Cotton 10 mM Mes buffer (pH 6.1)	Suttle, 1986
Citrus 50 mM K-phosphate buffer (pH 5.3)	Riov and Yang, 1982a
Oat Distilled H ₂ O	Gepstein and Thimann, 1980
Soybean Distilled H ₂ O	Bassi and Spencer, 1983
Sunflower Distilled H ₂ O	Bassi and Spencer, 1983
Tobacco 10 mM Mes buffer (pH 6.1) Distilled H ₂ O	Philosoph-Hadas <i>et al.</i> , 1986 Gepstein and Thimann, 1980
Tomato 50 mM Na-phosphate buffer (pH 6.1) Distilled H ₂ O	Philosoph-Hadas <i>et al.</i> , 1985 Gepstein and Thimann, 1980; Bassi and Spencer, 1983
Wheat Distilled H ₂ O	McKeon <i>et al.</i> , 1982
<i>Xanthium strumarium</i> L. Distilled H ₂ O	Grodzinski <i>et al.</i> , 1982
<i>Zea mays</i> L. Distilled H ₂ O	Grodzinski <i>et al.</i> , 1982

Table 1-2. Several methods in EFE measurement for saturating ACC into tissue discs.

Methods	Plants	References
Incubation for 2 hours	Granny Smith apple	Chaves and Thomas, 1984
	tobacco leaves	Philosoph-Hadas <i>et al.</i> , 1986
	wheat leaves	McKeon <i>et al.</i> , 1982
for 3 hours vacuum-infiltrated	oat leaves	Preger and Gepstein, 1984
	Granny Smith apple	Cheverry <i>et al.</i> , 1988
intermittent vacuum-infiltration	Golden Delicious apple	Saltveit <i>et al.</i> , 1978

In higher plants a number of plant enzyme preparations, such as IAA oxidase (Vioque *et al.*, 1981), peroxidase (Rohwer and Mader, 1981), lipoxygenase (Gardner and Newton, 1987; Nilsen *et al.*, 1988; Wang and Yang, 1987), etiolated pea seedling homogenates (Konze and Kende, 1979), carnation (Mayak *et al.*, 1980) and pea (McRae *et al.*, 1982) microsomal preparations, can convert ACC to ethylene in the presence of various cofactors, but they lack the specificity and high affinity for ACC characteristic of native EFE (Guy and Kende, 1984; McKeon *et al.*, 1984; Venis, 1984). These enzymic systems display K_m 's for ACC over the range of 3 mM to 389 mM, indicating a lower affinity for ACC relative to that found K_m for EFE, they also do not distinguish among the 2-ethyl-ACC stereoisomers as does EFE (Guy and Kende, 1984; McKeon *et al.*, 1984; Venis, 1984).

The main characteristics of EFE are:

(1) It has a high affinity for ACC, its substrate.

It was demonstrated that EFE had K_m values of 66 μ M for ACC in pea hypocotyl segments (McKeon *et al.*, 1982), 61 μ M in pea-leaf vacuoles (Guy and Kende, 1984) and 125 μ M in ripe kiwifruit (John *et al.*, 1989). Hoffman *et al.* (1982b) described a test to distinguish between artifactual and natural ACC-dependent ethylene-forming activities. When one of the ring hydrogens of ACC is substituted with an ethyl group, four stereoisomers of 1-amino-2-ethyl-cyclopropane-1-carboxylic acid (AEC) are generated. Only one of these, (1R,2S)-AEC, is converted preferentially to 1-butene by the same enzyme that oxidizes ACC to ethylene (EFE). Artifactual ethylene-forming systems do not show this stereospecificity (Venis, 1984).

(2) EFE is probably a membrane-bound enzyme.

It is difficult to measure EFE activity *in vitro* because its activity disappears when cell integrity is lost. Some direct information is available on the location of EFE in plant cells. Mattoo and Lieberman (1977) observed that slices of apple incubated in the presence of cell-wall degrading enzymes lost the ability to generate ethylene from endogenous substrates and from methionine supplied in the reaction medium. They suggested that the ethylene-synthesizing enzyme system is in the cell membrane-cell wall complex. This suggestion finds support in more recent work. Bouzayen *et al.* (1987) presented results which showed that the EFE activity of suspension cultured Acer cells was largely localized at the cell membrane-cell wall region. Intact protoplasts and vacuoles isolated from pea leaves possessed functional EFE properties and vacuoles produced 80% of the ethylene evolved by protoplasts (Guy and Kende, 1984). However when the plasma membrane or vacuolar membrane were ruptured, EFE activity was lost. The EFE activity of intact tissues was destroyed by homogenization and activity of EFE in the vacuole was destroyed by lysis of the vacuole (Guy and Kende, 1984; Mayne and Kende, 1986). Work using isolated vacuoles of *Vicia faba* provided evidence that EFE was associated with the inside face of the tonoplast and that the activity of this enzyme depended on membrane integrity (Mayne and Kende, 1986). In addition to the failure to isolate EFE except in intact vacuoles, protoplasts or cells, the sensitivity of EFE to detergents and to chilling-treatment suggests a requirement for an intact membrane. John *et al.* (1989) reported that EFE activity was largely retained when slices of apple (*Malus sylvestris*) tissue were incubated in the presence of the cell-wall degrading enzymes, Pectolyase and Caylase (a Cellulase), even though the action of these enzymes resulted in the loss of cell wall material so that more than 90% of the tissue could pass through a 0.2 mm mesh. Activity of the EFE disappeared after this treatment only when the cells and protoplasts were released from the tissue. John *et al.* (1989) also reported that membranes present in the juice squeezed from ripe kiwifruit (*Actinidia deliciosa*) possess an EFE activity which shows the two essential features (the low K_m and the stereospecificity for the substrate) of the enzyme activity observed *in vivo*. In addition, the EFE activity of the kiwifruit required membrane integrity, as the loss of activity occurred when the membrane vesicles were denied osmotic support. Fractionation of these membranes on continuous density gradients revealed that the EFE activity was confined to a particular species of membrane vesicle. When a variety of

marker enzymes characteristic of specific cell organelles were assayed in the fractions collected from the gradient, two enzymes were found to be associated with the fractions that contained the EFE activity. These were GOT (glutamate oxaloacetate transaminase), characteristic of plastids (John *et al.*, 1989; Mifflin, 1974), and α -mannosidase, characteristic of vacuolar sap (Boller and Kende, 1979). But when membranes were separated by flotation through a linear gradient of Nycodenz, there was no distinct band of protein, GOT or α -mannosidase associated with the EFE activity. In particular there was a significant lack of α -mannosidase activity in fractions in which the EFE was concentrated (John *et al.*, 1989). Using the response to osmoticum, Bouzayen *et al.* (1987, 1990) and Pech *et al.* (1989) distinguished different EFE localizations in cells of different grape cultivars. They reported that EFE was mainly located at the plasmalemma in cells of one grape cultivar (Muscat), whereas it was located at the tonoplast in cells of another grape cultivar (Gamay).

There are several possible functions for the membrane-bound nature of EFE. EFE activity may be coupled to a transmembrane proton flow (John, 1983) as EFE can be inhibited by 2,4-dinitrophenol, an uncoupler of proton transport system. Alternatively, EFE may require a membrane-bound electron transport system. Since the oxidation of ACC by EFE involves sequential electron transfers, disruption of the membrane could lead to loss of activity by interfering with the integrity of the electron transport chain. Finally, the membrane may protect the EFE by maintaining it in a high concentration in the presence of an as yet uncharacterized cofactor, or by otherwise protecting the EFE from inactivation (Thomas and Yang, 1987).

Many inhibitors of EFE activity have been reported. Cobaltous ion is a very effective inhibitor of EFE activity when applied in the range of 10 to 100 μ M (Lurssen *et al.*, 1979; Yu and Yang, 1979). Alpha-aminoisobutyrate, a structural analogue of ACC, competitively inhibits the conversion of ACC to ethylene (Sato and Esashi, 1980). Free radical scavengers such as *n*-propyl gallate and sodium benzoate represent another class of inhibitors (Lieberman, 1979). Other compounds such as Vitamin K₅ and menadione are effective in blocking the conversion of ACC to ethylene *in vivo*, although they lack the specificity for targeted action desired in enzyme inhibitors for physiological studies (Saltveit, 1980; Yang and Hoffman, 1984).

The level of EFE increases markedly following some stress conditions and ethylene treatment, or with physiological development (such as fruit ripening) and thus can effectively regulate ethylene production (Yang and Hoffman, 1984). In high temperatures (>35°C) EFE activity decreases and at 40°C is lost (Yang and Hoffman, 1984). In unripe fruits of cantaloupe and tomato, levels of EFE are low, but they increase markedly following treatment with ethylene (Liu *et al.*, 1985a).

1.2.3 Regulation of ethylene biosynthesis by ethylene

The development of ACC synthase and EFE differs during fruit ripening. Ethylene can regulate ethylene biosynthesis by autoinhibition or autocatalization, the nature of the response depending on the ripeness of the fruit. Treatment of an immature climacteric fruit with ethylene or propylene results in a decrease of ACC content followed by a decreased ethylene production (autoinhibition) (McMurchie *et al.*, 1972). This indicates that ethylene has an inhibitory effect on the synthesis of ACC synthase (Riov and Yang, 1982a). In such system there is also an increase of the malonylation of ACC (Liu *et al.*, 1985b). When fruit tissue becomes more sensitive to low ethylene (more than threshold), ethylene can induce a great deal of ethylene production (autocatalization) by stimulating of development of ACC synthase. Ethylene also stimulates EFE development during the various fruit ripening stage (Yang *et al.*, 1986).

Trewavas (1982) concluded that the limiting factor in plant development was the sensitivity of tissues to plant growth substances and not the change in concentration of these substances. McMurchie *et al.* (1972) suggested that in climacteric fruit (banana) ethylene biosynthesis is initiated by receptor System I. The ethylene produced reaches a threshold level in the fruit sufficient to initiate the respiratory climacteric and associated ripening changes. The rise in respiration is induced by a further large increase in autocatalytic ethylene production (System II). McGlasson (1985) subsequently defined that ethylene biosynthesis in climacteric fruit is regulated by two systems. System I is involved in the regulation of aging processes and is responsible for the low rate of ethylene production during growth, and System II is responsible for the autocatalytic increase in ethylene production which accompanied ripening. Nonclimacteric fruit has System I, but not System II. In the context of fruit ripening, the change in

sensitivity to ethylene can be explained as a change in the nature and concentration of receptors, which means development of System I (in non-climacteric fruit and preclimacteric fruit) and System II receptors (in climacteric stage of climacteric fruit), and increased synthesis of these receptors.

The difference between System I and System II ethylene biosynthesis does not lie in the ethylene biosynthetic pathway, but rather in the tissues capacity to develop ACC synthase, the rate-limiting enzyme in ethylene biosynthesis (Hoffman and Yang, 1980; Sitrit *et al.*, 1986; Yang, 1985). Although an immature fruit is responsive to ethylene with respect to the development of EFE, it is unresponsive with respect to the development of ACC synthase (Liu *et al.*, 1985a). Thus, the responsiveness of a fruit to develop ACC synthase is dependent upon not only the presence of ethylene, but also on the fruit developmental stage. Generally speaking, the increase in ACC synthase is preceded by the increase in EFE when preclimacteric fruits are exposed to exogenous ethylene (Liu *et al.*, 1985a); it is not known whether this is also true during natural ripening. Many workers have investigated the changes in ACC, ethylene, EFE activity, and ACC synthase activity during fruit ripening (Blankenship and Richardson, 1985; Bufler, 1986; Knee, 1984; 1985). Their results indicated that the increases in these are highly coordinated. In 'Golden Delicious' apples, EFE increases concurrently with the rise of internal ethylene concentration (System I ethylene). EFE increases not only earlier, but also greater in magnitude than ACC (Yang *et al.*, 1986). These data indicate that it is the increase in EFE which is responsible for this slight, but significant, increase in ethylene production rate before the climacteric rise in ethylene production. Knee (1984) also reported that in 'Cox's Orange Pippin' apples, the rise of ethylene slightly preceded the rise in ACC level. Blankenship and Richardson (1985) observed that EFE developed before the increase in ACC and ethylene synthesis during the cold storage required for ripening of d'Anjou pears. Similar conclusions were reported for apple (Bufler, 1986; Mansour *et al.*, 1985).

1.2.4 Ethylene action and its inhibitors

1.2.4.1 Ethylene action

One hypothesis of ethylene action is that the conversion of ethylene to ethylene oxide and some subsequent action of that metabolite is the mode of action of ethylene (Beyer, 1979). Although the correlation of ethylene oxidation and action was fairly good, it has been shown that inactivation of the ethylene-oxidizing system does not prevent the action of ethylene (Abeles, 1984). This seems to rule out the oxidation of ethylene as the primary mode of ethylene action.

Another theory of ethylene action is that as in the case of other hormones, ethylene was thought to bind to a receptor, forming an activated ethylene-receptor complex which in turn triggers the primary reaction (Sisler and Wood, 1987). In their work several lines of evidence indicate that ethylene is binding to a physiological receptor: (1) the type of compound that ^{pro}duces a ^{response} also competed with ethylene for binding; (2) the half maximum ^{for ethylene} and ethylene binding were very close; (3) the time of diffusion of ethylene to and from the site was similar to the more rapid ethylene responses, and (4) inhibitors of ethylene responses and inhibitors of ethylene action inhibited ethylene binding. Ethylene is thought to bind to a receptor, forming an activated complex which triggers the primary reaction. This primary reaction then initiates a chain of reactions which included modification of gene expression, and leading to various physiological responses (Yang, 1985, 1987a).

Veen (1986) suggested that the ethylene receptor is composed of a sub-unit A of proteinaceous nature and one or more enzymic sub-units (sub-unit B). Binding of ethylene to sub-unit A will cause its activating change. The activated sub-unit A leads to activation of sub-unit B and finally to the physiological and biochemical responses.

Yang (1985) has presented a model which suggested a possible mode of action of ethylene (Fig.1-3). This model proposed that ethylene initiated its effects in plants by binding reversibly to a metal-containing receptor site (Bengochea *et al.*, 1980a,b; Sisler, 1979). It has been

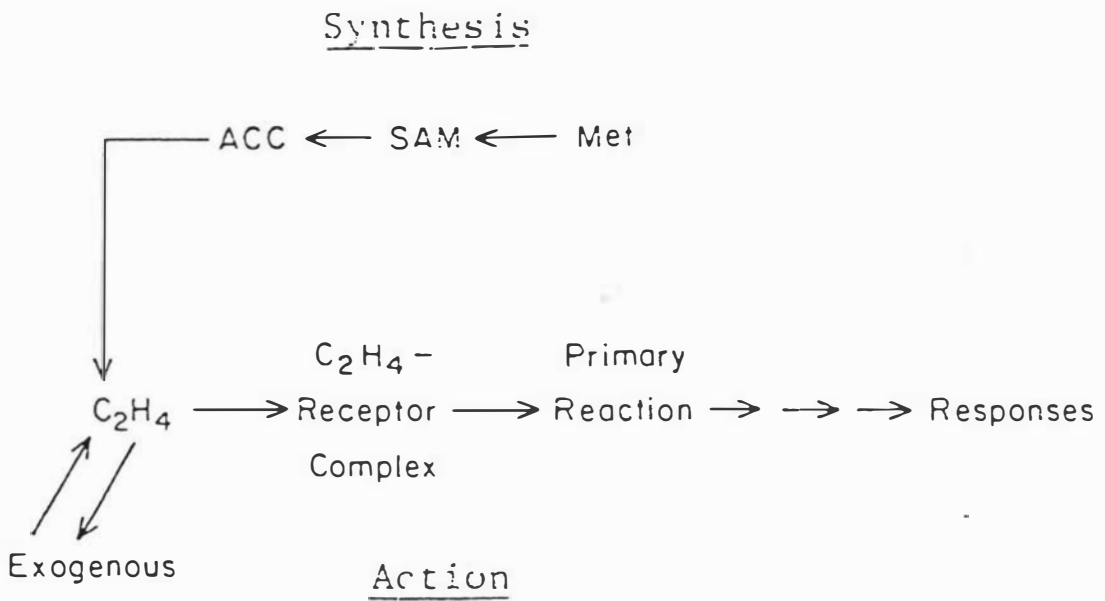


Fig.1-3 The chain of events between ethylene and its responses (From Yang, 1985).

suggested that the metal in the ethylene receptor might be copper (Burg and Burg, 1967; Yang, 1985). This ethylene-receptor complex then reacted further to produce the physiological and biochemical responses (Grierson, 1987). No details in Yang's model (1985) mentioned how the ethylene receptors regulate the activity and biosynthesis of enzymes.

Both models for ethylene action did not distinguish the nature of different characteristics and functions between System I and II ethylene receptors.

Yang (1985) pointed out that the increase in the sensitivity to ethylene action is the important factor triggering the onset of ripening. Measurements of ethylene production in climacteric fruit have revealed three types: type 1 fruit in which ethylene concentration increases before the onset of ripening as shown by the respiratory increase (such as banana, tomato), type 2 fruit in which a rise in ethylene production coincides with the onset of ripening (such as apple and apricot) and type 3 fruit in which ethylene production increases follows the onset of respiration (Fuerte avocado and feijoa) (Biale and Young, 1981). There is evidence that the ripening sensitivity of fruit response to ethylene increases during development, and that fruit attached to the plant are less sensitive to ethylene than detached fruits (Knee, 1985; Yang *et al.*, 1986). These results suggest that there may be high levels of a 'ripening inhibitor' in immature fruit which desensitize the fruit to respond to ethylene and, as a consequence, ripening is not initiated. The three types of fruit ripening indicated above could be explained by an increase in the sensitivity of fruit tissue to ripening with time as the fruit matures, due to a progressive disappearance of the 'ripening inhibitor' regulated by the low levels of System I ethylene. When the resistance to ripening decreases to a point at which the fruit tissues become responsive to their endogenous ethylene levels, the autocatalytic burst of System II ethylene production occurs and ripening proceeds.

Application of exogenous ethylene can accelerate a decrease in ripening resistance and consequently, the ripening process is accelerated. Conversely the decrease in resistance to ripening is arrested or delayed when ethylene synthesis or action is inhibited, resulting in little change in resistance to ripening; consequently the ripening process is not initiated (Yang, 1985).

Although resistance to ethylene action cannot be measured directly, it can be determined as the concentration of ethylene required to induce ripening. The more immature the fruit, the higher the ethylene level required to trigger fruit ripening (Peacock, 1972).

Yang (1987b) summarized the above observations and presented a model showing the sequence of ethylene action on the regulation of ethylene biosynthesis in fruit maturation and ripening (Fig.1-4). This model distinguished clearly the functions of the System I and System II ethylene receptors. The System I ethylene receptor which exists in nonclimacteric fruit, and immature and mature climacteric fruit is responsible for promoting development of EFE, ACC N-malonyltransferase as well as respiration rate. The System II receptor develops or becomes functional in mature climacteric fruits possibly as a result of inhibitor decline, and is responsible for the development of autocatalytic ACC synthesis and the subsequent development or induction of ripening-associated enzymes (e.g. polygalacturonase). Fruit development (maturation) can be regarded as a transition from a nonclimacteric (immature) state in which tissue lacks the System II receptor, to a climacteric (mature) state, where System II receptor develops in tissue (Yang and Hoffman, 1984). According to this model the lack of ethylene biosynthesis that occurs in nonclimacteric fruit following ethylene application, which does induce a respiration rise, can be interpreted as a lack of System II ethylene receptor.

1.2.4.2 Inhibitors of ethylene action

1.2.4.2.1 Carbon dioxide

It is well known that CO₂ delays ripening in fruit by reducing respiration rate as well as inhibiting ethylene action (Kader, 1986). This knowledge has been used commercially for enhancing storage life of fruits by development of CA or MA storage. The mode of action of CO₂ inhibition is not known. Burg and Burg (1967) have suggested that CO₂ reduced ethylene action and the effect appears to be competing with ethylene for the binding site on the ethylene receptor with a K_i of 15 ml/l. Sisler and Wood (1989) suggested that CO₂ is not the type of compound that usually binds to metals to which ethylene binds, thus CO₂ does not appear to inhibit ethylene binding competitively. Some work (Beyer, 1979) reported ethylene action

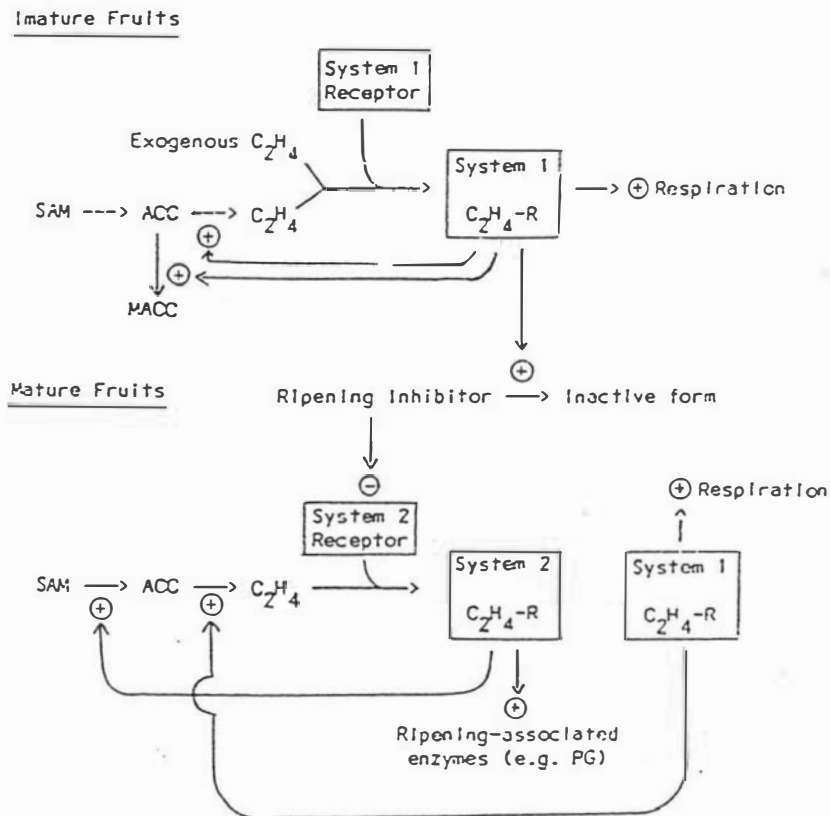


Fig.1-4 A model showing the sequence of ethylene action on the regulation of ethylene biosynthesis in fruit maturation and ripening. + and - indicate the metabolic processes are positively or negatively regulated by the specific ethylene receptor complex or the ripening inhibitor (Yang, 1987b).

involving ethylene metabolism, thus Beyer (1985) suggested that CO₂ can affect ethylene metabolism by inhibiting ethylene oxidation to CO₂ through a feedback inhibition mechanism.

1.2.4.2.2 Silver ions

The inhibitory effect of Ag⁺ on ethylene action has been used commercially to extend the shelf life of ~~the~~ cut flowers (Reid *et al.*, 1980; Veen, 1979). In fruits application of Ag⁺ resulted in varied results; ethylene production could be stimulated (Aharoni and Lieberman, 1979b; Aharoni *et al.*, 1979; Atta-Aly *et al.*, 1987), inhibited (Aharoni and Lieberman, 1979a; Saltveit *et al.*, 1978; Reid *et al.*, 1980; Veen, 1979) or not affected (Atta-Aly *et al.*, 1987) by Ag⁺ depending on species, tissue types and ripening stage of the tissues. A possible mechanism for the inhibitory effect of Ag⁺ on ethylene synthesis is that Ag⁺ interacts with the ligands on the ethylene receptor. ^{These} ligands normally facilitate the binding of ethylene to the receptor, but in the presence ^{of} Ag⁺ they are altered so that the receptor has a reduced capability to bind ethylene, or becomes biologically inactive (Yang, 1985).

1.2.4.2.3 Norbornadiene

Sisler and Pian (1973), and Sisler and Yang (1984) reported that some cyclic olefins counteracted ethylene-induced increases in the respiratory rate of tobacco leaves and compared the structure-activity relationship of a number of olefins which possess^{ed} anti-ethylene activity in the pea seedling bioassay. Among those tested, 2,5-norbornadiene was the most active compound and it inhibited ethylene action with a K_i of 170 ul/l. Ethylene was capable of counteracting the norbornadiene action, indicating a competitive inhibition (Peacock, 1972) and the inhibitory effect of norbornadiene on ethylene action is reversible. Recently, Liu *et al.* (1989) reported that in tomato fruit which have been treated with norbornadiene (NDE) for three days at any stage, the rate of respiration was gradually restored upon transfer to NDE-free air; NDE abolished the promotive effects of ethylene on both EFE and malonyltransferase in green tomatoes; NDE treatment simultaneously raised the levels of ACC and MACC; ACC synthase was also inhibited by NDE at all stages of ripening. Therefore, they concluded that the reduction in ethylene production in NDE treated tomato

fruits reflects the combination of all these effects of NDE. It is possible that norbornadiene attaches to the ethylene binding site to form a norbornadiene-receptor complex which is biologically inactive.

1.3 EFFECTS OF CARBON DIOXIDE ON ETHYLENE BIOSYNTHESIS

Controlled-atmosphere (CA) and modified-atmosphere (MA) storage and transport of fruits and vegetables have been used for a long time. Various aspects, including responses of fruits and vegetables to CA have been reviewed (Brecht, 1980; Dewey, 1983; Isenberg, 1979; Kader, 1980; Lipton, 1975; Smock, 1979). Burton (1974, 1978) discussed some of the biochemical, biophysical, and physiological aspects of modified atmospheres in relation to quality maintenance of fruits and vegetables. But much research has been directed toward determination of the optimum CA condition for a large number of fruits and vegetables and specific cultivars of each commodity (Dewey, 1977, 1983; Dewey *et al.*, 1969; Meheriuk and Richardson, 1982) rather than in investigating the specific mechanism of action. Only a few reports have dealt with the mode of action of reduced oxygen and elevated carbon dioxide concentrations, i.e., the biochemical and physiological basis for CA or MA effects on fruits and vegetables (Kader, 1986).

Generally, the effect of reduced O₂ and/or elevated CO₂ on reducing respiration rate has been assumed to be the primary reason for the beneficial effects of CA on fruits and vegetables. CO₂ has been shown to inhibit ethylene production of ripening fruit (Young *et al.*, 1962). This is an oversimplification, since postharvest deterioration of fresh produce can be caused by many factors in addition to high respiration rate, including: metabolic changes (biochemical changes associated with respiratory metabolism, ethylene biosynthesis and action, and compositional changes); physical injuries; water loss; physiological disorders; and pathological breakdown.

Reduced O₂ levels decrease ethylene production by fresh fruits and vegetables, and reduce their sensitivity to ethylene (Burg and Burg, 1967; Li *et al.*, 1983; McGlasson, 1985; Peacock, 1972; Yang, 1985). Burg and Burg (1967, 1969) demonstrated that O₂ is required for ethylene production and action. EFE is known to be sensitive to O₂ deprivation. Because this thesis

deals specifically with the effects of CO₂ (in an atmosphere where O₂ is not limiting) ~~supplied in atmosphere~~ on EFE activity and biosynthesis in nonclimacteric (Hosui) and climacteric (Granny Smith) fruits, the effects of O₂ on ethylene biosynthesis will not be reviewed further.

1.3.1 Effects of carbon dioxide on ethylene production of vegetative organs and tissues

Results of studies on the effect of elevated CO₂ levels on ethylene production are conflicting. Dhawan *et al.* (1981) indicated that CO₂ has been reported to promote, inhibit, or have no effect on the rates of ethylene production at different stages of plant growth and development. For example, CO₂ stimulated ethylene production of excised leaf segments in a closed system (Aharoni *et al.*, 1979; Aharoni and Lieberman, 1979b; Fuhrer, 1985; Gepstein and Thimann, 1981; Grodzinski *et al.*, 1982, 1983; Horton and Saville, 1984; Kao and Yang, 1982; McRae *et al.*, 1983; Philosoph-Hadas *et al.*, 1985; Preger and Gepstein, 1984) and in intact plants (Bassi and Spencer, 1982, 1985; Dhawan *et al.*, 1981). When CO₂ was removed from the atmosphere with KOH, ethylene production by infected sweet potato roots decreased (Imaseki *et al.*, 1968). During ethylene-stimulated elongation of aquatic plants, CO₂ may enhance the action of ethylene rather than counteract it (Suge and Kusamagi, 1975); CO₂ may act synergistically with ethylene under conditions where ethylene stimulates seed germination, or counteract ethylene under conditions where ethylene inhibits germination (Negm *et al.*, 1972).

Carbon dioxide can stimulate ethylene production in vegetative tissues and intact seedlings. In some reports, CO₂ stimulated both basal (Aharoni *et al.*, 1979; Aharoni and Lieberman, 1979b; Bassi and Spencer, 1982; Dhawan *et al.*, 1981) and ACC dependent (Bassi and Spencer, 1985; Fuhrer, 1985; Grodzinski *et al.*, 1983; Horton and Saville, 1984; Kao and Yang, 1982; McRae *et al.*, 1983; Philosoph-Hadas *et al.*, 1985; Preger and Gepstein, 1984; Tophof and Amrhein, 1985) ethylene production, but hardly affected ACC production (Kao and Yang, 1982). This suggests that CO₂ exerts its promotive effect on the step of ACC conversion to ethylene i.e. on EFE synthesis or activity. This effect is obtained when CO₂ is supplied either directly as a gas or indirectly as bicarbonate (Grodzinski *et al.*, 1983; McRae *et al.*, 1983).

Many investigators have observed that light markedly inhibited ACC-dependent ethylene production of excised leaf segments enclosed in flasks (Bassi and Spencer, 1983; De Laat *et al.*, 1981; Gepstein and Thimann, 1980; Grodzinski *et al.*, 1982; Kao and Yang, 1982; Preger and Gepstein, 1984; Wright, 1981). However, with intact plants in an open system the effect of light on ethylene production is marginal (Bassi and Spencer, 1983). The inhibitory effect of light observed with excised segments reflects partial depletion of endogenous CO₂ pools by photosynthetic fixation. When sufficient CO₂ is provided, light becomes a stimulating factor, suggesting that the amount of ethylene generated by photosynthetic tissues is controlled by the availability of CO₂ (Grodzinski *et al.*, 1982, 1983; Kao and Yang, 1982; Yang and Hoffman, 1984). The CO₂ effect on ethylene production from leaves does not seem to be a direct result of stomatal control (Horton and Saville, 1984). Philosoph-Hadas *et al.* (1986) reported that CO₂ exerts its effect by activating and enhancing the development of EFE *in vivo*.

1.3.2 Effects of carbon dioxide on ethylene production of whole fruits

Kader (1986) in his review observed that elevated CO₂ levels can reduce, promote, or have no effect on ethylene production rates by fruits, depending on the commodity and the CO₂ concentration.

It is possible that the increase in ethylene production by some commodities during and following exposure to CO₂ occurs only when the CO₂ concentration is at levels high enough to cause physiological injury to the tissue. It is not known whether this high CO₂-stress induced ethylene is due to a partial shift from aerobic to anaerobic conditions or to other mechanisms (Kader, 1986).

Carbon dioxide has been shown to inhibit ethylene production from ripening fruit (Potter and Griffiths, 1947; Young *et al.*, 1962). High CO₂ treatment of fruit during the first 10-15 days of storage can delay fruit senescence, improve fruit quality in some European pears, such as 'd'Anjou (12% CO₂ for 14 days) (Couey and Wright, 1977), Bartlett (20% CO₂ for 4-6 days) (Dangyang *et al.*, 1990) and Bosc (Chen *et al.*, 1985), apples, Golden Delicious (Couey and Olsen, 1977) and McIntosh (10-20% CO₂ for 10-15 days) (Bramlage, 1977) and Kiwifruit (10% or 30% CO₂ intermittent

treatment) (Nicolas *et al.*, 1989). The presence of 10% CO₂ abolishes the biological activity of 1 ppm ethylene (Burg and Burg, 1969). In certain fruits, CO₂ accumulates in the intercellular space and functions as a natural ethylene antagonist (Yang, 1985).

1.3.3 Effects of carbon dioxide on ethylene biosynthesis in fruit tissues

Very little work has been done on the effects of CO₂ on enzymes involving ethylene biosynthesis in fruit tissues.

Several researchers have used fruit discs to investigate this problem and different results have been reported on effects of CO₂ on EFE: Chaves and Tomas (1984) observed a reduction in ethylene production by entire 'Granny Smith' apples following exposure to 20% CO₂ for 2 hours. A similar result was observed in tissue slices. The inhibition continued even after an extended aeration period. There is also an inhibition of ethylene emission in tissue slices incubated with exogenous ACC (4 μ M and 100 μ M). This treatment increased the ACC content of the tissue, indicating a possible inhibitory effect of elevated CO₂ on the enzyme system responsible for the conversion of ACC into ethylene. These results did not really show the effect of CO₂ on EFE activity, because, on the one hand these authors used unsaturated ACC concentrations (0.04 and 0.1 mM) when measuring the conversion of ACC to ethylene, and, on the other, ACC can be converted to MACC by ACC N-malonyltransferase,

In contrast, Cheverry *et al.* (1988) reported that a high CO₂ concentration (20%) at 20°C inhibited the climacteric ethylene burst in 'Granny Smith' apple fruits, but did not change the ACC content. They also observed that that CO₂ stimulated EFE activity in discs of preclimacteric Granny Smith apple and avocado, but inhibited its synthesis.

Tan and Thimann (1989) investigated effects of CO₂ on ethylene production in several tissues including oat leaves, Golden Delicious apples and Anjou pears. The results indicated that there was a difference between ethylene production from endogenous and exogenous ACC (0.25 mM). The difference is in the CO₂ requirement. Lowering the CO₂ by 99% in the air (0.03%) decreased the ethylene production from exogenous ACC, but it did not decrease (in fact slightly increased) ethylene production from

endogenous ACC. This work also did not use the saturated concentrations of exogenous ACC when measuring the effect of CO₂ on the conversion of ACC to ethylene.

1.4 THE POSSIBLE MECHANISMS OF CARBON DIOXIDE EFFECTS ON EFE

1.4.1 The direct effect of carbon dioxide on EFE

McRae *et al.* (1983) indicated that CO₂ from bicarbonate directly facilitated the conversion of ACC to ethylene in model systems and intact tissues. Moyses (1974) strongly suggested a direct action of dissolved, unhydrated CO₂ gas rather than an indirect effect caused by a change in pH. The control exercised by the direct effect of CO₂ is also largely independent of CO₂ fixation. Based upon the knowledge that carbon dioxide can reversibly change the physical, chemical and biochemical properties of the cellular constituents, it has been proposed by Mitz (1979) that transient localized concentration changes of carbon dioxide within parts of the cell can markedly influence cell metabolism through dynamic changes in the constituents which react ^{with} CO₂ (Mitz, 1979).

Mitz (1979) summarized characteristics of direct CO₂ effects, and indicated that there were: (a) 'Highly selective effects': CO₂ may activate or inhibit one enzyme and may not influence another closely related or even the same enzyme in other tissues. One part of a membrane may respond to CO₂ but not another; (b) 'Threshold concentration requirement': different specific partial pressures of CO₂ are required to initiate specific activities, which means a threshold concentration of CO₂ is required; (c) 'A rapid, almost instantaneous response'; (d) 'A control range': as the CO₂ is slowly increased above a certain value, the response is in one direction and at a higher CO₂ concentration the response is in the opposite direction.

Carbon dioxide can react with primary amines, such as the amino acids, peptides and proteins in the cell to form carbamates (Mitz, 1979). The protein carbamate can cause the formation of internal ionic attractions or repulsions which could result in structural changes. One type of cross linkage could represent a salt bridge between the carbamic acid from one part of a protein and an amine on another part. Another form could be the

carbamic acid complex cross linking with bound heavy metals, such as iron, magnesium.

There are several possible ways by which CO_2 may act on membrane. The change in charge on the surfaces of membrane is caused by positive charged groups of free amino groups; on exposure to CO_2 these may become negatively charged carbamic acid ions by the diffusion of positive ions such as Na^+ and K^+ instead of negative ions such as Cl^- and HCO_3^- (Mitz, 1971, 1979). The change in charge may also cause an attraction of opposite or repulsion of similar charges to help open or close the holes or channels in the membrane (DeHoal and Defelice, 1978). CO_2 tension may also induce water movement into and out of the intermembrane spaces resulting in a membrane swelling.

Some proteins and complexes are known to dissociate, and can be fractionated, in the presence of CO_2 (Mitz, 1979). It is possible that an enzyme with its active site masked by the interaction with another protein or other polymeric structural element of the cell may be temporarily dissociated and consequently activated by a CO_2 tension increase. Another possible effect is the inhibition by CO_2 of coenzyme binding to an enzyme. The CO_2 response appears to have all the characteristics of reactions with an amino group at the enzyme active site for the coenzyme. The binding reaction with the CO_2 to form carbamate prevents the binding of the coenzyme until the CO_2 is removed (Mitz, 1979).

In animal sciences, some direct CO_2 effects on enzymes in whole tissues have been reported (Hastings, 1970; Longmore *et al.*, 1974). Turning enzymes on and off by a direct CO_2 effect was demonstrated by these investigators in rat liver carbohydrate and fatty acid metabolism. The CO_2 may change the surface properties (surface tension) between the lipid-rich mitochondria and the water solutions, which could result in the enzymes in one medium acting more freely on a substrate in another medium. In addition the lipids in mitochondria concentrate CO_2 which could cause either a direct change in the activity of the enzyme or a simple change in the solubility and transport of the metabolic intermediates in the mitochondria. Further, CO_2 may uncouple oxidative phosphorylation. This function of CO_2 is similar to the effect of 2,4-dinitrophenol, progesterone and thyroxine (Fanestil *et al.*, 1963).

EFE is a membrane bound enzyme or enzyme system which catalyzes the conversion of ACC to ethylene. Thus all above possible effects of CO₂ on enzymes could affect the activity of EFE.

1.4.2 The indirect effect of carbon dioxide on EFE

The rate of ACC conversion to ethylene is determined by the ACC concentration (which may be altered by malonylation or compartmentation) and the activity of EFE (McKeon and Yang, 1987). The rate of ethylene synthesis by plant tissues is sensitive to a wide array of environmental and developmental factors (Yang and Hoffman, 1984). Bradford, K. J. (Department of Vegetable Crops, University of California, Davis, personal communication) questions how such a diverse range of conditions, including auxins, hypoxia, wounding, chilling, water stress, fruit ripening and senescence, light, CO₂ and numerous chemicals, can influence the expression of ACC synthase or the conversion of ACC to ethylene. Boller and Kende (1980) pointed out 'It remains to be seen whether developmental, stress and hormonal stimuli all regulate the activity of ACC synthase through the same mechanism or whether different modes of control are exerted to modulate the activity of this important enzyme in ethylene synthesis'. After reviewing the literature, Bradford suggested that in many cases, alterations of intracellular pH occur during conditions which influence the rate of ethylene synthesis ^{and} could promote both the induction and activity of ACC synthase and the ethylene-forming enzyme. This would provide a sensitive means of integrating environmental and developmental factors into a single variable to regulate the rate of ethylene synthesis. It had been found that in illuminated green leaf tissues, ACC-dependent ethylene production is very sensitive responding rapidly to CO₂ and intracellular pH changes (Kao and Yang, 1982). These results suggested that EFE activity may be regulated by pH which in turn can be influenced by CO₂.

It has been suggested that EFE activity required a transmembrane proton gradient (John *et al.*, 1983; Mayne and Kende, 1986), but John *et al.* (1985) reported that the depolarisation resulting from KCl treatment in hypocotyl segments of mung bean and in discs of red beet did not affect the EFE activity; this was not consistent with his hypothesis above that EFE was located at the plasma membrane with its activity potential-dependent.

Thus there have been different opinions put forward to explain the mechanism of effects of CO₂ on the enzymes involved in the biosynthesis of ethylene. Further research is required to resolve this problem.

1.5 PROJECTS INVESTIGATED IN THIS THESIS

The aim of this thesis is to investigate the effects of CO₂ on regulation of EFE synthesis and activity in Hosui, Japanese pear and Granny Smith apple. In addition the interaction of CO₂ with EFE and ethylene action inhibitors at different ripening stages in nonclimacteric and climacteric fruit has been examined. From these studies it is hoped that further information will be obtained on the characteristics of the effects of CO₂ on the possible form of EFE, the possible binding sites of EFE, interactions between CO₂ and the substrate of EFE (ACC) and between CO₂ and ethylene receptors.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 MATERIALS

In this work, the optimum harvest date for climacteric fruit (Granny Smith apple) used is the date when fruits reach or come close to full maturity, but do not produce a significant quantity of ethylene, which means that fruits are at the preclimacteric stage of the transition between the maturation and ripening phase (Chu, 1984). The optimum harvest date for nonclimacteric Hosui fruit is the date when the fruit skin colour was getting colour grade 2-3 (Japanese Colour Chart) (Lallu, 1985a).

2.1.1 Japanese pear (*Pyrus serotina* cv. Hosui)

Fruit for the 1988 and 1990 experiments, and for the propylene treatment in 1989, were harvested from 6-8 year-old trees grown at the Ruakura Research Orchard, Ministry of Agriculture and Fisheries (MAF) in Hamilton. Fruit were harvested on 12 February, 1989 and 21 February, 1990. In 1989, fruit for the propylene treatment were harvested on 17 January, 26 January, 30 January, 8 February and 12 February which corresponded to 26, 14, 11, 4 and 0 days before the optimum harvest date, respectively. For other experiments in 1989, fruit were harvested from eight year-old trees grown at the MAF Manatuke Research Orchard, Gisborne on 17 January, 31 January, 7 February and 12 February. After each harvest fruit were stored at 0°C until 13 February when all fruit were transferred to Palmerston North.

After harvest, fruit were packed in Nashi (Japanese pear) Fruit Trays (Cardboard trays with pocket packs covered with a polyliner) and stored at 0°C for one night before air freighting to Palmerston North next morning. On arrival fruit, except those for the propylene experiment, were sorted into various colour grades according to the Japanese colour chart. Fruit of colour grade 2-3 and fresh weight (180 ± 5 g) were selected and then stored at 1 ± 1 °C until ready for use. Fruit for the propylene experiment were weighed and maintained for 24 hours at 21°C to equilibrate before the experiment

commenced. Internal ethylene concentrations in all experimental fruits harvested at different seasons were not detectable.

2.1.2 Apple (*Malus domestica* Borkh. cv. Granny Smith)

Fruit were harvested from twenty, 8-11 year-old trees located at the Fruit Crops Unit orchard, Massey University, Palmerston North on 30 April in 1987, 27 April in 1988, on 31 March, 20 April, 25 April, 4 May and 18 May in 1989 and on 25 April, 1990. The harvest dates in 1989 corresponded to 26, 14, 11, 4 and 0 days before the optimum harvest date. After harvest, fruit except those harvested at different days before the optimum harvest date in 1989 were weighed; those in the range 178-192g (means: 185g) were selected and placed onto cardboard tray, then packed into commercial cardboard cartons (100 fruit/carton) before coolstore at $1^{\circ}\pm 1^{\circ}\text{C}$. Internal ethylene concentrations were not detectable at harvest in any experimental fruit in the different seasons.

2.2 METHODS

2.2.1 Testing fruit maturity indices

2.2.1.1 Skin colour

Skin colour for Hosui fruit was measured at the two greenest parts of fruit equator using two methods: a MINOLTA Chromameter (CR-100) and the Japanese Colour Chart (Yamazaki and Suzuki, 1980). The relationship between colour measurement, (L,a,b mode), as determined on the Chromameter and the Japanese colour chart values is shown in Appendix 1; a high correlation was obtained between colour grades 1-6 of the Japanese colour chart and 'a' values and the hue angle from the Chromameter. Skin colour was measured as the 'L', 'a', 'b' values and then the hue angle was calculated. The values of the hue angle decreases as skin colour changes from green to yellow (Kajiura *et al.*, 1981; Little, 1975; Yamazaki and Suzuki, 1980).

For Granny Smith apples, skin colour was measured using a MINOLTA Chromameter (CR-100) (L,a,b mode) on the two greenest parts of the fruit at the equator.

2.2.1.2 Firmness

Firmness (Newton) was measured on pared surfaces from opposite sides of the fruit at the equator with an Effegi penetrometer fitted with ⁸8 mm diam. head after removal of a 1-2 mm thick slice of peel.

2.2.1.3 Soluble solids

An Atago refractometer was used to measure soluble solids(%) in juice squeezed from 5 mm thick slices taken from opposite sides of the fruit at the equator.

2.2.1.4 Starch contents as a maturity index for Granny Smith apple

Starch accumulated by the apple fruit during development is converted to sugar. An apple fruit was cut equatorially and placed one half face down into iodine solution for 30 seconds; the reaction between any starch present and the iodine causes a dark blue colour to develop. Iodine solution was made by adding 1 g potassium iodide and 0.25 g iodine to 100 ml water. The solution was stored in a sealed glass container in a dark place to prevent deterioration in the light. This starch test was carried out within 30 min after picking. The starch patterns, which indicated the relative amounts of starch decrease were scored on a scale of 0-6 (Reid *et al.*, 1982; Watkins, 1981).

2.2.2 Buffers

2.2.2.1 K⁺-phosphate buffer (pH 6.5)

Stock solutions of K₂HPO₄ (0.2 M) and KH₂PO₄ (0.2 M) were prepared separately. Appropriate amounts of the above solutions were mixed till pH value was 6.5 judged by an ORION (Watson Victor LTD, N.Z.) pH metre.

2.2.2.2 Na⁺-citric acid buffer

Stock solutions of 0.1 M C₆H₈O₇.H₂O (citric acid monohydrate) and 0.2 M Na₂HPO₄ were prepared. Appropriate amounts of these solutions were mixed until the pH values required were obtained.

2.2.3 Preparation of fruit discs

Radial plugs of Japanese pear (cv. Hosui) and apple (cv. Granny Smith) tissue were excised from the pericarp of the fruit at the equator with a cork borer (7 mm dia.). Peel and locule edge were discarded. Discs (7 mm in dia., 2 mm thick) were cut by razor blade, placed onto dry tissue paper and used immediately.

2.2.4 Incubation techniques

Vials (36 ml), each containing six fruit discs, were placed in the test tube racks and incubated at 27°C in a shaking waterbath (120 strokes/min).

2.2.5 Procedures for treatment with different gas (propylene or carbon dioxide) concentrations

2.2.5.1 Preparation of jars or vials

Agee jars (600 or 1100 ml), each containing one fruit, were used for measuring the respiration rate and ethylene production of whole fruit. The lid of each jar contained two holes into which were sealed rubber septum creating a gastight seal and through which gas sample could be taken.

Vials (36 ml), each containing six fruit discs, were used for measuring respiration rate and ethylene production of fruit discs. Each vial was sealed by a gastight lid which contained two holes which were sealed by a rubber septum under the lid and through which gas sample could be taken.

2.2.5.2 Procedures used for obtaining gas atmosphere

(a) Injecting with the gas mixture.

The appropriate gas mixtures, containing different CO₂ concentrations made up with O₂, were injected into the sealed vials, each of

which contained a second empty syringe into which the displaced air in the vial could flow to maintain atmospheric pressure in each vial.

(b) Flushing with the gas mixture.

Each vial was flushed for 20 seconds with ethylene-free gas containing 23% CO₂, 21% O₂, balance N₂, before being sealed with a gastight lid.

(c) Treating with propylene.

Jars (600 ml), each containing one whole fruit were flushed (flow rate: 15 ml/min) with the gas mixture containing 0.5% propylene in air which had been humidified by bubbling through water contained in a separated flask.

(d) Flushing with the air.

In some experiments, vials were flushed for 20 seconds with air by a fan to remove the accumulation of gases, such as CO₂, C₂H₄ or C₃H₆ from around the tissues in the vials.

(e) Removing gas (carbon dioxide) from tissues by vacuum.

In some experiments e.g. testing if the stimulatory effect of CO₂ on EFE activity is dependent on exogenous ACC (see Chapter 5), fruit discs were treated with CO₂ two times. Discs in the vials containing the medium were placed in a large container, and then vacuumed by vacuum pump at 90 Kpa for 1 min for removal of CO₂ from fruit tissues between treatments.

(f) Taking of gas samples from vials.

1 ml gas samples from each jar or vial were taken using a 1 ml gastight plastic syringe. The needle was inserted through the septum in the lid of jars or vials and the sample withdrawn. The syringe containing gas sample was then inserted into a rubber bung to prevent gas leakage from the needle. Gas samples were analysed as soon as possible by GLC.

2.2.6 Measuring respiration rate and ethylene production

2.2.6.1 Technique for measuring respiration rate and ethylene production of whole fruit

Fruit were equilibrated in the assay room (21°C) for 24 hours, before being sealed into jars (600 ml for Hosui, 1100 ml for Granny Smith) for

specific periods of time (0.5-2 hours) which depended on ^{the} rates of respiration or ethylene production of fruits. The higher respiration rate and ethylene production, the shorter the time required to accumulate enough CO₂ or C₂H₄ for quantitative determination. One ml gas samples were removed and analysed by a gas liquid chromatograph (GLC). Respiration rate (mlCO₂/Kg f.w./h) and ethylene production (ulC₂H₄/Kg f.w./h) of fruit were calculated according to the formula in Appendix 2. When respiration rate and ethylene production were measured at different temperatures, the units of respiration rate and ethylene production were calculated as mgCO₂/Kg f.w./h and ugC₂H₄/Kg f.w./h, respectively.

2.2.6.2 Technique for measuring respiration rate and ethylene production of fruit discs

Six fruit discs were sealed in each 36 ml vial and incubated at 27°C for specific period of time (0.5-1 hour) which depended on rates of respiration or ethylene production of the fruit discs. One ml gas samples were then taken and analysed by GLC. Respiration rate (ulCO₂/g f.w./h) and ethylene production (nlC₂H₄/g f.w./h) of fruit discs were calculated according to the formula in Appendix 2.

2.2.6.3 Technique for measuring internal ethylene concentrations in whole fruit

The needle of a 1 ml gastight plastic syringe was inserted into the fruit from the calyx end of fruit, which were submerged in water (Chu, 1984). A 1 ml sample was withdrawn and the ethylene concentration (ppm) in the gas sample was analysed by GLC.

2.2.7 Gas Liquid Chromatograph (GLC) for ethylene analysis

Ethylene concentrations (ppm) in a 1 ml gas samples were determined using either a Varian 3400 or a Pye Unicam Series 104 gas-liquid chromatograph fitted with a flame ionization detector (FID) and with a stainless steel activated alumina column (80/100 mesh, 6' long and 1/8" diameter). Temperature of column, injector and detector were 100°C, 100°C and 150°C respectively. The carrier gas was nitrogen. The flow rates for nitrogen, hydrogen and air were 30 ml/min, 30 ml/min and 300 ml/min, respectively.

2.2.8 Gas Liquid Chromatograph for carbon dioxide and oxygen analysis

Carbon dioxide (%) and oxygen (%) in 1 ml samples were determined using a Shimadzu GC-8A GLC, fitted with a thermal conductivity detector (TCD) and with a 6 ft concentric CTR 1 column (Cat. no. 8700, Alltech) which contained an outer column (1/4" diameter) packed with activated molecular sieve and an inner column (1/8" diameter) packed with Porapak. Temperatures of the detector (TCD) and column were 60°C and ambient respectively. Current for the TCD was 80 mA. Flow rate of carrier gas (hydrogen) was 30 ml/min.

2.3 CHEMICALS

All the chemicals used in this research were of Analar grade and were obtained from Sigma or BDH.

2.4 STATISTICAL METHODS

Each treatment had three or four replicates. Each experiment was repeated at least twice. An SAS programme (SAS/STAT User's Guide) was used to analyse data from each one experiment for ANOVA (analysis of variances), means and standard errors, paired comparison and Duncan's multiple comparison at 5% level, and the linear regression. Methods of comparison of two regression lines were calculated according to Neter and Wasserman (1974).

CHAPTER 3

MATURITY INDICES, RESPIRATION AND ETHYLENE PRODUCTION IN HOSUI (*PYRUS SEROTINA*) AND GRANNY SMITH APPLE (*MALUS DOMESTICA BORKH.*)

3.1 ABSTRACT

Changes of fresh weight, flesh firmness, soluble solids, skin colour, respiration and ethylene production were assessed for Hosui and Granny Smith fruits during maturation. In addition starch iodine pattern was evaluated for Granny Smith apple. The results demonstrated that skin colour for both species and starch index for Granny Smith apple were suitable maturity indices.

Respiration rate of Hosui fruit at each maturation stage was enhanced in the presence of 0.5% propylene and declined upon removal of the gas. Ethylene production was not enhanced by exposure of fruit to propylene, and remained at undetectable levels through the experiment. The results suggested that Hosui fruit grown in New Zealand is nonclimacteric.

Granny Smith fruit showed different respiration and ethylene production patterns. In 1987, fruit at 15°C showed typical climacteric respiration and ethylene peaks, and the onset of the respiration climacteric was 3 days earlier than that of ethylene production. Compared with the results in 1987, fruit respiratory and ethylene climacteric in 1989 was delayed. Fruit produced high levels of ethylene, but no typical peak occurred.

The ethylene production and respiration rate of Granny Smith fruit measured at 21°C after being stored at 1°+1°C for 20 days showed rapid rise without time lag compared to those in fruit immediately measured after harvest, and also ethylene production and respiration rate increased faster and greater in fruit after storage at 1°+1°C. The ethylene production and respiration rate of Hosui were not changed by coolstage.

At harvest, EFE was present in Hosui fruit, but not in Granny Smith apple. Neither fruit produced detectable level of ethylene at harvest.

Key words: climacteric, EFE, ethylene production, Granny Smith, Hosui, maturity indices, nonclimacteric, propylene, respiration rate.

Abbreviations: ACC: 1-aminocyclopropane-1-carboxylic acid; AOA: aminooxyacetic acid; GLC: gas liquid chromatograph; EFE: ethylene-forming enzyme;

3.2 INTRODUCTION

Based on observed respiratory and ethylene patterns during ripening, fruits can be classed as climacteric (Kidd and West, 1945) or nonclimacteric (Biale and Young, 1947). Different fruit and even different cultivars within a fruit species may respond differently in terms of the above definitions depending on their maturity or stage of ripening development at the time of harvest. Consequently processes and enzymes normally associated with ripening such as those involved in ethylene biosynthesis, respiration and fruit softening can be markedly different (Brady *et al.*, 1987; Sacher, 1973; Yang *et al.*, 1986; Yang, 1987). Maturity and ripening indices were investigated to aid selection of fruit at the same physiological stage.

For postharvest physiological research, it is important to use experimental material which is of the same physiological age with known ripening patterns. Watada *et al.* (1984) defined the terminology for the description of developmental stages of horticultural crops. The term, 'physiological maturity' was defined as the stage of development when a plant or plant part will continue ontogeny even if detached from the parent plant; the 'ripening' was the composite of the processes that occur from the latter stages of growth and development through the early stages of senescence which results in characteristic aesthetic and food quality, as evidenced by changes in composition, colour, texture, or other sensory attributes. Reid (1985) indicated that 'maturity' for fruits is defined as 'the stage at which a commodity has reached a sufficient stage of development that after harvesting and postharvest handling including ripening, where required, its quality will be at least the minimum acceptable to the ultimate consumer'.

A wide range of maturity and ripening indices have been evaluated for fruit species. The most common methods of assessing apple and pear

maturity include size, flesh firmness, soluble solids, starch content (iodine method), sugar content, ground colour, respiration rate, ethylene production, internal ethylene concentration, titratable acidity, calendar date, number of days from full bloom, accumulated heat units, colour of seeds and development of fruit abscission layers (Kingston, 1990; Reid, 1985; Watkins, 1981). Different species may have quite different suitable maturity indices (Kingston, 1990).

Variations in the time taken to reach maturity arise from a number of factors. Fruit are produced at many places within the tree canopy and this affects the amount of light, ambient temperature and endogenous hormone supply received by fruit (Kingston, 1990). Maturity indices can vary from country to country, district to district and season to season probably as a result of environmental variation (Chu, 1984; Reid *et al.*, 1982; Shaw and Rowe, 1982). Thus no one test can be used to predict fruit quality satisfactorily; in fact generally a combination of factors is used in British Columbia (Lau, 1985). ^{index} maturity indices used for pipfruit were the starch-iodine test, internal ethylene concentration, flesh firmness, soluble solids and seed colour. The Apple Maturity Program used in Washington, USA, (Bartram, 1986) used flesh chlorophyll level, skin colour, fruit firmness, soluble solids, acidity, starch level and ethylene production. Usually when three or four of these indices reach desired levels, harvest is initiated.

Different species and cultivars of pipfruit show different ripening characteristics. On the basis of postharvest respiration and ethylene production, Suzuki *et al.* (1981) and Kitamura *et al.* (1981) classified some Japanese pear cultivars (Shinsui, Kosui, Hosui, Yakumo and Kikusui) and Chinese pear cultivars (Ya-li, Rai-Yan-Tsu-li and Auto-li) as climacteric, while other Japanese pear cultivars (Nijisseiki and Niitaka) were classified as nonclimacteric. In their work, respiration rate and ethylene production of Hosui fruit were tested at a high temperature (30°C) without ethylene or propylene treatment. Preliminary experiments in 1986 demonstrated that during ripening, Hosui fruit from the DSIR Research Orchard in Nelson showed no respiration and ethylene peaks when maintained at 20°C for 20 days after harvest (data not shown). It has been reported that fruit like avocado can undergo the climacteric on the tree (Sitrit *et al.*, 1986). In order to clarify whether Hosui fruit grown in New Zealand is climacteric or non-climacteric, and whether fruit undergo a climacteric on tree, Hosui fruit were

harvested at different times before what was regarded as the optimum harvest date, and were treated with propylene before measurement of fruit respiration rate and ethylene production.

Studies involving treatment of fruit with exogenous ethylene or propylene have indicated that fruit response to ethylene may serve to distinguish whether fruit are climacteric or not (McMurchie *et al.*, 1972). The response of harvested fruit to applied ethylene depends on various factors, including tissue sensitivity and stage of maturation, as well as whether or not the fruit is climacteric (Biale and Young, 1981; Harkett *et al.*, 1971; Kitamura *et al.*, 1981; McGlasson, 1985). Application of ethylene to nonclimacteric fruit typically increases the respiration rate, which, upon removal of the gas, returns to basal level, but it does not stimulate ethylene synthesis (Biale and Young, 1981; Elkashif *et al.*, 1989). Propylene is an active analogue of ethylene which will induce a half maximal response at 130 times the concentration of ethylene required to achieve the same direct effect in apple (Sfakiotakis and Dilley, 1973a). Propylene has been used to stimulate the respiration of nonclimacteric and climacteric fruit as well as the autocatalytic ethylene production and ripening of climacteric fruit; this response is the same as that which occurs after application of ethylene (Bufler and Bangerth, 1983; McMurchie *et al.*, 1972; Eaks, 1980). Neither ethylene nor propylene is able to stimulate ethylene production of nonclimacteric fruit. By using propylene treatments, it is then known that any ethylene produced by the treatment is a result of fruit response.

McMurchie *et al.* (1972) suggested that in climacteric fruit ethylene biosynthesis is initiated by what they called ethylene receptor system I. In this system they suggested that the ethylene is produced at a low but constant rate and eventually reaches a level in the fruit sufficient to initiate the respiratory climacteric and associated ripening changes. The rise in respiration is accompanied by a further large increase in ethylene production due to the activation ^{or} development of ethylene receptor system II. In the context of fruit ripening, the change in sensitivity to ethylene can be interpreted as a change in development and of receptors. In a recent review, Yang (1987) suggested that only System I exists in nonclimacteric fruit and immature climacteric fruit in which ethylene or propylene can stimulate respiration rate, but not ethylene production. Yang postulated the existence of a ripening inhibitor which prevents development of the System II

receptor in immature climacteric fruit. As the 'ripening inhibitor', which desensitizes the fruit toward ethylene action and ripening, is inactivated, the fruit tissue begins to develop a functional System II receptor. Together with the preexisting ethylene, an ethylene-system II receptor complex is formed, which in turn induces the development of ACC synthase, the rate-limiting enzyme in ethylene biosynthesis (Hoffman and Yang, 1980; Sitrit *et al.*, 1986; Yang, 1986); Yang (1987) has suggested that the preexisting ethylene-system I receptor complex coordinates the development of EFE. Thus Yang (1987) has postulated that fruit development (maturation) can be regarded as a transition from a nonclimacteric (immature) state which lacks the System II receptor to a climacteric (mature) state, in which the System II receptor has developed. In this model, the difference between System I and System II ethylene production does not appear to lie in the ethylene biosynthetic pathway, but rather in the tissues ability to develop ACC synthase.

In this work EFE activity and response to CO₂ were investigated in both Hosui and Granny Smith fruits which had been stored at low temperature. It has been widely reported that low temperatures can influence fruit ripening behavior in several different ways which may depend on species: (a) For most fruit, low temperature storage delays fruit ripening by depressing respiration and ethylene production (Metzidakis and Sfakiotakis, 1989; Wills *et al.*, 1986); (b) For European pears, fruit ripening does not occur without low temperature treatments (Blankenship and Richardson, 1985; Hartmann *et al.*, 1987; Looney, 1972); (c) In peach, nectarine and other chilling sensitive fruits, extended periods of low temperature cause chilling injury. These fruits fail to ripen normally because of a loss in ability to synthesis ACC synthase when transferred to room temperature after storage (Anderson, 1982). Therefore, in this study respiration rate and ethylene production in the Japanese pear, Hosui, and apple, Granny Smith, were measured in fruit that were stored at ambient or low temperatures after harvest in order to investigate effects of low temperature on their ripening behaviors.

The maturation and ripening behavior of Hosui and Granny Smith fruit were investigated in several different ways:(a) Determination of the changes of different maturity indices during development and maturation in order to ensure that experimental fruit taken in different seasons were at the same ~~same~~

~~be~~ physiological stage when used in subsequent years; (b) Measurement of respiration rate and ethylene production of fruit harvested at several different dates before optimum harvest date in order to determine if fruit had reached the climacteric on the tree; (c) Measurement of respiration and ethylene production patterns of fruit kept at 21°C or 0°C after being harvested at optimum harvest date in order to determine the effect of temperatures on fruit ripening characteristics; (d) Determining the relationship between the onset of respiration, ethylene production and EFE development to try and understand the role of ethylene on fruit ripening and to see if ethylene synthesis was inhibited by tree inhibitors; (e) Treatment of Hosui fruit, picked at different times before optimum harvest, with propylene and measurement of respiration rate and ethylene production to check if this cultivar of Japanese pear was nonclimacteric when grown in New Zealand.

3.3 MATERIALS AND METHODS

3.3.1 Materials

3.3.1.1 Japanese pear (*Pyrus serotina* cv. Hosui)

Fruit for the following experiments in this chapter were harvested from Ruakura and Gisborne (See 'General Materials and Methods'). All fruit required for assessment of maturity parameters were evaluated following 24 hour equilibration at 21°C. All other fruit were immediately placed at 1°±1°C until used.

3.3.1.2 Apple (*Malus domestica* Borkh. cv. Granny Smith)

All fruit were harvested from the Massey Fruit Crop Unit Orchard and were treated the same as for Hosui except the fruit for measurement of the starch index (see 3.3.2.1).

3.3.2 Methods

3.3.2.1 Fruit maturity assessments

On arrival of the Hosui fruit at Massey University, Palmerston North or immediately after picking of Granny Smith fruit, ten sound fruit of equal size

were selected and placed at 21°C to equilibrate for 24 hours before assessment. Skin colour, firmness and soluble solids were measured. In 1989, immediately after harvest at the different dates, the starch index of the flesh of Granny Smith apples were assessed. All methods for measuring maturity indices were described in 'General Materials and Methods'.

3.3.2.2 Ethylene and carbon dioxide determination

Single fruit were sealed in 5 X 600 ml (Hosui) or 5 X 1100 ml (Granny Smith) jars at 21°C for 1-2 hours. The length of time when the jars were sealed depended on the amount of C₂H₄ (>0.1 ppm for Granny Smith apple) and CO₂ (>0.1% for both fruits) that accumulated in jars. Two 1 ml gas samples were withdrawn from each jar through a rubber septum with a gastight syringe. A one ml sample was used for both ethylene and CO₂ measurements. Ethylene and CO₂ concentrations were measured using GLC.

In 1988, respiration rate and ethylene production of Hosui fruit were measured within 36 hours of harvest and having been transferred to Massey University.

After transferring from Manatuke to Palmerston North in 1989, five uniform Hosui fruit were selected from each of the different harvest dates and maintained for 24 hours at 21°C before measuring respiratory rates and ethylene production.

In 1987, Granny Smith fruit were harvested and (a) stored at 2°C, 6°C and 15°C; (b) placed into coolstore for 20 days at 1°±1°C before being transferred to 2°C, 6°C and 15°C. All fruit were equilibrated for 24 hours at these temperatures prior to measuring respiration rates and ethylene production.

In 1988, respiration rate and ethylene production of Granny Smith apples were only measured once within 36 hours of harvest.

In 1989, Granny Smith apples were harvested on 20 April, 25 April, 4 May and 18 May. After harvest fruit were placed at 21°C, and fruit respiration rate and ethylene production were measured at regular intervals over 20

days. In another experiment, fruit were placed into coolstore at $1^{\circ}\pm 1^{\circ}\text{C}$ immediately after harvest on 25 April and left for 20 days before being transferred to 21°C , where respiration rate and ethylene production were measured at regular intervals over 20 days in order to investigate if low temperature influenced fruit respiration and ethylene production.

3.3.2.3 Propylene treatment

Hosui fruit were harvested from Hamilton at different dates in 1989 (see Materials). Five replicate fruit from each harvest were equilibrated at 21°C for 24 hours before treating with a current of humidified air or a humidified gas mixture containing 0.5% propylene in air. Each fruit was placed in a 600 ml jar which was flushed (flow rate: 15 ml/min) with the appropriate gas. After three days, gas flushing ceased and fruit were returned to air. For measuring the respiration rate and ethylene production during the propylene treatment, the gas flow to each jar was stopped, then gas samples were taken after 1 hour. CO_2 and C_2H_4 samples were taken, and measurements were carried out as described above.

3.3.2.4 Measurement of EFE activity

In 1988, EFE activity of both Hosui and Granny Smith fruits was measured immediately after harvest. Discs were prepared according to the method described in Chapter 2. Six discs in each of 5 X 36 ml vials were incubated for 2 hours at 27°C with 3 ml K^+ -phosphate buffer (pH 6.5) containing 1 mM ACC and 1 mM AOA, then removed from solution, dried on paper tissues and sealed in 36 ml vials at 27°C for 1 hour before ethylene production was measured by GLC.

3.3.3 Data analysis

All data were analysed using as SAS computing program (SAS/STAT User's Guide) which generated means, standard errors, paired comparisons or multiple comparisons (Duncan) (Steel and Torrie, 1981).

3.4 RESULTS

3.4.1 Maturity indices

Changes of fresh weight, flesh firmness, soluble solids, skin colour, fruit respiration rate and ethylene production for both Hosui and Granny Smith fruits were tested. Fruit skin colour were assessed using the Japanese colour chart and the MINOLTA Chromametre, L,a,b values for Hosui, and only L,a,b values for Granny Smith fruit. In addition, starch index was observed in Granny Smith fruit (Table 3-1 and Table 3-3).

3.4.1.1 Hosui fruit

During maturation of Hosui fruit, fresh weight and soluble solids increased while flesh firmness and respiration rate declined (Table 3-1). Skin colour 'a' value decreased from -2.52 at 26 days before harvest to -3.44 at 14 days and remained unchanged over the next 10 days before harvest, before increasing markedly in the 4 days immediately prior to harvest (12 February, 1989). As there was very little change in the 'b' values, the hue angle was calculated (see 'Appendix 1'); the values obtained mainly reflected the change in 'a' values with a gradual increase occurring over the period from 26 to 4 days prior to harvest followed by a rapid and dramatic reversal in the last 4 days before harvest.

The magnitude of the changes of fresh weight and colour grade ('a' value) were greater than for the other indices tested. Over the 26 days from 17 January to 12 February, fresh weight increased more than three times from 60.85 g to 184.62 g, while skin colour became more russet, colour 'a' value increased more than 2 times and Hue angle became lower. Skin colour as measured by the Japanese colour chart showed similar changes, with the colour grade changed from 1-2 to 2-3 during this period (Table 3-1). The most rapid change occurred in the last 4 days before optimum harvest. As it did increase soluble solids from 11.22% to 12.25%, skin colour changed as reflected in the increase in 'a' values from -4.13 to -1.08 and the decrease in hue angle from 97.56 to 91.87, and respiration rate decreased from 11.78 to 9.82 mlCO₂/Kg/h (Table 3-1).

Table 3-1. Maturity indices of Japanese pear (Hosui) harvested at different times before optimal harvest date in 1989.

Days before harvest	F.W. (g) n=10	Firmness (Newton) n=10	S.S. (%) n=10	Colour grade n=10	Skin			Colour Hue (m _l CO ₂ /Kg/h) Angle	Resp. rate n=5
					L	a	b		
26	60.85	52.39	10.86	1-2	55.08	-2.52	31.65	94.55	17.62
14	118.29	38.11	10.56	1	53.76	-3.44	30.36	96.36	12.56
11	165.26	31.83	10.00	1	57.68	-4.22	32.69	97.30	10.17
4	175.13	27.57	11.22	1	53.04	-4.13	31.46	97.56	11.78
0	184.62	27.37	12.25	2-3	53.87	-1.08	32.05	91.87	9.82

Colour grades were obtained from Japanese Colour Chart.

All values are means. Different letters within the same column represent significant differences at the 5% level (Duncan's multiple comparison).

S.S. is soluble solid content.

Soluble solids content and skin colour of Hosui fruit varied with district and between seasons (Table 3-2). In 1988 and 1989, fruit from both Hamilton and Manatuke were harvested at a colour grade between 2-3 as indicated on the Japanese colour chart. Soluble solids content of fruit from Hamilton in 1988 and 1989 were 11.3% and 12.27% respectively. In 1989, fruit from Manatuke with a colour grade of 2-3 were much larger and contained lower soluble solids content (11.52%) than fruit from Hamilton. Skin colour 'a' value and hue angle for Hamilton fruit in 1989 were -1.16 and 92.04 respectively. The 'a' value was higher, and the hue angle was lower than the values recorded in 1988 from the same orchard for fruit harvested at the same colour grade measured by the colour chart (Table 3-2). In 1989 fruit from Hamilton had a higher 'a' value, lower hue angle value and higher soluble solids content than fruit from Manatuke.

3.4.1.2 Granny Smith apple

Starch index of cortical tissue and respiratory rate of Granny Smith apples increased during maturation (Table 3-3). Starch index increased from 0 to 3-4 over the period tested. Respiration rate increased from 3.99 to 8.47 mlCO₂/kg/h in the period 26 to 4 days before the optimum harvest date, with no further change at harvest. Over the 26 days before optimum harvest date, fresh weight, soluble solids and respiration rate increased and firmness decreased. In the final four days before harvest, fruit flesh firmness decreased from 42.48 to 36.54 (Newton) and soluble solids increased by 10% from 11.62% to 12.77%. The hue angle value decreased significantly because the 'b' value increased and the 'a' value remained unchanged over these 4 days indicating a yellowing of the fruit (Table 3-3).

Comparing maturity indices between three seasons, Hue angle value was lower in 1988 than in 1987 and 1989, but 'a' value did not change with season (Table 3-4). Soluble solids were higher in 1989 than in 1987 and in 1988. Firmness of fruit harvested in 1988 and 1989 was lower than in 1987. While fruit weight were higher in 1989 than in 1987 and 1988. These data suggest that skin colour, especially 'a' value, may be a relatively stable index that could be of some value in developing a suitable harvest maturity index for Granny Smith apples.

Table 3-2. Differences of maturity indices of Hosui fruit from different orchards and in different seasons.

Orchard	Harvest date	F.W. (g)	Firmness (Newton)	S.S. (%)	Skin			Colour		Resp. rate (mlCO ₂ /Kg/h)
					L	a	b	Hue Angle		
			a	a	b	b	b	a	a	b
Hamilton	22/2/88	187.65	25.21	11.30	50.63	-3.62	31.85	96.49		5.77
			a,B	a,A	a,A	a,B	a,A	a,A	b,B	a,A
Hamilton	12/2/89	184.62	25.41	12.27	53.88	-1.16	32.24	92.04		9.82
			A	A	B	A	B	A	A	A
Manatuke	7/2/89	232.56	23.84	11.52	58.25	-2.24	33.14	93.89		8.90

All values are means. Values within the same column not followed by a letter in common are significantly different at the 5% level between different seasons in same orchard (small letter) or between different districts in same season (capital letter) (paired comparison).

Table 3-3. Maturity indices of Granny Smith apples harvested at different times before optimal harvest date in 1989.

Days before harvest	Starch grade	F.W. (g)	Firmness (Newton)	S.S. (%)	Skin Colour			Resp. rate (mlCO ₂ /Kg/h)	
					L	a	b		Hue Angle
	n=10	n=10	n=10	n=10	n=10			n=5	
26	0	158.08	42.04	10.59	55.43	-20.93	41.64	116.70	3.99
14	0	165.19	40.52	11.70	55.98	-20.07	38.66	117.46	4.73
11	1	169.20	41.01	12.09	55.23	-19.36	38.29	116.85	3.42
4	2-3	174.69	42.48	11.62	55.14	-19.92	37.88	117.78	8.47
0	3-4	176.73	36.54	12.77	56.99	-20.28	41.05	116.34	8.43

All values are means. Different letters within the columns represent significant differences at the 5% level (Duncan's multiple comparison).

Table 3-4. Differences in maturity indices of Granny Smith apples harvested at the optimal date in different seasons.

Harvest date	F.W.	Firmness	S.S.	Skin Colour			
	(g)	(Newton)	(%)	L	a	b	Hue Angle
	n=10	n=20	n=20			n=20	
30/4/87	165.57	39.63	10.81	57.47	-19.51	41.12	115.41
27/4/88	154.20	38.26	11.14	54.49	-19.99	43.91	114.45
25/4/89	176.58	36.59	12.57	56.37	-20.29	41.33	116.19

All values are means. Different characters within the same column represent significant differences at 5% level (Duncan's multiple comparison).

3.4.2 Respiration and ethylene production patterns

3.4.2.1 Hosui

In 1989 Hosui fruit from Manatuke from 4 different harvest dates, were stored for various periods of time at 0°C prior to transferring to 21°C where respiration was measured. As results from each harvest date were similar, the data were pooled (Fig.3-1). The average respiration rate was 7.07 mlCO₂/Kg/h over the 10 day test period. No climacteric respiration peak appeared and no ethylene was detected in the system over the 10 days of measurement.

3.4.2.2 Granny Smith apple

In 1987 respiration and ethylene production of Granny Smith fruit were measured at different temperatures, and thus the respiration rate and ethylene production were calculated as mgCO₂/Kg/h and ugC₂H₄/Kg/h. At low temperatures (2°C and 6°C), fruit respiration rates and ethylene production were suppressed (Fig.3-2) and no peaks occurred. At 2° respiration rates (2.69 mgCO₂/Kg/h) did not change in the first 4 days of storage, but increased to 4.2 after 8 days and to 5.26 mgCO₂/Kg/h after 13 days, with no change occurring over the 38 days test period. Ethylene production was not detectable at harvest. After 8 days at 2°C, trace amounts of ethylene were produced, and ethylene production then increased gradually from 0.09 ugC₂H₄/Kg/h at 10 days to 2.17 ugC₂H₄/Kg/h at 19 days. Thereafter it remained at this level. Compared with results at 2°C, fruit respiration and ethylene production at 6°C showed similar patterns, but their quantities were higher. At 6°no change of respiration rate (3.2 mgCO₂/Kg/h) occurred during the first 4 days storage. It increased from 4.91 at 8 days to 9 mgCO₂/kg/h at 14 days, then remained unchanged over the 38 days test period when the average respiration rate was 9.28 mgCO₂/Kg/h. Fruit at 6°C did not produce any detectable ethylene during the first 4 days of storage. Ethylene production gradually increased from 0.3 at 8 days to 20.86 ugC₂H₄/Kg/h at 38 days. Fruit stored at 15°C produced ethylene and showed a typical climacteric ethylene peak (100 ugC₂H₄/Kg/h) at 22 days after harvest. The respiratory rate increased immediately after harvest, reached a peak (20 mgCO₂/Kg/h) after 10 days, remained at this level for about 7 days before it declined slowly and erratically. The onset of the respiration climacteric occurred 3 days earlier than ethylene (Fig.3-2).

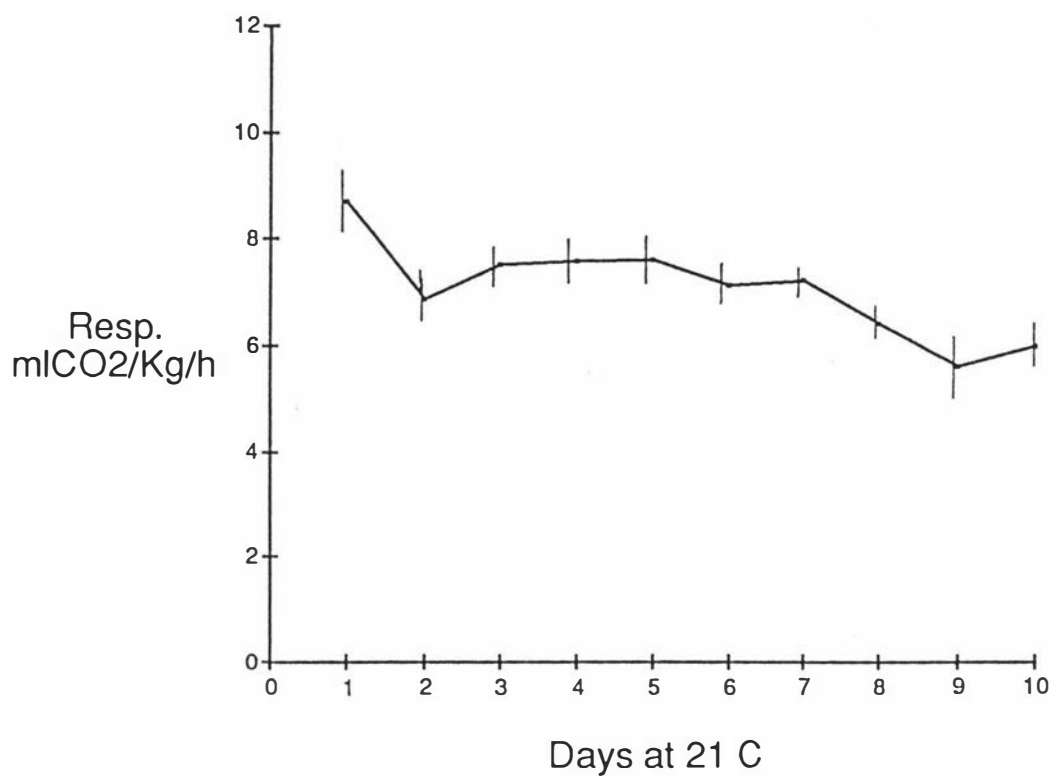


Fig.3-1 Respiration rate of Hosui fruit harvested from Manatuke (1989). Vertical bars indicate the standard error.

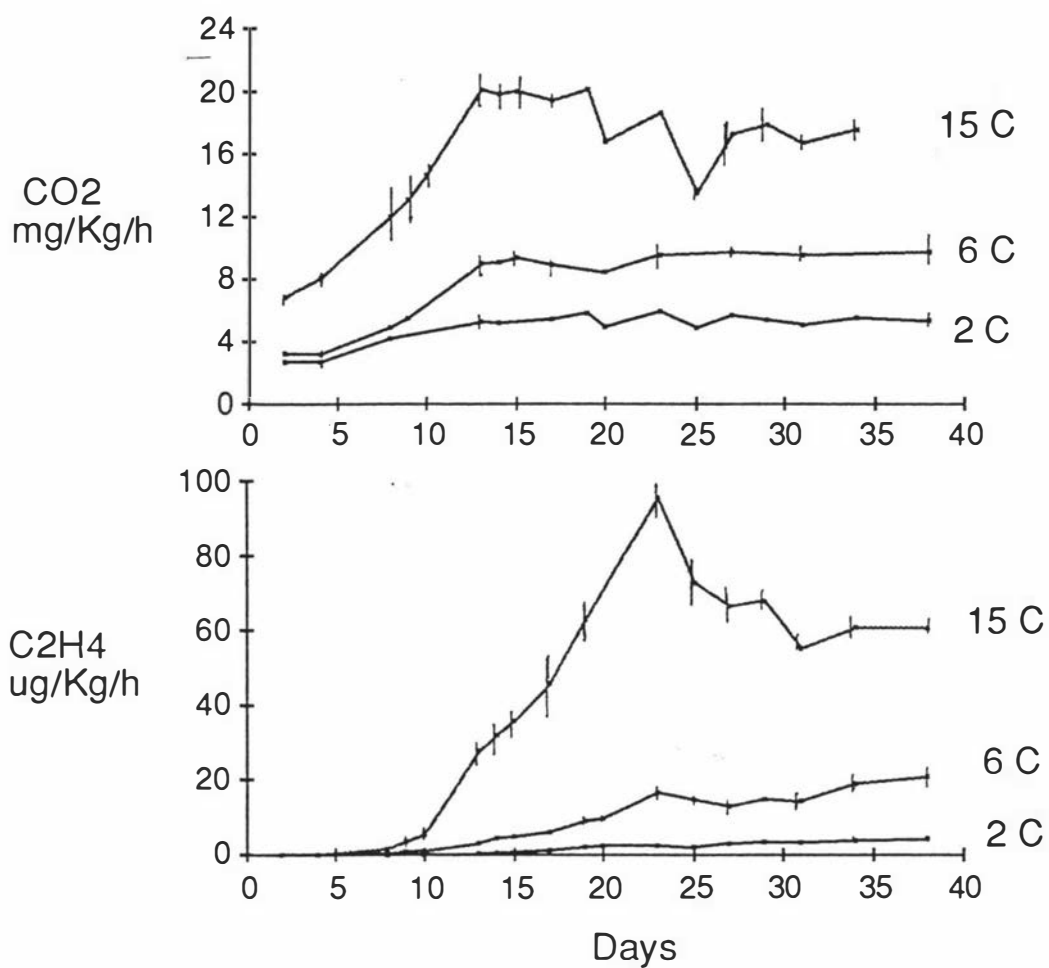


Fig.3-2 Respiration rate (a) and ethylene production (b) of Granny Smith fruit at different temperatures (1987). Vertical bars indicate standard error.

Fruit which had been maintained at $1^{\circ}\pm 1^{\circ}\text{C}$ for 20 days before being transferred to 2°C , 6°C or 15°C produced higher levels of ethylene and CO_2 than fruit which were placed at the same temperatures immediately after harvest (Fig.3-3). Those fruit also had shorter (at 2°C) or no time lag (at 6°C and 15°C) before the rise of respiration. Fruit respiration rate at 2°C did not change during the first 2 days ($6.8 \text{ mgCO}_2/\text{kg/h}$) but increased from $7.93 \text{ mgCO}_2/\text{Kg/h}$ after 4 days to $11.53 \text{ mgCO}_2/\text{Kg/h}$ at 15 days, and remained at this level thereafter. Ethylene production of fruit at 2°C did not change during the first 4 days ($15 \text{ ugC}_2\text{H}_4/\text{Kg/h}$), slightly increased to $20.61 \text{ ugC}_2\text{H}_4/\text{Kg/h}$ at 22 days before decreasing to $16.57 \text{ ugC}_2\text{H}_4/\text{Kg/h}$ at 37 days. Respiration rate of fruit measured at 6°C increased from $10.99 \text{ mgCO}_2/\text{Kg/h}$ at the first day to $16.2 \text{ mgCO}_2/\text{Kg/h}$ after 4 days, then did not change over the period tested (37 days). No change of ethylene production ($23.8 \text{ ugC}_2\text{H}_4/\text{Kg/h}$) occurred within the 15 days after fruit were transferred from $1^{\circ}\pm 1^{\circ}\text{C}$ to 6°C . Ethylene production then increased slightly to $30.6 \text{ ugC}_2\text{H}_4/\text{Kg/h}$ at 22 days and remained the same over the period tested. Respiration rate of fruit at 15°C increased from 20.2 to $27 \text{ mgCO}_2/\text{Kg/h}$ within the first 4 days, then remained at the same level for 11 days before gradually decreasing to $18 \text{ mgCO}_2/\text{Kg/h}$ at 37 days. Ethylene production of Granny Smith rapidly increased from 32.5 to $81.2 \text{ ugC}_2\text{H}_4/\text{Kg/h}$ within the first 2 days at 15°C and remained at the same level for 13 days. Thereafter ethylene production increased to $98.5 \text{ ugC}_2\text{H}_4/\text{Kg/h}$ at 22 days before decreasing to $74 \text{ ugC}_2\text{H}_4/\text{Kg/h}$ at 32 days. No change occurred from 32 to 37 days.

In order to clarify whether Granny Smith apples undergo a climacteric on the tree, fruit were picked on 20/4, 25/4 and 4/5 in 1989, and the respiration rate and ethylene production were measured immediately after harvest (Fig.3-4). Fruit produced low CO_2 ($8\text{-}9 \text{ mlCO}_2/\text{Kg/h}$) at harvest and the respiration rate declined in these fruit during 20 days at 21°C . No respiration peak was found and no ethylene was detected during the test period.

Respiration rate of fruit harvested on 18 May, 1989 was $8.03 \text{ mlCO}_2/\text{Kg/h}$; this increased steadily to $10.79 \text{ mlCO}_2/\text{kg/h}$ during 9 days, but no obvious climacteric peak occurred over 20 days at 21°C . In contrast to earlier harvested fruit, ethylene production occurred within 2 days at 21°C and increased rapidly during the next 6 days; this was followed by a slower rate of increase over the next 10 days before a peak of $45.76 \text{ ulC}_2\text{H}_4/\text{Kg/h}$ was reached after 17 days (Fig.3-5).

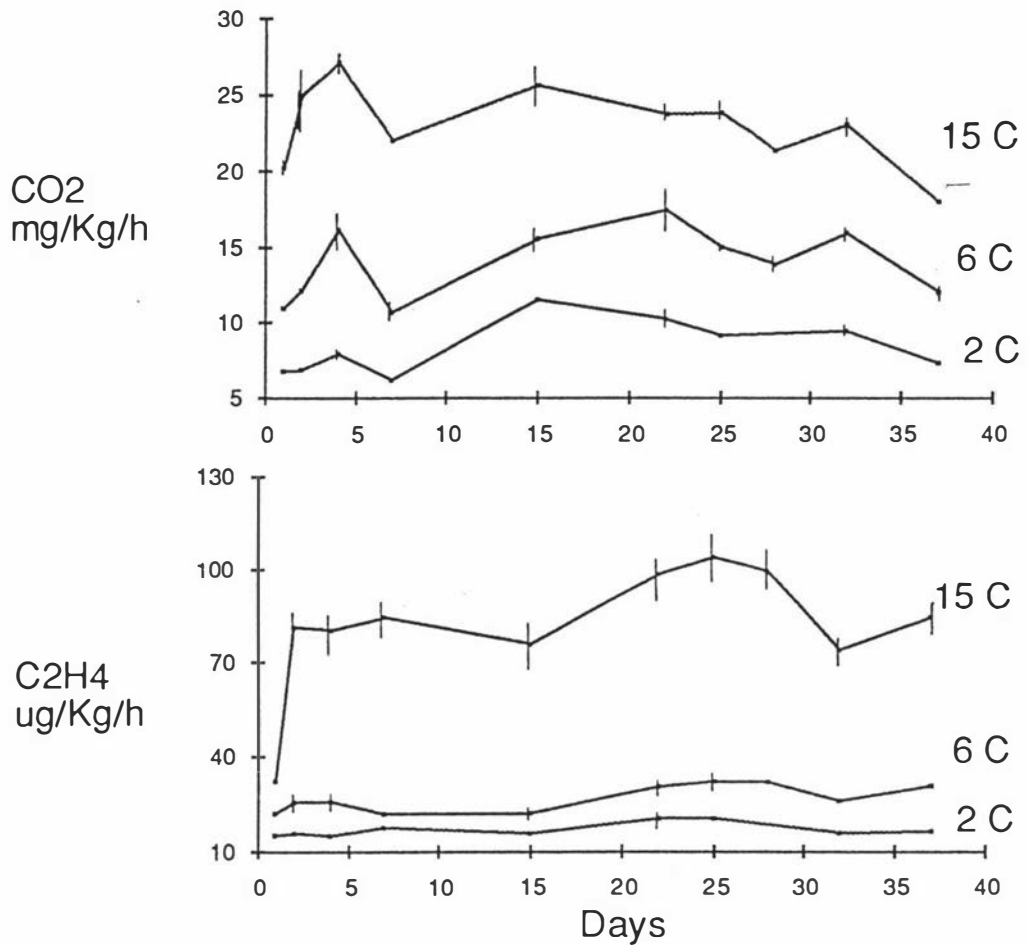


Fig.3-3 Respiration rate (a) and ethylene production (b) of Granny Smith fruits maintained at 2 , 6 and 15 C after transfer from 20 days at 1 ± 1 C, in 1987. Vertical bars indicate the standard error.

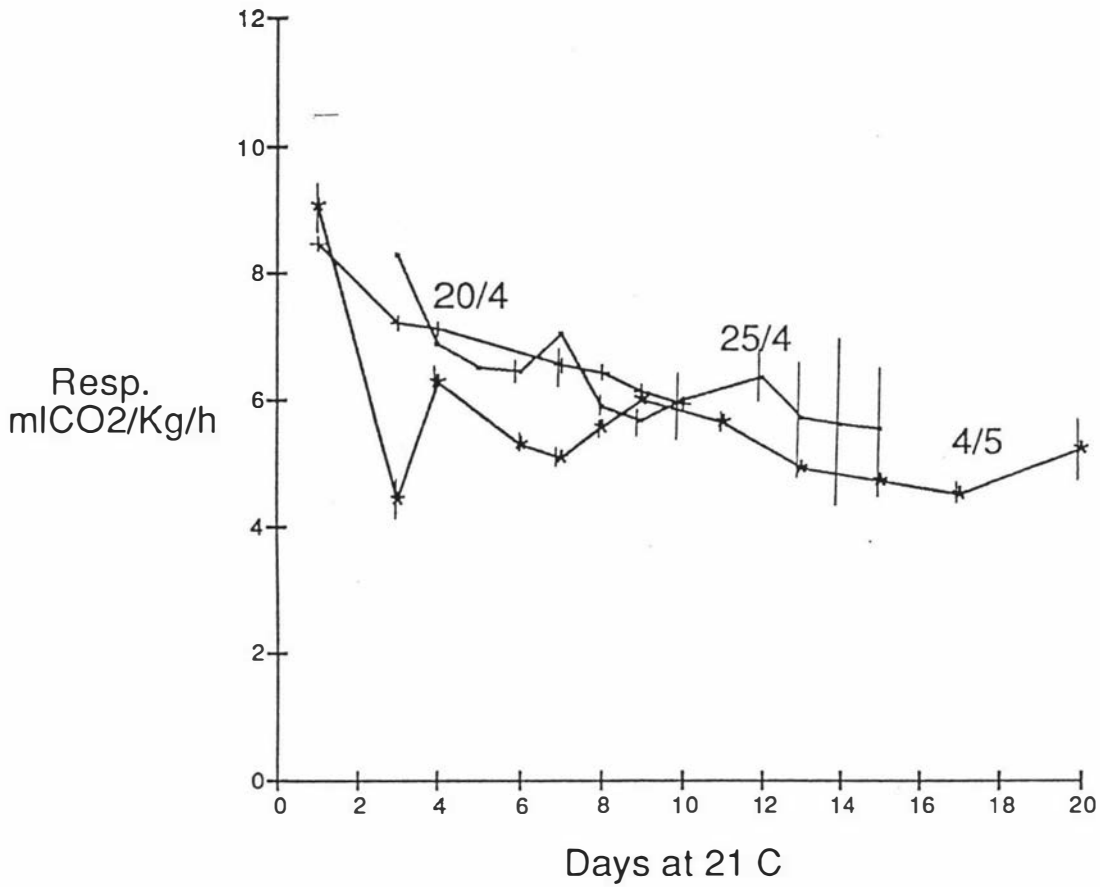


Fig.3-4 Respiration rate of Granny Smith fruit harvested on different dates (1989). Vertical bars indicate the standard error.

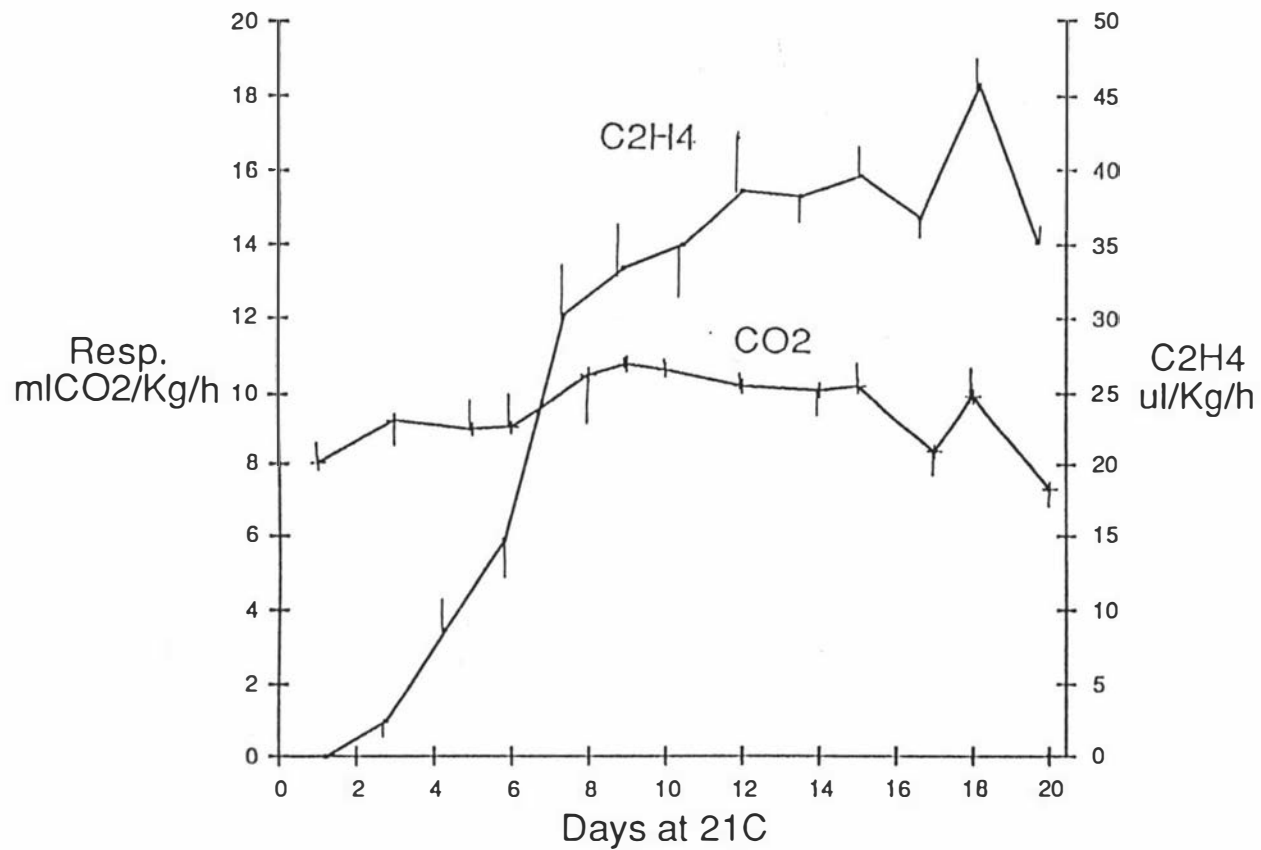


Fig.3-5 Respiration rate and ethylene production of Granny Smith fruit harvested at 18 May, 1989. Vertical bars indicate the standard error.

Fruit that was harvested on 25 April, 1989 then stored at $1^{\circ}\pm 1^{\circ}\text{C}$ for 20 days before being transferred to 21°C showed a respiration response that was typical of climacteric fruit (Fig.3-6). Ethylene production and respiration rate increased immediately on transfer to 21°C and reached peaks of $91.2 \mu\text{C}_2\text{H}_4/\text{Kg/h}$ and $14.9 \text{ mlCO}_2/\text{Kg/h}$ respectively after 5 days at 21°C . In contrast, fruit harvested on the same date and placed at 21°C immediately, without a period of exposure to $1^{\circ}\pm 1^{\circ}\text{C}$, showed a relatively low rate of CO_2 production (about $6 \text{ mlCO}_2/\text{kg/h}$), no respiration peak developed and no ethylene was detected during measurement over 16 days (Fig.3-4).

3.4.2.3 Respiration and ethylene production of Hosui fruit treated with propylene

The initial respiration rates of Hosui fruit harvested at five different times was less at each successive harvest date (Fig.3-7). The postharvest respiratory drifts varied in fruit from different harvest, but reached a ~~same~~ *similar* level of $7.6 \text{ mlCO}_2/\text{Kg/h}$ (means) after 3-9 days. Respiration rate was stimulated by propylene; the more immature the fruit, the greater the respiration increase induced. Respiration rate was increased immediately after exposure of fruit to propylene and this stimulation was maintained as long as propylene was present. Removal of propylene resulted in a reduction of the respiration rate (Fig.3-7). With the exception of fruit from the first harvest (Fig.3-7a), respiration had returned to basal levels 7 days after removal of propylene (Fig.3-7b-e).

Propylene treatment did not stimulate ethylene production above the basal levels of $0.1 \mu\text{C}_2\text{H}_4/\text{Kg/h}$ during or after 3 days treatment of fruit from any harvest date.

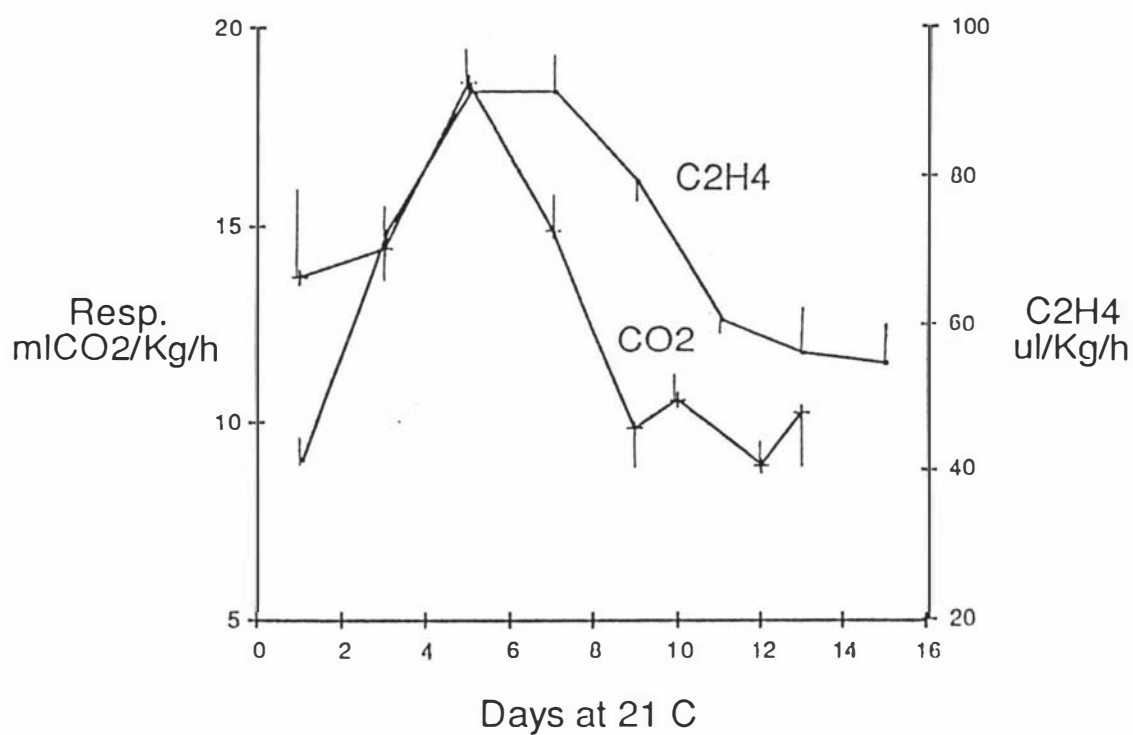


Fig.3-6 Respiration rate and ethylene production of Granny Smith fruit harvested at 25 April, 1989 and measured at 21 C after being stored at 1 ± 1 C for 20 days. Vertical bars indicate the standard error.

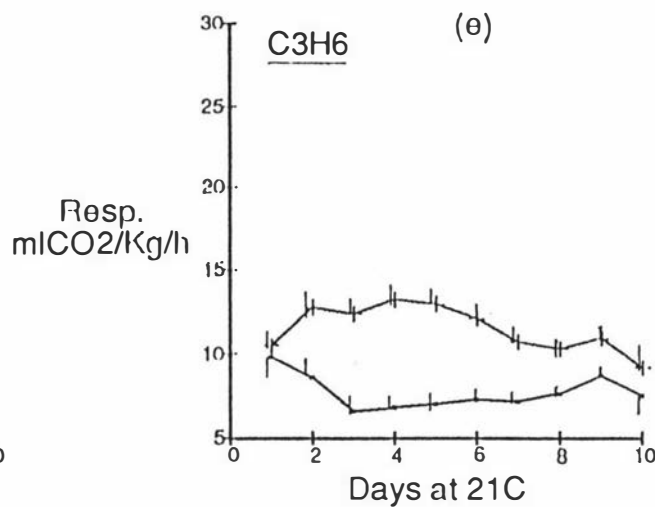
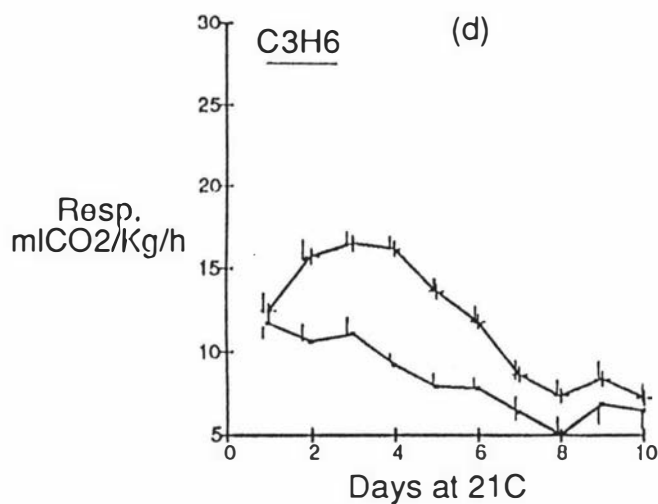
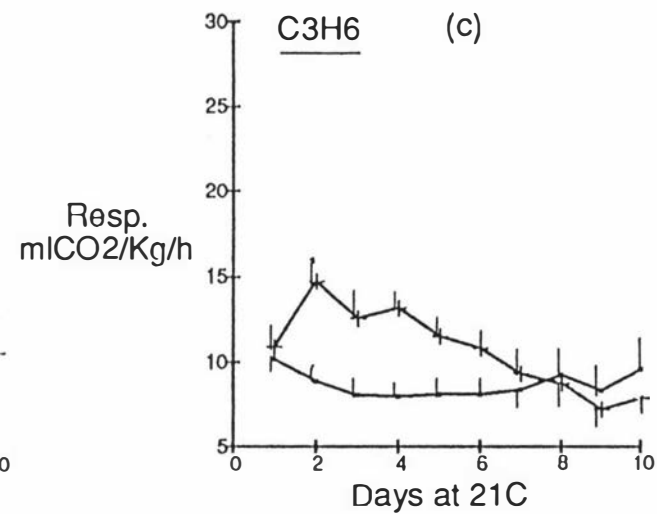
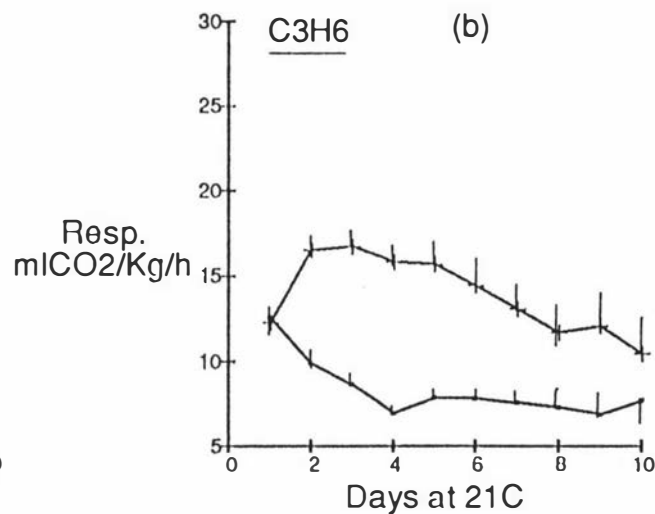
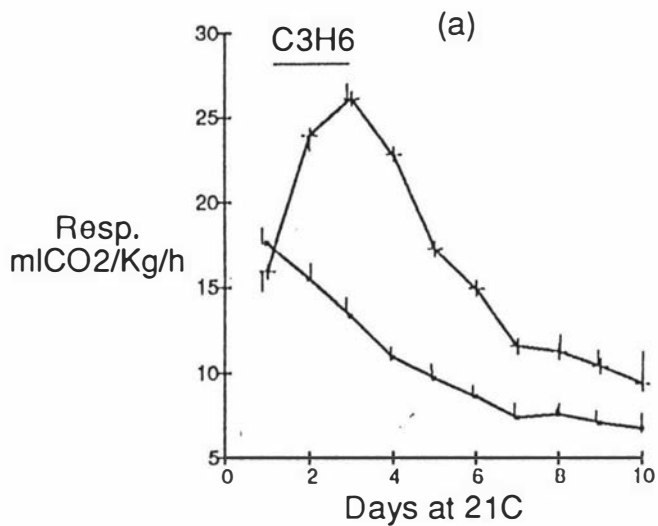


Fig.3-7 Respiration rate of Hosui fruit harvested at different dates treated with propylono (0.5%).
 The harvest dates in 1989 were:
 a:17 January.
 b:23 January.
 c:31 January.
 d:8 February.
 e:12 February.
 - - Control.
 +- propylene treatment.
 Vertical bars indicate the standard error.

3.4.2.4 Respiration, ethylene production and EFE activity at harvest time

Hosui fruit harvested on the 22 February, 1988 had a respiration rate of 5.76 mlCO₂/Kg/h at 21°C. Ethylene production was not detectable in this fruit, even though EFE activity was present (5.67 nC₂H₄/g/h) (Table 3-5).

Respiration rate of Granny Smith apple harvested on 27 April, 1988 was 6.83 mlCO₂/Kg/h; neither ethylene production nor EFE activity were detectable (Table 3-5).

3.5 DISCUSSION

Some postharvest physiological research has been done on Japanese pear and Granny Smith fruits in an attempt to define maturity indices, fruit storage requirements and physiological storage disorders of Hosui (Kitamura *et al.*, 1981; Lallu, 1985a,b; Suzuki *et al.*, 1981) and Granny Smith fruits (Reid *et al.*, 1982; Shaw and Rowe, 1982; Watkins, 1981), but there are very few, or conflicting reports on their ripening characteristics.

Suzuki *et al.* (1981) measured respiration rate and ethylene production of Hosui fruit at 30°C. Respiration (160 µMCO₂/100g/h) and ethylene peaks (8-9 nMC₂H₄/100g/h) were detected during measurement. No details were reported on how rapidly peaks occurred after harvest or how long they were maintained. Fruit was not exposed to ethylene or propylene with respiration and ethylene production being measured after fruit harvest. They concluded that Hosui was a climacteric fruit in Japan. No further work has been done evaluating the response of this cultivars to ethylene or propylene treatment in order to confirm that it was climacteric. No evidence has been found in these experiments to indicate that Hosui grown in Hamilton and Gisborne in New Zealand is a climacteric fruit as reported for this cultivar in Japan (Suzuki *et al.*, 1981). Respiration rate after harvest of fruit picked at a range of maturities showed a similar pattern, a gradual decline over time with no evidence of the rise in CO₂ production which is one of the characteristics of climacteric fruit (Biale and Young, 1981). After harvest, low temperature storage did not induce respiration and ethylene production climacteric peaks in Hosui fruit from Manatuke. Propylene treatment (0.5%) of fruit of different maturities stimulated respiration rates

Table 3-5. Respiration rates, ethylene production and ethylene forming enzyme (EFE) activity in Hosui and Granny Smith fruits at harvest time (in 1988).

Species	Harvest date	Respiratory rate (ml CO ₂ /Kg/hr) n=5	Ethylene production (ul C ₂ H ₄ /Kg/hr) n=5	EFE Activity (ml C ₂ H ₄ /g/hr) n=4
Hosui	22/2/88	5.76	ND	5.67
Granny	28/4/88	6.83	ND	ND

All values are means.

ND: not detectable.

with the increase being more marked in the less mature fruit. Removal of the propylene resulted in a decline in respiration rate. Propylene did not induce any ethylene production during or after treatment. These results indicate that the mechanisms for stimulation of respiration and autocatalysis of ethylene production are different. These results support Yang's model which suggests that ethylene acts by regulating of ethylene biosynthesis in maturing and ripening fruit (Yang, 1987). The model hypothesizes that in immature fruit and nonclimacteric fruit, ethylene or propylene bind to the System I receptor which in turn stimulates fruit respiration. When climacteric fruit are mature, they develop the System II receptor which regulates autocatalytic ethylene production. Because there appears to be no System II receptor in nonclimacteric fruit and immature climacteric fruit, autocatalytic ethylene production does not occur in these fruit.

Because Granny Smith fruit harvested at different maturities or exposed to low temperature did produce respiratory and ethylene climacteric peaks, it is classified as a climacteric fruit. It has been observed that cold storage was required for ripening of some pipfruit such as European pears cv. d'Anjou (Blankenship and Richardson, 1985; Knee, 1987). Knee (1987) also found that EFE developed before the increase in ACC and ethylene production. It is possible that ACC synthase and EFE development can be induced by low temperature (Knee, 1987). The present results could indicate that low temperatures accelerated ethylene production in Granny Smith apples by stimulating biosynthesis of both ACC synthase and EFE.

Depending on the relationship between ethylene evolution and the respiratory rise, climacteric fruit can be classified into three types (Biale and Young, 1981). Increase in ethylene production can (1) precede, (2) coincide with or (3) follow the respiratory rise. In these results, the onset of the rise in ethylene production of Granny Smith apple occurred later than the respiration climacteric. This suggests that Granny Smith apple belongs to the third type. It is possible that a 'ripening inhibitor' ^{was} presented in this type of fruit and prevented autocatalytic ethylene synthesis (Yang, 1987). In 1987 and 1989, typical ethylene peaks occurred in Granny Smith fruit. Therefore while Granny Smith apple may still be classified as a climacteric fruit, its initial increase in respiration rate does not seem to be very sensitive to an ethylene increase ~~to proceed~~. Different respiration and ethylene production patterns of Granny Smith apple in 1987 and 1989 might be explained by

different environmental factors during the growing season prior to harvest which could possibly: (a) delay the development of enzymes involved in ethylene biosynthesis directly, and/or (b) induce some ripening inhibitors which inhibit autocatalysis of ethylene production (Rhodes and Reid, 1975; Sfakiotakis and Dilley, 1973b).

Because of the difference in EFE development in the two species at harvest time, the presence^{CR} or absence of EFE in fruit at harvest time does not allow us to distinguish whether fruit will subsequently ripen in a climacteric or nonclimacteric manner. This result supports Yang's model (1987) which suggested that EFE is regulated by the System I receptor which exists in both climacteric and nonclimacteric fruits. The main difference between climacteric and nonclimacteric fruit is the capacity for autocatalytic ethylene production to develop in climacteric fruit as a result of the biosynthesis of the enzyme ACC synthase in response to the presence of the System II ethylene receptor.

The study on aspects of maturity has been conducted on Hosui fruit from Hamilton and Manatuke, and Granny Smith apples from Fruit Crop Units Research Orchard, Massey University. In Granny Smith apple fresh weight, starch grades and respiration rate increased, while changes of skin colour, as expressed by hue angle decreased significantly during the stages of fruit development and maturity. Skin colour 'a' value was not significantly different between seasons. In Hosui fruit, fresh weight and soluble solids increased, and firmness and respiration rate decreased. Colour grades as measured by the Japanese colour chart increased with maturity, and correlated highly with 'a' value and hue angle (Appendix 1.). These limited studies suggest that skin colour 'a' value (L, a, b mode) and starch index for Granny Smith fruit, and colour grade (Japanese colour chart) for Hosui fruit may be useful aids in the assessment of maturity.

Both fruit, Hosui and Granny Smith apple harvested in 1989 contained higher soluble solids and lower firmness than those in 1987 and 1988. Data on firmness, soluble solids and colour index (Table 3-1, 3-2, 3-3 and 3-4) suggested that if fruit had been harvested 4 days earlier in 1989 there would have been no significant difference between the three years in these maturity parameters.

INDEX SHOWED NO SIGNIFICANT DIFFERENCE. IT HAS BEEN SHOWN THAT INCREASED

preharvest temperatures influenced the quality of kiwifruit (Hopkirk *et al*, 1989) and grape (Coombe, 1987; Koblet, 1985). It is possible that because of environmental factors during the growing season in 1989, fruit were more mature than those in 1987 and 1988.

It can be concluded that the Hosui, Japanese pear grown in New Zealand is a nonclimacteric fruit and that Granny Smith apple is a climacteric fruit. Skin colour for both fruit and starch index for Granny Smith apple were suitable maturity indices. Both fruit can ripen on the tree. Fruit respiration and ethylene production can be varied by seasons and districts and affected by postharvest storage conditions.

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CHAPTER 4

DEVELOPMENT OF ASSAY METHODS FOR ESTABLISHING ETHYLENE-FORMING ENZYME (EFE) ACTIVITY

4.1 ABSTRACT

In this work, several methods for measuring EFE activity *in vivo* in fruit of Hosui and Granny Smith apple were tested. These involved evaluation of the effects of pH levels, sugars and their osmotic potentials in the medium, and methods of saturating the tissue with ACC.

Discs produced wounded ethylene at 4 or 2 hours after discs ~~were~~ ^{ed} prepared by cutting Hosui or Granny Smith fruits, respectively.

The isotonic buffers of K⁺-phosphate or Na⁺-citric acid (pH 6.5) were tested. The isotonic concentration for mannitol solution was 0.4 M.

The saturating concentration of ACC for EFE activity was 1 mM for discs of both Hosui and Granny Smith fruits. The apparent K_m values of EFE for ACC were 0.166 mM and 0.193 mM for Hosui and Granny Smith, respectively. Several methods were suitable for saturating fruit discs with ACC. They were : (a) incubation of discs in a medium which contained >1 mM ACC for 2 hours at 27°C; (b) vacuum-infiltration of discs 3 times for 1 minute at 90 Kpa; (c) vacuum-infiltration of discs for 1, 2 or 3 min at 90 Kpa in the medium at 27°C. EFE activity was measured within 2 hours.

Three methods for measuring EFE activity were tested. The most suitable method involved saturation of the discs with ACC, then drying of the discs on tissue paper, placing them into sealed vials at 27°C for a appropriate period of time before measuring EFE activity.

The pH experiment showed that the low pH treatment stimulated EFE activity in discs of Granny ~~S~~ ^{ed} Smith apple within 1 hour ^{of} treatment, then EFE activity decreased. The optimum pH values of EFE in Granny Smith and Hosui fruits changed with fruit ripening.

Based on these results, a quick, reliable and consistent EFE assay for Japanese pear and apple has been developed. The effects of various measuring methods upon the activity of EFE was discussed in terms of the enzyme location *in vivo*.

Key words: ACC saturation, buffers, EFE activity, EFE localization, Granny Smith, Hosui, isotonic, K_m , pH, sugars, V_{max} , wounding.

Abbreviations: ACC: 1-aminocyclopropane-1-carboxylic acid; AOA: aminooxyacetic acid; CHI: cycloheximide; EFE: ethylene-forming enzyme; GLC: gas liquid chromatograph; K_m : the Michaelis-Menton constant; V_{max} : the maximum reaction rate.

4.2 INTRODUCTION

The conversion of ACC to ethylene is carried out by an oxidative enzyme or enzyme system which is generally referred to as the ethylene-forming enzyme (EFE). So far the EFE in plant organs has not been isolated independently of intact cellular material (vacuoles, protoplasts or tissues); characterization of EFE has been done only *in vivo* (Bouzayen *et al.*, 1990; Guy and Kende, 1984; John *et al.*, 1989; McKeon and Yang, 1987). Membrane vesicles in juice squeezed from the pericarp of ripe kiwifruit possessed EFE activity which showed the essential features of the *in vivo* enzyme (Mitchell *et al.*, 1988).

EFE activity *in vivo* is expressed by measurement of ethylene production from tissues which are saturated by ACC at mM concentrations (John *et al.*, 1989). EFE shows two main features *in vivo*: EFE activity requires membrane integrity. Treatments which perturb membrane integrity can inhibit EFE activity (Apelbaum *et al.*, 1981; John *et al.*, 1989; Porter *et al.*, 1986) and it has a relatively high affinity for its stereoselective substrate, ACC (Hoffman *et al.*, 1982). The reported K_m values for ACC are varied ranging from 8.7 μ M in dark grown *Lemna Minor* L. (Fuhrer, 1985), 61 μ M in pea-leaf vacuoles (Guy and Kende, 1984), 66 μ M in pea hypocotyl segments (McKeon and Yang, 1984), to 125 μ M in ripe kiwifruit (John *et al.*, 1989; Mitchell *et al.*, 1988).

Many factors can stimulate or inhibit EFE activity. They are listed in Table 4-1. From this review on the effects of various factors on EFE activity,

it can be deduced that EFE has a high sensitivity to various chemical and physical stimuli, high requirement for membrane integrity and a high energy (ATP) requirement for action. A range of various methods have been published for measuring EFE activity in fruit and vegetative tissues, but few ~~of~~ attempts have been made to compare and contrast these methods for consistency and efficiency. At least 3 major types of variable may affect the efficiency of EFE quantification. These indicate:

(a) Buffer, pH and osmotica: EFE activity has been measured in various buffer, sugar and osmotic solutions with different pH values (see Table 1-1). No report has been found to investigate or compare their effects on measurement of EFE activity.

(b) ACC incorporation: Several methods for saturating ACC have been used (see Table 1-2). No work had been done to compare different methods for ACC incorporation into discs from different species. Vacuum-infiltration methods increase the amount and rate of ACC uptake and reduce the risk of disc aging, although it is possible that vacuum might induce membrane damage and thus reduce EFE activity.

(c) EFE measurements: EFE activity has been measured in tissues floating in medium such as buffer and water; on filter paper wetted with buffer; or in air with no buffer (see Chapter 1). EFE catalyzes the conversion of ACC to ethylene and is an oxidative reaction. It is necessary to test if these various methods give different results due to limitation in oxygen availability.

The procedures that have been used to measure EFE activity in both fruit and leaf materials are very varied and have not been quantitatively or qualitatively compared for a given plant material. Therefore prior to commencing research to study the response of EFE to various conditions, it was necessary to devise a quick, reliable and consistent EFE assay procedure that could be used for Hosui and Granny Smith fruits.

Table 4-1. A listing of factors which influence EFE activity in plant tissues.

Factors	Effects	References
Osmotic shock	inhibition	Burg and Thimann, 1960
Vacuum	inhibition	Saltveit and Dilley, 1978b
Wounding	inhibition	Kende and Boller, 1981 Saltveit and Dilley, 1979
High temperature	inhibition	Adam and Mayak, 1984
Cold shock	inhibition	Yu <i>et al.</i> , 1980
Light	inhibition	Bassi and Spencer, 1983 De Laat <i>et al.</i> , 1981 Gepstein and Thimann, 1980 Grodzinski <i>et al.</i> , 1982, 1983 Jiao <i>et al.</i> , 1987
Low O ₂ level	inhibition	Adams and Yang, 1979
Cell-wall degrading enzymes (Pectolyase and Caylase)	inhibition	Porter <i>et al.</i> , 1986
Uncoupling of oxidative phosphorylation DNP (2,4-dinitrophenol CCCP (carbonyl cyanide <i>m</i> -chlorophenylhydrazone)	inhibition	Mayne and Kende, 1986
Free radical scavengers n-propyl gallate	inhibition	Konze <i>et al.</i> , 1980
Various inhibitors cobaltous ion a-aminoisobutyric acid	inhibition	McKeon and Yang, 1987
Carbohydrates	inhibition stimulation	Philosoph-Hadas <i>et al.</i> , 1987 Colcasure and Yopp, 1976 Kim <i>et al.</i> , 1987 Meir <i>et al.</i> , 1985 Philosoph-Hadas <i>et al.</i> , 1985 Riov and Yang, 1982
High CO ₂	stimulation (activity) stimulation (synthesis) inhibition (synthesis)	Cheverry <i>et al.</i> , 1988 Philosoph-Hadas <i>et al.</i> , 1986 Philosoph-Hadas <i>et al.</i> , 1986 Cheverry <i>et al.</i> , 1988
The pH values	*	Mayne and Kende, 1986;

* The optimum pH values could be a wide range.

4.3 MATERIALS AND METHODS

4.3.1 Materials (See 'General Materials and Methods')

at which

The time ~~of~~ experiments carried out were written in the titles of tables or figures. Fruit of maturity at harvest time, colour grade 2-3 (Japanese pear colour chart), fruit weight 180 ± 5 g for Hosui, and 'a' value (-20 ± 0.5), starch grade 2-3 and fresh weight (means:185g) for Granny Smith were used for experiments.

4.3.2 Preparation of fruit discs (See 'General Materials and Methods')

4.3.3 Ethylene and carbon dioxide measurement (See 'General Materials and Methods')

4.3.4 Methods

4.3.4.1 Wound ethylene production by discs

Six discs were placed in each of 5 X 36 ml vials and incubated in a shaking waterbath (120 strokes/min) at 27°C. Ethylene concentrations in vials were measured after 0.5, 1, 2, 3, 4, 6, 8 hours incubation. For each measurement, vials were sealed for 30 minutes before withdrawing a 1 ml gas sample for ethylene measurement using GLC. Immediately after each sampling, vials were flushed with air for 20 seconds to prevent CO₂ accumulation and then returned to incubate at 27°C until the next ethylene determination was required.

4.3.4.2 Isotonic solution assessment

The osmotic potential of the tissue was determined using a gravimetric method (Harker, 1986). Five discs were placed in each of three petri dishes and incubated in the buffers or mannitol solution for 2 hours at 27°C. Buffers used were K₂HPO₄-KH₂PO₄ buffer (pH 6.5); and Na₂HPO₄-citric acid buffer (pH 6.5) (see Chapter 2). Mannitol (pH 6.5) concentrations used were 0.2 M, 0.3 M, 0.4 M and 0.8 M for Hosui and 0.4 M and 0.8 M for Granny Smith tissues. Fresh weight of each disc was measured accurately before and after incubation by electronic balance (Mettler PE 360) to obtain

an indication of the osmotic potential of the tissues.

4.3.4.3 EFE activity in 0.4 M or 0.8 M mannitol solution at 27°C

Six discs were immersed in each of 4 X 36 ml vials containing 3 ml of 0.4 M or 0.8 M mannitol solution which contained 3 mM ACC and 1 mM cycloheximide (CHI); the vials were incubated for 2 hours at 27°C, before being sealed for 1 hour at 27°C when a 1 ml sample were withdrawn from each vial for ethylene analysis by GLC.

4.3.4.4 Comparison of methods for saturating fruit tissue with ACC

To determine the concentration of ACC which saturated Hosui and Granny Smith tissues, six discs were placed in each of 3 X 36 ml vials containing 3 ml of medium and incubated at 27°C for 2 hours. The medium contained K⁺-phosphate buffer (pH 6.5) and a range of ACC concentrations (0, 0.005, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 mM). Discs were then dried on tissue paper and each of six disc replicates were placed dry and sealed in each of 3 X 36 ml vials for a further 1 hour incubation at 27°C. A 1 ml sample was then taken from each vial and ethylene concentration was measured by GLC.

To evaluate the most efficient method of saturating tissue with ACC, several methods were used to expose discs to a medium containing 5 mM ACC in K⁺-phosphate buffer (pH 6.5).

The methods used were:

(a) Immersion method: as a control, discs were immersed in above medium for 2 hours at 27°C.

(b) Vacuum-infiltration method: Discs were infiltrated with the medium by vacuum (90 Kpa) followed by incubation for 2 hours at 27°C. The vacuum-infiltration time were:

- 3 minutes;
- 2 minutes;
- 1 minute;
- Intermittent-vacuum: 1 min vacuum, then release, repeated for 3 times.

(c) Discs were incubated in the medium for 2 hours at 27°C, then vacuum-infiltrated with the same medium. The vacuum-infiltration time were:

- 3 minutes;
- 2 minutes;
- 1 minute;
- Intermittent-vacuum: 1 min vacuum, then release, repeated for 3 times.

After treatment with ACC, discs were dried on tissue paper. Six discs were then sealed in each of 3 X 36 ml vials for 1 hour at 27°C before ethylene concentration in a 1 ml sample withdrawn from each vial, was measured by GLC.

4.3.4.5 Comparison of three methods for measuring EFE activity

To obtain the highest EFE activity, three methods for measuring EFE activity were tested. After saturation with 5 mM ACC by immersion of discs in the medium for 2 hours at 27°C, six discs were sealed in each of 4 X 36 ml vials with the following conditions:

- (a) Discs were immersed in 3 ml medium.
- (b) Discs were put on filter paper wetted by the medium.
- (c) Discs were dried on filter paper, then placed into vials containing no medium.

After incubation for 1 hour at 27°C, 1 ml gas was taken from each vial for ethylene analysis. Fresh weight of the six discs from each vial was determined and EFE activity was expressed as $\text{nIC}_2\text{H}_4/\text{g/h}$.

4.3.4.6 Optimum media pH for determination of EFE activity in Japanese pear and apple tissues

To determine the optimum pH for EFE activity in Hosui and Granny Smith fruit discs, Na^+ -citric acid buffer was used to obtain different pH values for the incubation media. The pH values used for Hosui discs were 3.0, 4.0, 5.0, 5.7, 6.2, 6.5, 7.2, and 8.2, while those for Granny Smith discs were 2.5, 3.0, 3.5, 4.0, 4.3, 4.8, 5.2, 5.7, 6.2, 7.2 and 8.2.

Six discs were saturated by incubation in each of 4 X 36 ml vials containing 3 ml of the buffer containing 5 mM ACC at each pH level for 2 hours at 27°C, then vacuum-infiltrated at 90 Kpa for 1 min to reduce the possible difference of ACC uptake caused by different pHs. Discs were then sealed in the same vials and incubated for 1 hour before 1 ml gas was removed from each vial for ethylene analysis.

The pH values in the media were measured before and after incubation.

4.3.4.7 Time course of effect of pH levels on EFE activity

Six discs were immersed and incubated at 27°C in each of 4 X 36 ml vials containing 3 ml Na⁺-citric acid buffer with 5 mM ACC. The pH values used for Hosui discs were 6.2, 7.2 and 8.2, those for Granny Smith discs were 4.0, 6.2 and 8.2. Ethylene concentrations were measured after 0.5, 1, 2 and 3 hours incubation. For each measurement, vials were sealed for 30 minutes before withdrawing a 1 ml gas sample for ethylene measurement using GLC. Immediately after each sampling, vials were flushed with air for 20 seconds to prevent CO₂ accumulation and then returned to incubate at 27°C until the next ethylene determination was required when the procedure was repeated.

4.3.4.8 Effect of sugars on ethylene production and EFE activity

The influence of 0.4 M sucrose, fructose, glucose, mannitol and sorbitol on EFE activity and ethylene production was tested by adding each sugar separately to the incubation medium with (for EFE activity measurement) or without (for ethylene production measurement) 1 mM CHI and 5 mM ACC, respectively. For EFE activity measurement a K⁺-phosphate buffer containing 1 mM CHI and 5 mM ACC was used as a control. For ethylene production, the same buffer was used but without ACC and CHI. The pH value of all solutions was 6.5. Six discs were placed in each of 3 X 36 ml vials containing 3 ml of the appropriate sugar solution for each treatment. After saturation of the discs with the appropriate media using the immersion method for 2 hours, discs were dried on tissue paper. Six discs were sealed and incubated in each of 3 X 36 ml vials for each treatment for 1 hour at 27°C before a 1 ml sample was removed for ethylene analysis.

4.3.4.9 Statistical analysis

Data were analysed by Duncan's multiple comparison, paired comparison and linear regression (Steel and Torrie, 1986) using the SAS statistical programs (SAS/STAT User's Guide, Release 6.03 Edition).

4.4 RESULTS

4.4.1 Wound ethylene production

Hosui fruit discs did not produce detectable ethylene in the first 3 hours after cutting (Table 4-2). After 4 hours incubation, trace amounts of ethylene were produced which increased to 5.3 and 7.24 nl/g/h after 6 and 8 hours at 27°C, respectively. In Granny Smith, fruit discs produced ethylene immediately after cutting; production increased to a maximum of 55.78 nl/g/h after 5 hours before decreasing to the initial level after 10 hours.

4.4.2 Isotonic solution for fruit discs

Fresh weights of Hosui fruit discs increased significantly in 0.2 M and 0.3 M mannitol (Table 4-3), but decreased in both Hosui and Granny Smith discs exposed to 0.8 M mannitol after 2 hour incubation (Table 4-3). No significant changes occurred in the fresh weights of either Hosui or Granny Smith fruit discs during the two-hour incubation in different buffers or 0.4 M mannitol solutions at 27°C. This indicated that the K⁺-phosphate buffer, the Na⁺-citric acid buffer tested and the 0.4 M mannitol solution at pH 6.5 were isotonic for fruit discs of both Hosui and Granny Smith fruits (Table 4-3).

4.4.3 EFE activity in 0.4 M and 0.8 M mannitol solution

EFE activity in both ~~of~~ Hosui fruit and Granny Smith fruits ^{was} ~~were~~ higher in 0.8 M mannitol than in 0.4 M (Table 4-4).

Table 4-2. Wound ethylene production by fruit discs of Hosui and Granny Smith at 27°C (1989).

Time (hour)	Ethylene production (nl/g/h)	
	Hosui	Granny Smith
0.5	0	23.93 ^{cd}
1.0	0	23.17 ^{cd}
2.0	0	31.94 ^c
3.0	0	41.46 ^b
4.0	trace	-
5.0	-	55.78 ^a
6.0	5.30 ^b	-
7.0	-	44.95 ^b
8.0	7.24 ^a	-
10.0	-	23.31 ^{cd}

*All values are means (n=5). Different characters within the same column represent significant differences at the 5% level (Duncan's multiple comparison).

Table 4-3. Fresh weight of Hosui and Granny Smith cortical discs incubated in different buffers (pH 6.5) and mannitol solutions (pH 6.5) for 2 hours at 27°C (1988).

(a). Hosui.

Time	Fresh weight (g)					
	K ⁺	Na ⁺	Mannitol solution			
	-phosphate	-citric	0.0 M	0.4 M	0.3 M	0.2 M
initial	a 0.2701	a 0.2780	a 0.2805	a 0.2908	b 0.2696	b 0.2915
final	a 0.2808	a 0.2838	b 0.2418	a 0.3044	a 0.2979	a 0.3356

(b). Granny Smith.

Time	Fresh weight (g)			
	K ⁺	Na ⁺	Mannitol solution	
	-phosphate	-citric	0.0 M	0.4 M
initial	a 0.2596	a 0.2259	a 0.2080	a 0.2307
final	a 0.2545	a 0.2297	b 0.1770	a 0.2328

*All values are means (n=3). Different characters within the same column for each fruit represent significant differences at the 5% level between initial and final values (paired comparison).

Table 4-4. EFE activity in fruit discs of Hosui and Granny Smith in 0.4 M and 0.8 M mannitol solution at 27°C (1990).

Species	EFE activity (nlC ₂ H ₄ /g/h) in mannitol solution	
	0.4 M	0.8 M
Hosui	53.34 ^b	82.61 ^a
Granny Smith	90.12 ^b	125.18 ^a

All values are means (n=4). Different characters in the same line represent significant differences at the 5% level (paired comparison).

4.4.4 Comparison of different methods to saturate ACC

In Hosui fruit, EFE activity increased from 12 nl/g/h at 0.02 mM ACC to 112 nl/g/h (V_{max}) at 1 mM ACC concentrations (Fig.4-1). In Granny Smith apple, the activity increased from 20 nl/g/h at 0.04 mM ACC to 103 nl/g/h (V_{max}) at 1 mM (Fig.4-2). When ACC concentrations were higher than 1 mM, EFE activity in discs of both fruits remained unchanged. Thus the saturated concentration of ACC for EFE was 1 mM for both fruits. Results of a double reciprocal plot of EFE activity vs. ACC concentrations indicated that the apparent K_m values of EFE for ACC were 0.166 mM for Hosui and 0.193 mM for Granny Smith apple (Fig.4-1 and Fig.4-2).

In some experiments, it is necessary to use a vacuum-infiltration method to ensure that (a) the discs were saturated with ACC, quickly to shorten the experimental period to minimize disc ageing; (b) ions as Ag⁺ and Co⁺⁺ ions penetrated into the fruit tissue; (c) CO₂ or C₂H₄ were removed from fruit tissue after treatment. Discs were vacuum-treated before or after a 2 hour incubation with the medium at 27°C to determine the effect of vacuum-infiltration on EFE activity.

medium at 27°C.

No significant change of EFE activity occurred when discs of Hosui fruit were vacuum-infiltrated with 5 mM ACC before or after incubation compared with control discs which were incubated for 2 hours in the same medium (Table 4-5A).

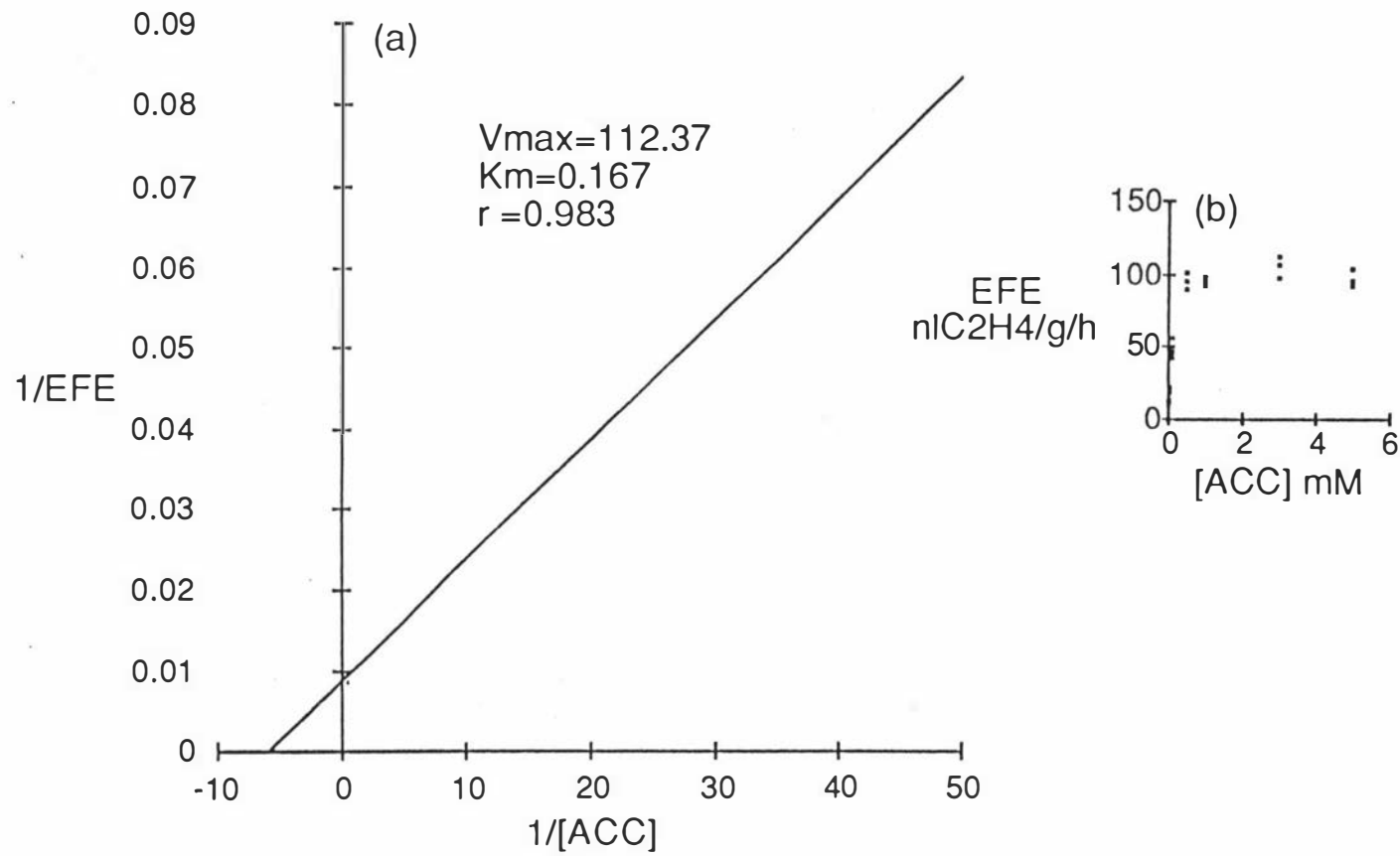


Fig.4-1 Double reciprocal plot (a) and data (b) of EFE activity against ACC concentrations in Hosui fruit discs.

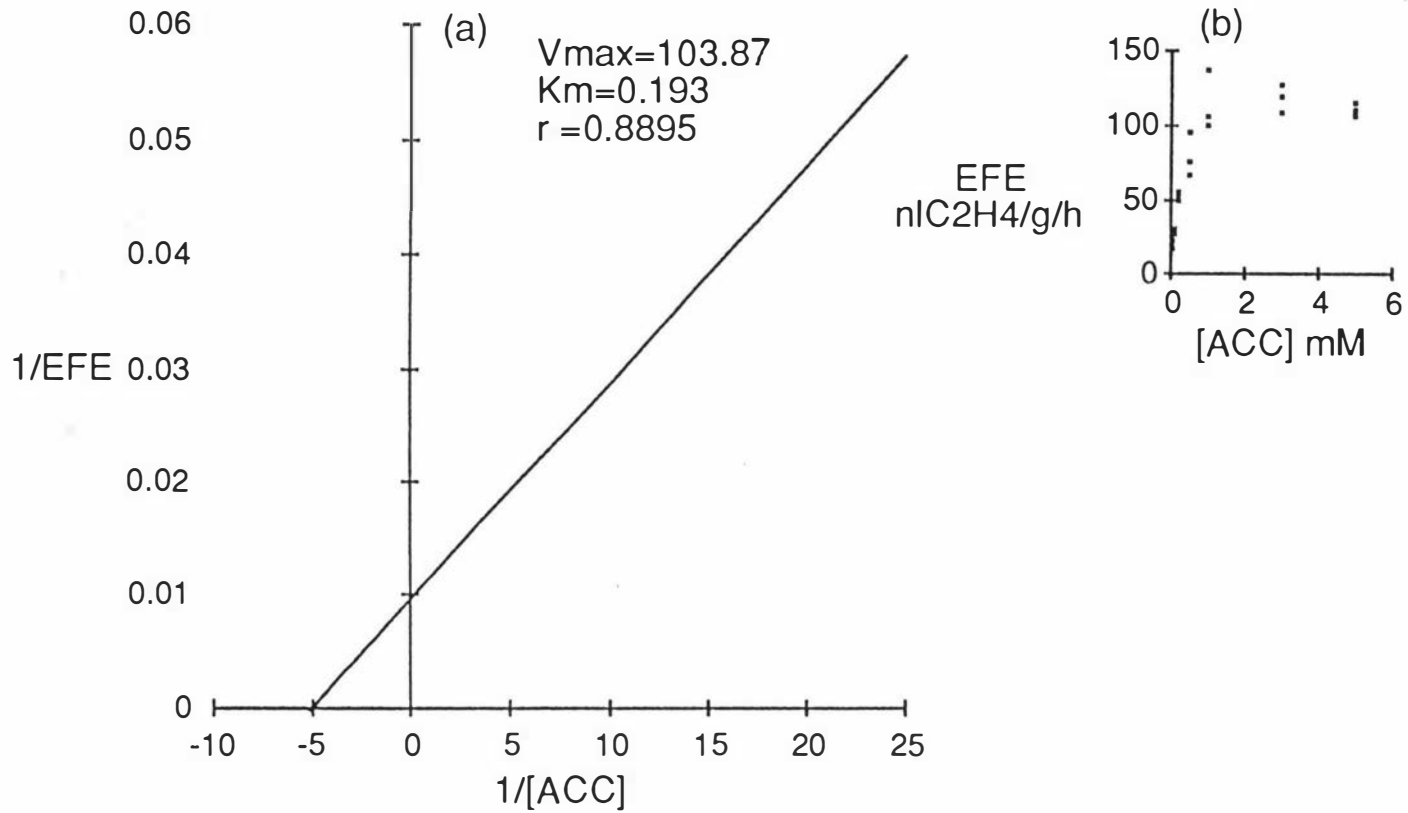


Fig.4-2 Double reciprocal plot (a) and data (b) of EFE activity against ACC concentrations in Granny Smith fruit discs.

Table 4-5. EFE activity of Hosui and Granny Smith fruit discs after vacuum-infiltration of 5 mM ACC for different periods of time (1989).

(A). Hosui.

Treatment	EFE activity (nlC ₂ H ₄ /g/h)	
	Vacuum after incubation	Vacuum before incubation
Control	50.84	50.84
Vac. 3 min	49.82	47.22
Vac. 2 min	49.68	53.33
Vac. 1 min	50.94	51.85
3 X Vac. for 1 min	49.26	53.61

(B). Granny Smith.

Control	120.28	102.37
Vac. 3 min	134.13	97.71
Vac. 2 min	128.14	91.43
Vac. 1 min	124.19	94.74
3 X Vac. for 1 min	120.99	102.32

All values are means (n=4 for Hosui, n=6 for Granny Smith). Different characters within each column for the same species represent significant differences at the 5% level (Duncan's multiple comparison).

Granny Smith fruit discs, infiltrated with ACC by vacuum for 1, 2 and 3 minutes before incubation had lower EFE activities than control fruit discs which were immersed in the medium for 2 hours (Table 4-5B); there was no effect when discs were vacuum-infiltration after incubation.

No significant difference was found between the EFE activity in control discs and in discs exposed to the intermittent vacuum treatments for either Hosui and Granny Smith.

4.4.5 EFE activity measurement

The most efficient method for measuring EFE activity was found to occur after incubation of discs in ACC followed by placement into a dried vial for both Hosui and Granny Smith. This treatment was 10% and 14% more effective than when wet filter paper was used, and 63% and 47% more effective than when discs were fully immersed in the medium for Hosui and Granny Smith, respectively (Table 4-6).

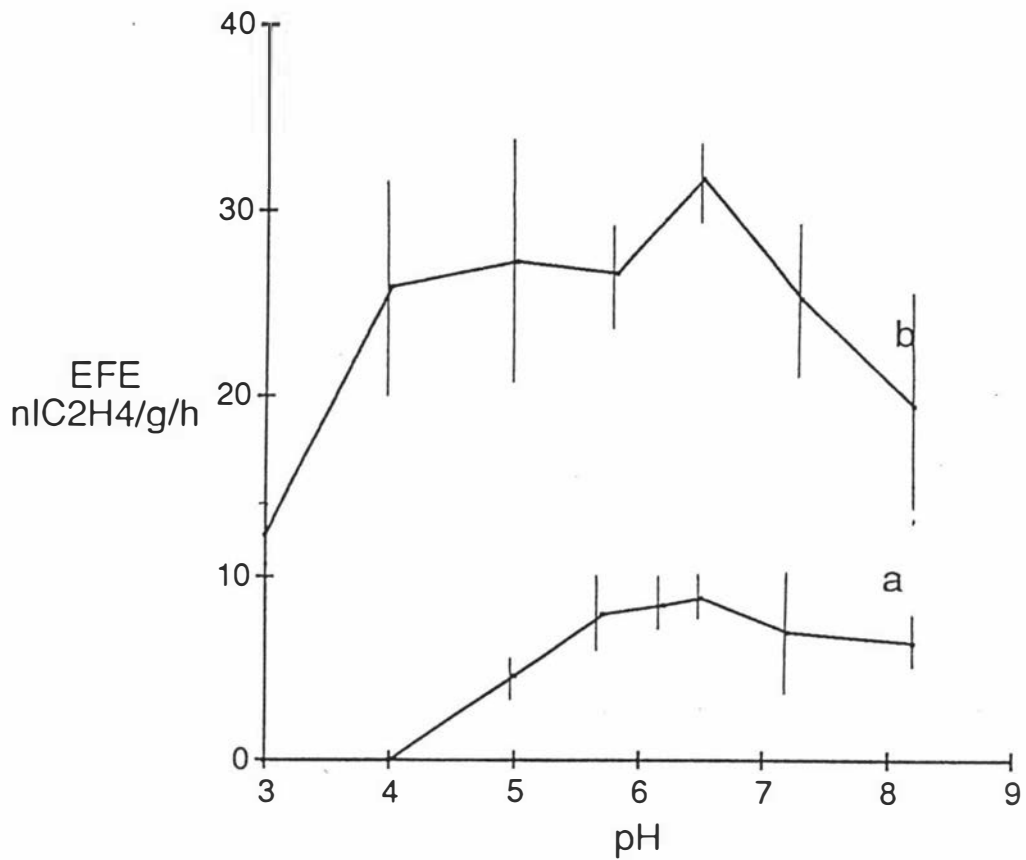
4.4.6 Effect of pH on EFE activity

EFE activity in both species was influenced by pH of the incubation medium (Fig.4-3 & Fig.4-4). In Hosui fruit which had been stored for 9 days at $1^{\circ}\pm 1^{\circ}\text{C}$, EFE activity was not detectable at pH 3 to 4; EFE activity increased at pH 5 reaching a plateau²⁴ of activity between pH 5.5 to 6.5, before falling away at higher pH levels (Fig.4-3). After 17 days storage at $1^{\circ}\pm 1^{\circ}\text{C}$, EFE activity increased from 12.31 nl/g/h at pH 3 to a maximum of 31.77 nl/g/h at pH 6.5, then fell to 19.49 nl/g/h at pH 8.2 (Fig.4-3).

Table 4-6. EFE activity in fruit discs of Hosui (1989) and Granny Smith (1988) after 2 hours incubation in 5 mM ACC followed by 1 hour immersion in medium, placement on medium impregnated filter paper or placement in air.

Species	EFE activity (nlC ₂ H ₄ /g/h)		
	Immersion	With filter paper	Dry discs
Hosui	41.70 ^b	102.64 ^a	113.97 ^a
Granny Smith	102.55 ^c	140.95 ^b	163.93 ^a

All values are means (n=4). Different characters in the same species represent significant differences at the 5% level (Duncan's multiple comparison).



Incubation time	pH values							
	3.0	4.0	5.0	5.7	6.2	6.5	7.2	8.2
initial	3.0	4.0	5.0	5.7	6.2	6.5	7.2	8.2
final	3.2	4.2	5.2	5.7	6.2	6.5	7.2	8.1

Fig.4-3. Effects of pH on EFE activity of Hosui fruit in 1990. Fruit were stored for 9 days (a) and 17 days (b) at $1^{\circ}\pm 1^{\circ}\text{C}$; The pH values of medium were measured at initial and complete time of incubation in the medium in (b). Vertical bars indicate standard error.

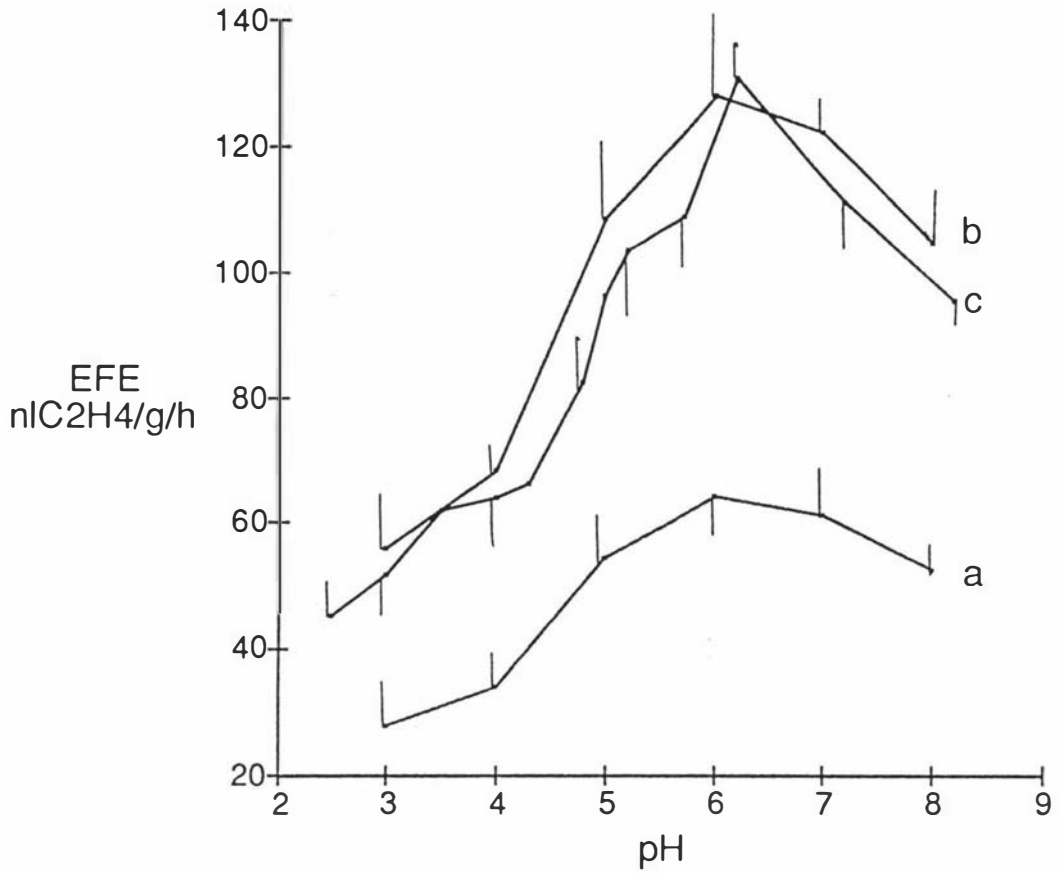
In Granny Smith apples stored for 30 days at $1^{\circ}\pm 1^{\circ}\text{C}$, EFE activity increased steadily from 27.94 at pH 3 to a maximum of 64.10 nl/g/h at pH 6.0-7.0 before it fell to 52.47 nl/g/h at pH 8.0. After 70 and 80 days storage at $1^{\circ}\pm 1^{\circ}\text{C}$, EFE activity in fruit discs was 100% higher than in fruit only stored for 30 days. Although the pattern of response to various pH levels was similar. In fruit stored for 70 and 80 days at $1^{\circ}\pm 1^{\circ}\text{C}$ activity increased from 70.44 at pH 3 to 130.81 nl/g/h at pH 6.2, then decreased to 111.19 nl/g/h at pH 7.2 before falling to 95.62 nl/g/h at pH 8.2 (Fig.4-4).

In spite of the reaction taking place in a buffered system, small pH changes (0.1-0.3 pH units) occurred in the medium during the 2 hour incubation at 27°C (Fig.4-3 & Fig.4-4). With Hosui discs, there was a slight increase in pH when the initial pH values of the medium were in the range 3.0-5.0; there was no pH change when the initial pH was 5.7-7.2 (Fig.4-3). There was 0.1 pH unit decrease in the medium at pH 8.2 after 2 hours incubation. With Granny Smith discs, a slight increase in pH developed when the initial values of the medium were 2.5-4.0; there was slight decreased (0.1 pH unit) in the media pH after incubation at pH 4.8-8.2 (Fig.4-4).

EFE activity in discs of Hosui fruit decreased during the first hour of incubation although rate of decrease was different at different pH's. In discs of Granny Smith, EFE activity did not change during the first hour of incubation at different pH's.

During the first hour, EFE activity in Hosui was the highest at pH 6.2. After 2 hours incubation, EFE activity was not significantly different between discs incubated at pH 6.2, 7.2 and 8.2 (Table 4-7). Compared with the 0.5 hour incubation, EFE activity after 2 hours decreased 54% at pH 6.2, 45% at pH 7.2 and did not change at pH 8.2.

The initial EFE activities in Granny Smith fruit during the first hour after immersion were not significantly different for a given different pH's (Table 4-7). After 2 hours incubation, EFE activity was highest at pH 6.2 and there was no significant difference in activity between pH 4.0 and 8.2. Compared with 0.5 hour, EFE activity decreased 52% for pH 4.0 and 34.6% for pH 8.2 after 3 hours incubation. EFE activity did not change during 3 hours incubation at pH 6.2 (Table 4-7).



Incubation time	pH values											
	initial	2.5	3.0	3.5	4.0	4.3	4.8	5.2	5.7	6.2	7.2	8.2
final	2.8	3.2	3.6	4.2	4.3	4.7	5.1	5.6	6.1	7.2	8.1	

Fig.4-4. Effects of pH on EFE activity of Granny Smith fruit in 1988. Fruit were stored for 30 days (a), 70 days (b) and 80 days (c) at $1^{\circ}\pm 1^{\circ}\text{C}$. The pH values of medium were measured at initial and complete time of incubation in the medium in (b). Vertical bars indicate standard error.

Table 4-7. EFE activity of Hosui (1989) and Granny Smith (1988) cortical discs after incubation in Na⁺-citric acid buffer containing 5 mM ACC of different pH values over 3 hours at 27°C.

PH values		EFE activity ($\mu\text{C}_2\text{H}_4/\text{g/h}$) after time (hours)			
		0.5	1.0	2.0	3.0
Hosui	6.2	a, A 63.04	a, B 39.39	a, C 29.25	a, D 17.26
	7.2	b, A 44.70	b, B 24.24	a, B 29.46	a, B 24.11
	8.2	c, A 24.09	c, B 18.18	a, A 24.24	a, AB 20.99
Granny Smith	4.0	a, A 132.55	a, A 120.92	b, B 85.83	b, B 63.50
	6.2	a, A 103.70	b, A 105.99	a, A 103.72	a, A 92.97
	8.2	a, A 99.30	b, A 91.39	b, A 79.30	b, B 64.94

All values are means (n=4). Different small characters within the same column or different capital characters in the same line for each species represent significant differences at the 5% level (Duncan's multiple comparison).

4.4.7 Effects of different sugars (0.4 M) on EFE activity and ethylene production from fruit discs

In this experiment, CHI, as a protein synthesis inhibitor was added to the medium for EFE activity measurement in order to prevent the synthesis of new enzymes during the testing period.

Compared with the K⁺-phosphate buffer, sugars stimulated ethylene production and EFE activity in discs from both fruit (Table 4-8). Hosui fruit discs did not produce detectable ethylene in K⁺-phosphate buffer. In sucrose and glucose solutions, ethylene production was more than 7 nl/g/h, while in fructose, mannitol and sorbitol solutions, it was half this. EFE activity of Hosui fruit was approximately doubled when each sugar was added to the incubation medium. There were no significant differences in EFE activity between the different sugar treatments (Table 4-8).

In buffer solution Granny Smith fruit discs produced a small amount of ethylene (4.92 nl/g/h); in addition EFE activity was higher at 96.61 nl/g/h (Table 4-8). Ethylene production and EFE activity were stimulated 2-3 fold by individual sugars (Table 4-8). Glucose treatment produced significantly less ethylene than other sugars. Fructose was slightly less effect on stimulating EFE activity than the other sugars tested.

4.5 DISCUSSION

Ethylene production in plants can be stimulated by various environmental factors, e.g. wounding, physical load (bruising, mechanical/physical damage), disease, drought, water-logging, chilling temperature and exposure to various chemicals, all of which result in production of stress-induced ethylene (Abeles, 1973).

Wounding is one of the most common factors inducing ethylene production in plants (Abeles, 1973). In this work, there were different responses in wound ethylene production from Hosui and Granny Smith fruits. Fruit discs of Hosui, a nonclimacteric fruit, produced trace amounts of ethylene, but only 4 hours after excision. Ethylene production was still low but continued to increase gradually even after 8 hours. In discs from Granny Smith

Table 4-8. Ethylene production and EFE activity of Hosui and Granny Smith fruit discs immersed for 2 hours in 0.4 M sugar solutions at pH 6.5 (1989).

Species	Treatment	Ethylene production (nlC ₂ H ₄ /g/h)	EFE activity (nlC ₂ H ₄ /g/h)
Hosui	Control	0	47.18
	Sucrose	7.27	96.48
	Fructose	3.62	86.86
	Glucose	8.45	87.45
	Mannitol	3.40	92.01
	Sorbitol	3.21	92.32
	Granny Smith	Control	4.92
	Sucrose	12.84	171.62
	Fructose	10.26	163.51
	Glucose	9.34	180.42
	Mannitol	10.59	183.90
	Sorbitol	11.47	169.35

All values represent means (n=3). Different characters within the same column for the same species represent significant differences at the 5% level (Duncan's multiple comparison).

apples, a climacteric fruit, ethylene was produced immediately after cutting, increasing to a peak of 55.78 nl/g/h after 5 hours, then decreasing to initial production levels after 10 hours.

Since the discovery of the role of ACC in ethylene synthesis by Adams and Yang (1979) and Lüssén *et al.* (1979), the ACC pathway has been established as a major route for ethylene biosynthesis in higher plants (Yang and Hoffman, 1984). In this pathway, ACC synthase, which catalyzes the conversion of S-adenosylmethionine (SAM) to ACC, is the key enzyme. It can be induced by various kinds of stimuli, including wounding, and its increase is associated with the induction of ethylene synthesis (Boller and

Kende, 1980; Kende and Boller, 1981; Saltveit and Dilley, 1978a). Wounding stimulated biosynthesis of ACC synthase because the activity of ACC synthase in wounded pericarp of green and ripening tomatoes declined when CHI (a mRNA coding inhibitor) was added to the incubation medium (Hyodo *et al.*, 1989; Kende and Boller, 1981). The present results showed that wound ethylene was produced more rapidly and in larger amounts in wounded tissue from Granny Smith, a climacteric fruit, than from wounded Hosui as a nonclimacteric fruit. It might cause by mRNA coding for ACC synthase ^{after wounding being} was slower in Hosui than in Granny Smith fruit.

The experiment using different osmoticum showed that 0.4 M sugar was the isotonic concentration for discs of both Hosui and Granny Smith fruits, and EFE activity in discs of both fruits was higher in 0.8 M (high osmotica) than in 0.4 M (isotonic) mannitol solution. These results were similar to those found by other workers. Bir and Bramlage (1973) found that ion leakage from apple fruit tissue (Richared Delicious) was minimized in solutions of 0.3 M to 0.7 M sucrose, and Ferguson and Watkins (1981), and Harker (1986) used 0.3 M and 0.35 M sucrose to protect cells in Granny Smith fruit tissue from rupturing. John *et al.* (1989) found that EFE activity was higher in an 0.8 M solution than in 0.4 M, and reported that the maximum EFE activity in kiwifruit was at 0.7 M mannitol or greater; however the reason why EFE activity was completely lost when osmotic support was removed was not understood. It has been suggested that EFE activity requires a close association of particular sub-cellular organelles that are maintained within an osmotically sensitive membrane vesicle (John *et al.*, 1989) or an environmental where transmembrane gradients of ions or electrical potential exist (John, 1983).

Carbohydrates, including sucrose, galactose, sorbitol, fructose, glucose, and mannitol have been shown to enhance ethylene production by stimulating ACC synthase activity and conversion of ACC to ethylene in various vegetative tissues, such as leaf discs (Meir *et al.*, 1985; Philosoph-Hadas *et al.*, 1985; Riov and Yang, 1982) and mung bean hypocotyls (Colclasure and Yopp, 1976). Galactose stimulated ethylene production of tomato fruit, but sucrose, fructose, glucose and sorbitol did not (Kim *et al.*, 1987). In the present experiments, all sugars tested stimulated EFE activity in discs of both fruits, and no significant difference of EFE activity was observed between different sugar treatments. However ethylene production

of Hosui discs treated with sucrose and glucose ^{were} ~~were~~ higher than those treated with other sugars. Ethylene production of Granny Smith discs treated with glucose was lower than those treated with other sugars. It has been reported that mannitol and sorbitol did not penetrate, or only slowly penetrated the plasma membrane, while other sugars moved more rapidly across cell membranes (Berliner and Martindale, 1981; Evans and Ting, 1973; Ruesink, 1978). Bouzayen *et al.* (1990) suggested that EFE in grape is probably located in both the tonoplast and the plasma membrane. Thus the present results might be interpreted to indicate that (a) all sugars tested stimulated EFE activity (tested by adding saturated exogenous ACC) located in the plasma membrane; (b) some sugars (e.g. glucose for Hosui and sucrose for Hosui and Granny Smith) stimulated ACC synthase activity more than others, and (c) those sugars penetrating into the cytoplasm, could stimulate activity of the EFE (only limited endogenous ACC available) located at tonoplast, thus they would stimulate ethylene production more than other sugars. The mechanism of the stimulative effect of sugars on ethylene production is unknown although it has been suggested that sugars stimulate the enzymic hydrolysis of indole-3-acetyl-L-alanine to produce IAA which in turn stimulates ACC synthase (Meir *et al.*, 1989).

The timing of vacuum-infiltration of ACC had a significant effect on EFE activity in Granny Smith fruit discs, but not in Hosui fruit discs. Infiltration before incubation reduced EFE activity in discs of Granny Smith fruit compared with infiltration after incubation. EFE activity was higher in discs of both fruits kept dry after incubation than those immersed in medium. It is likely that these treatments reduced the availability of O₂ to the active site of EFE either by reducing O₂ levels at the surface of discs (immersion) or by reducing O₂ levels within discs (Rajapakse *et al.*, personal communication). Low O₂ concentration inhibited the formation of C₂H₄ from ACC and it is possible that vacuum-infiltration and immersion of tissues may result in internal O₂ levels being low enough to reduce ethylene production (Saltveit and Dilley, 1978b). Burg and Thimann (1960) have shown that EFE activity is reduced by vacuum treatment which was thought to have damaged membranes to which the EFE may have bound.

EFE activity was less sensitive to vacuum treatment, but ~~reduced~~ ^{affected by wetting} more ~~measured in the medium than in air~~ in Hosui than in Granny Smith. These results could be explained by suggesting that EFE was distributed in

different ratios in the tonoplast and plasma membrane in discs of Hosui and Granny Smith fruits, and that these two sites had different sensitivities to vacuum treatment and O_2 levels in tissues. The ratio of forms of EFE on the tonoplast/plasma membrane might be higher in Hosui than in Granny Smith. This result supported to the idea that EFE may be located at different sites in the cell (Bouzayen *et al.*, 1990).

It has been suggested that EFE activity requires a transmembrane proton gradient (John, 1983; Mayne and Kende, 1986). In isolated vacuoles, EFE activity depended upon the pH of the suspending medium, and ionophores, such as valinomycin, nigericin, and A23187, inhibited ethylene-stimulated ethylene formation (Mayne and Kende, 1986). In the present work, EFE activity in Granny Smith fruit tissue was stimulated by low pH values (pH 4.0) within a short time (1 hour) after excision, but thereafter activity decreased. Simultaneously, pH values in the incubating medium increased. EFE activity in discs of both fruits were lower at higher pH (8.2) than at lower pH (< 8.2) within 0.5 and 1 hour incubation. Simultaneously, pH values in the incubating medium for discs of both fruits decreased slightly. The stimulation of EFE activity by low pH may possibly be a result of a temporary increase in the transmembrane proton gradient which then abates due to various internal regulatory mechanism, such as intracellular buffers, ATP-dependent H^+ transport and bicarbonate uptake (Smith and Raven, 1979; Hager and Moser, 1985). The inhibition of EFE activity by high pH in both fruits might be caused by the opposite effects of low pH.

Compared with EFE activity at optimum pH, after 2 hours incubation at high pH (8.2) EFE activity in Hosui was no significant different, but was slightly lower in Granny Smith. For both fruits, the optimum pH for EFE occurred over a wide range after long time incubation (2 hours). Some workers (Smith and Raven, 1979; Felle, 1987; Roberts, *et al.*, 1984) reported that cytoplasmic pH is maintained within fairly narrow limits, although it can change up to 0.5 units due to various environmental or metabolic influences without serious damage to cell function. The present results might suggest that there was a high capacity for buffering in cells from both Hosui and Granny Smith fruit discs. As the EFE activity measured *in vivo* after 2 hours incubation was similar in all 3 pH treatments, this might indicate that the cytoplasmic pH either did not change in response to external pH changes or that in the 2 hours incubation period the cytoplasmic pH reversed to the optimum value (pH 6.5) from the higher or lower pH's in the experimental medium. The optimum pH for EFE in Granny Smith

might be slightly lower than that in Hosui, and it took longer time to return back from pH 8.2 to the optimum pH, thus after 2-3 hours incubation at pH 8.2 EFE activity in Granny Smith was still slightly lower than at optimum pH.

This work has shown that EFE activity in both Hosui and Granny Smith fruit discs can be markedly affected by a range of varied treatments. Different sugars, which may or may not penetrate into cytoplasm, uniformly stimulated EFE activity (with exogenous ACC) without significant difference; High osmotic medium (0.8 M) increased EFE activity compared with isotonic medium (0.4 M). EFE activity decreased during the 2 hours following vacuum treatment, was affected by the pH in the medium and EFE showed the differential sensitivity to low O_2 levels in the medium. Those results suggest that it is possible that there may be different forms of EFE *in vivo* which could be located at the plasma membrane and at the tonoplast. The range of K_m values of EFE that have been obtained by other authors from different plants might be caused by different affinity for ACC due to the isozymes of the EFE or different forms during the various ripening stages between species. Various sugars, high osmotic potential, vacuum treatment and different pH's might readily influence the EFE located at the plasma membrane. This suggestion may appear to conflict with the kinetic analysis in which only one apparent K_m value of EFE for ACC was attained in both fruits. These could be several reasons for this result. Because EFE activity was measured *in vivo*, the apparent K_m values may have represented the combined K_m 's for different EFE forms or perhaps the different forms of EFE may have the same apparent K_m values. It is also possible that one EFE form may have had a very low K_m , which was not easy to assay *in vivo* in the experimental system used.

From the present results the following procedure provides the more simple, consistent and effective method of assaying for EFE in Hosui and Granny Smith fruit discs. Discs incubated for 2 hours; vacuum-infiltrated for 1 minute with isotonic buffer or 0.4 M mannitol solution (pH 6.5) containing 3-5 mM ACC, dried on paper tissue and sealed in 36 ml vial at 27°C for 0.5-1 hours before a gas sample is removed for ethylene analysis measured by

GLC. For optimum pH experiment, EFE activity has to be measured in fruit discs immersed in the medium with different pH, in case pH changed by intracellular buffers (Smith and Raven, 1979; Mayne and Kende, 1986). Cycloheximide (1 mM) would need to be added to the medium in order to differentiate between treatment's effects on EFE synthesis or activity.

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CHAPTER 5

EFFECTS OF CARBON DIOXIDE ON EFE (ETHYLENE-FORMING ENZYME) IN HOSUI AND GRANNY SMITH FRUITS

5.1 ABSTRACT

In both ~~α~~ Hosui and Granny Smith fruits, EFE activity was stimulated by CO₂. In Hosui fruit, EFE activity remained unchanged upto 5% CO₂, but increased with CO₂ levels which were higher than 5%. In Granny Smith fruit, EFE increased gradually as CO₂ increased from 5 to 30%.

Characteristics of EFE development at low temperature (1°±1°C) and the response to CO₂ (20%) treatment were tested. The results demonstrated that EFE developed with storage time at 1°±1°C in both fruits. EFE in short-term storage Hosui and Granny Smith fruits were more sensitive to CO₂ treatment than long term storage fruits. When Hosui fruit were stored at 1°±1°C for long period, CO₂ no longer stimulated EFE activity, but in Granny Smith fruit stored for 176 days at 1°±1°C, CO₂ still stimulated EFE activity.

Treatment discs with cycloheximide showed that CO₂ induced EFE biosynthesis in Granny Smith apple stored for short term, but not in Hosui fruit.

Several different methods were used to analyse kinetic data from the interaction of CO₂ and ACC or EFE activity. Results indicated that the EFE in both fruits was probably not an allosteric enzyme because the plot of $\log[V/(V_{\max}-V)]$ vs. $\log[S]$ gave n_H values that were around 1. CO₂ increased the V_{\max} of EFE in discs of Hosui and Granny Smith fruit without changing the apparent K_m values of EFE for ACC significantly.

EFE activity in Hosui fruit discs was stimulated by CO₂ only when ACC was added exogenously ~~ACC was available~~.

EFE activity was lower in an isotonic mannitol solution (0.4 M) than it was at 0.8 M, but CO₂ stimulated EFE activity in discs of both Hosui and Granny Smith fruits in 0.4 M mannitol, but not in a 0.8 M solution.

EFE activity and the response to CO₂ were pH-dependent in fruit discs of Granny Smith. At pH 6.2 ~~of EFE~~, the stimulatory ^{by} ~~percentage~~ ^{being added to} of EFE activity by CO₂ was the lowest.

The present results can be explained ~~that~~ ^{by} CO₂ ^{being added to} ~~may~~ stimulate the activity of EFE located in the plasma membrane. The possible mechanism of the stimulatory effect of CO₂ on EFE activity may be that CO₂ combined with EFE or ACC to form a CO₂-EFE-ACC or EFE-ACC-CO₂ complex which enhanced the maximum reaction rate of conversion of ACC to ethylene.

Key words. ACC, AOA, carbon dioxide, CHI, EFE, ethylene production, Granny Smith, Hosui, K_m, pH, V_{max}.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AOA, aminooxyacetic acid; CHI, cycloheximide; EFE, ethylene-forming enzyme; K_m, the Michaelis-Menton constant; V_{max}, the maximum reaction rate.

5.2 INTRODUCTION

There have been many reports on the effects of CO₂ on ethylene biosynthesis in vegetative tissues. In photosynthetic tissues the promotive effect of CO₂ was observed with isolated protoplasts (McRae *et al.*, 1983; Tophof and Amrhein, 1985), excised leaf segments in closed systems (Aharoni *et al.*, 1979; Aharoni and Lieberman, 1979; Fuhrer, 1985; Gepstein and Thimann, 1980; Grodzinski *et al.*, 1983; Horton and Saville, 1984; Kao and Yang, 1982; McRae *et al.*, 1983; Philosoph-Hadas *et al.*, 1985, 1986; Preger and Gepstein, 1984), and intact plants (Bassi and Spencer, 1982, 1985; Dhawan *et al.*, 1981). CO₂ stimulated both basal (Aharoni *et al.*, 1979; Aharoni and Lieberman, 1979; Bassi and Spencer, 1982; Dhawan *et al.*, 1981) and ACC dependent ethylene production (Bassi and Spencer, 1985; Fuhrer, 1985; Grodzinski *et al.*, 1983; Horton and Saville, 1984; Kao and Yang, 1982; McRae *et al.*, 1983; Philosoph-Hadas *et al.*, 1985; Preger and Gepstein, 1984; Tophof and Amrhein, 1985), but did not affect ACC production (Kao and Yang, 1982). CO₂ exerted its stimulatory effect on the conversion of ACC to ethylene by enhancing not only the activity, but also the synthesis, of EFE in leaf discs (Philosoph-Hadas *et al.*, 1986).

There have been very few studies on the effect of CO₂ on ethylene biosynthesis in fruit tissues. Chaves and Tomas (1984) reported that ethylene production in both whole fruit and in tissue slices of Granny Smith apple were inhibited by 20% CO₂. There was also an inhibition of ethylene emission in tissue slices incubated with exogenous ACC (0.04 mM and 0.1 mM) and CO₂ treatment increased the ACC content of the tissue. They suggested that the action of CO₂ was directed toward the enzyme system responsible for the conversion of ACC to ethylene. Cheverry *et al.* (1988) obtained different results. In their work, 20% CO₂ inhibited the development of the climacteric ethylene burst in Granny Smith apple and avocado fruits, but did not change ACC content. In the autocatalytic process, 20% CO₂ antagonized the stimulation of EFE synthesis by ethylene, but promoted EFE activity. Tan and Thimann (1989) reported that a clear difference existed between the endogenous production of ethylene and its production from applied ACC, a difference which holds equally for leaves and for fruit tissue. The difference is in the CO₂ requirement; lowering the CO₂ level by 99% or more decreased the production of ethylene from applied ACC by 50-60%, but it did not decrease (it even slightly increased) its production from endogenous precursors. The conclusion is that while the need for CO₂ has not been explained, it has at least been delimited.

It is clear that the several aspects of the effects of CO₂ on ethylene synthesis in fruit tissues need to be clarified: does CO₂ inhibit ACC synthase activity and/or its synthesis; does CO₂ stimulate or inhibit EFE activity and/or its synthesis; does CO₂ have different effects on the EFE in tissues from nonclimacteric and climacteric fruits at various stages of maturity and ripeness; what is the mechanism of action of CO₂ on EFE activity and synthesis in fruit tissues.

Cycloheximide (CHI) is a protein synthesis inhibitor which inhibits both the development of ACC synthase (Boller and Kende, 1980; Yu and Yang, 1980; Kende and Boller, 1981) and the conversion of ACC to ethylene (Hyodo and Nishino, 1981; Riov and Yang, 1982; Yu *et al.*, 1979) in various excised plant tissues. Thus CHI was used for distinguishing between the effects of CO₂ on EFE activity and synthesis.

Analysis of the kinetics of reactions provides a useful tool to investigate the mechanism of enzyme reactions (Smith *et al.*, 1983). Information on the nature of enzymes can be obtained according to the following equations:

$$(a) \log[V/(V_{\max}-V)] = n_H \log[S] - \log K$$

Where V and V_{\max} are the reaction rate and the maximum reaction rate respectively; $[S]$ is the substrate concentration; K is a complex constant as $K = [S]^{n_H}[(V_{\max}/V)-1]$; n_H is the Hill coefficient with numerical values which indicates if the enzyme is allosteric.

When $n_H=1$, it is not allosteric. When $n_H > 1$ or $n_H < 1$, the enzyme is allosteric.

$$(b) 1/V = (K_m/V_{\max}) * 1/[S] + 1/V_{\max}$$

$$(c) V = -K_m[V/S] + V_{\max}$$

where K_m is the Michaelis-Menton constant, a useful parameter characteristic of each enzyme and its substrate, which is equal to the substrate concentration that gives one-half the numerical maximum velocity (V_{\max}). From equations (b) and (c), the nature of the enzyme (affinity for its substrate) and types of its inhibitors, such as the competitive, noncompetitive and uncompetitive inhibitors, can be inferred depending on the values of K_m and V_{\max} (Smith *et al.*, 1983).

Kinetics studies of EFE for ACC had^{ve} been used to give some indication of the characteristics of EFE, e.g. its high affinity and stereospecificity for its substrate, ACC (Hoffman *et al.*, 1982; Venis, 1984), localization of EFE (Guy and Kende, 1984; Mitchell *et al.*, 1988). But no work has been reported on the mechanism of the CO_2 effect on EFE activity in fruit tissues.

To further understand the relationship between fruit ripening and the EFE response to CO_2 , EFE development at low temperature ($1^\circ \pm 1^\circ C$) and its response to CO_2 were tested. To distinguish between the effects of CO_2 on EFE activity and on EFE biosynthesis, cycloheximide, a protein synthesis inhibitor was used. In order to obtain some information on EFE localization and the form which responded to CO_2 , EFE activity in 0.4 M and 0.8 M mannitol with and without CO_2 treatment was measured. For understanding the mechanism of the CO_2 stimulatory effect on EFE activity, EFE kinetics with and without CO_2 treatment, and the effect of pH on the response were investigated.

5.3 MATERIALS AND METHODS

5.3.1 Experimental materials (See 'Chapter 2')

5.3.2 Methods

5.3.2.1 Measurement of EFE activity (See 'Chapter 4')

5.3.2.2 Carbon dioxide treatment

In all of the experiments involving CO₂ treatment, the O₂ level was maintained at 20±1%.

In those experiments involving a CO₂-free atmosphere, the CO₂ was absorbed by 0.2 ml of 20% KOH solution contained in a small plastic center well hung in each vial as control.

For the CO₂ kinetic experiment, six discs were sealed in each of 4 X 36 ml vials. The appropriate gas mixtures, containing different CO₂ concentrations made up with O₂, were injected into vials, each of which contained a second empty syringe into which the displaced air in the vial could flow to maintain atmospheric pressure in each vial. The CO₂ concentrations used were 5%, 10%, 20%, 30% and 38% for Hosui, and 5%, 10%, 15%, 20% and 30% for Granny Smith apple.

For other experiments involving CO₂, fruit discs were sealed in vials after which they were flushed for 20 seconds with C₂H₄ free gas containing 23%CO₂ ± 21%O₂ with the balance being N₂. When required 1 ml gas samples were removed by a syringe inserted through a septum located in the top of each sealed vial, and CO₂ and O₂ concentrations were measured by GLC.

5.3.2.3 EFE development at low temperature (1° ± 1°C)

After harvest, fruit was stored at 1°±1°C. Fruit was removed from coolstore at different times and tissue discs were prepared as previously described (Chapter 2). For Hosui, fruit was stored for 2, 18, 21, 58, 70, 90,

105 and 117 days, and for Granny Smith 2, 8, 26, 50 72, 142 and 176 days before removal. EFE activity and the response to CO₂ were measured by sealing discs in vials, with or without 20% CO₂, for 2 hours after saturation of the discs with 5 mM ACC using the intermittent vacuum-infiltration method described previously (Chapter 4).

5.3.2.4 Effect of 1 mM cycloheximide on EFE and the response to CO₂

To distinguish between the effects of CO₂ on EFE activity and its synthesis, CHI was used in this experiment.

Discs sampled from Hosui fruit stored for 4 and 40 days at 1°±1°C were incubated for 2 hours at 27°C in a K⁺-phosphate buffer (pH 6.5) medium containing 5 mM ACC, 5 mM AOA and plus or minus 1 mM CHI. Discs were then treated with 20% CO₂ for 1 hour.

Discs sampled from Granny Smith fruit stored at 1°±1°C for 8, 26 and 50 days in 1988, and 10 and 35 days in 1989 were incubated in the same medium and given the same CO₂ treatment described for Hosui.

For both Hosui and Granny Smith fruit discs a separate series of discs were incubated in a K⁺-phosphate buffer (pH 6.5) medium without ACC and AOA for measurement of ethylene production.

After 1 hour exposure to CO₂, 1 ml of gas was sampled from each vial and ethylene concentration was measured by GLC. EFE activity or ethylene production were calculated as described previously.

5.3.2.5 Detecting if the stimulatory effect of CO₂ on EFE activity is dependent on exogenous ACC in Hosui

Ethylene production was measured in Hosui fruit discs treated without adding exogenous ACC, while EFE activity was measured in discs which had been supplied with exogenous ACC (5 mM).

To measure ethylene production, six discs were placed into each of 4 X 36 ml vials and incubated in air at 27°C. After 1 hour vials were flushed with 20% CO₂ for 20 seconds, sealed and incubated for a further 1 hour

before a 1 ml gas sample was taken from each vial for ethylene analysis. As control, vials were sealed for a further hour without being flushed with CO₂ before sampling for ethylene analysis.

EFE activity was measured after exposing discs to CO₂ at different times after cutting in order to test if the stimulatory effect of CO₂ on EFE activity was ACC-dependent; i.e. did CO₂ stimulate EFE activity only when exogenous ACC was added. All discs were treated with the same medium containing 5 mM ACC, 5 mM AOA and 1 mM CHI at 27°C. The following treatments were evaluated:

(1) Control. After cutting discs were kept in air for 1 hour before they were vacuum-infiltrated at 90 Kpa with the medium for 1 minute. Discs were then dried on paper tissue and sealed in vials for 1 hour before a 1 ml sample was taken for measuring ethylene concentration and calculation of EFE activity;

(2) CO₂ treatment. The same procedure as for the control treatment was used, except that the discs were flushed with 20% CO₂ for 20 seconds before sealing the vials. Thus discs were treated with CO₂ after saturation with ACC;

(3) Pretreatment with CO₂. In this treatment discs were treated with CO₂ before saturation with ACC. Discs were pretreated in vials with 20% CO₂ for 1 hour after cutting before being flushed with air. They were then vacuum-infiltrated at 90 Kpa with the medium for 1 minute, before being dried on paper tissues and sealed in vials for 1 hour. A 1 ml sample was then taken from each vial for measuring ethylene and calculation of EFE activity;

(4) Double CO₂ treatment. Discs were treated the same as the 'Pretreatment with CO₂' except that they were flushed with 20% CO₂ for 20 seconds before sealing the vials for 1 hour prior to ethylene measurement.

5.3.2.6 EFE activity and response to CO₂ in 0.4 M and 0.8 M mannitol solution in discs of Hosui and Granny Smith fruits

Six discs were incubated for 2 hours at 27°C in each of 3 X 36 ml vials containing 3 ml of 5 mM ACC, 1 mM CHI and 0.4 M or 0.8 M mannitol

solution. CHI in the medium prevented new enzymes being synthesized during incubation. After incubated in the same medium for a further hour, with or without 20% CO₂, a 1 ml sample was taken from each vial for measurement of ethylene concentration and EFE activity was calculated.

5.3.2.7 EFE kinetics

Fruit discs were taken from Hosui or Granny Smith fruit in which internal ethylene and ethylene production were not detectable. Six discs were incubated for 2 hours at 27°C in each of 3 X 36 ml vials containing 3 ml of medium containing 1 mM CHI, 5 mM AOA in K⁺-phosphate buffer (pH 6.5) with various ACC concentrations (0, 0.02, 0.04, 0.1, 0.2, 0.5, 1.0, 3.0 and 5.0 mM). Discs were then dried on the paper tissues before being incubated for a further hour in an atmosphere with or without 20% CO₂ before a 1 ml gas sample was removed from each vial for determination of ethylene concentration was measured and calculation of EFE activity.

5.3.2.8 EFE activity at different pH levels

Six discs from Granny Smith fruit were incubated for 2 hours at 27°C in each of 4 X 36 ml vials containing 3 ml of 5 mM ACC, 1 mM CHI in Na⁺-citric acid buffer at pH 4.0, 6.2, 7.2 or 8.2. Discs were then vacuum-infiltrated at 90 Kpa for 1 min to reduce possible differences in ACC concentrations caused by different uptake at the various pH levels. After incubation for a further 1 hour at 27°C in the same medium, with or without CO₂, a 1 ml sample was taken from each vial for measurement of ethylene concentration and calculation of EFE activity.

5.3.3 Statistical analysis

Each treatment had three or four replicates. Each experiment was repeated at least twice. An SAS computing programme (SAS/STAT User's Guide) was used to analyse data for means and standard errors, the linear regression, paired comparison and Duncan's multiple comparison (Steel and Torrie, 1981). Methods of comparison of two regression lines were calculated according to Neter and Wasserman (1974).

5.4 RESULTS

5.4.1 EFE activity at different carbon dioxide levels

EFE activity of Hosui fruit discs was positively correlated to CO₂ levels in vials (Fig.5-1). There was no significant difference in response between 0 and 5% CO₂ treatments, but EFE activity increased steadily with CO₂ concentrations up to 70.85 nC₂H₄/g/h at 38%.

EFE activity in fruit discs of Granny Smith treated with 5% CO₂ was increased 31% (125.85 nC₂H₄/g/h) compared with control (95.89 nC₂H₄/g/h) (Fig.5-2). There was no further increase on EFE as CO₂ was increased from 5 to 20% CO₂. At 30% EFE activity increased to 161.78 nC₂H₄/g/h.

5.4.2 EFE development and its response to carbon dioxide in fruits stored at low temperature

Because EFE activity was only measured *in vivo*, in this measurement any increase ⁱⁿ EFE that occurred in fruits during storage ~~would be~~ ^{the} combined results of EFE activity and EFE synthesis.

In both Japanese pear and apple, EFE developed steadily during coolstorage (Fig.5-3 and Fig.5-4). In Hosui fruit, EFE activity was 5.77 nC₂H₄/g/h at harvest time. It increased steadily until 105 days after which it increased rapidly to 264.02 nC₂H₄/g/h (Fig.5-3). EFE activity was enhanced by CO₂ treatment. The proportional increase was greater in fruit stored for short periods (a 50-60% increase in fruit stored for 2-18 days) than in fruit stored for long periods (a 20-30% increase in fruit stored for 21-105 days). The CO₂ induced increase was not significant in discs from fruit stored 117 days.

EFE activity was not detectable in Granny Smith fruit at harvest time or 8 days after storage (Fig.5-4). After 26 days storage, EFE activity appeared, after which there was an almost linear increase with time until a level of 163.25 nC₂H₄/g/h was measured after 176 days. During short time storage (about 30-50 days), EFE synthesis was stimulated by CO₂. CO₂ stimulated EFE activity to 7.74 nC₂H₄/g/h in discs from fruit that had been

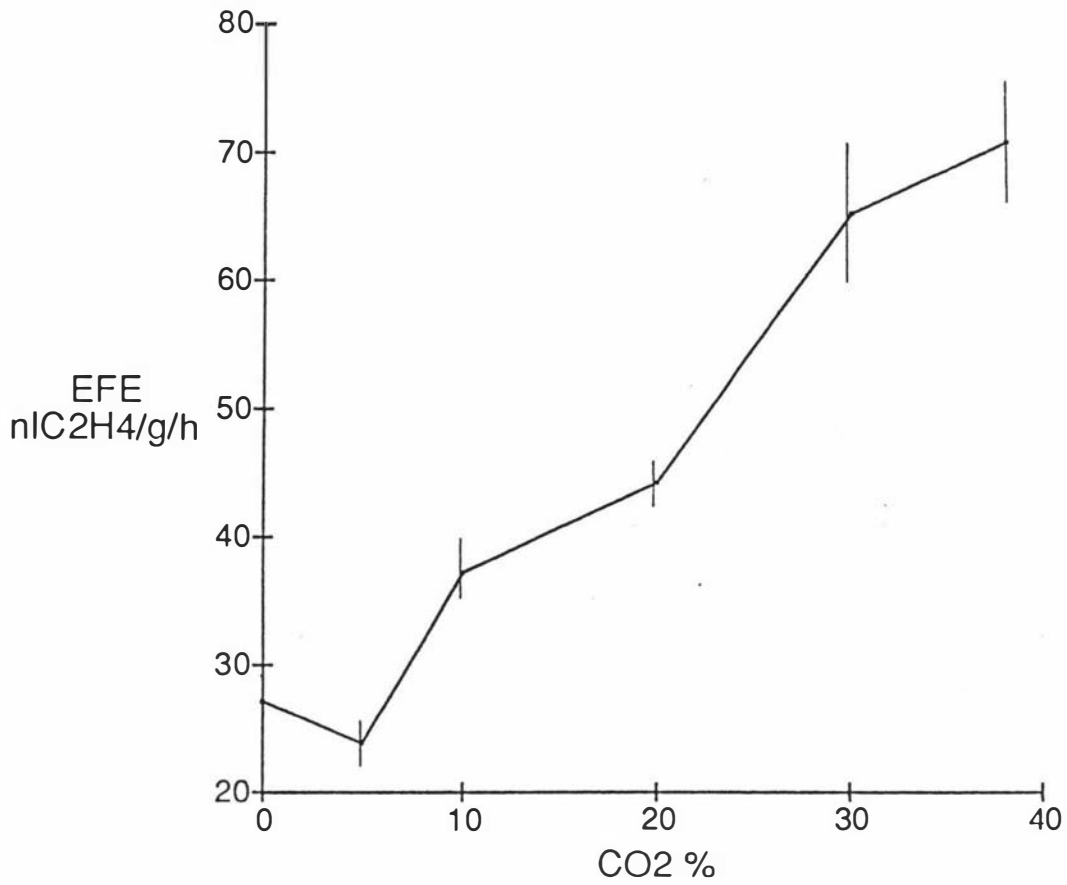


Fig.5-1 EFE activity at different CO₂ levels in Hosui fruit discs (1988). Vertical bars indicate standard error.

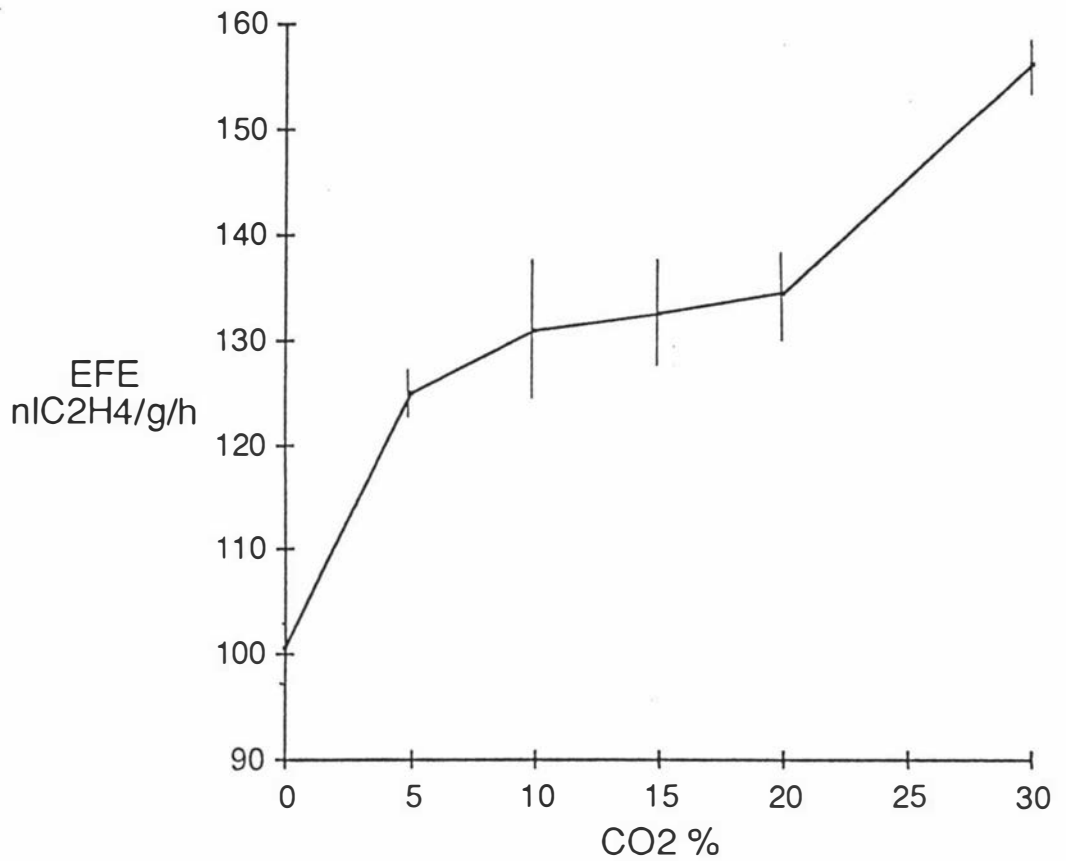


Fig.5-2 EFE activity at different CO₂ levels in Granny Smith fruit discs (1988). Vertical bars indicate standard error.

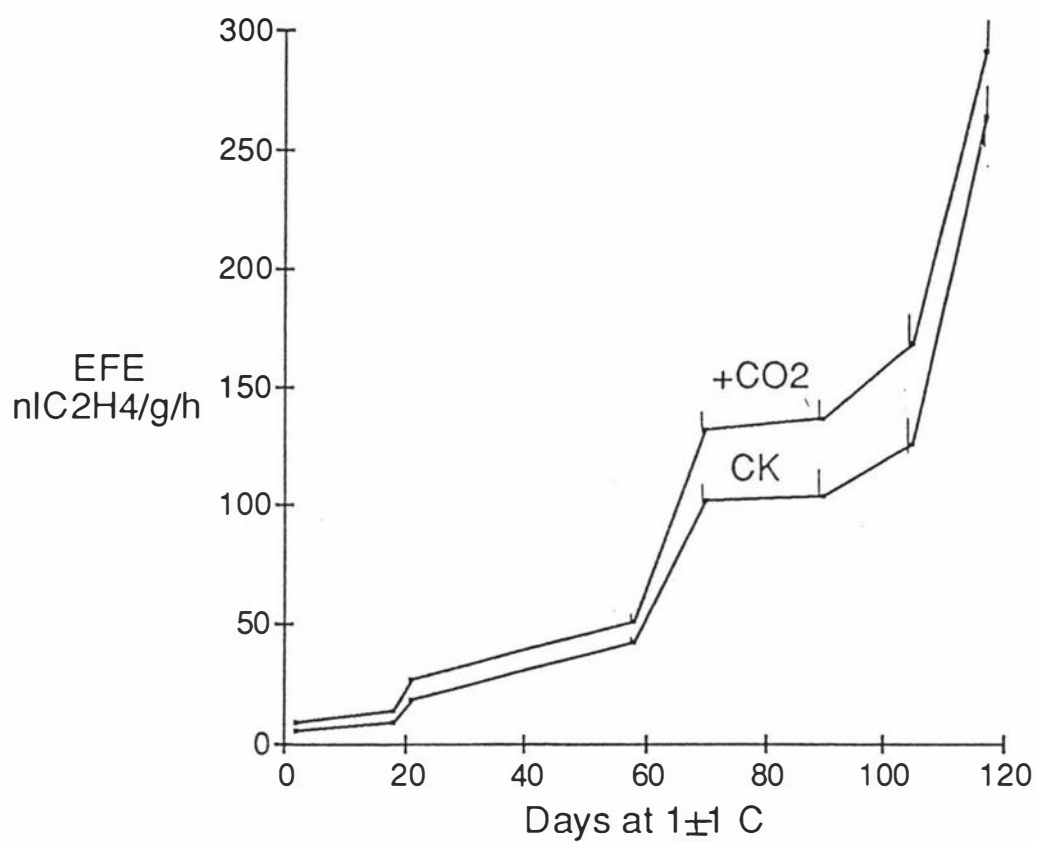


Fig.5-3 EFE development and response to CO₂ in Hosui fruit (1988). Vertical bars indicate standard error.

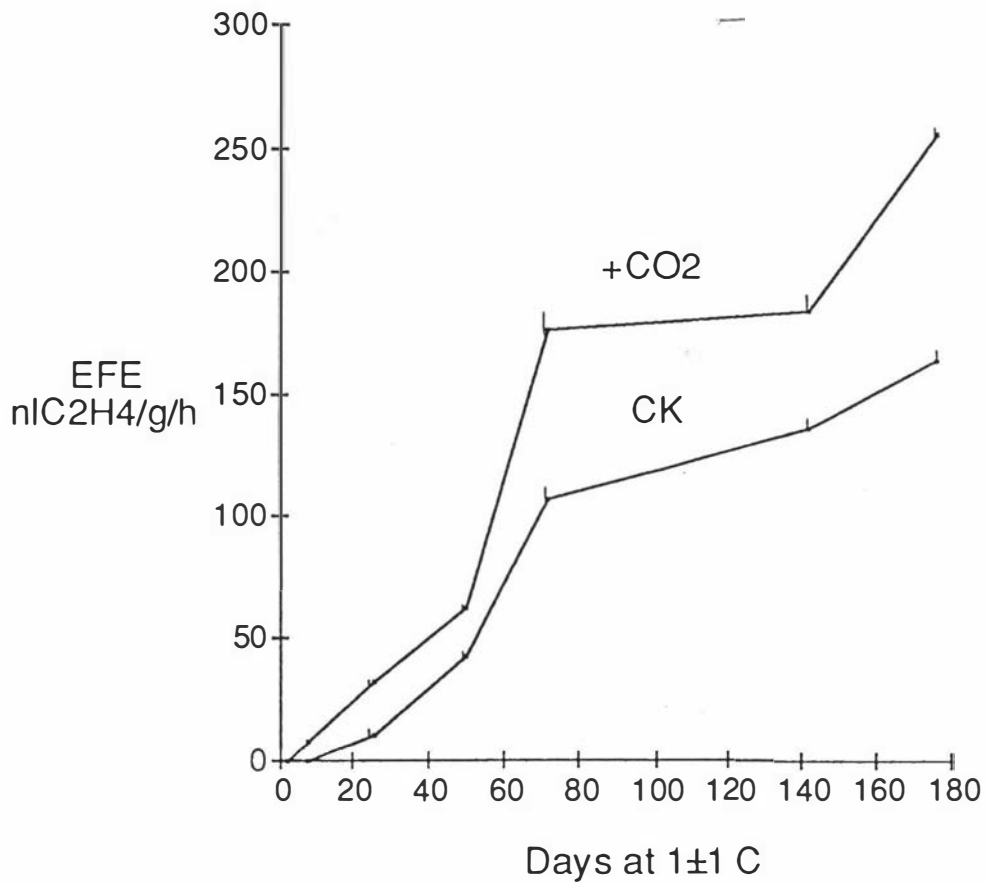


Fig.5-4 EFE development and response to CO₂ in Granny Smith fruit (1988). Vertical bars indicate standard error.

stored for 8 days (Fig.5-4). After 26 days, EFE activity had increased 2 fold after CO₂ treatment compared with control discs (Table 5-2). Subsequently, the percentage increase in EFE activity induced by CO₂ treatment was between 35-65%. In contrast to Hosui, the percentage increase in EFE activity varied, and there was no obvious change with fruit storage periods.

5.4.3 Does carbon dioxide stimulate EFE activity and its synthesis?

To further investigate the effects of CO₂ on EFE activity and its synthesis in both fruit types, the effect of 1 mM cycloheximide (CHI) on EFE activity was evaluated.

Ethylene production from discs of Hosui fruit stored for 4 or 40 days was not detectable. However after 4 days storage CO₂ increased EFE activity by 86% ^{from 6.75 to 12.6} to 6.75 nlc₂H₄/g/h (Table 5-1). After 40 days storage, EFE activity in fruit discs had increased more than ~~12~~ fold to 75.58 nlc₂H₄/g/h and while CO₂ treatment still increased EFE activity it did so by only 45% (Table 5-1). CHI treatment affected neither the base level nor the CO₂ induced increase of EFE activity measured in discs from fruit stored for either 4 or 40 days.

Table 5-1. EFE from fruit discs of Hosui stored for 4 days and 40 days at 1°±1°C incubated in a medium containing 5 mM AOA and 5 mM ACC treated with 20% CO₂ and 1 mM cycloheximide (CHI) (1990).

Treatments	EFE activity (nl/g/h)	
	days at 1°±1°C	
	4 days	40 days
+AOA, +ACC	6.75 ^b	75.58 ^b
+AOA, +ACC, +CO ₂	12.60 ^a	109.23 ^a
+AOA, +ACC, +CHI	7.60 ^b	78.63 ^b
+AOA, +ACC, +CHI, +CO ₂	14.95 ^a	106.14 ^a

All values are means (n=3). Different characters within the same column represent significant differences at the 5% level (Duncan's multiple comparison).

Table 5-2. Ethylene production and EFE activity in fruit discs of Granny Smith (stored for various lengths of time at $1^{\circ}\pm 1^{\circ}\text{C}$) treated with 20% CO_2 and 1 mM cycloheximide (CHI) in 1988 and 1989.

(a) 1988.

Treatment	EFE activity ($\text{nLC}_2\text{H}_4/\text{g/h}$) Days at $1^{\circ}\pm 1^{\circ}\text{C}$		
	8	26	50
+ACC, +AOA	b 0	c 10.33	b 42.41
+ACC, +AOA, +CO ₂	a 7.74	a 32.28	a 61.75
+ACC, +AOA, +CHI	-	c 9.35	b 44.87
+ACC, +AOA, +CHI, +CO ₂	-	b 21.33	a 68.02

(b) 1989.

Treatment	Ethylene Production ($\text{nLC}_2\text{H}_4/\text{g/h}$) Days at $1^{\circ}\pm 1^{\circ}\text{C}$	
	10	35
Control	0	a 22.18
+CO ₂	0	a 23.86
+AOA, +CHI	0	b 12.25
+AOA, +CHI, +CO ₂	0	b 13.23

Treatment	EFE activity ($\text{nLC}_2\text{H}_4/\text{g/h}$) Days at $1^{\circ}\pm 1^{\circ}\text{C}$	
	10	35
+ACC, +AOA	c 8.08	b 34.52
+ACC, +AOA, +CO ₂	a 31.90	a 62.50
+ACC, +AOA, +CHI	c 8.82	b 25.06
+ACC, +AOA, +CHI, +CO ₂	b 19.05	a 71.96

All values are means. Different characters within the same column at the same date represent significant differences at the 5% level (Duncan's multiple comparison).

In 1988, EFE activity was not detectable in Granny Smith discs after 8 days storage, but it increased to 10.33 and 42.41 nC₂H₄/g/h after 26 and 50 days storage respectively (Table 5-2). CO₂ treatment resulted in EFE activity of 7.74 nC₂H₄/g/h after 8 days storage. This stimulatory effect was maintained and CO₂ increased EFE activity by 212% and 45% after 26 and 50 days respectively. CHI treatment had no effect on base level of EFE activity after 26 and 50 days. The stimulatory effect of CO₂ was reduced by 33.9% in presence of CHI after 26 days storage, but the stimulation was not affected by CHI after 50 days storage (Table 5-2a).

In 1989 ethylene production of discs and EFE activity were measured after 10 and 35 days storage (Table 5-2b). Ethylene production was not detectable in discs sampled from 10 day stored fruit, but 22.18 nC₂H₄/g/h was produced in fruit stored for 35 days. Ethylene production was not affected by CO₂ treatment, but it was inhibited about 55% by CHI. EFE activity increased from 8.08 nC₂H₄/g/h in 10 day fruit to 34.52 nC₂H₄/g/h in 35 day fruits (Table 5-2b). EFE activity was increased 2.9 and 0.8 fold by CO₂ treatment in 10 and 35 day fruit respectively. CHI again had no effect on the base level of EFE activity. CO₂ increased EFE activity by only 2 fold after 10 days storage in CHI-treated discs compared with the 2.9 fold increase in the absence of CHI. However in fruit from 50 days storage CHI did not affect the CO₂ induced EFE stimulation.

5.4.4 Is the stimulatory effect of carbon dioxide on EFE activity dependent on exogenous ACC ?

Ethylene production was not detectable in control Hosui fruit discs and it was not stimulated by CO₂ (Table 5-3).

When discs were saturated with the medium containing ACC, AOA and CHI, EFE activity was 29.63 nC₂H₄/g/h and this was stimulated 54% by treatment with CO₂ applied after incubation. When discs were exposed to CO₂ before incubation, EFE activity was slightly but significantly reduced compared with control discs. However exposure to CO₂ after incubation reversed this inhibitory effect of pretreatment CO₂. Thus the stimulation of CO₂ on EFE activity in Hosui fruit discs was dependent on exogenous ACC.

Table 5-3. Effects of CO₂ treatments on ethylene production and EFE activity of Hosui fruit discs (1990).

(a) Ethylene production of discs treated with CO₂.

Treatment	C ₂ H ₄ Production (nlC ₂ H ₄ /g/h)
Control	0
Treatment with CO ₂	0

(b) EFE activity measured after exposing discs to CO₂ at different times.

	EFE activity (nlC ₂ H ₄ /g/h)
Control	29.63 ^b
CO ₂ treatment	45.53 ^a
Pretreatment with CO ₂	26.90 ^c
Double CO ₂ treatment	45.01 ^a

All values are means (n=4). Different characters within the column represent significant differences at the 5% level (Duncan's multiple comparison).

5.4.5 EFE activity and the response to carbon dioxide in 0.4 M and 0.8 M mannitol solutions

EFE activity of Hosui fruit discs was greater in 0.8 M mannitol than in 0.4 M mannitol. CO₂ increased EFE activity by 41.3% in 0.4 M mannitol, but did not affect activity at 0.8 M (Table 5-4).

Similar results were obtained in Granny Smith fruit. EFE activity increased by 39% in 0.8 M mannitol compared to 0.4 M. CO₂ stimulated EFE activity by 17.3% in 0.4 M mannitol, but had no effect on EFE activity in an 0.8 M solution (Table 5-4).

5.4.6 Effect of carbon dioxide treatment on EFE kinetics

To prevent new protein biosynthesis during incubation EFE activity was measured in the medium containing CHI.

Table 5-4. EFE activity and the response to CO₂ in 0.4 M and 0.8 M mannitol solutions at 27°C in fruit discs of Hosui and Granny Smith (1990).

Treatment	EFE activity (nlC ₂ H ₄ /g/h) in mannitol solution	
	0.4 M	0.8 M
Hosui		
	Control	51.57
+CO ₂		
		72.87
Granny Smith		
	Control	90.12
+CO ₂		
		105.74

All values are means (n=3). Different characters (lower case) within the same column for each fruit and different characters (upper case) within the same line represent significant differences at the 5% level (paired comparison).

Data obtained on EFE activity in discs exposed to different ACC levels were transformed and plotted in several ways to investigate the mechanism of the CO₂ effect on EFE activity.

In Hosui discs, plotting of $\log(V/V_{\max}-V)$ vs. $\log[S]$ of EFE showed that n_H values for control and CO₂ treatment were 0.9266 and 0.9151, respectively, and not significantly different (Table 5-5).

A plot of V vs. $V/[S]$ showed that CO₂ increased V_{\max} from 109.99 nlC₂H₄/g/h to 150.54 nlC₂H₄/g/h. However there was no significant effect of CO₂ on the apparent K_m values for ACC which were 0.152 mM in the control and 0.170 mM in the CO₂ treatment (Table 5-5).

Results of a double reciprocal plot also showed that CO₂ increased V_{\max} from 112.37 nlC₂H₄/g/h to 137.10 nlC₂H₄/g/h (Fig.5-5 and Table 5-5). The apparent K_m value of EFE was 0.167 mM and CO₂ treatment did not change this K_m value.

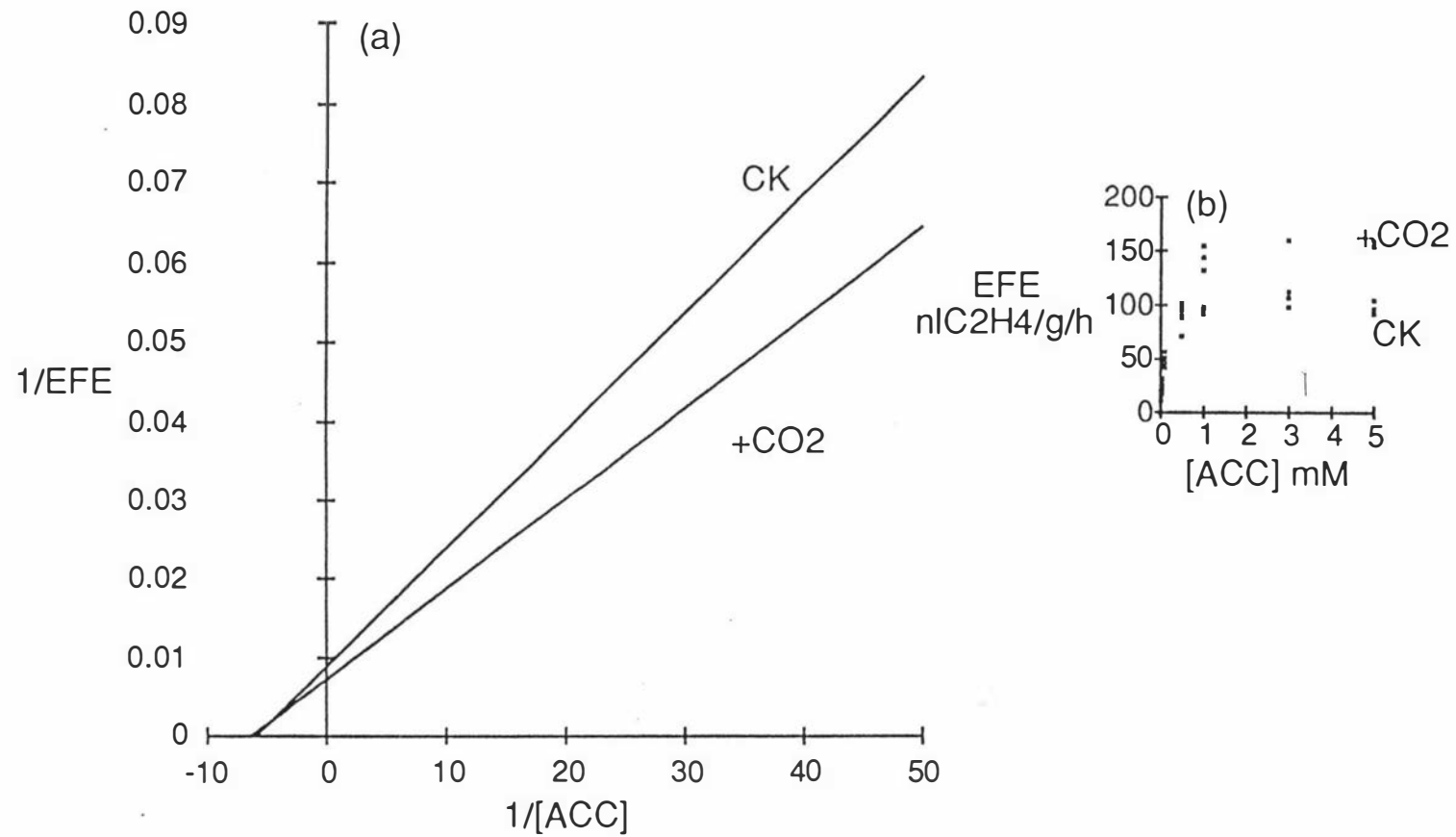


Fig.5-5 Double reciprocal plot (a) and data (b) of EFE activity against ACC concentrations in Hosui fruit discs.

Table 5-5. Linear regression results of EFE kinetics for ACC with 20% CO₂ in fruit discs of Hosui (1989).

Plot methods		Control	+CO ₂	
log(V/V _{max} -V) vs. log[S]	η_H	0.9266	0.9151	ND*
	r^2	0.7633	0.8937	-
V vs. V/[S]	V _{max}	109.99	150.54	SD*
	K _m	0.152	0.170	ND
	r^2	0.8856	0.7863	-
1/V vs. 1/[S]	V _{max}	112.37	137.10	SD
	K _m	0.167	0.156	ND
	r^2	0.9830	0.9354	-

All values are the results of the linear regression (n=3 for each of eight ACC concentrations). SD represents significant differences and ND represents no significant differences between treatments at the 5% level. r^2 is the regression coefficient for the linear regression.

V (nlC₂H₄/g/h): the reaction rate; V_{max} (nlC₂H₄/g/h): the maximum reaction rate; S (mM): concentrations of the substrate (ACC).

In Granny Smith apple, a plot of log(V/V_{max}-V) vs. log[S] indicated that there was no significant difference between the η_H values of control (0.8595) and CO₂ treated (1.200) fruit tissues (Table 5-6).

Results of the V vs. V/[S] plot showed that CO₂ stimulated V_{max} by 146% from 118.95 (control) to 293.12 nlC₂H₄/g/h. The apparent K_m changed slightly from 0.227 mM to 0.484 mM, but this difference was not significant.

Results of the double reciprocal plot also showed that CO₂ treatment increased the V_{max} of EFE from 103.87 nlC₂H₄/g/h to 293.86 nlC₂H₄/g/h, while the apparent K_m increased slightly from 0.193 mM to 0.508 mM, but the difference was not significant (Fig.5-6 and Table 5-6)

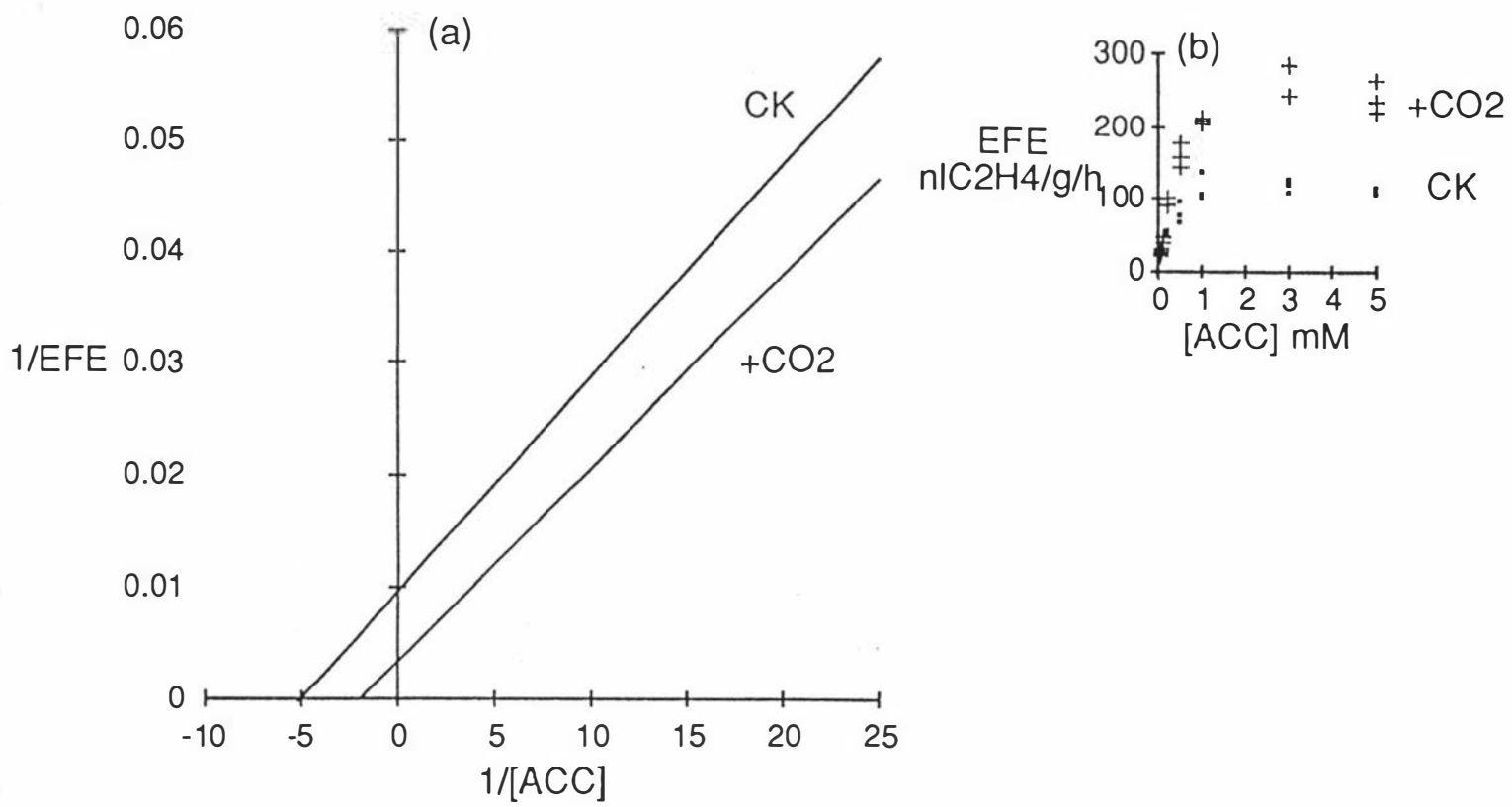


Fig.5-6 Double reciprocal plot (a) and data (b) of EFE against ACC concentrations in Granny Smith fruit discs.

Table 5-6. Linear regression results of EFE kinetics for ACC treated with 20% CO₂ in fruit discs of Granny Smith (1989).

Plot Methods		Control	CO ₂	
log(V/V _{max} -V) vs. log[S]	n _H	0.8595	1.2000	ND
	r ²	0.6343	0.7668	-
V vs. V/[S]	V _{max}	118.95	293.12	SD
	K _m	0.2268	0.4838	ND
	r ²	0.7745	0.8908	-
1/V vs. 1/[S]	V _{max}	103.87	293.86	SD
	K _m	0.1928	0.5075	ND
	r ²	0.8895	0.9833	-

All values are the results of the linear regression (n=3 for each of eight ACC concentrations). SD represents significant differences and ND represents no significant differences between treatments at the 5% level. r² is the regression coefficient for the linear regression.

V (nlC₂H₄/g/h): the reaction rate; V_{max} (nlC₂H₄/g/h): the maximum reaction rate; S (mM): concentrations of the substrate (ACC).

5.4.7 Effect of pH on response of EFE activity to carbon dioxide

EFE activity of Granny Smith fruit discs showed different sensitivity to CO₂ treatment at various pH values (Table 5-7). In the range of pH 3.5-6.2, EFE activity increased gradually from 36.84 to 106.1 nlC₂H₄/g/h, after which it remained constant through to pH 8.2. CO₂ stimulated EFE activity at each of the pHs' tested in this experiment. CO₂ increased EFE activity more at low pHs (3.5-4.5) than at the higher pHs tested. The lowest response to CO₂ of EFE was at pH 6.2 where a 24% increase was measured (Table 5-7).

5.5 DISCUSSION

EFE in both Hosui and Granny Smith fruits developed earlier than ACC synthesis. Discs from Hosui, a nonclimacteric fruit, did not produce ethylene at harvest indicating that they lacked either ACC synthesis or EFE or both. However when these same discs were treated with exogenous ACC, ethylene^{was} produced indicating that EFE was present and that ACC synthase was absent. Granny Smith, a climacteric fruit, did not produce ethylene at harvest, but in contrast to Hosui, EFE was not present in this fruit.

Table 5-7. EFE activity in fruit discs of Granny Smith incubated in Na⁺-citric acid buffer containing 5 mM ACC and 1 mM CHI at different pH levels treated with 20% CO₂ (1989).

pH	EFE (nl C ₂ H ₄ /g/h)		Increase (%)
	-CO ₂	+CO ₂	
3.5	36.84 ^{cB}	70.94 ^{dA}	92.60
4.5	41.18 ^{cB}	114.33 ^{bcA}	177.60
5.5	63.94 ^{bB}	98.94 ^{cA}	54.74
6.2	106.08 ^{aB}	131.89 ^{bA}	24.33
7.2	99.72 ^{aB}	170.36 ^{aA}	70.84
8.2	104.68 ^{aB}	160.23 ^{aA}	53.07

All values are means (n=3). Different characters (lower case) within the same column for same treatment (Duncan's multiple comparison) and different characters (upper case) within the same line (paired comparison) represent significant differences at the 5% level.

as exogenous ACC was not converted to ethylene. Ethylene production appeared at least 10 days later after harvest than EFE activity in Granny Smith apple discs (Table 5-2b). Blankenship and Richardson (1985) reported that during the cold storage period required for ripening of d'Anjou pears, EFE developed before the increase in ACC and ethylene synthesis. Similar conclusions were reported for apple (Bufler, 1986; Mansour, *et al.*, 1985). No work on the characteristics of EFE development in nonclimacteric Japanese pears has been reported. The present results indicate that the main difference between nonclimacteric and climacteric fruits in relation to ethylene synthesis was the capability for developing ACC synthase, but not EFE. It is also possible that a ripening inhibitor was present in Granny Smith fruit which prevented development of ACC synthase during the preclimacteric stage.

The activity of EFE increased gradually in Hosui and Granny Smith fruits during storage at 1°±1°C. CO₂ treatment increased EFE activity in discs of both fruits. EFE was not detectable in fruit discs of Granny Smith

during short-term storage after harvest, but EFE synthesis was induced by CO₂. This was demonstrated by using CHI, a protein synthesis inhibitor, ~~which~~ ^{which} could reduce CO₂ stimulation on EFE activity in Granny Smith. Applied along with ACC and AOA thus creating conditions where both ACC synthesis and EFE synthesis were inhibited by CHI, and ACC synthase activity was inhibited by AOA, EFE activity remained the same as in control discs. Application of CO₂ to discs from fruit up to 26 days in storage after harvest stimulated EFE ^{activity} ~~measured~~; a large proportion of this ^{increase in} ~~additional~~ EFE ^{activity} which was due to EFE synthesis was reduced as it occurred when CHI was added in the incubation medium. The CO₂ stimulation of EFE synthesis became ~~the~~ less the longer the fruit was in store. These results are similar to those found by Philosoph-Hadas *et al.* (1986) in tobacco leaf discs which suggests ^{act} that EFE in discs from preclimacteric fruit and vegetative leaf tissues are regulated by CO₂ through the same mechanism.

It is possible that CO₂ not only regulates EFE activity directly but also influences EFE activity and/or biosynthesis indirectly through regulating the binding capability of the ethylene receptor and thus perhaps affecting the regulation of ethylene action (Yang, 1987). Bufler (1986) demonstrated that CO₂ promoted ethylene action in preclimacteric apple peel tissue. Similar effects of CO₂ have been reported in ethylene-stimulated growth rate of rice coleptiles (Ku *et al.*, 1970) and in overcoming thermodormancy of lettuce seeds (Negm *et al.*, 1972). At this time it was not possible to show which ethylene receptor was involved in the CO₂ promotive effect on ethylene action or which ethylene biosynthetic enzyme might have been regulated by this receptor. It is possible that in Hosui, a nonclimacteric fruit, ethylene biosynthesis was only regulated by the System I ethylene receptor (McMurchie *et al.*, 1972; Yang, 1987) and that this receptor was not affected by CO₂. Thus CO₂ did not stimulate EFE synthesis through interaction with the System I ethylene receptor in Hosui fruit discs. In Granny Smith apple, a different result was obtained. CO₂ stimulated EFE synthesis in discs sampled from the short-term storage fruit (10 days after harvest) which did not produce ethylene. It is most likely therefore that these fruit were at the preclimacteric stage (only possessed ^{ed} the System I ethylene receptor), ~~did~~ ^{at} respond to CO₂ by stimulating EFE synthesis. CO₂ is thought to be a structural analogue of ethylene (Burg and Burg, 1967). It is possible that CO₂ could combine with the ethylene binding site of the System I ethylene receptor or alternatively CO₂ would possibly increase the affinity of ethylene

to bind to the receptor and as a consequence stimulate EFE synthesis at this stage of development. As fruit ripened and produced more ethylene, this stimulatory effect of CO₂ on EFE biosynthesis was lost. It seems likely that, because there is more ethylene in the tissue arising from natural production, the System I ethylene binding site is saturated with endogenously produced ethylene, and thus CO₂ no longer has a stimulatory effect on EFE synthesis (Burg and Burg, 1967; Sisler, 1979; Sisler and Wood, 1987).

These results indicate that the regulation of EFE by the System I ethylene receptor in Granny Smith apple and Hosui fruit were different. In Hosui fruit, CO₂ could only regulate EFE activity through interaction with the System I ethylene receptor, but in Granny Smith fruit it could regulate both EFE activity and its biosynthesis at the preclimacteric stage. This explanation differs from that proposed by Yang in his model on regulation of ethylene biosynthesis by ethylene (Yang, 1987). In his model EFE was regulated by the System I ethylene receptor and no difference of EFE regulation by the ethylene receptor between climacteric and nonclimacteric fruit was mentioned.

To further develop an understanding of the mechanism by which CO₂ directly stimulated EFE activity, several hypothesis were proposed and checked by measuring EFE activity in discs that were incubated in a range of ACC concentrations with or without CO₂ present.

It is possible that EFE is an allosteric enzyme, its activity being regulated by CO₂ as its effector. Generally, an allosteric enzyme will show the following characteristics (Barman, 1969): (a) The plot of V (the reaction rate) vs. S (the substrate concentration) will often yield sigmoid rather than hyperbolic curves; (b) these enzymes are usually unstable and therefore, difficult to purify because of their structural complexity. It is possible that several highly purified enzymes possessing normal Michaelis-Menton kinetics are in fact fragments of more complex allosteric systems; (c) The catalytic function of allosteric enzymes may be affected and controlled by interaction with a small molecular ligand, such as in the haemoglobin-oxygen system.

EFE has some of the characteristics of allosteric enzymes. For example, EFE had not yet been purified from plant tissues because any extraction treatment which perturbed membrane integrity during purification caused a loss of EFE activity (Yang and Hoffman, 1984). In research with carnation petals and oat leaves, sigmoid-like curves were found when EFE kinetic studies were undertaken even though EFE was not identified as an allosteric enzyme (Adam *et al.*, 1985; Preger and Gepstein, 1984). CO₂ as a small molecule could bind and interact with the EFE enzyme, as it can react with amino groups of proteins, peptides or amino acids; such reactions could change enzyme activity through forming a carbamate (Mitz, 1979). Therefore for the above reasons, EFE might be an allosteric enzyme in fruits and that CO₂, as an effector, could promote its activity.

It is possible to test this hypothesis by investigating the kinetics of the production of an end product (in this case C₂H₄) in response to varying concentrations of a substrate (in this case ACC) where the rate of the reaction is determined by the activity of an enzyme (in this case EFE) which may be affected by the presence or absence of a particular cofactor (in this case CO₂). It is possible to gain an insight into the nature of the interaction by studying the results obtained from application of the following equation (Smith, *et al.*, 1983):

$$\log[V/(V_{\max}-V)] = n_H \log[S] - \log K$$

When $\log[V/(V_{\max}-V)]$ is plotted against $\log[S]$, the result is a straight line with slope equal to n_H . When $n_H = 1$, there is no cooperativity which means that the enzyme is not allosteric; if $n_H = 2$, it is positively cooperative allosteric; when $n_H = 0.5$, it is negatively cooperative allosteric (Smith, *et al.*, 1983). Results from this research showed that the n_H values of EFE in both fruit types were approximately 1 which indicated that EFE was probably not an allosteric enzyme. Because EFE activity was tested *in vivo*, it might have been influenced by many factors. For example pH levels could cause dissociation of an allosteric enzyme into its component parts thus changing the sigmoid kinetics curve to an hyperbolic curve; there might be the variation of EFE activity in different types of fruit discs; different endogenous ACC levels might result in different EFE levels or EFE activities being measured especially at low ACC levels. It probably will not be possible to determine the nature of the enzyme until EFE is finally purified.

It has been shown that Hosui fruit discs did not synthesize detectable ethylene, and that CO₂ did not affect ethylene production (in the absence of exogenous ACC). EFE activity did exist in the discs, but 'Pre-CO₂ treatment' did not stimulate EFE activity; 'CO₂ treatment' stimulated EFE activity only when discs had been saturated with ACC, and 'Double CO₂ treatment' stimulated EFE activity at the same level of 'CO₂ treatment'. These results indicated that the stimulatory effect of CO₂ on EFE activity was dependent on an exogenous supply of ACC, which is in agreement with the work of Tan and Thimann (1989). They found that CO₂ is needed to stimulate conversion of exogenous ACC to ethylene, but not for the endogenous formation of ethylene which is generally considered to proceed via ACC. They suggested that when ethylene is produced from a modified form, e.g., 'bound' or better, 'activated' ACC, in the endogenous system, a cycle of reactions leads to ACC releasing one of the carbon atoms of methyl thioribose as CO₂ (Miyazaki and Yang, 1987); it is possible that this CO₂ might somehow remain associated with the ACC so that it satisfies the CO₂ requirement. When exogenous ACC is added, more CO₂ is required to form this ACC-CO₂ complex. It is possible that CO₂ might combine reversibly with the EFE-ACC complex to increase V_{max}.

In order to test if CO₂ combined to the binding site of EFE, data from the EFE kinetic experiment using a range of ACC concentrations were transformed according to the following Lineweaver-Burk equation:

$$1/V = (K_m/V_{max}) * 1/[S] + 1/V_{max}$$

A plot of 1/V vs. 1/[S] (a double reciprocal plot) yields a straight line with slope K_m/V_{max} and ordinate intercept 1/V_{max}.

Alternatively, multiplying the above equation by V_{max} gives:

$$V = -K_m[V/S] + V_{max}$$

which can be used graphically for determining K_m (-K_m is the slope) and V_{max} (intercept).

Using the results obtained from the above relationships a plot of V vs. V/[S] for EFE in both Hosui and Granny Smith fruits showed that treatment with CO₂ increased V_{max} without causing any change in K_m values of EFE. That is, CO₂ only increased the values of the intercepts without changing the slopes.

A double reciprocal plot showed that CO_2 significantly decreased the intercepts ($1/V_{\max}$) and slopes (K_m/V_{\max}) for both fruits. These results were similar, but opposite to the mechanism of noncompetitive inhibition which increases intercept ($1/V_{\max}$) and slope (K_m/V_{\max}) without changing K_m . A noncompetitive inhibitor decreases the V_{\max} without changing K_m , because the inhibitor does not bind with the enzyme at the substrate binding site (Smith *et al.*, 1983). Combining the above results together with the fact that the CO_2 stimulation of EFE was dependent on exogenous ACC; it is suggested that (a) EFE possesses more than one substrate binding site, for example, an ACC binding site and an ACC- CO_2 binding site which might be located on the same or on different subunits. (b) CO_2 combines with a non-substrate binding site of EFE to increase EFE activity. When CO_2 reacts with different binding sites to form an EFE-ACC- CO_2 complex and/or an CO_2 -EFE-ACC complex, the V_{\max} of the reaction of conversion of ACC to C_2H_4 would be increased.

Carbon dioxide is reviewed to have marked effects on metabolism in plant cells. Mitz (1979) has summarized the characteristics of direct CO_2 effects individually that of the dissolved, unhydrated form of CO_2 . These include: (1) the direct CO_2 effects are highly selective. There may be activation or inhibition of one enzyme and not of another closely related or even the same enzyme in another tissue. One part of a membrane may respond to CO_2 but not another; (2) different specific partial pressures of CO_2 are required to initiate specific activities. This represents a minimum concentration or "threshold" effect; (3) response is rapid, and may be almost instantaneous; (4) there may be a control range, so that as the CO_2 is slowly increased above a given value, the response is in one direction and at a still higher CO_2 concentration the response is in the opposite direction.

In this present work some of these characteristics were observed. For example, CO_2 stimulated EFE activity in Hosui fruit from short-term storage, but not in fruit from long-term storage. CO_2 also stimulated EFE activity in discs incubated in 0.4 M mannitol, but not in 0.8 M mannitol. In Hosui fruit discs a low concentration of CO_2 (<5%) did not stimulate EFE activity, but a higher concentration (>5%) did. The stimulatory effect of CO_2 on EFE activity in both Hosui and Granny Smith fruit discs and on EFE synthesis in the preclimacteric Granny Smith fruit discs occurred within 1 hour. Thus it is possible that CO_2 as a dissolved gas directly stimulated EFE activity or its biosynthesis.

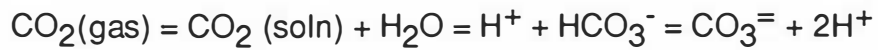
The direct effect of CO₂ on enzymes can occur in different ways: (1) an enzyme with its active site masked by interaction with another protein or polymeric structural element of the cell may be temporarily dissociated and, consequently, activated by an increase in CO₂ tension (Mitz, 1956); (2) the binding reaction of CO₂ with the active site of enzyme to form carbamate may prevent the binding of an essential coenzyme, which occurs in carbohydrate metabolism (Chance and Park, 1967). In the present work, CO₂ stimulation of EFE activity may be the consequence of dissociation of EFE from a masking protein or other elements.

John *et al.*(1989) indicated that maximum EFE activity of Kiwifruit discs was observed with sucrose, mannitol and sorbitol supplied at a strength of 0.7 M or higher, but EFE activity was completely lost when osmotic support was removed. In the present experiments EFE activity was higher in 0.8 M mannitol solution than in 0.4 M mannitol, although in 0.8 M solution, EFE did not respond to CO₂ as it did in 0.4 M mannitol. It was shown previously (Chapter 4) that 0.4 M mannitol was the isotonic concentration for both Hosui and Granny Smith fruit discs. Thus although EFE activity was higher in 0.8 M mannitol, this was not the normal osmotic condition of the cells. Bouzayen *et al.*(1987, 1990) and Pech *et al.*(1989) reported that grape (cv. Muscat) cells in suspension culture were osmoticum sensitive suggesting that EFE activity was mainly located at the plasmalemma with a small degree of activity inside the cell, probably at the tonoplast. Cells from another grape (cv. Gamay) were osmoticum insensitive, the bulk of ethylene production apparently being intracellular with the tonoplast being the most probable site for location of the EFE (Guy and Kende, 1984; Pech *et al.*, 1989). EFE activity in Hosui and Granny Smith fruit discs was sensitive to osmoticum changes. Based on the grape work it is thus possible that one of EFE forms in these fruits was located at the plasmalemma. Because of the change in EFE activity and loss of the response to CO₂ that occurred simultaneously when tissue was incubated in 0.8 M mannitol, which penetrates the plasma membrane slowly (Berliner and Martindale, 1981), it is possible that the plasmalemma is the site of the EFE form which responded to CO₂.

EFE activity had been shown to increase in Hosui fruit maintained in low temperature storage at $1^{\circ}\pm 1^{\circ}\text{C}$. It appeared that in whole fruit that had been stored for a short time the stimulatory effect of CO_2 on EFE activity was higher than that in fruit stored for long periods. The response of EFE activity to CO_2 showed two phases, which were that EFE activity was stimulated by CO_2 , and EFE activity in fruit after long-term storage was no longer response to exogenous CO_2 . One possible explanation for these results is suggested by Tan and Thimann (1989). They suggested that ethylene may be produced from an ACC- CO_2 complex which would be a modified form of ACC. When measuring EFE activity in fruit at the preclimacteric stage of development, excess exogenous ACC required more CO_2 to form the ACC- CO_2 complex. But when fruit was riper, more CO_2 would be produced from respiratory metabolism, and this might be sufficient to allow the action of the ACC- CO_2 complex without the need for exogenous CO_2 . Under these conditions CO_2 would not be expected to stimulate EFE. Another possible explanation is that EFE may possess more than one substrate binding site, e.g. a site which binds ACC alone, and a site which can bind a slightly differently shaped ACC which develops when it is combined with CO_2 . When high levels (20%) of CO_2 exist in the tissue, an EFE-ACC- CO_2 complex could be formed which enhanced the rate of conversion of ACC to C_2H_4 . When there is a change in the nature of the cell membrane (e.g. change in degree of saturation or fluidity) resulting from senescence or caused by treatment with a high osmotic solution, ~~these~~ ^{there} would be an increase in leakiness and which ^{could} affect the activity of proteins such as EFE. The EFE-ACC- CO_2 binding site may lose its activity because of a change of EFE configuration such as an alternation of quaternary structure of the enzyme.

Mattoo *et al.* (1982) suggested that EFE can change from a membrane-associated form to a soluble form. The latter form had a higher activity which was inhibited by CoCl_2 during aging of the homogenates of etiolated pea subhook segments. But the K_m value of EFE for ACC was high (2.99 to 5.58 mM) suggesting that the conversion of ACC to ethylene may have occurred in a cell-free system. In the present work, EFE in Hosui fruit stored for long term showed high activity, but did not respond to CO_2 . It is possible that a change of form of EFE, for example, from the membrane-bound form to a soluble form occurred. The soluble form of EFE still had high activity using ACC as substrate, but it no longer responded to CO_2 .

Brown (1985) reported that high levels of CO₂ (5%) probably affected both the internal and external pH of cells. CO₂ in tissues can exist in several forms:



At pH 6.3 and 25°C, equal amounts of dissolved CO₂ and bicarbonate occur. As the pH decreases, relatively more CO₂ would be present, and as the pH increases relatively more bicarbonate would occur. Because cytoplasmic pH is commonly close to 6.5, and CO₂ levels of 5% can occur within fruit tissue, it is possible for the active species to be either CO₂ or HCO₃⁻ (Sisler and Wood, 1988).

In Granny Smith apple, CO₂ increased EFE activity more at low pH (<5.5) than at high pH suggesting that the main functional form of CO₂ *in vivo*, which stimulated EFE activity, was CO₂ in a dissolved form. At low pH, more CO₂ would exist in the medium and thus CO₂ would stimulate EFE activity directly, rather than indirectly by changing internal and external pH of cellular solution.

Carbon dioxide also may act at the membrane level. If the amino groups on the membrane and structural proteins react with CO₂, positive ions (RNH₃⁺) may become negative (RNHCOO⁻). This temporary change in charge from (+) to (-) would change the environment which acts as a control mechanism that selectively favours the transport of positive ions such as Na⁺, K⁺ and H⁺, through a membrane and inhibits the transport of negative ions such as Cl⁻ and HCO₃⁻. (Mitz, 1979). The change in charge can also cause an attraction of opposite or repulsion of similar charges to help open or close the holes or channels in the membrane (DeHoal and Defelice, 1978). John (1983) hypothesized that EFE activity involved proton transfer across the membrane. Thus CO₂ stimulated EFE activity may occur through the above mechanisms causing an increased H⁺ transport.

In conclusion, the results presented here show that in both the nonclimacteric Hosui and the climacteric Granny Smith fruit, CO₂ can have a dual effect on EFE, and thus may influence ethylene biosynthesis in multiple ways. In Granny Smith fruit, CO₂ promoted EFE synthesis at the preclimacteric stages, and stimulated the activity at all of the ripening stages tested. In Hosui fruit, CO₂ only stimulated EFE activity in fruit stored at

$1^{\circ}\pm 1^{\circ}\text{C}$ till 105 days after harvest, and had no effect in the fruit stored for 117 days after harvest. When discs of both fruits were treated with high (0.8 M) osmotic strength solutions, EFE activity was enhanced, but there was no stimulatory CO_2 response, which suggested that the EFE which responded to CO_2 may be located at the plasma membrane. Results from EFE kinetic studies showed that EFE is probably not an allosteric enzyme. The promotive effect of CO_2 on EFE activity increased V_{max} without changing K_m for ACC in discs of both Hosui and Granny Smith fruits. The mechanism of CO_2 stimulation of EFE activity may be 'direct' through interaction with the active binding sites located at the same or different subunits of EFE or by increasing H^+ transport across membrane associated with EFE activity. The mechanism could be 'indirect' through changing the internal pH levels in cells but this appears less likely.

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CHAPTER 6

INTERACTION BETWEEN CARBON DIOXIDE AND THE EFE INHIBITOR (CoCl₂), AND BETWEEN CARBON DIOXIDE AND THE ETHYLENE ACTION INHIBITORS (AgNO₃ AND 2,5-NORBORNADIENE) ON EFE ACTIVITY

6.1 ABSTRACT

Ethylene production occurred from fruit discs of Granny Smith, but was not detectable from excised fruit discs of Hosui Japanese pear. Carbon dioxide had no effect on ethylene production from fruit discs of either Hosui or Granny Smith. Carbon dioxide stimulated EFE activity in discs of Hosui and Granny Smith fruits. Cobalt ions (CoCl₂) is an inhibitor of EFE activity. It inhibited ethylene production in Granny Smith fruit discs. Low concentrations (0.01 mM) of Co⁺⁺ ions either stimulated, or had no effect on EFE activity in discs of both fruits, but higher concentrations (>0.5 mM) inhibited EFE activity. The combination of CO₂ and Co⁺⁺ did not reverse the inhibition caused by Co⁺⁺ ions.

AgNO₃, a potent ethylene action inhibitor, inhibited ethylene production of fruit discs in Granny Smith apple. Low concentrations (<0.25 mM) Ag⁺ stimulated, but higher levels (0.5 and 1 mM) inhibited EFE activity in discs of both fruits. The combination of CO₂ and Ag⁺ did not reverse the inhibitory effect of Ag⁺ (1 mM). In ~~both of fruit~~ ^{from both fruit} discs, respiration rate was stimulated by 0.5 and 1 mM Ag⁺.

2,5-Norbornadiene is an ethylene action inhibitor. At 0.5% (V/V), it inhibited EFE activity in fruit discs from both fruits. CO₂ partially reversed the NDE inhibition in discs of Hosui fruit stored for 50 days, and Granny Smith fruit stored for 60 days at 1°±1°C, but not in discs of Granny Smith fruit stored for 102 days at 1°±1°C.

The possible mechanisms of the interactions between CO₂, the inhibitors on EFE activity and ethylene receptors are discussed.

Key words. carbon dioxide; cobalt ion; ethylene action; ethylene forming enzyme; ethylene production; Granny Smith apple; Hosui; 2,5-norbornadiene; silver ion.

Abbreviation. ACC: 1-aminocyclopropane-1-carboxylic acid; AOA: aminooxyacetic acid; CHI: cycloheximide; EFE: ethylene-forming enzyme; NDE: 2,5-norbornadiene; STS: silver thiosulphate.

6.2 INTRODUCTION

Carbon dioxide is known to be involved in the regulation of ethylene biosynthesis and action in several different ways: (a) CO₂ regulates ethylene synthesis by affecting the enzymes in the ethylene biosynthetic pathway (Philosoph-Hadas *et al.*, 1986; Chaves ^{and Tomas} *et al.*, 1984; Cheverry *et al.*, 1988); (b) CO₂ influences ethylene action by competing for the ethylene binding site(s) on ethylene receptors (Burg and Burg, 1967); (c) CO₂ as one of the products of ethylene metabolism, inhibits ethylene metabolism through a negative feed-back mechanism (Beyer, 1979, 1985).

Carbon dioxide has been shown to stimulate EFE activity and its synthesis in many vegetative tissues (Kao and Yang, 1982; Philosoph-Hadas *et al.*, 1986; McRae *et al.*, 1983), but in fruit tissues, conflicting results have been observed. Chaves and Tomas (1984), using Granny Smith fruit tissues showed that CO₂ increased ACC content although ethylene production decreased even after adding 0.04 and 0.1 mM ACC to fruit discs. These authors concluded that CO₂ inhibited the ethylene enzyme(s) responsible for conversion of ACC to ethylene. Cheverry *et al.* (1988) demonstrated that a high CO₂ concentration enhanced EFE activity in excised tissues of apple (Granny Smith) and avocado incubated with ACC (2 mM) and CHI (1 mM) or NDE (5 ml/l). In the autocatalytic phase of ethylene production, 20% CO₂ antagonized the stimulation of EFE synthesis by C₂H₄, but promoted EFE activity.

Carbon dioxide has rather different effects on ethylene binding. Burg and Burg (1967) studied the interaction between CO₂ and C₂H₄ using the pea stem straight growth test. They showed that CO₂ was a competitive inhibitor of ethylene action, and suggested that CO₂ delays fruit ripening by competing with ethylene for the receptor binding site. Sisler (1979) indicated

that CO₂ displaced bound C₂H₄ in tobacco leaves; at 2% CO₂ displaced about 35% of the bound ethylene, but increasing the CO₂ concentration to 10% did not displace the remaining ¹⁴C-labelled C₂H₄. He concluded that CO₂ acted, not only by direct competition, but in some indirect manner not yet determined. Thomas *et al.* (1984) indicated that CO₂ appeared to promote ethylene binding at the high CO₂ concentrations (8%) in extraction of the ethylene-binding site from *Phaseolus vulgaris* L. cotyledons.

As one of ^{the} end products of C₂H₄ metabolism, it is also possible that CO₂ could inhibit C₂H₄ metabolism by a negative feed-back mechanism and result in C₂H₄ accumulation in the tissue (Smith *et al.*, 1985).

Inhibitors of both EFE activity and ethylene action are useful tools for testing mechanisms of synthesis, activity of ethylene biosynthesis enzymes and the regulation of ethylene production by ethylene receptors (Veen, 1987; Yang, 1985).

Cobalt ion effectively inhibited ethylene production induced by IAA or kinetin in mung bean hypocotyl segments (Lau and Yang, 1976) and ACC-dependent ethylene production in hypocotyl segments of mung bean (Yu and Yang, 1979). Yu and Yang (1979) showed that Co⁺⁺ inhibited the reaction converting ACC to C₂H₄ and suggested that Co⁺⁺ may inhibit ethylene production by interacting with the sulfhydryl group thought to be part of the EFE.

Compounds inhibiting ethylene action, such as the silver ion and 2,5-norbornadiene (NDE), can be used in studying the mechanism of ethylene action (Veen, 1985, 1986 and 1987; Wang, 1987).

The silver ion can inhibit (Saltveit *et al.*, 1978; Reid *et al.*, 1980; Veen, 1979a,b), stimulate (Aharoni and Lieberman, 1979; Aharoni *et al.*, 1979; Atta-Aly *et al.*, 1987) or have no effect (Atta-Aly *et al.*, 1987) on ethylene production, depending on species, tissue type and developmental stage of the tissue.

The silver ion has been shown to be a potent ethylene antagonist on etiolated pea stems, cotton plants and orchid flowers (Beyer, 1979). Lau and Yang (1976) reported that C₂H₄ production by post-climacteric apple tissue

and by etiolated mung bean hypocotyl tissue was inhibited by Ag^+ . Silver nitrate (0.1 mM) inhibited ethylene production from mature green banana fruit slices, from pericarp discs of mature green tomato fruit and cortical tissue from post-climacteric apples (Idared) (Saltveit *et al.*, 1978).

In carnation flower petals, STS completely blocked the ethylene surge preceding the wilting of the petals (Reid *et al.*, 1980; Veen, 1979a,b). In tomato discs from attached fruit or from mature green fruit, long-term (1 or 2 days) treatment with 1 mM STS stimulated both the conversion of ACC to ethylene and the synthesis of ACC, while short-term (2 hours) silver (1 mM STS) treatment had no effect (Atta-aly *et al.*, 1987).

However in contrast to the above results, Ag^+ stimulated ethylene production in senescing tobacco leaf discs (Aharoni *et al.*, 1979; Aharoni and Lieberman, 1979) and tomato fruit tissue (Atta-Aly *et al.*, 1987).

Explanations given for the mechanisms of Ag^+ effects on ethylene synthesis are conflicting. Atta-aly *et al.* (1987) and Saltveit *et al.* (1978) suggested that in fruit tissues, the stimulation or inhibition of ethylene production by Ag^+ depended on the climacteric stage of the fruit; silver may have blocked the autoinhibition of ethylene synthesis, and thereby promoted the increase in ethylene production in nonclimacteric tissue, or alternatively it may have blocked the autocatalytic process of ethylene synthesis by ethylene, to reduce ethylene production. Aharoni *et al.* (1979) suggested that Ag^+ stimulated ethylene production through preserving IAA in the tissue or through accelerating the mechanism by which IAA or kinetin stimulated ethylene biosynthesis in leaf discs.

Norbornadiene (NDE), a cyclic olefin, is another powerful inhibitor of ethylene action which is thought to competitively block the physiological ethylene-binding site (Sisler and Yang, 1984; Sisler *et al.*, 1985). Because NDE is a gas and can be applied and removed sequentially, giving reversible effects, it has proved to be a useful tool to study ethylene action (Yang, 1987a). Among several cyclic olefins tested, NDE had the highest inhibitory effect on ethylene production (Sisler and Yang, 1984). NDE retarded senescence of green tomato (Liu *et al.*, 1989), apple (Blankenship and Sisler, 1989; Treccani *et al.*, 1986) and carnation flowers (Sisler *et al.*, 1983; Peiser, 1989; Wang and Woodson, 1989), and inhibited abscission of

citrus leaves (Sisler *et al.*, 1985). In vegetative tissue, ethylene production was found to increase after long exposure, 24 h or longer, to NDE (Sisler *et al.*, 1985). The different responses of ethylene production to NDE may result from different durations of NDE treatment of different tissues (Peiser, 1989).

There have been very few reports on ~~effects of~~ the interaction between CO₂ and CoCl₂, between CO₂ and Ag⁺, and between CO₂ and NDE on EFE in fruit tissues. Cheverry *et al.* (1988) showed that the maximal inhibition of EFE activity in avocado fruit at the autocatalytic stage of ethylene production occurred in the presence of NDE alone, while less inhibition was measured with a mixture of NDE and CO₂. They confirmed that, in the presence of NDE, ethylene production in tissues excised from climacteric avocado fruits is no longer autocatalytic, and that CO₂ in the absence of any autocatalytic ethylene production stimulated conversion of ACC to ethylene.

If Ag⁺ and NDE are ethylene action inhibitors, their inhibiting effect on ethylene biosynthesis should occur through inhibition of ethylene binding to the ethylene receptor. Only two hypotheses have been suggested indicating how Ag⁺ or NDE may interact with the ethylene receptor and inhibit ethylene action (Yang, 1985; Veen, 1986). Yang (1985) presented a model of action of ethylene which assumed that one or more of the coordination ligands in the receptor site facilitated the binding of ethylene to its receptor, resulting in a biologically active complex. Antagonists such as Ag⁺ are thought to interact with these ligands, resulting in a receptor having little capability to bind ethylene, or in an ethylene-receptor complex which is biologically inactive or less active. This model also assumed that NDE competes with ethylene for the same binding site with the resulting NDE-receptor complex being biologically inactive. NDE may act through a double bond which binds a metal such as copper which is thought to be contained in the ethylene receptor (Burg and Burg, 1967).

Veen (1986) presented another model to explain the competition between ethylene and NDE and between ethylene and the silver ion. This showed some resemblance to the calcium-calmodulin scheme which has been proposed (Smith, 1983). This model hypothesized that the ethylene receptor is composed of a sub-unit A, of a proteinaceous nature, and one or more enzymic sub-units B, which contain the copper. Binding of ethylene to

sub-unit A causes an active allosteric change in that protein. The activated sub-unit A will act as a regulatory system for the sub-unit B. The type of response caused by ethylene may depend on which enzymic sub-units B are present. Ethylene binding to sub-unit A leads to activation of sub-unit B and finally to a response. Ag^+ may displace the copper in the receptor sub-unit B, preventing sub-unit A from activating sub-unit B, thus blocking the action of ethylene. NDE and ethylene compete for the same site in sub-unit A. Binding of NDE to sub-unit A does not cause activation of sub-unit A, whereas ethylene-binding does.

Results from Chapter 5 have shown that CO_2 stimulated EFE activity in discs of both Hosui and Granny Smith fruits. CO_2 also stimulated EFE biosynthesis in discs of preclimacteric Granny Smith fruit, but not in Hosui fruit discs. In order to investigate the mechanism of CO_2 direct action on EFE activity, the interaction of Co^{++} and CO_2 was studied. In addition the interactions between CO_2 and the two ethylene action inhibitors, Ag^+ and NDE on EFE were tested to elucidate the effect of CO_2 on the ethylene receptor binding sites.

6.3 MATERIALS AND METHODS

6.3.1 Materials (See Chapter 2)

6.3.2. Methods

6.3.2.1 Preparation of fruit discs (see Chapter 2)

6.3.2.2 Measurement of ethylene production, EFE activity and respiration of fruit discs

EFE activity, ethylene production and respiration of discs were measured by sealing six discs in each of 3 or 4 X 36 ml vials for a certain time after vacuum-infiltration with 0.4 M mannitol solution containing 5 mM AOA to inhibit ACC synthase, 1 mM CHI to inhibit EFE synthesis and with (for EFE), or without (for ethylene production) 5 mM ACC (as the substrate for EFE). After incubation at 27°C, 2 X 1 ml gas samples were removed from each vial. One sample was used for ethylene analysis, the other for CO_2 analysis using GLC (See Chapter 2).

6.3.2.3 Carbon dioxide treatment

To test the influence of CO₂ treatment on EFE activity, ethylene production and respiration of fruit discs, 3 or 4 X 36 ml vials and containing six discs were flushed for 20 seconds with a gas mixture which contained 23% CO₂, 21% O₂ and 66% N₂. In control (CO₂-free) vials, small plastic tubes which contained a suspended piece of filter paper, wetted with 0.1 ml 10% KOH, was hung in each vial to prevent accumulation of CO₂. After incubation for a certain time, 1 ml gas was sampled for CO₂ measurement by GLC.

6.3.2.4 Cobalt ion treatment

In order to reduce possible variation of internal cobalt which might have been caused by different tissue permeability to Co⁺⁺, fruit discs were vacuum-infiltrated with a medium containing 5 mM ACC, 5 mM AOA and various Co⁺⁺ concentrations.

For ethylene production measurements, six fruit discs for each treatment were vacuum-infiltrated (a vacuum of 90 Kpa was applied for 1 min then released; this procedure was carried out 3 times to ensure full penetration of the solution) in 3 X 36 ml vials containing 3 ml 0.4 M mannitol and the following concentrations of CoCl₂: 0, 0.01, 0.05, 0.1, 0.5, 1 and 5 mM for Hosui and 0, 0.01, 0.1, 1, 3, 5 and 10 mM for Granny Smith.

To measure EFE activity, 5 mM ACC, 5 mM AOA and 1 mM CHI were added to the above 0.4 M mannitol solutions.

After vacuum-infiltration, the fruit discs were dried on filter paper then six discs were sealed in each of 3 X 36 ml vials for 2 hours at 27°C before removal of 1 ml gas for C₂H₄ measurement.

To test the effect of CO₂ on EFE activity of discs in the presence of Co⁺⁺-treated discs, the same method of Co⁺⁺ application was used as above with only one concentration, 1 mM CoCl₂. After saturation with the medium, discs were treated with or without CO₂ for 1 and 4.5 hours for Hosui fruit, and sealed for 2 hours for Granny Smith fruit at 27°C before removal of 1 ml gas for C₂H₄ measurement (method described above).

6.3.2.5 Silver ion treatment

Silver nitrate solutions were applied to fruit discs using the same method as for the cobalt treatment. Concentrations of AgNO_3 in 3 ml of 0.4 M mannitol medium were 0, 0.02, 0.05, 0.1, 0.25, 0.5, 1.0, 3.0 and 5.0 mM without (for ethylene production measurement) or with (for EFE activity measurement) 5 mM ACC, 5 mM AOA and 1 mM CHI. After treatment six discs were dried and sealed in each of 4 X 36 ml vials for 2 hours at 27°C before removal of gas samples for C_2H_4 and CO_2 measurement.

To test the effect of CO_2 on EFE activity in Ag^+ -treated discs, the same method was applied except that only one concentration of Ag^+NO_3 (1 mM) was used, and that the time of CO_2 treatment was 2 hours.

6.3.2.6 2,5-Norbornadiene treatment

In Hosui, discs were sampled from fruit that had been stored for 30 days at $1^\circ \pm 1^\circ\text{C}$. In Granny Smith apple, discs were sampled from fruit that had been stored for 60 and 102 days at $1^\circ \pm 1^\circ\text{C}$.

Six discs were placed in 4 X 36 ml vials containing 0.05 M K^+ -phosphate-sucrose buffer (pH 6.5), 5 mM ACC, 5 mM AOA and 1 mM CHI and vacuum-infiltrated for 3 minutes before removal and drying on filter paper. Six discs were sealed in each of 4 X 36 ml vials. Carbon dioxide was applied using the method described above. After sealing, NDE was injected into each vial with a syringe to give a concentration of 0.5% (v/v). After 2 hours incubation at room temperature (about 20°C) in the fume cabinet, 1 ml gas was sampled for ethylene analysis by GLC.

6.3.3 Statistical analysis of results

Each treatment had three or four replicates. Each experiment was repeated at least twice. A computing programme (SAS/STAT User's Guide) was used to analyse data for means and standard errors, and Duncan's multiple comparisons (Steel and Torrie, 1981).

6.4 RESULTS

6.4.1 Effects of CoCl_2 on EFE activity

EFE activity in both fruits was stimulated, inhibited, or unaffected depending on the concentrations of cobalt applied (Fig.6-1 and Fig.6-2).

In Hosui fruit, EFE activity in discs increased from 16.14 (control) to 18.57 $\text{nIC}_2\text{H}_4/\text{g/h}$ at 0.01 mM CoCl_2 (Fig.6-1), before declining to the control value at 0.05 mM. At higher concentrations, EFE activity decreased as Co^{++} increased. There was a rapid 58% decrease in EFE activity as Co^{++} increased from 0.05 to 1 mM, followed by a slower 31% decrease between 1 to 5 mM CoCl_2 (Fig.6-1).

In contrast no significant increase in EFE occurred in Granny Smith apple discs between control and 0.01 mM Co^{++} treatment (Fig.6-2). Over the range of 0.1-3 mM, EFE activity decreased rapidly by 64.9% from 32.32 to 11.33 $\text{nIC}_2\text{H}_4/\text{g/h}$ followed by a slower fall off (30.2%) in EFE activity between 3 and 10 mM CoCl_2 .

6.4.2 Interaction between CoCl_2 and Carbon dioxide on EFE activity

Because 1 mM CoCl_2 significantly reduced EFE activity, this concentration was selected for the experiment to test the interaction between Co^{++} and CO_2 on EFE activity.

In Hosui, ethylene production from discs was not detectable after 1 hour incubation at 27°C and only trace amounts of ethylene were measured during 4.5 hour incubation, thus the effect of Co^{++} on ethylene production could not be measured. After a 1 hour incubation CO_2 increased EFE activity by 42.76% (Table 6-1), but Co^{++} did not significantly reduce EFE activity. The combination of Co^{++} and CO_2 did not increase or decrease EFE activity compared with control but the addition of CO_2 did overcome the inhibitory effect of Co^{++} alone.

EFE activity in control discs did not change between 1 and 4.5 hours incubation. CO_2 also increased EFE activity after 4.5 hours. While Co^{++} again reduced EFE activity at this time, by 62%. However after 4.5 hours the combination of CO_2 and CoCl_2 did not reverse the inhibitory effect of CoCl_2 .

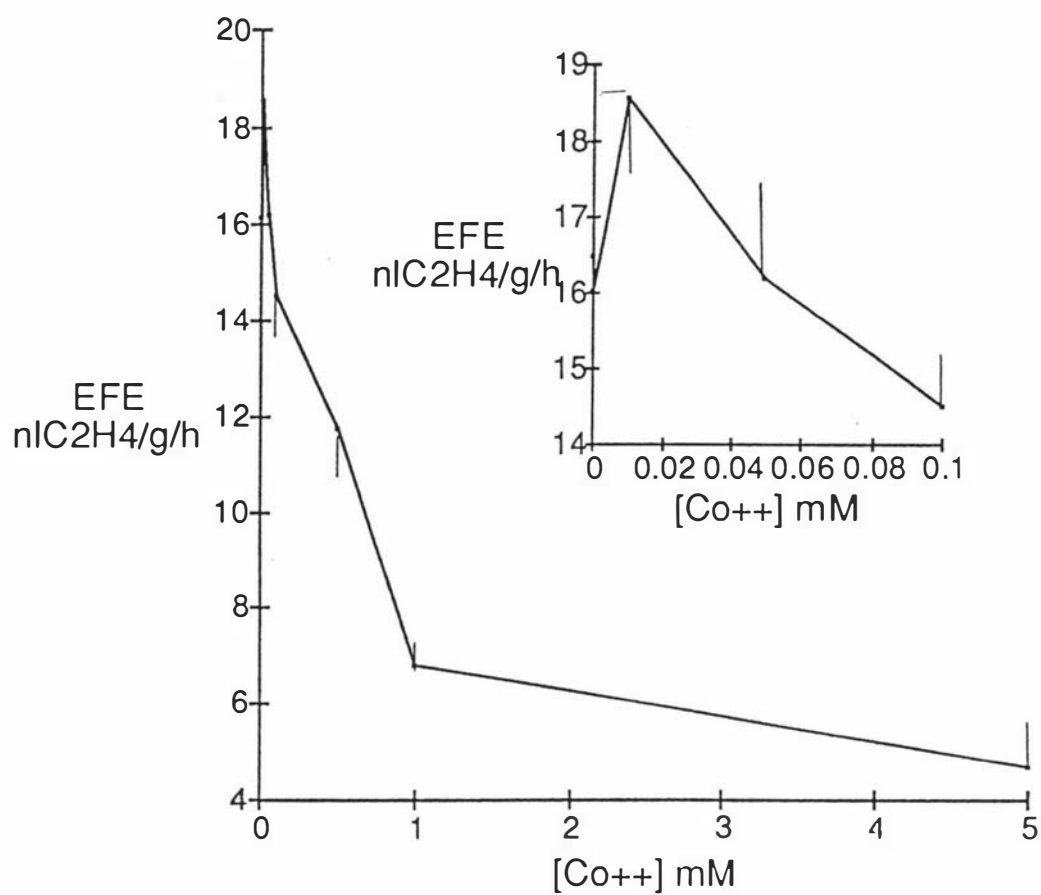


Fig.6-1 EFE activity of Hosui discs treated with various CoCl₂ concentrations (1989). Vertical bars indicate standard error.

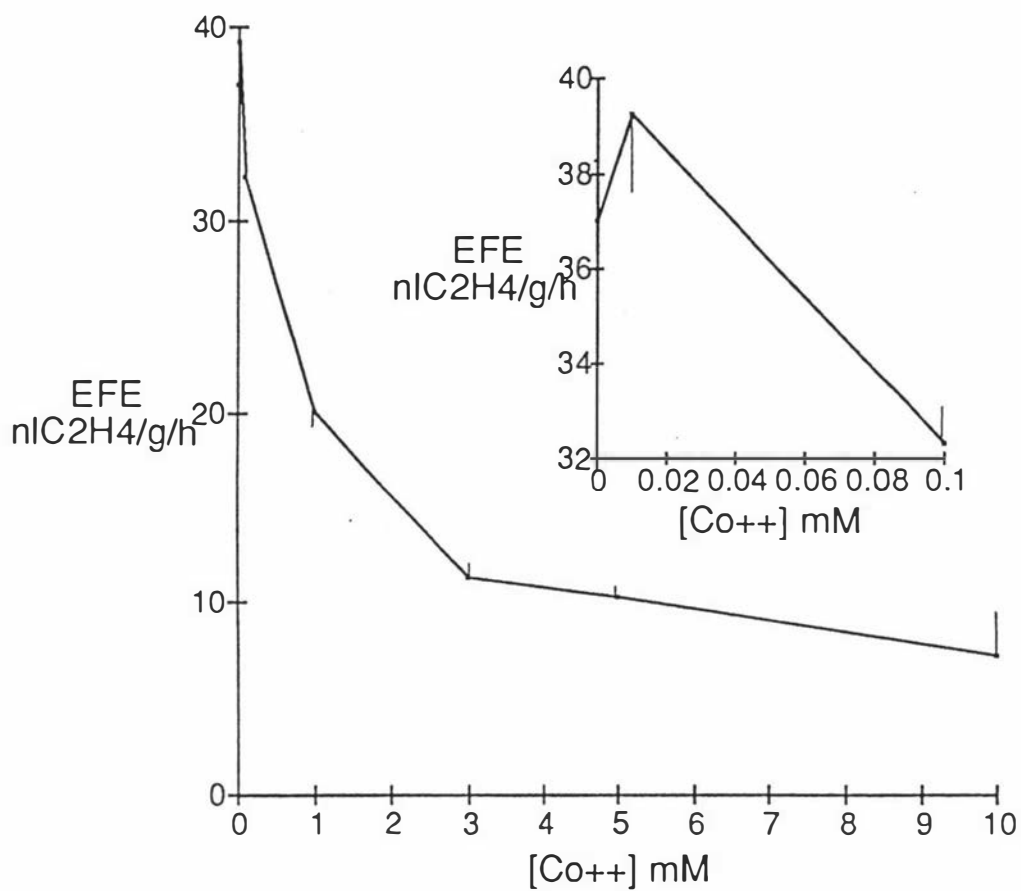


Fig.6-2 EFE activity of Granny Smith discs treated with various CoCl₂ concentrations (1989). Vertical bars indicate standard error.

Table 6-1. EFE activity in Hosui fruit discs treated with 1 mM CoCl_2 and 20% CO_2 for 1 and 4.5 hours at 27°C (1990).

Treatments	EFE activity (nlC ₂ H ₄ /g/h)		
	Incubation time at 27°C		
	1 h	4.5 h	
Control	20.56 ^{bc}	20.99 ^b	NS
CO_2	31.73 ^a	26.17 ^a	NS
1 mM CoCl_2	14.68 ^c	8.10 ^c	SD
CO_2 + 1 mM CoCl_2	23.21 ^b	12.82 ^c	SD

All values are means (n=3).

Values within a column followed by different characters represent significant differences at the 5% level (Duncan's multiple comparison).

SD and NS represent significant and non significant differences at the 5% level between 1 and 4.5 hours for each treatment (Duncan's multiple comparison).

In Granny Smith fruit, CO_2 did not affect ethylene production, but incubation with 1 mM Co^{++} decreased it by 65.4% (Table 6-2). The combination of CO_2 and Co^{++} did not reverse the inhibitory effect of Co^{++} on ethylene production. CO_2 stimulated EFE activity, and Co^{++} reduced it by 40.27%. CO_2 did not reverse the inhibitory effect of Co^{++} .

6.4.3 Effect of AgNO_3 on EFE activity and respiratory rate

In control Hosui discs, EFE activity was 82.85 nlC₂H₄/g/h, and this did not change when discs were treated with 0.02 to 0.1 mM Ag^+ (Fig.6-3). A large decrease (66.82%) in EFE activity occurred at higher Ag^+ concentrations, particularly between 1 and 3 mM. At concentrations of 3 mM and above tissue browning was found, thus the decline in EFE activity above 3 mM AgNO_3 can be attributed to acute toxicity and probably death of tissue.

Respiration rate of Hosui fruit discs was not affected by Ag^+ in the range of 0.02-0.1 mM (Fig.6-3). Respiration rate increased at 0.5 and 1 mM, dropped significantly at 3 mM and then remained relatively constant between 3-10 mM AgNO_3 .

Table 6-2. Ethylene production and EFE activity of Granny Smith fruit discs treated with 1 mM CoCl₂ and 20% CO₂ (1989).

Treatment	C ₂ H ₄ Prod. (nlC ₂ H ₄ /g/h)	EFE activity (nlC ₂ H ₄ /g/h)
Control	15.28 ^a	142.26 ^b
CO ₂	14.99 ^a	170.89 ^a
1 mM CoCl ₂	5.29 ^b	84.94 ^c
CO ₂ + 1 mM CoCl ₂	5.21 ^b	71.32 ^c

All values are means (n=3). Ethylene production of fruit discs were measured without ACC and AOA. EFE activity was tested after discs were infiltrated with ACC (5mM), AOA (5mM). Values within a column followed by different characters represent significant differences at the 5% level (Duncan's multiple comparison).

In Granny Smith fruit discs, similar results were obtained (Fig.6-4). Compared with control, EFE activity increased marginally at 0.05 mM AgNO₃ with concentrations greater than 0.5 mM markedly inhibiting EFE activity. There was no activity at 3 mM and higher, and tissue browning occurred at 3 and 5 mM.

Respiration rate in Granny Smith fruit discs was unchanged at 0.02 and 0.05 mM Ag⁺, but it increased gradually over the range of 0.1-1 mM and reached a peak at 0.5 mM AgNO₃ (Fig.6-4) before declining to 1.31 ulCO₂/g/h at 3 and 5 mM concentration (Fig.6-4). This was probably caused by toxicity.

6.4.4 Interaction between AgNO₃ and carbon dioxide on EFE activity

Because 1 mM Ag⁺ significantly decreased EFE activity without reducing respiration in each fruit type, this concentration was selected to test the interaction between Ag⁺ and CO₂ on EFE activity.

Carbon dioxide increased EFE activity of Hosui fruit discs (Table 6-3). Addition of 1 mM AgNO₃ to the medium reduced EFE activity by 37.3% and this inhibition was not reversed by addition of CO₂.

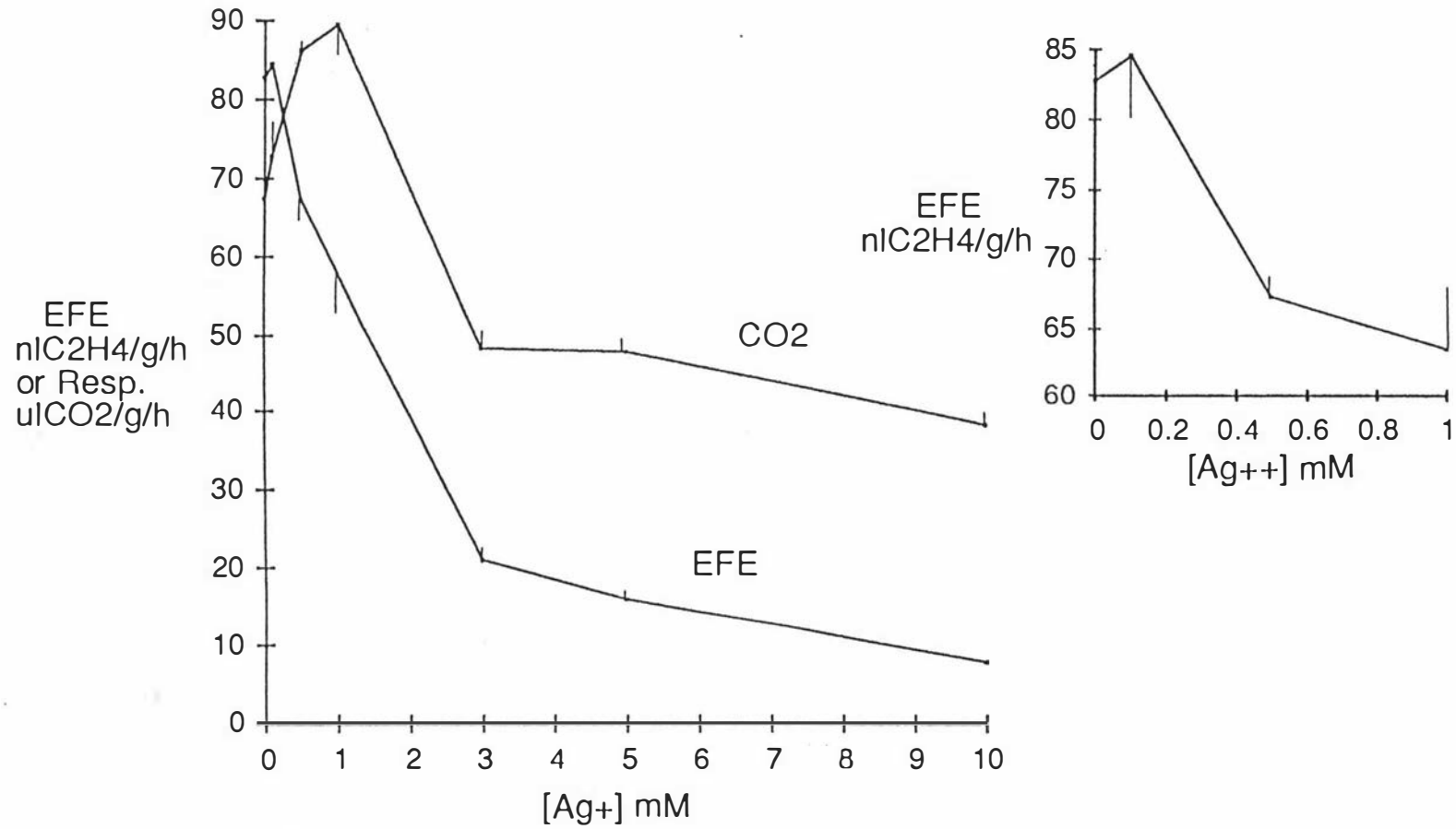


Fig.6-3 Effects of $AgNO_3$ on EFE activity and respiration rate of Hosui fruit discs (1990). Vertical bars indicate standard error.

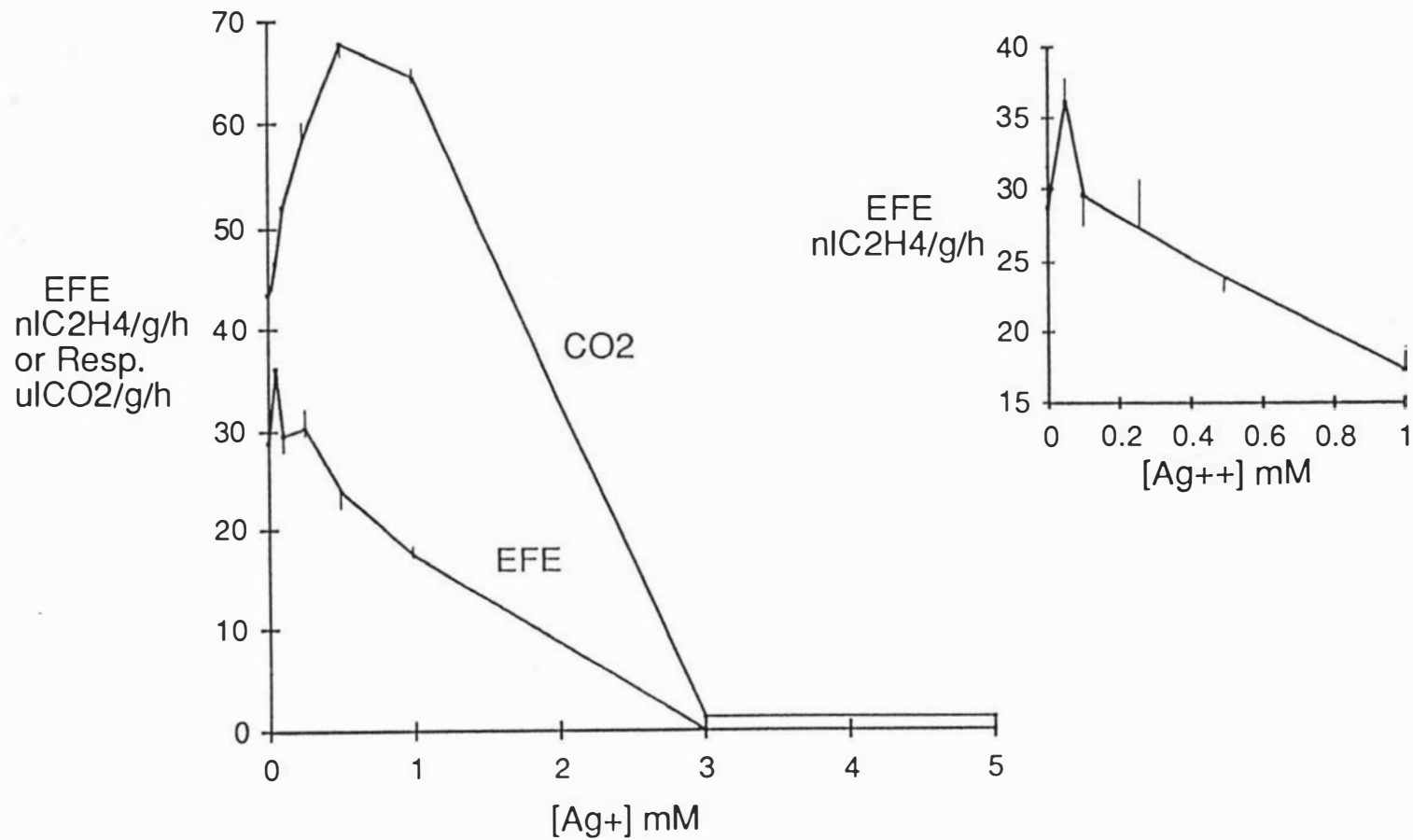


Fig.6-4 Effects of $AgNO_3$ on EFE activity and respiration rate of Granny Smith fruit discs (1989). Vertical bars indicate standard error.

Table 6-3. EFE activity of Hosui fruit discs treated with 1 mM AgNO₃ and 20% CO₂ (1990).

Treatment	EFE activity (nlC ₂ H ₄ /g/h)
Control	35.52 b
CO ₂	54.91 a
1 mM AgNO ₃	13.25 c
CO ₂ + 1 mM AgNO ₃	13.94 c

All values are means (n=4). Values within a column followed by different characters represent significant differences at the 5% level (Duncan's multiple comparison). Ethylene production of discs was not detectable.

Ethylene production of Granny Smith fruit discs was unaffected by CO₂ (Table 6-4), but almost completely inhibited by 1 mM Ag⁺. CO₂ did not reverse this inhibitory effect.

EFE activity in Granny Smith fruit discs was stimulated 29.5% by CO₂. Ag⁺ reduced EFE activity by 68.2% and again CO₂ was not able to reverse the inhibitory effect of AgNO₃.

Table 6-4. Ethylene production and EFE activity in Granny Smith fruit discs treated with 1 mM AgNO₃ and 20% CO₂ (1989).

Treatment	C ₂ H ₄ Prod. of discs (nlC ₂ H ₄ /g/h)	EFE activity (nlC ₂ H ₄ /g/h)
Control	18.69 a	42.59 b
CO ₂	19.88 a	55.17 a
1 mM AgNO ₃	0.98 b	13.46 c
CO ₂ + 1 mM AgNO ₃	0.54 b	12.05 c

All values are means (n=3). Values within a column followed by different characters represent significant differences at the 5% level (Duncan's multiple comparison).

6.4.5 Effects of NDE and carbon dioxide on EFE activity

In Hosui discs, CO₂ enhanced EFE activity by 56% and NDE treatment decreased it by 48.8% (Table 6-5). When discs were treated with the mixture of NDE and CO₂, the inhibitory effect of NDE was reversed, although the full stimulatory effect of CO₂ was not achieved.

In Granny Smith fruit which had been stored for 60 days, CO₂ increased EFE activity in discs by 33.5%, while NDE inhibited it by 71.6% (Table 6-6). CO₂ slightly reversed the inhibition caused by NDE. EFE activity increased 102% during the period from 60 to 102 days in coolstore. In fruit stored for 102 days CO₂ increased EFE activity by 36.3%. NDE reduced it by 60.6% and CO₂ did not reverse the inhibitory effect of NDE (Table 6-6).

Table 6-5. EFE activity of Hosui fruit discs treated with 0.5% norbonadiene (NDE) and 20% CO₂ (1989).

Treatment	EFE activity (nLC ₂ H ₄ /g/h)
Control	37.42
CO ₂	58.40
NDE	19.17
CO ₂ + NDE	31.71

All values are means (n=4). Values within a column followed by different characters represent significant differences at the 5% level (Duncan's multiple comparison). Ethylene production of discs was not detectable.

Table 6-6. EFE activity of Granny Smith fruit discs treated with 0.5% norbornadine (NDE) and 20% CO₂ (1989).

Treatment	EFE activity (nlC ₂ H ₄ /g/h)	
	60 days	102 days
Control	36.47 ^b	85.75 ^b
CO ₂	48.70 ^a	116.84 ^a
NDE	10.37 ^d	33.77 ^c
CO ₂ + NDE	15.22 ^c	40.85 ^c

All values are means (n=4). Values within a column followed different characters represent significant differences at the 5% level (Duncan's multiple comparison).

6.5 DISCUSSION

It has been reported that there were several cell-free ethylene-forming systems *in vivo* such as peroxidase (Rohwer and Mader, 1981), lipoxygenase (Gardner and Newton, 1987; Nilsen *et al.*, 1988) in higher plants (See Chapter 1). It is essential to test if CO₂ stimulated ACC-dependent ethylene production only through stimulating EFE activity. Lau and Yang (1976) have observed that Co⁺⁺ inhibited all ethylene production systems tested including those of fruit tissues and vegetative tissues treated with IAA, kinetin, IAA plus kinetin, Ca⁺⁺, kinetin plus Ca⁺⁺ or Cu⁺⁺. Yu and Yang (1979) identified that Co⁺⁺ affected ethylene production by inhibiting the conversion of ACC to ethylene (i.e. EFE activity) and suggested that Co⁺⁺ may interact with the sulfhydryl group of the enzyme which is responsible for the oxidation of ACC to ethylene (EFE). Thus Co⁺⁺ was used to test if CO₂ had a direct effect on EFE activity.

~~the~~ In the present results CO₂ stimulated EFE activity in fruit discs of Hosui and Granny Smith, CoCl₂ (1 mM) significantly inhibited EFE activity in both fruit discs, and the combination of CO₂ and Co⁺⁺ was not able to reverse the inhibitory effect of Co⁺⁺ on EFE activity. These indicated that CO₂ stimulated ACC-dependent ethylene production in both fruit discs occurred only through the pathway proposed by Adams and Yang's (1979). That

Co^{++} showed similar effects on EFE activity in discs from both Hosui and Granny Smith fruits indicated that the inhibitory effect of Co^{++} on EFE activity was through the same mechanism. CO_2 did not interact with Co^{++} at the same binding site (sulfhydryl group), thus the stimulatory effect of CO_2 on EFE activity must occur as a result of interaction at a different site and have another mechanism. Because EFE showed stereospecificity towards (1R,2S)-AEC, one of the four stereoisomers of 1-amino-2-ethylcyclopropane-1-carboxylic acid (AEC) (Hoffman *et al.*, 1982), further evidence on the direct stimulatory effect of CO_2 on EFE activity could be obtained by testing specific capability of tissue conversion of AEC to ethylene with CO_2 treatment.

Beyer (1979) suggested that CO_2 increased ethylene production through inhibiting ethylene metabolism. Results from this present work did not support this suggestion because CO_2 treatment did not increase ethylene accumulation in the air of sealed vials after using Co^{++} to block ACC dependent-ethylene production of both fruit discs.

Silver nitrate (1 mM) significantly reduced ethylene production and stimulated the respiration to the maximum rate in both fruit discs. These results confirmed those of Saltveit *et al.* (1978) with post-climacteric apples, but the Ag^+ concentration for getting the maximum respiratory rate was lower (0.1 mM AgNO_3) than in our experiments (0.5-1 mM AgNO_3). These differences could be a result of the senescent tissue being more permeable to AgNO_3 than immature tissue, thus allowing Ag^+ ions easier across to ethylene binding sites. It is also possible that the tissues from different species, cultivars or organs at different ripening stages have ~~had~~ different sensitivities to Ag^+ treatment. Generally, compared with immature tissue, more mature tissues are more sensitive to ethylene and the inhibitors (Yang, 1985).

In this work, AgNO_3 inhibited EFE activity in both Hosui (as a nonclimacteric fruit) and Granny Smith apple (as a climacteric fruit). These results did not agree with the work of Atta-Aly *et al.* (1987) in which Ag^+ stimulated EFE activity in immature tomato fruit and inhibited it in tomato at a stage of response where ethylene was autocatalytic. The present results demonstrated that Ag^+ did not stimulate ethylene production in either types of fruit discs. Thus Ag^+ was not able to block autoinhibition of ethylene

production, if it existed in the Hosui and Granny Smith fruit discs. Because the inhibitory pattern of Ag^+ on EFE activity in Hosui and Granny Smith fruit discs was similar, it is suggested that Ag^+ inhibited EFE activity in non- and climacteric fruits through the same mechanism. McGlasson (1985) and Yang (1987b) stated that ethylene biosynthesis was regulated by the System I receptor in nonclimacteric and immature climacteric fruit tissues, and by System I and System II receptors in mature climacteric fruit tissues. Because ethylene biosynthesis in nonclimacteric fruit is thought to be regulated only by the System I receptor it is logical to assume that EFE is also regulated by the System I ethylene receptor *in vivo*. This is in agreement with Yang's model (1987b) in which EFE is regulated by the System I ethylene receptor, while ACC synthase is regulated by the System II ethylene receptor. Ethylene production was inhibited more by Ag^+ than was EFE activity in discs of Granny Smith fruit at the early climacteric stage. This may indicate that Ag^+ inhibited both ACC synthesis and conversion of ACC to ethylene by inactivating both ethylene System I and System II receptors. The combination of CO_2 and Ag^+ did not reverse the inhibitory effect of Ag^+ on EFE activity and ethylene production. This result at least indicated CO_2 did not compete with Ag^+ for the same binding site on the ethylene receptors.

In this work, CO_2 stimulated EFE activity in discs of Hosui and Granny Smith fruit (Table 6-5 and Table 6-6). NDE significantly inhibited EFE activity. This inhibitory effect of NDE was partially reversed by CO_2 in discs of Hosui fruit, and in discs of Granny Smith fruit stored for 60 days ~~at~~ ^{in the} ~~store~~, but not in fruit that had been stored for 102 days.

Because the same inhibitory pattern of NDE on EFE activity was obtained in discs of both Hosui and Granny Smith fruits, this indicated that NDE probably regulated EFE activity through the same mechanism. Yang (1987a) suggested that NDE competitively inhibits ethylene action through reversibly binding to the same binding site of the receptor as does ethylene. The present work indicates that NDE competes with ethylene to bind to the System I receptor in Hosui fruit (as a nonclimacteric type) and Granny Smith fruit (as a climacteric type) at preclimacteric stage. In this case the combination of CO_2 and NDE could reverse, at least partially, inhibitory effect of NDE on EFE activity in discs of Hosui, and in discs of Granny Smith fruit stored for 60 days. This result indicated that CO_2 can compete with NDE at the same binding site on the ethylene receptor. In Granny Smith fruit

the effects of CO₂ on EFE in NDE-treated fruit discs depended on the stage of fruit ripeness (Table 6-6). When Granny Smith fruit had been in storage for 102 days, CO₂ stimulated EFE activity, but did not reverse the NDE inhibitory effect on EFE activity. This result indicated that CO₂ stimulated EFE activity directly in discs of 102-day Granny Smith fruit, but did not stimulate it indirectly by competitively binding to the ethylene receptor with NDE. That CO₂ lost its capacity to reverse the inhibitory effect of NDE on EFE may be caused by an increased ethylene binding affinity of the receptors developing with time or by an increase in the amount of ethylene receptors occurring in ripening tissue which could make it more sensitive to ethylene (Yang, 1987b). Both NDE and CO₂ are competitive ethylene action inhibitors which bind to the same binding site with ethylene (Yang, 1985 and 1987a), thus the binding interactions between C₂H₄, CO₂ and NDE are complicated. It is possible that CO₂ increased the affinity of ethylene binding to the receptor (Thomas *et al.*, 1984) in Granny Smith fruit stored for 60 days at 1°±1°C, which produced a small amount of ethylene, but did not in 102 days fruit which produced more ethylene than 60 days fruit. CO₂ partially reversed the inhibitory effect of NDE on EFE activity, but did not reverse that of Ag⁺. These results suggest that NDE and Ag⁺ bind at different sites on the ethylene receptor, ~~and~~ thus inhibiting EFE activity.

The mechanism by which ethylene receptors regulated EFE is unknown. Yang (1985) suggested that the silver ion was a noncompetitive ethylene action inhibitor which combined with and modified the receptor molecule thus preventing the primary ethylene-receptor binding; NDE was a competitive ethylene action inhibitor which combined with the receptor at the ethylene binding site to form an inactive NDE-receptor complex. In his model, one or more of the coordination ligands in the receptor site facilitates the binding of ethylene to the receptor, resulting in a biologically active complex. Ag⁺ interacts with these ligands, resulting in a receptor having little capacity to bind ethylene, or in an ethylene-receptor complex which becomes biologically inactive or less active. In this model no suggestion was made to explain how the ethylene receptor regulated relevant enzyme activity and its synthesis. Veen's work (1986) indicated that Ag⁺ was a competitive inhibitor of ethylene action, and he presented a model explaining the interactions between the ethylene receptor and its inhibitors, NDE and Ag⁺; this was described in the introduction to this chapter. His model indicated how the ethylene receptor might regulate the activity of enzymes

involved in ripening and the mechanism by which those inhibitors affect ethylene action. Neither model described any basic differences between the System I and the System II ethylene receptors.

According to Veen's model (1986), these present results can be explained by suggesting that the System I ethylene receptor is composed of a subunit A and the subunit B (in this case, subunit B includes EFE). Binding of ethylene (or CO_2) to subunit A leads to activation of subunit B (EFE). Silver ions binds to the System I ethylene receptor sub-unit B, preventing sub-unit A from activating sub-unit B, thus inhibiting EFE activity. Binding of NDE to sub-unit A does not activate sub-unit A, again preventing activation of sub-unit B, hence inhibiting EFE activity.

EFE activity could be inhibited directly by Co^{++} , and indirectly by Ag^+ and NDE through their binding to the ethylene receptor. Inhibition of EFE activity may be caused by the inactivated enzyme catalytic site losing the capacity to convert ACC partially and the ACC- CO_2 complex completely to ethylene (See Chapter 5).

In this work, results showed that CO_2 stimulated EFE activity in discs of both Hosui and Granny Smith fruits. Co^{++} inhibited EFE activity in both types of fruits disc, and CO_2 was not able to reverse the Co^{++} -inhibitory effect. These results indicated that CO_2 only stimulated ACC-dependent ethylene production (EFE activity) through Adams and Yang's ethylene biosynthesis pathway. CO_2 also was not able to reverse the inhibition of Ag^+ on EFE activity, but it could partially reverse NDE-induced inhibition on EFE in discs of Hosui and of Granny Smith stored for 60 day at coolstore. Thus Ag^+ and NDE probably inhibited EFE activity by inhibiting ethylene action through binding to different binding sites on the ethylene receptors. Both CO_2 and NDE probably compete between molecules as well as with ethylene for the same binding site at the ethylene receptor. Because the inhibitory patterns of Ag^+ or NDE on EFE activity in discs of both fruit types (non- and climacteric fruits) were similar, and only the System I ethylene receptor occurred in nonclimacteric fruit and preclimacteric fruit, it suggests that EFE activity is regulated by the same mechanism: Ag^+ and NDE interact with ethylene on the System I ethylene receptor to form an inactive inhibitor-receptor complex which is biologically inactive or less active.

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CHAPTER 7

FINAL DISCUSSION

The use of a low oxygen and high carbon dioxide atmosphere during CA and MA storage can delay fruit senescence and extend storage life of many different fruits. Short periods of exposure to high CO₂ treatments before storage can delay fruit senescence, improve fruit quality and control insects efficiently in some European pears, such as 'd'Anjou (12% CO₂ for 14 days, Couey and Wright, 1977), Bartlett (20% CO₂ for 4-6 days, Danyang *et al.*, 1990) and Bosc (Chen *et al.*, 1985), and apples, Golden Delicious (Couey and Olsen, 1977) and McIntosh (10-20% CO₂ for 10-15 days) (Bramlage, 1977). So far very little research has been done to investigate the biochemical and physiological mechanisms by which CO₂ delays fruit ripening through its effects on processes, such as respiration rate and ethylene synthesis (Kader, 1986; Kerbel *et al.*, 1988). The known effect of CO₂ is thought to inhibit ethylene action (Burg and Burg, 1967), but recent work has indicated that CO₂ may also inhibit biosynthesis of ethylene in fruit, such as apple (Chaves and Tomas, 1984; Chevery *et al.*, 1988; Looney, 1975), avocado (Marcellin and Chaves, 1983) and tomato (Buescher, 1979; Zamponi *et al.*, 1990).

In higher plants it is known that ethylene synthesis occurs through the Met - SAM - ACC - C₂H₄ pathway (Adams and Yang, 1979; Lüssen *et al.*, 1979). Two enzymes, ACC synthase and EFE catalyze the reaction from SAM to ACC and conversion of ACC to ethylene respectively. Some research has been done in an attempt to understand the effects of CO₂ on ethylene biosynthesis. It has been reported that CO₂ did not change internal ACC content in the fruit of Granny Smith and avocado (Chevery *et al.*, 1988) or tomato (Zamponi *et al.*, 1990). But there have been several studies which report that CO₂ stimulated EFE synthesis and activity in many vegetative tissues, organs and seedlings. Several different results have been reported on the effects of CO₂ on EFE in fruit discs.

Chaves and Tomas (1984) reported that CO₂ increased the ACC content of Granny Smith fruit tissues and concluded that CO₂ inhibited the enzyme responsible for conversion of ACC to ethylene. But their technique

for measuring the conversion of ACC to ethylene used unsaturated ACC concentrations (0.04 and 0.1 mM). Generally EFE activity *in vivo* has been measured by ethylene production from plant tissues saturated with exogenous ACC (Yang and Hoffman, 1984) and thus their results did not really show the full effect of CO₂ on EFE activity.

Cheverry *et al.* (1988) indicated that CO₂ stimulated EFE activity in discs of preclimacteric Granny Smith apple and avocado, but inhibited its synthesis induced by C₂H₄.

Tan and Thimann (1989) investigated the effects of CO₂ on ethylene production in oat leaves, Golden Delicious apples and Anjou pears. Their results indicated that there was a difference between ethylene production from endogenous and exogenous ACC (0.25 mM) sources. They showed that by lowering the CO₂ in the air by 99%, there was a decrease in the ethylene production in fruit discs when exogenous ACC was used, but there was no decrease (in fruit there was even slight increase), in ethylene production when endogenous ACC was used in the same CO₂ environment.

The above reports indicate that there may be several different results in effects of CO₂ on ethylene production or EFE activity and its synthesis in Granny Smith fruit discs : (1) CO₂ inhibited, stimulated or did not change ethylene production from endogenous ACC; (2) adding CO₂ (20%) or reducing endogenous CO₂ decreased ethylene production from unsaturated (0.04-0.25 mM) exogenous ACC; (3) adding CO₂ (20%) stimulated ethylene production from saturated exogenous ACC (2 mM) by stimulating EFE activity, and inhibited EFE synthesis induced by exogenous C₂H₄. No further work was found to clarify these effects of CO₂ on EFE in climacteric fruit tissues, and no similar work using nonclimacteric fruit tissues has been done.

Tissues (discs) may have some differences from whole plant organs, such as fruit and leaves in their physiological behaviour. For example, preparing discs by cutting fruit or leaves can enhance or inhibit ethylene synthesis in wounded citrus peel discs (Riov and Yang, 1982b), citrus leaf discs (Riov and Yang, 1982a) and mung bean hypocotyls (Yoshii and Imaseki, 1982). Some workers have reported that CO₂ might have different effects on whole fruit and fruit tissues. Generally CO₂, an ethylene action

inhibitor, inhibited ethylene synthesis (Burg and Burg, 1967). Zamponi *et al.* (1990) reported that CO₂ has different effects on ethylene production of the whole tomato fruit and fruit discs. CO₂ inhibited ethylene production of the whole fruit, but did not affect it in fruit discs. They suggested that it is possible that the lack of a CO₂ effect on ethylene production in discs was due to a compensation between the decrease in the quantity and an increase of the activity of EFE.

Although differences in response to CO₂ have been found between tissues and whole fruit, excised tissues could still reflect some *in vivo* physiological changes. So far EFE has not been purified from plant tissues and EFE activity has only been measured by the production of ethylene. A lot of information has been obtained on the characteristics of EFE (Yang, 1987b). Thus in this work discs of both Hosui and Granny Smith fruits were used as experimental material after carefully testing techniques of EFE activity measurement.

This thesis investigated the effects of CO₂ on EFE synthesis and its activity in Hosui, a nonclimacteric fruit and Granny Smith, a climacteric fruit. Based on the results obtained, this chapter discusses: the possible direct effects of CO₂ on EFE activity, and the indirect effects of CO₂ on EFE activity and synthesis through interaction with an ethylene receptor; the possible form of EFE responding to CO₂; the interaction between CO₂ and ACC; the effects of interactions between CO₂ and various inhibitors (Co⁺⁺, Ag⁺ and NDE) on EFE activity and its synthesis. A model suggesting a possible mechanism of the CO₂ effects on EFE is presented.

7.1 EFE MAY BE REGULATED BY THE SYSTEM I ETHYLENE RECEPTOR

Based on the knowledge of ethylene biosynthesis and its regulation in climacteric and nonclimacteric fruits, Yang (1987b) presented a model which hypothesized that: the difference between the System I and the System II ethylene synthesis appears to lie in the ability of the tissue to develop ACC synthase, the rate-limited enzyme in the ethylene biosynthesis pathway; EFE is regulated by the System I ethylene receptor, and ACC synthase is regulated by the System II ethylene receptor. However Yang's model does not indicate: (1) the nature of the different characteristics of the System I and

the System II ethylene receptors; (2) whether the regulation of EFE by the System I receptor in nonclimacteric and climacteric fruits is exactly the same mechanism; (3) whether there are multiple forms of EFE and whether they are regulated in different ways; (4) whether and how the System I receptor regulates EFE synthesis or its activity; (5) whether autoinhibition of ethylene synthesis in climacteric fruit is regulated by one or both of the ethylene receptors.

One of the aim^s of the present work was to investigate the effects of CO₂ on EFE activity and its synthesis in two different fruit types (nonclimacteric and climacteric) for determining if EFE development is regulated by the same mechanism i.e. by the System I receptor as suggested by Yang (1987b), and to determine if there was a difference in EFE regulation by the System I receptor in nonclimacteric and climacteric fruits.

Ethylene has been shown to stimulate the conversion of ACC to ethylene in excised tissues of preclimacteric cantaloupe fruit (Hoffman and Yang, 1982), citrus and tobacco leaves (Chalutz *et al.*, 1984; Riov and Yang, 1982b). Liu *et al.* (1985a) found that in preclimacteric tomato and cantaloupe fruits (unripe), ethylene accelerated EFE development, but not ACC synthase. Thus in preclimacteric stage fruit, ethylene inhibits ethylene synthesis by autoinhibition through repression of the synthesis of ACC synthase and inhibition of its activity, and simultaneously stimulates EFE development. Liu *et al.* (1985a) also reported that ethylene induced EFE development in *rin* and *nor* tomato fruits (which are classified as nonclimacteric fruits) though the magnitude of promotion was not as great as in the normal tomato. These results suggested that EFE development in preclimacteric fruit and in nonclimacteric fruit was regulated by the same mechanism. Thus Yang (1987b) suggested that EFE is regulated by the System I receptor in both climacteric and nonclimacteric fruits.

7.1.1 Hosui is a nonclimacteric fruit in New Zealand

Respiration rate of Hosui fruit increased following propylene treatment and returned to normal rates upon removal of the propylene. Ethylene production was barely detectable at a range of maturities and did not increase after harvest, and it was not induced by exposure to propylene. Ethylene production of Hosui fruit discs was also undetectable. These

results suggested that Hosui fruit grown in New Zealand is nonclimacteric. By comparing the effects of CO₂ on EFE in a nonclimacteric (Hosui) and climacteric (Granny Smith) fruit it should be possible to determine whether the regulation of EFE by the System I ethylene receptor has the same mechanism in both types of fruit.

7.1.2 EFE development in fruit stored at low temperature

Because EFE activity was measured *in vivo* from fruit that had been in storage at 1°±1°C for long periods of time, it was not possible to determine if the measurement changes in EFE resulted from increased synthesis or activity (or both) of the enzyme. Thus results from such experiments probably reflected a combination of EFE synthesis and activity changes during storage.

7.1.2.1 EFE development in Hosui fruit

During cool storage, EFE in Hosui developed during the time fruit was in storage at 1°±1°C, but ethylene production of fruit was not detectable or was only present in trace amounts, during the storage (more than 150 days at 1°±1°C).

Because Hosui is a nonclimacteric fruit, and because only the System I ethylene receptor is thought to exist in nonclimacteric fruit (McMurchie *et al.*, 1972), it is possible that EFE development in Hosui is regulated by the System I ethylene receptor. Ethylene could bind with the binding site of the System I ethylene receptor to regulate EFE development. Because ethylene production in Hosui was very low, it is likely that System I ethylene receptor has a very high affinity for trace amount ethylene.

The effects of ethylene on ripening in nonclimacteric fruit are not clear. McMurchie *et al.*(1972) and McGlasson (1985) suggested that the System I ethylene system is involved in the regulation of the aging process and is responsible for the low rate of ethylene production during growth, and that the System II ethylene system is responsible for the autocatalytic increase in ethylene production which accompanies ripening. McGlasson (1985) suggested that nonclimacteric fruit have System I but not System II. Yang (1987b) also indicated that ethylene does not affect the ripening

processes in nonclimacteric fruit. But Cohen (1978) reported that ethylene treatment could accelerate skin colour change from green to yellow in citrus fruit (a nonclimacteric fruit) without changing any other ripening processes. No results were presented to indicate whether ethylene accelerated this colour change in nonclimacteric fruit directly by binding with the System I ethylene receptor, or affected it indirectly by influencing fruit ripening through stimulation of fruit metabolism (eg. respiration). No information was found on the effects of ethylene on Hosui fruit ripening. Thus the question arises as to what is the significance of EFE development in nonclimacteric fruit. Several possible explanations can be suggested.

It is possible that EFE does have some, as yet unidentified, role in the regulation of ripening of nonclimacteric fruit. Recently Hartmann (1989) found that changes in ethylene production, ACC and MACC levels occurred during ripening in the nonclimacteric cherry fruit which were similar to those found in climacteric fruit. He suggested that the ripening of the nonclimacteric cherry fruit may be linked with ethylene, thus EFE might have some role in ripening of nonclimacteric fruit, but this role was not defined.

Another possible explanation for the presence of EFE in nonclimacteric fruit is that for ethylene to be produced as a result of various stresses, such as wounding, physical damage, drought, water-logging (which mainly affect induction of ACC synthase (Abeles, 1973; Yang and Hoffman, 1984)), development of EFE is necessary to convert ACC to ethylene.

For fruit attached to the tree, ACC could be synthesized in other organs and transferred in the xylem to the fruit (Osborne, 1989). Hosui fruit had EFE activity at harvest time. Thus EFE development in attached fruit may be responsible for conversion of ACC, synthesized in other organs, to ethylene in the fruit, which may be an important factor involved in fruit growth (McGlasson, 1985).

7.1.2.2 EFE development in Granny Smith fruit

It has been shown that EFE was able to develop in Granny Smith fruit stored at $1^{\circ}\pm 1^{\circ}\text{C}$, and that it had a similar developmental pattern to that of Hosui. This might indicate that EFE development in both types of fruit was

regulated by the same mechanism or the same ethylene receptor. Because only System I ethylene receptor existed in nonclimacteric fruit, it is likely that the System I receptor regulated EFE development in both nonclimacteric and climacteric fruits.

Granny Smith apples picked at different dates in 1989 showed neither ethylene production nor EFE activity at harvest time. Fruit harvested on 25 April, 1989 showed no respiration peak and no ethylene developed during 25 days at 21°C. However fruit harvested on the same date and then stored at 1°±1°C for 20 days, showed rapid ethylene production when transferred to 21°C. This behaviour was similar to that shown by other pears and apples. In the European pear variety, Conference, and at least one apple variety, Golden Delicious, rapid ethylene production occurred over a shorter period and in a more uniform manner after two periods of 48 hours exposure to 3°C than it did at the higher temperature of 18°-20°C (Knee *et al.*, 1983). Blankenship and Richardson (1985) demonstrated that the capacity to convert ACC to ethylene in 'd'Anjou pear developed at least 4 days earlier than the increase in ACC synthase that occurred at 20°C, after fruit has been stored for 41 and 50 days at -1°C. Because the rate of synthesis of ACC synthase activity increased more rapidly in discs from pears held at -1°C than from pears immediately after harvest, and because cycloheximide (CHI) inhibited these increases and reversed increases resulting from pre-incubation of discs with CHI, Knee (1987) concluded that the mRNA for ACC synthase was formed at -1°C. The mechanism of low temperature stimulated synthesis of ethylene biosynthesis enzymes remains unknown. It is possible that low temperatures stimulate the synthesis of ethylene receptors, leading to the induction of EFE synthesis. Results showed that low temperature could induce ethylene production (caused by the development of ACC synthase and EFE) in Granny Smith (a climacteric fruit). This has potential for accelerating fruit ripening for commercial purposes.

7.2 THE EFFECT OF CARBON DIOXIDE ON EFE BIOSYNTHESIS

7.2.1 Carbon dioxide stimulates EFE biosynthesis

Carbon dioxide stimulates EFE synthesis in some vegetative tissues (Aharoni and Lieberman, 1979b; Horton and Saville, 1984; Kao and Yang, 1982; Philosoph-Hadas *et al.*, 1986). High CO₂ levels inhibited the induction

of both ACC synthase and EFE in Golden Delicious apple fruit (Bufler, 1984; 1986), preclimacteric tomatoes (Liu *et al.*, 1985a; Zamponi *et al.*, 1990), Granny Smith apple and avocado (Cheverry *et al.*, 1988). In fruit discs different results were found. Cheverry *et al.* (1988) reported that EFE induction by ethylene in apple and avocado discs was inhibited by CO₂, but the EFE activity was increased by it. In the present work the stimulatory effect of CO₂ on EFE synthesis was found in preclimacteric Granny Smith apple without pre-treatment with ethylene, but not in Hosui. Granny Smith fruit were tested at the preclimacteric stage because they did not produce detectable ethylene and the EFE activity was not detectable. At this ripening stage, fruit ethylene synthesis was probably regulated by the System I ethylene receptor (Yang, 1987b). After CO₂ treatment, EFE activity appeared, indicating that EFE synthesis was stimulated by CO₂, perhaps through interacting with the System I receptor. It is possible that at this ripening stage CO₂ might be a structural analogue of C₂H₄ which could combine with the ethylene binding site on the System I receptor and stimulate ethylene synthesis. Alternatively CO₂ might increase the affinity of ethylene to bind to the ethylene receptor (Thomas *et al.*, 1984). In other case it is possible that the ethylene receptor, which was previously inactive or blocked, became activated, resulting in the stimulation of EFE synthesis.

7.2.2 The possible mechanism of the stimulatory effect of carbon dioxide on EFE biosynthesis

Sisler *et al.* (1983) suggested that the mode of ethylene action in plant cells involved enzyme synthesis. Based on other plant and animal hormonal responses, they presented a simplified hypothetical model of the mode of action of C₂H₄ in plant cells, which suggested that the ethylene molecule attached to a specific binding site on the ethylene receptor. This interaction released a 'second message', a molecule which itself, or through the mediation of subsequent messenger molecules, resulted in transcription of new mRNA material from the gene. The proteins coded by this RNA were the enzymes which caused the symptoms of ethylene treatment. Several pieces of evidence support this model. Cycloheximide (CHI), an inhibitor of protein synthesis on 80-S ribosomes, inhibited the effects of ethylene (Frenkel *et al.*, 1968), and a number of plant tissues appeared to contain a specific ethylene binding site (Sisler, 1979; Bengochea *et al.*, 1980a,b).

However this model (Sisler *et al.*, 1983) did not attempt to distinguish between the differences of the functions of System I and System II ethylene receptors.

The present work suggests that CO₂ does stimulate EFE synthesis through the System I receptor in preclimacteric Granny Smith fruit, but not in Hosui nor in ripe Granny Smith fruit. But Yang (1987b) did not mention any difference between System I receptors in nonclimacteric and climacteric fruits. This difference could be interpreted by suggesting that System I ethylene receptors have different functions, firstly regulation of EFE activity, and secondly regulation of its synthesis. If this were so, C₂H₄ would bind separately to each of these two types of System I ethylene receptors to stimulate EFE activity and synthesis. In preclimacteric Granny Smith fruit, CO₂ and ethylene had similar effects on promoting both EFE activity and synthesis, it might mean that CO₂ could bind with both of the System I ethylene receptors.

Of course, it is possible that the stimulatory effect of CO₂ on EFE synthesis in Granny Smith apple might be through other mechanisms which do not involve the ethylene receptor. CO₂ may indirectly stimulate EFE synthesis by changing intracellular pH or some other protein synthesis regulators. Perhaps different forms of EFE occur in different species, which are regulated by different receptors. In this case CO₂ might stimulate synthesis of one of the EFE forms which does not occur in Hosui and ripe Granny Smith fruit.

7.3 THE EFFECT OF CARBON DIOXIDE ON EFE ACTIVITY

Carbon dioxide might stimulate EFE activity directly by binding to the EFE binding site, or indirectly by interaction with the System I ethylene receptor which regulates EFE activity.

7.3.1 The direct stimulatory effect of carbon dioxide on EFE activity

The main stimulatory effect of CO₂ on EFE activity was caused by directly binding of CO₂ to the EFE binding site in discs of Granny Smith apple at all stages tested and unripe Hosui.

Table. Characteristics of ethylene biosynthesis in Hosui and Granny Smith apple fruits.

	Non-climacteric Hosui (unripe)		Climacteric apple (unripe) (ripening)
(A) C ₂ H ₄ or C ₃ H ₆ effect on respiration	CO ₂ ↑ while C ₂ H ₄ or C ₃ H ₆ present	—	Permanent rise No effect
(B) C ₂ H ₄ production	Low		Low High
C ₂ H ₄ or C ₃ H ₆ effect on C ₂ H ₄ production	No effect		↑ No effect
(C) Endogenous C ₂ H ₄ level	Low		Low High
(D) ACC	Very low		Very low High
ACC synthase	Very low		Low High
(E) EFE	Low		Absent High
EFE after low temperature storage	↑		↑
CO ₂ effect on EFE activity in short storage	↑↑		↑↑ ↑
after long storage	No effect		↑ ↑
CO ₂ effect on EFE biosynthesis	No effect		↑ No effect
(F) C ₂ H ₄ receptor	System I		System I System I & II

Bengochea *et al.* (1980a,b), and Thomas and Yang (1987) indicated that enzyme preparations such as IAA oxidase, peroxidase, lipoxygenase, pea stem homogenates and microsomal preparations were able to convert ACC to ethylene although these enzymes display high K_m values for ACC, from 3 to 389 mM. The present results showed that CO_2 directly stimulated EFE activity and this stimulatory effect of CO_2 on EFE activity was dependent on exogenous ACC when the EFE activity assay was carried out in the presence of CHI (which inhibited possible EFE biosynthesis during incubation) and high (1-5 mM) ACC concentrations. Thus it was necessary to test whether CO_2 stimulated ethylene production through other pathways. The Co^{++} ion which is known to act as an EFE activity inhibitor (Yu and Yang, 1979), completely blocked the stimulatory effect of CO_2 . Enzyme kinetics results showed that CO_2 significantly increased the maximum reaction rate of EFE without changing the K_m for ACC. This suggests that CO_2 stimulated EFE activity through the formation of an CO_2 -EFE-ACC complex and/or EFE-ACC- CO_2 complex in Hosui and Granny Smith fruits (See 'Chapter 5').

7.3.2 Carbon dioxide may stimulate EFE located at plasma membrane

Recently, Pech *et al.* (1989) and Bouzayen *et al.* (1990) demonstrated *in vitro* cultured cells of *Vitis vinifera* L. cv. Muscat and in leaf tissues of *Hordeum vulgare* L. and *Triticum aestivum* L. that there were probably two sites of ethylene production: (1) An external site located at the plasma membrane, capable of converting apoplastic ACC to ethylene, which was very sensitive to high osmotica (0.8 M mannitol); and (2) An intracellular site, possibly located in the tonoplast, which was capable of converting internal ACC to ethylene and which remained unaffected even under severe plasmolysis. In other grape cells cultured *in vitro* (*Vitis vinifera* L. cv. Gamay) and in pea leaves (*Pisum sativum* L.), only the intracellular site was thought to operate as ethylene production was almost unaffected by plasmolysis. Thus they concluded that in cells sensitive to osmotica, ethylene forming activity is mainly located at the plasmalemma with a small activity inside the cell probably at the tonoplast. In osmoticum insensitive cells, the bulk of ethylene production is intracellular.

In this work, mannitol which cannot, or can only slowly, penetrate into the cell (Berliner and Martindale, 1981; Evans and Ting, 1973; Ruesink, 1978), stimulated EFE activity in Hosui and Granny Smith fruit discs when treated at an isotonic concentration (0.4 M). This suggests that mannitol stimulated EFE located at the plasma membrane. In discs of both fruit, EFE activity was higher in 0.8 M mannitol solution than in 0.4 M, but the tissue lost its capacity to respond to CO₂ at the high mannitol concentration. It could mean that activity of EFE, located at plasma membrane, is only stimulated by CO₂ when in an isotonic solution. Pech *et al.* (1989) and Bouzayen *et al.* (1990) concluded that the EFE located in the plasma membrane was dependent on ACC present in the apoplast. Because discs from Granny Smith produced ethylene without the addition of exogenous ACC, it is possible that in this fruit another form of EFE is located at the tonoplast, which mainly converted endogenous ACC (stored in the vacuole) to ethylene (Pech *et al.*, 1989).

The isotonic potentials varied with species. The isotonic potentials of mannitol solution was shown to be 0.35-0.4 M for Granny Smith apple flesh tissues (with no significant changes occurring during ripening) (Harker, 1986), 0.7 M for Kiwifruit (John *et al.*, 1989) and 0.7-0.9 M for Delicious apple (Mattoo and Lieberman, 1977). The effects of high osmotic potentials on EFE activity also varied by either inhibiting or not affecting EFE activity; this effect would depend on whether EFE was located in plasma membrane or tonoplast (Pech *et al.*, 1989). John *et al.* (1989) reported that the maximum EFE activity in kiwifruit membranes was observed when sucrose, mannitol and sorbitol were present in the medium at 0.7 M or at higher concentrations. Indeed their data showed that the maximum EFE activity was obtained at 1.0 M mannitol. They also reported that the EFE activity was completely lost when the osmotic support was removed.

Although the mechanism by which high osmotica stimulate EFE activity is unknown (John *et al.*, 1989), the explanation could be that some sugars, including mannitol, can stimulate EFE activity (see 'Chapter 3'). Thus the higher apparent EFE activity obtained in 0.8 M mannitol might be the consequence of the stimulatory effect of 0.8 M mannitol on EFE activity being greater in discs of both Hosui and Granny Smith fruits than the inhibitory effect that occurred at this higher (0.8 M) osmotic potential.

7.3.3 Carbon dioxide may stimulate EFE activity through altering the nature of the ethylene receptor.

Libbenga and Mennes (1987) reviewed hormone binding and its role in hormone action. They indicated that in hormonal systems, cells of different tissues and organs not only transmit signals, but they are also capable of detecting signals which they receive from other parts of the system, and responding to those signals in their own characteristic way. Receptor activation triggers a molecular program which is simply a direct activation of a distinct set of enzymes. The normal experience with animal hormones is that receptors are confined to specific target organ. It might be anticipated that receptors for plant hormones would be localized in hormone-responsive tissues, hence governing tissue sensitivity (Venis, 1985). Libbenga and Mennes (1987) also indicated that, in general, chemical signals ultimately influence target cells, either by altering the activities or rates of synthesis of existing proteins or by altering the synthesis of new ones. If the set of responsive enzymes is different in another type of target cell, then the same signal elicits a different response *via* a similar receptor-and-transduction chain.

In plant/fruit systems, plant hormones (the present work, ethylene) could bind to their receptors (in this case, the System I receptor) governing tissue responses (in this case, ethylene biosynthesis). Chemical signals (in this case, CO₂) could influence target cells (in Hosui and Granny Smith fruits) either by altering the activity or rates of synthesis of existing proteins or by altering the synthesis of new ones (in this case, EFE).

Veen (1986) presented a theoretical model to explain the anti-ethylene effects of silver thiosulphate and 2,5-norbornadiene. This model suggested how the receptors regulate ripening enzyme activity and possible mechanisms of ethylene-action inhibitors, but it did not give any explanation for distinguishing between System I and System II ethylene receptors.

Silver ions and NDE are ethylene action inhibitors which inhibit ethylene action by binding at the ethylene receptor. In this work, using the nonclimacteric fruit, Hosui (in which only the System I ethylene receptor is thought to occur); EFE lost the capacity to respond to CO₂ in the presence of Ag⁺. This suggested that Ag⁺ ions in some way inhibits the System I receptor which in turn regulates EFE activity.

It has been reported that NDE is a competitive inhibitor of autocatalytic ethylene synthesis (Sisler and Yang, 1984) and ethylene action (Yang, 1985; Yang and Hoffman, 1984). Cycloheximide, a protein synthesis inhibitor, inhibited the conversion of ACC to ethylene in mung bean hypocotyls (Yu *et al.*, 1979), in preclimacteric cantaloupe tissue (Hoffman and Yang, 1982) and in flavedo and albedo tissues of citrus fruits (Riov and Yang, 1982b). In discs of Hosui and preclimacteric Granny Smith fruit, the combination of NDE and CHI inhibited EFE activity, and its inhibitory effect could be partially reversed by CO₂. In Granny Smith, when fruit was ripe, CO₂ could no longer reverse NDE inhibitory effect on EFE activity. Because EFE synthesis was inhibited by CHI, these experiments only measured the effect of CO₂ on EFE activity. NDE, an ethylene action inhibitor, inhibited EFE activity by binding to the System I receptor at the ethylene binding site, suggesting that at this more advanced ripening stage, the System I ethylene receptor regulates EFE activity. It has been reported that CO₂ might competitively bind to ethylene receptor with NDE at the ethylene binding site (Sisler and Yang, 1984). A combination of NDE and CO₂ reversed the inhibitory effect of NDE on EFE activity suggesting that CO₂ might reverse the inhibitory effect of NDE on EFE activity by binding to System I ethylene receptor at the ethylene binding site. When Granny Smith was ripe CO₂ still directly stimulated EFE activity, but lost its capability to stimulate EFE indirectly by binding to the System I receptor.

In the present work, EFE developed during coolstorage at $1^{\circ}\pm 1^{\circ}\text{C}$ in both Hosui and Granny Smith fruit. The response of EFE to CO₂ in Hosui and Granny Smith fruit discs were different; CO₂ stimulated EFE activity and its synthesis in preclimacteric Granny Smith fruit discs, but it only stimulated EFE activity in discs of more ripe Granny Smith and Hosui fruits. These results indicated that EFE was able to develop during storage in Hosui and Granny Smith. The difference between the nonclimacteric and climacteric fruits was their response to CO₂. The following hypothesis is presented to explain the above results:

EFE was measured in fruit tissue which consisted of very uniform cells in both Hosui and Granny Smith fruits. EFE is capable of developing in both fruits, but there is a difference in response to CO₂ between Hosui and preclimacteric Granny Smith fruits, and between Granny Smith fruit at different ripening stages. It is suggested that EFE is regulated by the same receptor (System I ethylene receptor) but that ethylene receptor has different functions which regulates EFE synthesis and EFE activity separately.

It is suggested that there are two different forms of System I receptors, System I-A₁ ethylene receptor which regulates EFE biosynthesis, and System I-A₂ ethylene receptor which regulates EFE activity, both of which are thought to be located in the same target cell. Unripe Granny Smith fruit and Hosui fruit possess both System I-A₁ and System I-A₂ receptors. Ethylene could bind with both of them to stimulate EFE synthesis and activity. CO₂ could combine with System I-A₁ receptor in preclimacteric Granny Smith fruit to form an activated receptor which stimulates EFE synthesis. CO₂ can bind to System I-A₂ receptor in both preclimacteric Granny Smith and unripe Hosui fruits, but not from ripe Hosui and Granny Smith fruits, to stimulate EFE activity (Table 7-1).

Table 7-1. Summary of effects of CO₂ on EFE synthesis and its activity.

Effects of CO ₂	Hosui		Granny Smith	
	Unripe	Ripe	Preclimacteric	Ripe
Direct effect:	+	-	+	+
Indirect effect:				
System I-A ₁	-	-	+	-
System I-A ₂	+	-	+	-
Type number of cell	1	2	3	4

*Direct effect means that CO₂ directly affected EFE activity.

**Indirect effects mean that CO₂ regulated EFE activity or synthesis through binding with ethylene receptors, in which the System I-A₁ regulates EFE synthesis, and the System I-A₂ regulates EFE activity.

*** '+' and '-' represent CO₂ stimulatory effect and no effect on EFE respectively.

****Type number of cell: See text.

Osborne (1989) has indicated that there are numbers of target cells in plants where ethylene can induce specific effects. It is possible that even within uniform tissue, such as that found in the fruit ^{cortex} cortical, there may be cells, or organs within cells, which may respond differently and selectively to ethylene, or treatments which influenced ethylene action. If it is suggested that there are 4 types of cells, or regions in cells, in fruit cortical tissue, then the above results could be explained according to their response to CO₂ and the mechanisms of the responses:

Type 1 cell: (to be found in unripe Hosui fruit).

CO₂ stimulated EFE activity directly by interacting with the EFE binding site;
 CO₂ did not stimulate EFE biosynthesis indirectly through interacting with the System I-A₁ ethylene receptor;
 CO₂ stimulated EFE activity indirectly through interacting with the System I-A₂ ethylene receptor;

Type 2 cell: (to be found in ripe Hosui fruit).

CO₂ did not stimulate EFE activity directly by interacting with the EFE binding site;
 CO₂ did not stimulate EFE biosynthesis indirectly through interacting with the System I-A₁ ethylene receptor;
 CO₂ did not stimulate EFE activity indirectly through interacting with the System I-A₂ ethylene receptor;

Type 3 cell: (to be found in preclimacteric Granny Smith fruit).

CO₂ stimulated EFE activity directly by interacting with the EFE binding site;
 CO₂ stimulated EFE biosynthesis indirectly through interacting with the System I-A₁ ethylene receptor;
 CO₂ stimulated EFE activity indirectly through interacting with the System I-A₂ ethylene receptor;

Type 4 cell: (to be found in climacteric or in ripe Granny Smith fruit).

CO₂ stimulated EFE activity directly by interacting with the EFE binding site;
 CO₂ did not stimulate EFE biosynthesis indirectly through interacting with the System I-A₁ ethylene receptor;
 CO₂ did not stimulate EFE activity indirectly through interacting with the System I-A₂ ethylene receptor;

Hosui fruit may have Type 1 and 2 cells. When fruit develops from unripe to ripe, the type of cell changes from Type 1 to 2. Granny Smith fruit may have Type 3 and 4 cells. Fruit at the preclimacteric stage have Type 3 cells, while fruit at all climacteric stages have Type 4 cells.

It is possible that the types of cells described are morphologically distinct. However it is more likely that these types as described represent regions of the cells which change in their responsiveness to CO₂ with development of maturation and ripening.

7.4 A POSSIBLE MODEL OF THE REGULATION OF EFE BY CARBON DIOXIDE

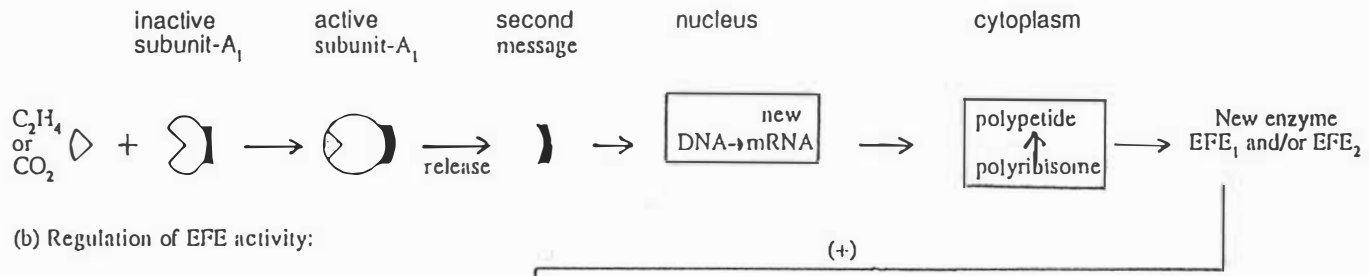
The following model (Fig.7-1), derived from the above suggestions, provides an explanation of the mechanism of regulation of EFE by CO₂.

This model suggests that EFE exists in two forms, EFE₁, located at the plasma membrane and EFE₂, located at the tonoplast. EFE₁ is thought to be the main form responding to CO₂ treatment.

The results from the EFE kinetics experiment indicated that CO₂ might stimulate EFE₁ activity directly by forming a CO₂-EFE₁-ACC complex and/or EFE₁-ACC-CO₂ complex which increases the reaction rate of ACC conversion to ethylene.

EFE is thought to be regulated by the System I ethylene receptor. CO₂ may stimulate EFE synthesis and activity by binding with the System I receptor. The effects of CO₂ on EFE varied, depending on the types of cell in the fruits at a given stage of development (Table 7-1). The System I ethylene receptor may occur in at least two forms which have different structures and the different functions. The System I-A₁ receptor contains the subunit-A₁ and a second message. This complex is responsible for initiating EFE synthesis by interacting with the nuclear DNA to produce mRNA which codes for the production of new EFE located at either the plasma membrane or the tonoplast. The System I-A₂ receptor consists of subunit A₂ together with another subunit B-EFE1 or subunit B-EFE2 depending on whether the System I-A₂ receptor is located at the plasma membrane. Both subunit A₂ and subunit B together *are thought to be necessary* and regard to regulate EFE1 and/or EFE2 activity. Both System I-A₁ and System I-A₂ receptors could be located in the same target cell.

(a) Regulation of EFE biosynthesis:



(b) Regulation of EFE activity:

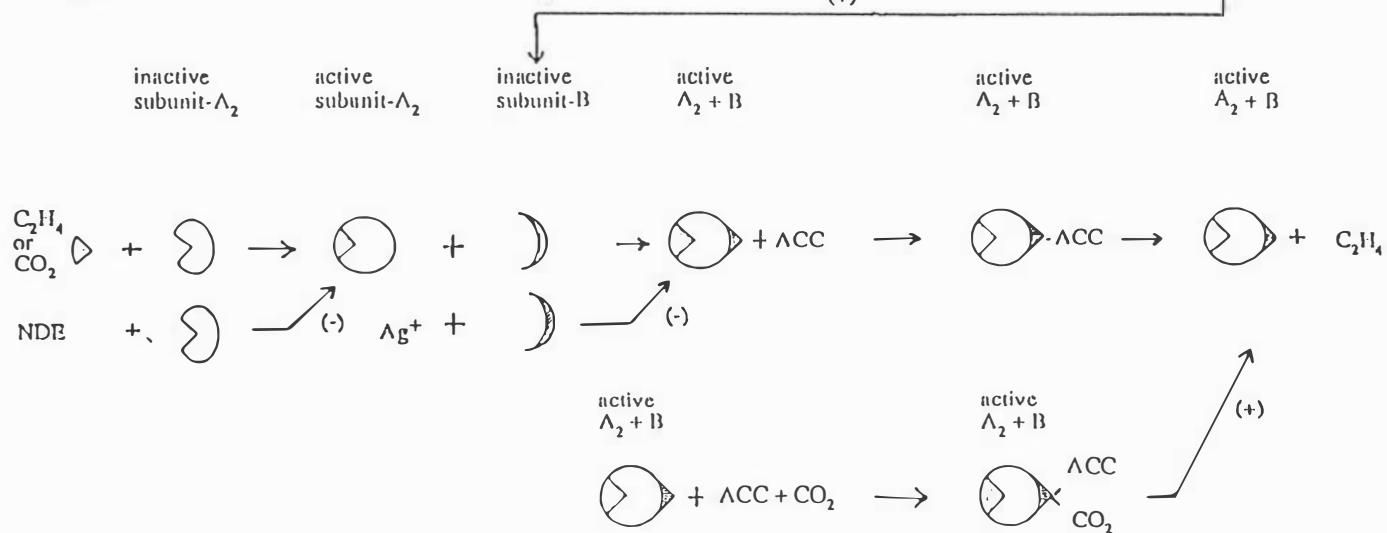


Fig. 7-1 Model outlining possible mechanisms by which CO_2 regulates the biosynthesis and regulation of the EFE located at the plasma membrane. EFE₁ located at plasma membrane; EFE₂ located at tonoplast:
 System I- A_1 ethylene receptor = subunit- A_1 + second message for EFE biosynthesis:
 System I- A_2 ethylene receptor = subunit- A_2 + subunit B+EFE₁;
 (+) = stimulation; (-) = inhibition.

In preclimacteric Granny Smith fruit (Type 3 cell), CO₂ could bind to subunit-A₁ changing it from an inactive to an active conformation. This could release a second message which is necessary for the synthesis of EFE₁ and/or EFE₂. When the fruit is ripe (Type 4 cell), the System I-A₁ receptor becomes more specific (ie. only responds to C₂H₄, but not CO₂). Thus the stimulatory effect of CO₂ on EFE synthesis in ripe Granny Smith fruit disappears. In Hosui fruit (Type 1 and 2 cells) CO₂ does not bind to the System I-A₁ receptor at any stage of development. Thus CO₂ does not stimulate EFE synthesis in Hosui.

In unripe Hosui fruit (Type 1 cell) and preclimacteric Granny Smith (Type 3 cell), CO₂ could bind to the subunit-A₂ of System I-A₂ receptor at the ethylene binding site, changing it from an inactive to an active form. The active subunit A₂ could then combine with the subunit B-EFE₁ to form active subunit B-EFE₁ which catalyzes ACC conversion to ethylene. When Hosui (Type 2 cell) and Granny Smith (Type 4 cell) tissues become more mature or ripe, CO₂ can not bind to the subunit-A₂ of System I-A₂ receptor and thus can not stimulate EFE activity indirectly.

Inhibitors of ethylene action, such as NDE and Ag⁺, are thought to act at the different binding sites of the ethylene receptor to inhibit ethylene action. *The present work only to investigate the inhibitory effects of these inhibitors of only* In this work only to investigate the inhibitory effects of these inhibitors on EFE activity, but not on EFE synthesis. In this model proposed, it suggests that NDE binds at the ethylene binding site of subunit-A₂ of the System I-A₂ receptor, preventing activation of subunit-A₂. Silver ions bind to subunit B-EFE₁ (and B-EFE₂) of the System I-A₂ receptor to prevent EFE becoming the active form. In both cases, EFE₁ activity was inhibited. Because ethylene action inhibitors inhibited reversibly, EFE activity still existed partially. In all Types of cell, CO₂ did not reverse the inhibitory effect of Ag⁺ on EFE activity because it did not bind to the same binding site as Ag⁺. In Type 1 and 3 cells which produced trace amount of ethylene, CO₂ competed more successfully than NDE for the ethylene binding site and stimulates EFE activity. Thus CO₂ can reverse the inhibitory effect of NDE on EFE. In Type 2 and Type 4 cells, which produced more endogenous ethylene than Type 2 or 3 cells, CO₂ could not stimulate EFE activity through binding to the System I-A₂ receptor. It also lost its capability to compete with NDE to bind to the System I-A₂ receptor, thus could not stimulate EFE activity indirectly.

7.5 FINAL CONCLUSION

To understand the effects of CO₂ on EFE and its regulation, two pome fruits, Hosui Japanese pear and Granny Smith apple were selected as experimental material. Their ripening patterns and characteristics were tested. Results showed that Hosui grown in New Zealand is a nonclimacteric fruit, and that the Granny Smith apple is a climacteric fruit. Granny Smith fruit showed some ripening patterns which were similar to those European pears. Fruit stored at low temperature developed the capacity for rapid ethylene production much more than fruit stored at a higher temperature.

After comparing varied assay methods of EFE activity done by other workers, a reliable method for testing EFE activity in Hosui and Granny Smith fruit was developed, which was that fruit discs were incubated for 2 hours, vacuum-infiltrated ^{with} intermitted, or vacuum-infiltrated 1 to 3 minutes with isotonic buffer or 0.4 M mannitol solution (pH 6.5) containing 3-5 mM ACC. Discs were then dried on paper tissues and sealed in 36 ml vial at 27°C for 0.5-1 hours before taking gas sample for measurement of ethylene production. Cycloheximide (1 mM) was added into the tested medium to distinguish the effects of treatments on EFE activity and synthesis.

EFE and its regulation in both types of fruit showed some similar characteristics:

EFE was capable of developing in both fruits stored at low temperature, and showed similar developmental patterns;

Carbon dioxide stimulated EFE activity in discs of unripe Hosui, and Granny Smith fruit during the period tested. Co⁺⁺ inhibited EFE activity. CO₂ did not reverse this inhibition indicating that CO₂ had a direct stimulatory effect on EFE activity. The mechanism of this effect was that CO₂ increased the maximum reaction rate of conversion of ACC to ethylene without changing the K_m of EFE for ACC. It might have been caused by CO₂ binding to EFE to form a CO₂-EFE-ACC or EFE-ACC-CO₂ complex.

EFE activity was greater when measured in a high osmoticum medium (0.8 M) than in isotonic solution, but EFE lost the capability to respond to CO₂ in the higher osmoticum. The stimulatory effect of CO₂ on EFE activity was dependent on exogenous ACC. These results indicated that CO₂ stimulated activity of the EFE probably located at the plasma membrane rather than EFE located in the tonoplast.

Silver ions and NDE are both ethylene action inhibitors. Their inhibitory effect on EFE activity in both fruit discs, presumably by binding to the ethylene receptor, indicated that EFE activity is regulated by the ethylene receptor.

Carbon dioxide did not reverse the inhibitory effect of Ag⁺ on EFE activity in the unripe preclimacteric Granny Smith fruit. This showed that CO₂ did not bind competitively to the ethylene receptor at the same binding site as Ag⁺.

Carbon dioxide could partially reverse the inhibitory effect of NDE (a competitive ethylene action inhibitor) on EFE activity in unripe and the preclimacteric Granny Smith fruit. It indicated that CO₂ had an indirect stimulatory effect on EFE activity by competitively binding to the ethylene receptor at the ethylene binding site. Because it has been suggested that in nonclimacteric fruit only the System I ethylene receptor regulates ethylene synthesis, CO₂ may stimulate EFE activity indirectly through interacting with the System I ethylene receptor at the ethylene (or NDE) binding site.

In ripe Hosui fruit CO₂ did not induce either a direct or an indirect stimulatory effect on EFE activity. In ripe Granny Smith fruit CO₂ had a direct stimulatory effect on EFE activity, but it no longer had its indirect stimulatory effect. Thus when both fruits were ripe, CO₂ did not indirectly stimulate EFE activity by interacting with the System I ethylene receptor.

These results agree with the idea that EFE in both nonclimacteric and climacteric fruits is regulated by the System I ethylene receptor as proposed by Yang (1987b) who developed a model showing the sequence of ethylene action on the regulation of ethylene biosynthesis during fruit maturation and ripening. Additional results have been presented to show the possible mechanisms of the direct and indirect effects of CO₂ on EFE activity.

In this work different effects of CO₂ on EFE in two types of fruit were found. Firstly, CO₂ stimulated EFE biosynthesis in preclimacteric Granny Smith fruit, but not in Hosui, thus the System I ethylene receptor had different responses to CO₂. CO₂ could bind to the ethylene binding site of the System I receptor which was responsible for EFE synthesis in preclimacteric Granny Smith fruit, but not in Hosui, a nonclimacteric fruit. Thus according to the responses to CO₂ System I ethylene receptor has at least two different forms. CO₂ could stimulate EFE synthesis through binding to a System I-A₁ receptor, and CO₂ could indirectly stimulate EFE activity by binding to the System I-A₂ receptor. Secondly, CO₂ stimulated EFE activity directly in preclimacteric and ripe Granny Smith fruit, and in unripe Hosui, but not in ripe Hosui fruit. Thus EFE had different characteristics in the ripe nonclimacteric fruit and in ripe climacteric fruit.

Depending on the effects of CO₂ on EFE synthesis and EFE activity, it is suggested that 4 different types of reaction centres (types of cells, or regions within cells) were suggested to exist (Table 7-1). Hosui fruit had Type 1 or 2 cells and Granny Smith fruit had Type 3 or 4 cells. The existence of a cell type depended on the characteristics of their EFE and System I receptors at the different ripening stages. These results extend Yang's (1987b) model. He did not elaborate on whether there were differences in the way that the System I ethylene receptor could regulate EFE. He also did not indicate that EFE might have different characteristics which could change during maturation and ripening, and that the System I ethylene receptor differently regulated EFE during fruit ripening in nonclimacteric and climacteric fruits.

Enhanced CO₂ levels are commonly used for CA storage of fruit, and high levels (10-15%) of CO₂ have also been used as prestorage treatments for apple to enhance quality. It has been suggested that CO₂ inhibits ethylene synthesis in the whole fruit due an inhibition of ethylene action. This present work has shown that the short-time CO₂ treatment might stimulate EFE synthesis and its activity depending on the types of fruit and their ripening stage. The stimulatory effect of CO₂ on EFE activity in fruit discs

was dependent on exogenous ACC, thus this stimulatory effect might not occur in whole fruit in which the endogenous ACC concentration was limited by ACC synthesis. However if ACC biosynthesis was induced by various stresses, such as chilling or mechanical damage, fruit kept in high CO₂

atmosphere before transfer to ambient atmosphere might produce more ethylene than undamaged fruit, and thus show acceleration of senescent or damaging physiological processes.

Attempting to elucidate the role of CO₂ in regulating the biosynthesis of ethylene in this research, it is clear that further work is needed before a firm conclusion can be made. A number of different research areas need further development and some of these are suggested as followed:

Further work is needed to compare the response of CO₂ on ethylene synthesis and its regulation between whole fruit and fruit discs;

This work investigated the effects of the short-term high concentration CO₂ treatments on EFE, but it is necessary to compare the effects of short-term and long-term CO₂ treatments as well as low and high CO₂ concentration treatments on EFE synthesis, activity and regulation.

To further understand the mechanism of the CO₂ effect on the ethylene receptor, the effects of interactions between CO₂ and ethylene action inhibitors, such as Ag⁺ and NDE on the kinetics of EFE need to be investigated.

There is a need to determine the extent to which different forms of EFE are located at the tonoplast, the plasma membrane or elsewhere and to see if they responded differently to CO₂ and/or ethylene inhibitors. There is also a need to investigate the possible changes of EFE forms during ripening, and to test their response to CO₂ in different species of nonclimacteric and climacteric fruit.

It should be possible to investigate the influence of high CO₂ on EFE configuration by changing intracellular pH or characteristics of membranes (potentials across membranes, fluidity, permeability to various ions, interactions between membrane lipids and proteins).

For fully understanding the effects of CO₂ on ethylene biosynthesis, it is necessary to clarify the effects of CO₂ on other ethylene synthetic enzymes, such as ACC synthase and ACC N-malonyltransferase.

To clarify characteristics of EFE localization and its regulation, the purification of EFE and ethylene receptors ~~are~~ required.

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Appendix 1
Colour grades of Japanese colour chart and their relevant L a b values
of chromameter

Colour grade (Japanese colour chart)	L	a	b	Hue*** Angle
	(chromameter)			
	c	f	b	f
1	48.58	-2.64	25.57	95.90
	c	e	b	e
2	49.32	-1.82	24.50	94.25
	ab	d	a	d
3	50.77	-0.44	29.00	90.88
	a	c	a	c
4	51.71	2.40	28.74	85.18
	bc	b	a	b
5	49.48	6.40	28.37	77.28
	d	a	b	a
6	45.35	8.25	25.97	72.37
c.e.**	-0.3236	0.9762	0.2858	0.9780

* All values are means (n=3). Different characters within the same column represent significant differences at 5% level (Duncan multiple comparison).

** Correlation coefficient of L, a, b and ratio of a/b with colour grades (Japanese colour chart).

***Hu angles are calculated according to following equations:

$$H^0 = \tan^{-1}(b/a) \text{ when } a > 0$$

$$H^0 = 180^0 + \tan^{-1}(b/a) \text{ when } a < 0$$

Appendix 2
Calculation of fruit respiratory rate and ethylene production

Respiratory rate is calculated according to the following formula:

$$\text{Resp. rate} = \frac{[\text{volume of jar(ml)} - \text{volume of fruit(ml)}] * \text{CO}_2(\%)}{\text{f.w. (Kg)} * \text{hours}}$$

(ml CO₂/Kg/h)

or

$$\text{Resp. rate} = \frac{[\text{volume of jar(ml)} - \text{volume of fruit(ml)}] * \text{CO}_2(\%) * 1.976 * \frac{273}{273+t^\circ\text{C}}}{\text{f.w. (Kg)} * \text{hours}}$$

(mg CO₂/Kg/h)

Ethylene production is calculated according to the following formula:

$$\text{C}_2\text{H}_4 \text{ prod.} = \frac{[\text{volume of a jar(ml)} - \text{volume of the fruit(ml)}] * \text{C}_2\text{H}_4(\text{ul})}{\text{f.w. (Kg)} * \text{hours} * 1000}$$

(ul/Kg/h)

or

$$\text{C}_2\text{H}_4 \text{ prod.} = \frac{[\text{volume of a jar(ml)} - \text{volume of the fruit ml}] * \text{C}_2\text{H}_4(\text{ul}) * \frac{1.26 * 273}{273+t^\circ\text{C}}}{\text{f.w. (Kg)} * \text{hours} * 1000}$$

(ug/Kg/h)

In calculation, the density of fruit is assumed to be 1.0 g/ml, thus the absolute value of fruit volume (ml) is same with its fresh weight (g).

For fruit discs, the unit of ethylene production and EFE activity was nlC₂H₄/g/h, and the unit of respiration rate was ulCO₂/g/h.