THE ROLE OF ETHYLENE IN KIWIFRUIT SOFTENING

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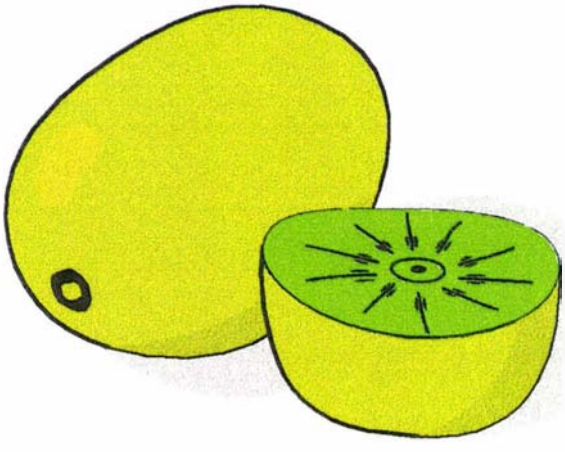

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ABSTRACT

Premature fruit softening during storage at 0°C is a serious and costly problem for the New Zealand kiwifruit industry. Ethylene gas (C_2H_4) is a potent promoter of fruit softening; it is involved in regulation of fruit ripening and influences a number of processes, including ethylene production, respiration rate and fruit softening. Exogenous ethylene increases softening rate in kiwifruit at 20°C and at 0°C; a concentration of 0.01µl/l will enhance softening at 0°C. The precise relationship between kiwifruit softening and endogenous ethylene concentration is not known. The influences of low temperature, an ethylene synthesis inhibitor (aminovinylglycine (AVG)), an inhibitor of ethylene action (1-methylcyclopropene (1-MCP)) and application of ethylene at different maturities were investigated in an attempt to elucidate ethylene's role in initiating kiwifruit softening.

Exposing fruit to 0°C for more than 52 days hastened ethylene production upon removal to 20°C compared with fruit maintained at 20°C continuously after harvest; this enhanced ethylene was associated with increased 1-aminocyclopropane-1-carboxylic acid (ACC) concentration and ACC oxidase (ACO) activity.

Kiwifruit softening at 0°C occurred even though ethylene production was low and constant between 0.02 to 0.06µl/kg/h (corresponding to internal ethylene concentrations (IEC) 0.2 to 0.6µl/l). This softening was associated with low ACC concentrations varying between 0.2 to 0.5nmole/g and ACO activity varying between 0.01 to 0.66nl/g/h. These results indicate that very low concentrations of ethylene may play an essential role in kiwifruit softening.

In kiwifruit treated with AVG (500ppm) 4 weeks before harvest, ethylene biosynthesis, manifested as reduced ACC concentration, ACO activity and ethylene production, was significantly inhibited both at 20°C and after storage at 0°C. AVG application resulted in a slower softening rate and firmer fruit than in untreated controls. Rates of softening

were 0.4N/day and 1.9N/day in the AVG treated fruit and control fruit respectively during 14 days at 20°C. However, the AVG effect was reduced after storage at 0°C for 14 days, indicating that the AVG effect was only temporary and may not be sufficient to warrant possible commercial use for longer storage.
Application of 250ppm AVG 2 or 4 weeks before harvest, and 500ppm AVG 2 weeks before harvest had no effect on ethylene production or fruit firmness.

Fruit infected with *B. cinerea* produced more ethylene than non-infected fruit at 0°, 4°, 10° and 20°C. An increase in ethylene production, induced by *B. cinerea* infection, occurred in tissue slices from the invasion zone (the infection front containing both infected tissue and sound tissue immediately ahead of the infection front) and adjacent zone (sound tissue ahead of the invasion zone) of kiwifruit. The increased ethylene in infected fruit was associated with increased ACO activity in tissue from adjacent and distal (sound tissue at the distal end of the fruit) zones.

Application of 500ppm AVG to kiwifruit vines before harvest reduced ACC concentration and ACO activity and ethylene production induced by *B. cinerea* at 20°C. However this AVG reduced ethylene production from *B. cinerea* infected fruit after 4 weeks at 0°C was insufficient to prevent rapid growth of *B. cinerea*. Therefore, such an AVG treatment can not be used to reduce *B. cinerea* infection during storage at 0°C.

As kiwifruit matured, softening was enhanced increasingly by exogenous ethylene, indicating that tissue sensitivity to this growth regulator increased with time. The sensitivity of kiwifruit was reduced by treatment with 1-MCP, an inhibitor of ethylene action. When applied to kiwifruit at harvest, 1-MCP reduced ethylene production and respiration rate, resulting in a slower fruit softening and firmer fruit during storage at both 0°C and 20°C. Kiwifruit treated with 1-MCP plus ethylene at harvest, remained firmer than fruit exposed to ethylene alone for 4 days at 20°C and 8 days at 0°C, after which this 1-MCP effect disappeared with firmness being the same for both ethylene plus 1-MCP and ethylene treated fruit at the two temperatures. When both 1-MCP plus

ethylene were applied after storage at 0°C, 1-MCP negated the ethylene-induced softening with treated fruit having a softening rate 4 times less than for fruit treated with ethylene alone. Softening rate of control and 1-MCP treated fruit was the same as those treated with 1-MCP plus ethylene (approximately 0.06 ('Kiwifirm'unit)/day) compared with 0.2 ('Kiwifirm'unit)/day for fruit treated with ethylene alone. Since 1-MCP binds to the ethylene receptor sites irreversibly, it is suggested that kiwifruit can synthesis new ethylene receptors with time during storage at 0°C and 20°C, making kiwifruit increasingly sensitive to endogenous ethylene.

Over several different experiments in 3 seasons, kiwifruit softened from ≈ 90N to 10~19N while endogenous ethylene production remained low (below 0.1~0.2 μl/kg/h) and constant at 20°C. Increased ethylene production only occurred as fruit softened from 10N~19N to eating ripe (6~8N).

These results have led to a model being proposed for ethylene action during ripening of kiwifruit. Kiwifruit softening (phases 1 and 2) occurs even though ethylene production is low as is ACS and ACO activity. It is possible that this basal level of ethylene corresponds to System 1 ethylene production which is thought be associated with basal metabolic maintenance; this ethylene probably binds to System 1 ethylene receptors. The changing ability of fruit to soften with increased maturity is due to increasing sensitivity to such low ethylene concentration resulting from the progressive formation of new ethylene receptors in the fruit. Application of AVG and 1-MCP reduced ethylene production, leading to a delay in ethylene-induced softening. It is possible that low endogenous ethylene (System 1 ethylene) is sufficient to induce starch degradation and solubilization of pectins in cell walls caused by hydrolase enzymes such as amylase, β-galactosidase and xyloglucan endotransglycosylase early in ripening. Alternatively oligomers derived from cell wall breakdown may induce ethylene production even though such oligomer elicitors have not been reported to exist in kiwifruit. Maximal cell wall swelling, depolymerization of the solubilized pectin by polygalacturonase and breakdown

of the middle lamella only occur when kiwifruit already have softened to <20N (phase 3 of softening), and these events are associated with or co-ordinated by System 2 autocatalytic ethylene. This autocatalytic ethylene is associated with high ACS and ACO activity and may bind to System 2 ethylene receptors, leading to ethylene dependent responses such as PG activation which results in ready-to eat fruit with a firmness of 6~8N.

In conclusion, kiwifruit softening from 90N to 10~19N occurs with low and constant ethylene production. Because new ethylene receptors of kiwifruit can be formed with time at both 0°C and 20°C, it appears that kiwifruit sensitivity to low ethylene concentration also increases with time in storage. It is possible that different cultivars of kiwifruit with different softening rates, may have different amounts or rates of formation of new ethylene receptors. By comparing physiological, biochemical and molecular attributes of Hayward and other kiwifruit cultivars and selections, it should be possible to provide information on their responsiveness and sensitivity to ethylene. This will allow plant breeders to create new cultivars that have both low ethylene production and low sensitivity to ethylene that would provide a range of commercial cultivars with prolonged and different storage lives for the international kiwifruit market.

Application of inhibitors of ethylene biosynthesis (AVG) and ethylene action (1-MCP) reduced ethylene production and softening rate of kiwifruit with 1-MCP being more effective for longer than AVG. Although 1-MCP showed promise as a tool to delay softening during storage at 0°C and shelf life at 20°C, further research is required to determine optimum concentration, time and frequency of application, and efficiency when applied at 0°C in order to derive treatments that may have significant commercial applications.

Keywords: *Actinidia deliciosa*, kiwifruit, softening, maturity, ethylene, ethylene receptors, sensitivity, low temperature, aminoethoxyvinylglycine (AVG), 1-methylcyclopropene (1-MCP), *Botrytis cinerea*.

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CHAPTER ONE

INTRODUCTION

The kiwifruit industry has grown to be an important horticultural export earner in New Zealand. In 1998, 60 millions trays (NZ$ 429 million, F.O.B.) were exported to Europe (52%), Japan (30%), East Asia (8%) including Taiwan, Korea and China and Hong Kong, and emerging markets (10%) including the Americas, Southeast Asia and Australia (Anon., 1998, 1999). This represented 41% of New Zealand fresh fruit exports and 26% of total New Zealand horticultural exports. Among the many attributes of kiwifruit contributing to its remarkable popularity is its unusually good storage life (Reid and Harris, 1977). The kiwifruit (*Actinidia deliciosa* (A.Chev) C.F.Liang et A.R.Ferguson cv Hayward) can be stored at 0°C ~ 0.5°C, 95% RH (relative humidity) in air with ethylene at < 0.03 µl/l for at least 6 months (McDonald, 1990).

Premature fruit softening during storage is a serious and expensive problem for the New Zealand kiwifruit industry (Banks *et al.*, 1992; Davie *et al.*, 1996). Fruit are not acceptable for export if firmness value is below 9.8N or if there are localised areas of the fruit with firmness below 9.8N (called soft patches). Premature softening of kiwifruit may be responsible for up to 70% of fruit losses after harvest (Banks *et al.*, 1992). Considerable variation in fruit softening rates exist between seasons, districts and orchards, but the reasons for this have not yet been determined and hence cannot easily be predicted. Postharvest diseases are also significant contributors to fruit losses. *Botrytis* storage rot, caused by *Botrytis cinerea*, is one of the most important postharvest disease problems facing the kiwifruit industry. In addition to direct and indirect grower losses due to infected fruit there are two further serious implications for fruit quality: first, ethylene produced by infected fruit, even prior to the appearance of visible symptoms, can induce softening of other healthy fruit in the same tray, resulting in reduced storage life. Second, fruit are often exported before the first rots appear which create the need for repacking overseas to remove both rotted and soft fruit (Manning and Pak, 1993).

Ethylene gas is known to be a potent promoter of fruit softening. It is produced naturally by ripening fruit and induces unripe fruit to ripen rapidly (Abeles *et al.*, 1992; Puig *et al.*, 1996). Kiwifruit are very sensitive to ethylene; concentrations as low as 0.01 µl/l reduced storage potential by 46 % at 0°C and thus very low ethylene levels have the potential to cause the majority of loss of storage life of fruit (Jeffery and Banks, 1996). Although the minimum threshold limit of kiwifruit sensitivity to ethylene has not been determined (Harris, 1981), scrubbing ethylene levels to near zero should delay kiwifruit softening rate (McDonald and Harman, 1982; Banks *et al.*, 1991).

The overall objective of the research presented in this thesis was to investigate factors that initiate the postharvest production of ethylene in kiwifruit and to attempt to elucidate the relationship ethylene production and softening in this important fruit.

1.1 Softening in kiwifruit

Kiwifruit softening occurs non-uniformly as the fruit has several distinctly different fruit tissue types (Hallett *et al.*, 1992; MacRae, 1988) (Fig. 1-1): core, seed area (inner pericarp) and flesh (outer pericarp). Shape of individual cells, and the way in which they are put together, differ in each tissue type. The core consists of small regularly shaped cells (dimensions between 0.1-0.2mm, 2.4µm wall thickness) packed closely together with very few air spaces between them. Cells (inner pericarp) from the seed cavity are of two types, locule and locule wall. The locule area contains both seeds and radially elongated thin-walled cells (dimensions 0.2-0.4mm and up to >1mm, 2µm wall thickness). The locule walls consist of smaller thicker-walled radially elongated cells (0.05-0.1mm x 0.2-0.4mm, 2.9µm wall thickness) fitted closely together. Cells of the outer pericarp are irregular in shape and size (cross-sectional diameter 0.5-0.8mm and 0.1-0.2 mm for large and smaller cells respectively, mean thickness of cell walls 2.5µm) and are loosely arranged together. There are relatively large air spaces between them. These different tissue types in kiwifruit have different effects on tissue firmness; cell wall breakdown, triggered by ethylene, was more pronounced in the outer pericarp and locule wall area of the inner pericarp than in the locule area of the inner pericarp and core tissues (Hallett *et al.*, 1992; Redgwell *et al.*, 1990). When a penetrometer is used to

measure fruit firmness, it is measuring an overall response to the three tissue types. Therefore, if any one of these tissue is abnormally hard or soft, it can influence the fruit penetrometer reading. The relative amounts of each cell type in the individual fruit may also affect the fruit firmness measurement.

There are also chemical differences between the tissues (Hallett *et al.*, 1995; MacRae, 1988). At harvest, the core has more starch than the other tissues, including the outer

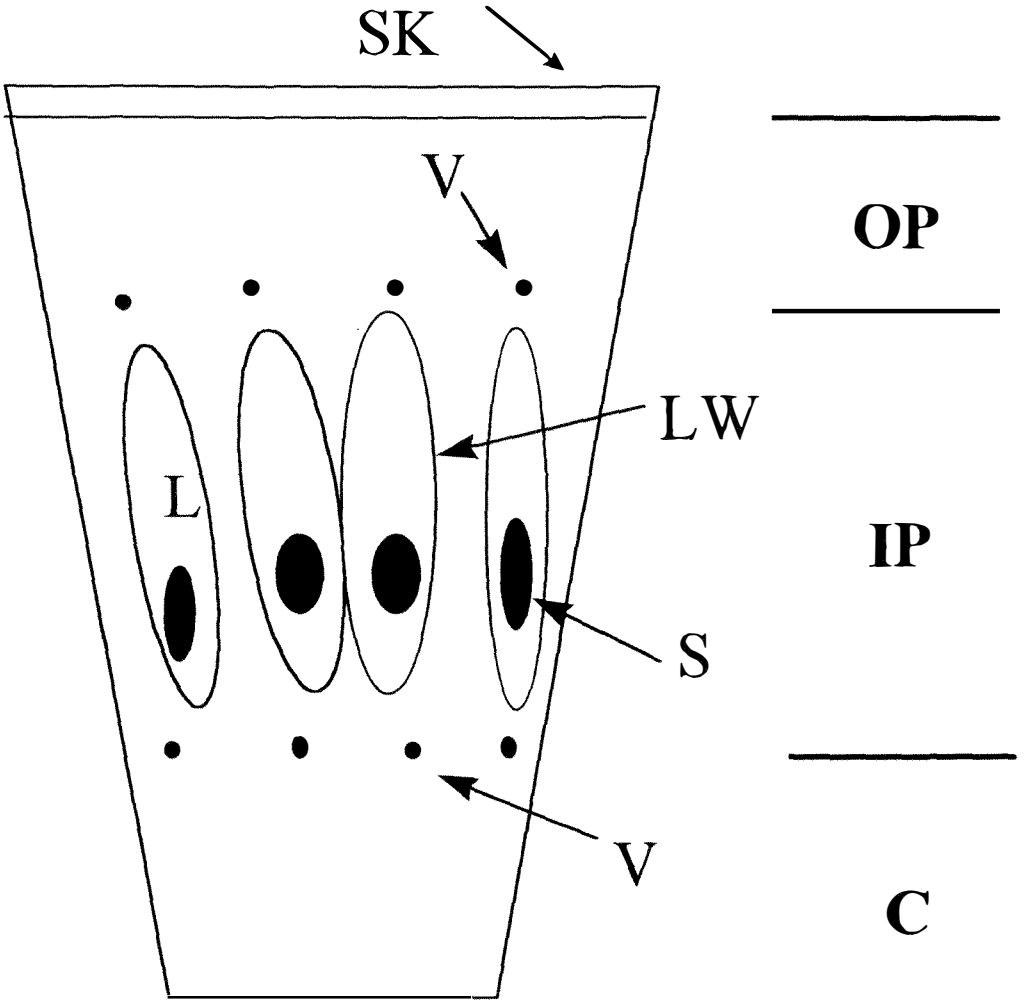

Fig. 1-1. Diagrammatic cross-section of a segment of kiwifruit. *C* = core, *IP* = inner pericarp, *L* = locule, *LW* = locule wall, *OP* = outer pericarp, *S* = seed, *SK* = skin layers (epicarp), *V* = location of major vascular trace (Hallett *et al.*, 1992).

pericarp and the seed area, and it has a higher sugar/acid ratio (1.93) than the outer pericarp (1.14) or the seed area (0.86). During fruit ripening starch is converted to sugar. Logically, the core, with the highest starch content at harvest, is sweet but the seed area is sour when eaten. Much of the starch loss occurs during the early stages of kiwifruit softening. Fruit taste is known to be influenced by sugar, acid and starch composition in each of the tissues (MacRae, 1988).

Firmness is a critically important physical factor that determines fruit quality. Fruit softening occurs during storage and postharvest handling as cell walls lose their integrity during ripening. Fruit tissue is composed of hundreds of cells, each enclosed by a wall. Cells are bound together by a complex cementing material, the middle lamella. The plant cell wall consists of cellulose fibers embedded in a matrix or paste of pectins and another type of polysaccharide called hemicelluloses, which largely determine the shape, firmness and texture of fruit tissue, and assists it to resist physical damage and microbial attack (Carpita and Gibeaut, 1993; Salisbury and Ross, 1992). Cellulose consists of linear chains of ß-1,4-glucosyl residues bound tightly via hydrogen bonding to form microfibrils which form a highly structured framework, giving rise to a structure of considerable tensile strength. This fraction represents 25-35% in kiwifruit cell walls (Redgwell *et al.*, 1991). Pectins which consist of an α-1,4-galacturonan backbone with 2- and 2,4-linked rhamnosyl residues, are often the major type of heterogeneous polysaccharide in fruit cell walls and are an important component of the middle lamella (Salisbury and Ross, 1992); pectins represent 40-50% of the cell wall in kiwifruit (Redgwell *et al.*, 1991). Hemicelluloses are composed of a variety of polymers such as xyloglucans, glucomannans and galactoglucomannans. The hemicellulose is linked to pectin covalently and to cellulose non-covalently via strong hydrogen bonding; it represents 15-25% of kiwifruit cell walls (Redgwell *et al.*, 1991). The cell wall has two important but seemingly incompatible properties. The first is its plasticity, which enables it to expand as the cell enlarges during fruit development. The second is its rigidity, which confers strength and determines cell shape. As kiwifruit ripen naturally or after ethylene treatment, their cell walls undergo dramatic changes in both structure and composition, including solubilisation and degradation of cell wall pectins, loss of galactose from the

sidechains of the pectic polymers, and a marked swelling of the cell walls, so that they become thinner and less rigid. These changes cause the fruit tissue to soften and the texture to change (Hallett *et al.*, 1992; Redgwell *et al.*, 1992; Redgwell and Harman, 1988; Redgwell and Percy, 1992). Starch is also lost as softening progresses in kiwifruit (Arpaia *et al.*, 1987; Hallett *et al.*, 1995); much starch loss occurs during early stages of kiwifruit softening associated with high amylase activity (Bonghi *et al.*, 1996; MacRae, 1988; MacRae *et al.*, 1989; Matsumoto *et al.*,1983). At maturation, fruit starch content is about 5-7% fresh weight, whereas after ripening, only traces are detectable and sugars increase up to 12-15% (Beever and Hopkirk, 1990).

Softening is one of the most significant quality alterations consistently associated with ripening of fleshy fruits. Kiwifruit texture changes dramatically during fruit ripening, and firmness can decline by as much as 94%, from about 60-90 N at harvest to 5-8 N when ripe (Beever and Hopkirk, 1990; Harker and Hallett, 1994). Softening in kiwifruit at 0°C occurs in two or three phases, depending on maturity at harvest (Lallu *et al.*, 1989; MacRae *et al.*, 1989; MacRae *et al.*, 1990; McDonald, 1990) (Fig. 1-2). The first phase (1 in Fig. 1-2) is a lag period when very little softening occurs. This lag disappears in more mature fruit; fruit harvested early in the season and stored for a short period will be firmer than fruit harvested late in the season and stored for the same length of time. The second phase (2 in Fig. 1-2) is a rapid phase of softening which occurs immediately after harvest in late harvested fruit or after a lag period in early harvest fruit; it continues for up to 6 weeks and is where the largest reduction in fruit firmness takes place from 60-90N to 20N. In this phase, there is little or no swelling of the cell wall (Hallett *et al.*, 1992), loss of starch (Arpaia *et al.*, 1987) and very low ethylene production (Bonghi *et al.*, 1996; Tonutti *et al.*, 1993). The third phase (3 in Fig. 1-2) is a slow rate of softening which begins at about 20 N and continues until fruit are overripe with a firmness of less than 7 N regardless of harvest time. There is some evidence that a relatively sharp reduction in firmness (the fourth phase ?) occurs at the very end of this phase when final tissue disintegration is taking place and the fruit is no longer edible. In phase 3, expansion and swelling of the cell wall, and ethylene production reach their maximum (Bonghi *et*

al., 1996; Hallett *et al.*, 1992); breakdown of the middle lamella becomes evident only during this phase of kiwifruit softening (MacRae and Redgwell, 1992).

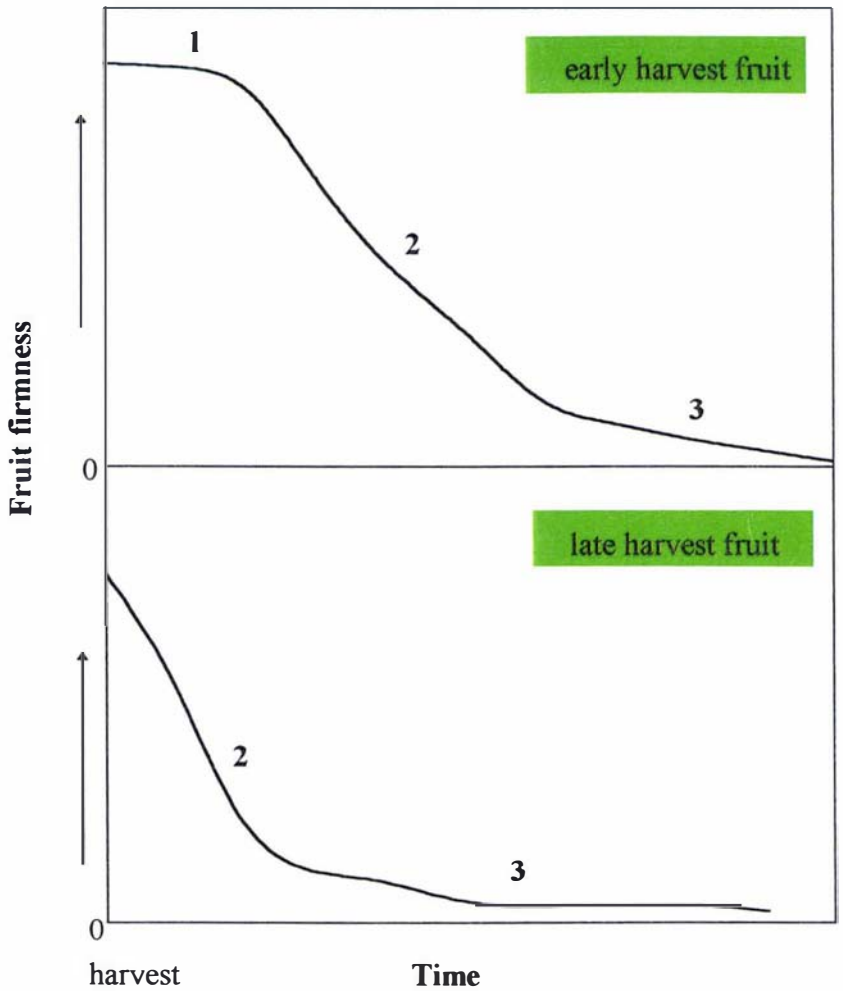

Fig. 1-2. Diagrammatic representation of softening in kiwifruit (MacRae *et al.*, 1990). 1 indicates the first phase of softening, 2 the second phase and 3 the third phase.

A number of studies have examined changes in the cell wall chemistry and the enzymes involved in degradation of the kiwifruit cell wall (Fig. 1-3) (Bonghi *et al.*, 1996; Gallego and Zarra, 1997, 1998; MacRae *et al.*, 1990; MacRae and Redgwell, 1992; Redgwell *et al.*, 1990, 1991, 1992, 1997; Redgwell and Harman,1988; Redgwell and Harker, 1995; Ross *et al.*, 1993; Schroder *et al.*, 1998). Early in ripening, starch degradation occurred

coincident with high amylase activity; starch degradation could play a role in the early events of fruit softening (Arpaia *et al.*, 1987; MacRae *et al.*, 1989). During the rapid softening phase, large amounts of pectin are solubilised in the cell wall, resulting in reducing adhesion between cells and thus separating cells at the middle lamella; most cell

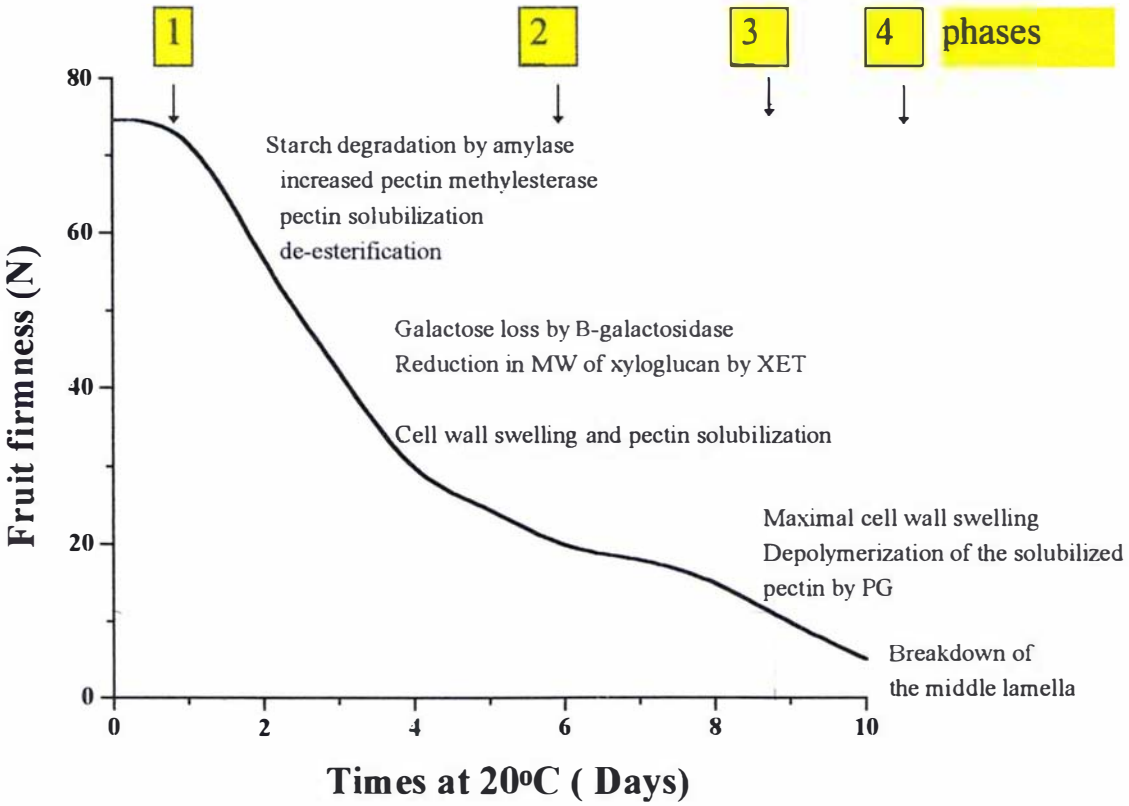

Fig. 1-3. Schematic representation of postharvest kiwifruit softening in relation to the timing of key events in the softening process. This figure is modified after MacRae and Redgwell (1992). XET: xyloglucan endotransglycosylase; PG: polygalacturonase.

wall galactose is lost before pectic solubilisation during ripening, probably due to an exo-β-galactosidase enzyme system (Ross *et al.*, 1993). Redgwell and Harker (1995) also found that the loss of cell wall-associated galactose and pectin solubilisation in ripening kiwifruit are separate processes and that galactose loss, in part, may be independent of ethylene. The further reduction in cell to cell adhesion lessens the ability to maintain integrity of the wall and thus contributes to fruit softening. There was a correlation between swelling and degree of pectin solubilisation (Redgwell *et al.*, 1997); cell wall swelling occurs as a result of changes to the viscoelastic properties of the cell wall during

pectin solubilisation. Cell wall swelling is associated with movement of water into voids left in the cellulose-hemicellulose network by the solubilisation of pectin (Redgwell *et al.*, 1997). In the slow softening phase (phase 3), most of the pectins have been solubilised through depolymerization and degalactosidation; polygalacturonase (PG) is involved in depolymerising the solubilized pectin (MacRae and Redgwell, 1992). Breakdown of the middle lamella becomes evident only during the last phase of kiwifruit softening (MacRae and Redgwell, 1992). Several enzymes are thought be involved in degradation of cell wall pectic polymers; β-galactosidase (β-GAL), pectin methyl esterase (PME) and polygalacturonase (PG). These enzymes break the connecting bonds of the pectin polysaccharides and are involved in degradation of the middle lamella so that the individual fruit cells separate because of reduced adhesion between neighboring cells (MacRae *et al.*, 1990; Hallett *et al.*, 1992; Harker and Hallett, 1994). Xyloglucan endotransglycosylase (XET) which has six cDNA clones (AdXET1-6), and endo β-1,4-D-glucanase (EGase or cellulase) are involved in degradation of hemicellulose and cellulose of cell wall respectively; ripe kiwifruit XET was encoded by AdXET6 (Schroder *et al.*, 1998). These changes during kiwifruit softening can be accelerated by ethylene which is known as the ripening hormone (MacRae *et al.*, 1989; Matsumoto *et al.*, 1983; Redgwell *et al.*, 1990, 1992); ethylene induced ripening was accompanied by a decrease in starch and amylose content and an increase in soluble solids and sugars. PME activity increased 2 to 3 fold during ethylene exposure, indicating that PME action may be a key to the rapid softening induced by exposure to ethylene (MacRae *et al.*, 1989; Redgwell *et al.*, 1990, 1992).

1.2 Ethylene biosynthesis in higher plants

Ethylene is involved in the regulation of many developmental processes and stress responses in higher plants including fruit ripening, seed germination, seedling growth, leaf abscission, flower senescence, pathogen responses and environmental stress (Abeles *et al.*, 1992). Ethylene can be beneficial to horticultural crops by improving postharvest quality of the product by inducing uniform ripening, but it usually has deleterious effects including accelerating senescence and reducing shelf-life. It is essential to control

ethylene production during postharvest handling to avoid these deleterious effects. Thus it is important to know how ethylene is synthesized and how ethylene acts in plants.

Ethylene in higher plants is formed from L-methionine via S-adenosyl-L-methionine (AdoMet) and the cyclic, nonprotein amino acid 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1979; Lurssen *et al.*, 1979). The enzymes catalyzing the individual steps of this pathway are Ado-Met synthetase, ACC synthase (ACS) and ACC oxidase (ACO) which catalyse the conversion of L-methionine to AdoMet, AdoMet to ACC and ACC to ethylene, respectively (Yang and Hoffman,1984). ACS seems to be a pyridoxal dependent enzyme requiring pyridoxal phosphate as a cofactor for maximal activity; ACS is strongly inhibited *in vivo* and *in vitro* by aminooxyacetic acid (AOA) and aminoethoxyvinylglycine (AVG) which are well known inhibitors of pyridoxal phosphate-dependent enzymes. ACS activity is enhanced by factors that promote ethylene formation such as ethylene, auxins, pathogens and stress conditions including drought, wounding and chilling injury. ACS is encoded by a highly divergent gene family and up to 9 different ACS genes are differentially regulated in response to a variety of developmental, environmental and chemical factors (Kende, 1993). For example, tomato genes LE-ACS2 and LE-ACS4 are induced during fruit ripening by ethylene and after wounding, while LE-ACS3 does not accumulate in a ripening fruit but is induced after wounding (Fluhr and Mattoo, 1996). The conversion of AdoMet to ACC is the key rate-limiting reaction in most plant tissues and is the main site of control of ethylene production (Boller *et al.*, 1979; Cameron *et al.*, 1979; Fluhr and Mattoo, 1996; Kim and Yang, 1992); for example ethylene production of tissues excised from root, stem, leaf and fruit of 16 plant species greatly increased following the application of ACC (Cameron *et al.*, 1979). In addition to ACC, ACS produces 5'-methyl-thioadenosine (MTA), which is utilized for the synthesis of new methionine via a modified methionine cycle or Yang cycle (Yang and Hoffman, 1984). This pathway allows high rates of ethylene production without accumulation of high intracellular concentrations of methionine because of the recycling of MTA to methionine. Thus high rates of ethylene biosynthesis can be maintained even when the free methionine pool is small (Yang and Hoffman, 1984; Kende, 1993).

The last step in the ethylene biosynthetic pathway is conversion of ACC to ethylene. This is an oxygen dependent reaction catalysed by ACO (Adams and Yang, 1979) which is also known to play an important role in the regulation of ethylene biosynthesis in most ripening fruit including melon (Yamamoto *et al.*, 1995), pears (Lara and Vendrell, 1998) and tomato (Barry *et al.*, 1996; Hamilton, 1990). In *in vivo* assays, ACO seems to be a membrane-bound enzyme (Guy and Kende, 1984; John *et al.*, 1989; Mattoo and Lieberman,1977; Mitchell *et al.*, 1988) since ACO activity requires membrane integrity (John, 1997). When the plant cell was treated with cell wall degrading enzymes or the plasma membrane or vacuolar membrane were ruptured, ACO activity was lost (Kende, 1989; Mitchell *et al.*, 1988). In addition, ACO is damaged by prolonged exposure to chilling temperature (Abeles *et al.*, 1992; Wang and Adams, 1980, 1982) because of severe disruption to cellular membranes. However, it was recently discovering that ACO is a member of the Fe(II)-dependent dioxygenase family and the enzyme resembles flavanone 3-hydroxylase; its activity did not depend on membranes because the enzyme was extracted in a soluble form from melon fruit (Ververidis and John, 1991). The membrane requirement for ACO is attributable to a need for a charged plasma membrane to maintain ascorbate in the reduced state for an ACO located in the apoplast (John, 1997). The antigen corresponding to ACO is located primarily in the cell wall in ripening tomato and apple fruit (Rombaldi *et al.*, 1994). Recently, it has been proposed that there are two potential subcellular localization of ACO in plant cells: a site external to the plasma membrane (cell wall), and a site internal to the plasma membrane, presumably cytosolic (Rombaldi *et al.*, 1994). However, the mechanism by which ACO is activated remains unclear.

Besides being converted to ethylene, ACC is also metabolized to a presumably biologically inactive end-product, 1-(malonylamino) cyclopropane-1-carboxylic acid (MACC) by ACC *N*-malonyltransferase. While malonylation is thought to be irreversible under physiological conditions, high levels of MACC induce conversion of MACC to ACC in watercress stem sections and tobacco leaf discs which have low ACS activity (Jiao *et al.*, 1986). Thus, this malonylation of ACC participates in the regulation of ethylene biosynthesis (Abeles *et al.*, 1992) since MACC may remove ACC from the

metabolic pool or it may serve as a source of ACC under certain stress conditions (Jiao *et al.*, 1986).

1.3 Stress ethylene in higher plants

Production of stress ethylene in plants is induced by various factors including wounding, physical load, disease, drought, cold temperature and exposure to various chemicals (Abeles *et al.*, 1992). Stress ethylene is one of the general phenomena observed in plant tissues subjected to various unfavorable conditions (Hyodo, 1991). Ethylene production and ACC content are low before stress treatment but rapidly increase following stress. The conversion of SAM to ACC is a key reaction controlling the production of stress ethylene. Application of AVG, a potent inhibitor of ACS, effectively eliminates the increase in ACC formation and the production of stress ethylene. Therefore, stress induces the synthesis of ACS, which in turn causes rapid accumulation of ACC and a marked increase in the production of stress ethylene (Yang and Hoffman, 1984); wounding stress stimulated the expression of specific ACS genes including ACC2 and ACC4 genes in *Arabidopsis* plants (Liang *et al.*, 1992) and LE-ACS3 in tomato (Yip *et al.*, 1992).

Infection of fruit by pathogens induced production of ethylene as a result of contributions both by the host and the pathogen (Mattoo and Suttle, 1991). Achilea *et al.* (1985) described the origin of the ethylene produced during infection of grapefruit with *P. digitatum*; at the initial phase of infection, enhanced ACC-mediated ethylene production originating from the peel cells as a result of the physical stress that the invading fungal hyphae imposed on the plant tissue that caused cells to plasmolyse and cell walls to swell in the apparently healthy zone (Barmore and Brown, 1979). As infection proceeded, the tissue was killed; the ethylene biosynthetic pathway in the infected regions was impaired due to loss of membrane and cell wall integrity, and thus ACC accumulated. Therefore, the increased production of ethylene at this stage was probably of fungal origin as large amounts of ethylene were produced from radioactive glutamate but not from methionine in the infected regions (Achilea *et al.*, 1985). Ethylene production in cucumber and beans infected with *Botrytis cinerea* decreased with increase in disease severity and maximum

levels were emitted by leaves showing a low disease index (Elad, 1990); thus stress ethylene production is associated with living tissue, indicating that this stress ethylene production is a likely response to physiological stress caused by the fungal infection (Takeda and Nakamura, 1990). Severe stress results in cell death and cessation of ethylene production.

It is possible that in some cases ethylene originates from microorganisms invading plant tissues. Ilag and Curtis (1968) found that 58 of 228 species of fungi examined produced ethylene and ethylene was a metabolic product of fungi using methionine as a precursor of ethylene in *Penicillium digitatum* and *Botrytis cinerea* (Chalutz *et al.*, 1977; Qadir, 1994). In addition, this ethylene from microorganisms may be derived through a pathway not involving ACC (Qadir, 1994). Exogenously applied ACC led to a marked increase in ethylene production in healthy sweet potato root tissue adjacent to the area infected with actively growing *Ceratocystis fimbriata* but not in the necrotic tissue (Hyodo and Uritani, 1984). Hayward kiwifruit infected with *Botrytis cinerea* produced ethylene and accumulated ACC even at low temperatures (0°C and 10°C) where uninfected kiwifruit did not produce ACC or ethylene (Niklis *et al.*, 1993). These authors suggested that this increased ethylene was closely associated with mycelial growth in the *Botrytis* infected tissues. Recently, Qadir *et al.* (1997) found that *B. cinerea* had the capacity to produce ethylene in the presence of methionine. However, they did not know whether the enhanced ethylene production in *B. cinerea* infected kiwifruit arose from the pathogen or from kiwifruit tissue as a result of infection.

Low temperature storage is a useful method to maintain quality of horticultural crops since low temperature reduces respiration rate, ethylene production, ripening, senescence and decay (Wang, 1994). Although low temperature storage has many beneficial effects, it has some detrimental effects in chilling sensitive tropical and subtropical crops. Tropical and subtropical plants exhibit various physiological and biochemical alterations when exposed to low or nonfreezing temperatures below about 10°C to 12°C (Lyons, 1973; Morris, 1982) resulting in a reduction of their market life because of their susceptibility to chilling injury (Saltveit and Morris, 1990).

There are two different types of response to low temperature on induction of ethylene production in plants. The first response is chilling stress. Exposure to low temperatures, at which plants are susceptible to chilling injury, stimulates ethylene production. Production of ethylene is enhanced when plants are transferred to warmer temperatures after exposure to chilling temperatures (Abeles *et al.*, 1992). Chilling resulted in development of injury symptoms, including skin pitting, and caused a parallel increase in tissue ACC with increasing stress, resulting in high ethylene production. However, cucumber tissues exposed to excessive chilling (more than 4 days at 2.5°C) produced very little ethylene upon transfer to 25°C. Such fruit showed a large increase in ACC levels but little ethylene production, even in the presence of exogenous ACC, indicating that ACO was reduced markedly by severely disrupting cellular membranes (Wang and Adams, 1980, 1982). In addition, Cabrera *et al.* (1992) found that chilling-induced ethylene production in cucumber was higher and loss of ACO activity greater in chilling sensitive lines than from chilling resistant lines.

The second type of response to low temperature is an enhancement of ethylene production upon transfer from low temperature to room temperature apparently without development of chilling injury symptoms. This effect has been studied intensively in European pears where a period of low temperature is used commercially to induce ripening in many cultivars including Bosc, Conference, Passe-Crassane and D' Anjou (Blankenship and Richardson, 1985; Knee, 1987; Knee *et al.*, 1983; Sfakiotakis and Dilley, 1974). D' Anjou pears, harvested at commercial maturity, required about 46 days at -1°C to induce ripening and produce ethylene (Blankenship and Richardson, 1985). Preclimacteric Bosc pears held continuously at 20°C produced ethylene at a very low rate and resisted ripening for 12 days. However, if held at 5 or 10°C for the first 7 days, ethylene production at 20°C began to increase almost immediately; storage at 0°C was considerably less effective (Sfakiotakis and Dilley, 1974). Knee *et al.* (1983) also found that the time taken from harvest to rapid ethylene production was shorter and more uniform at 3°C than at 18-20°C for Conference pears. Lelievre *et al.* (1997b) evaluated the role of chilling on ethylene biosynthetic gene expression in Passe-Crassane pear fruits.

These fruits require a 3-month chilling treatment at 0°C to be able to produce ethylene and ripen. The chilling treatment strongly stimulated ACO activity, and to a lesser extent ACS activity. At the same time, the levels of mRNAs hybridizing to ACS and ACO probes increased dramatically. However, fruit stored at 18°C immediately after harvest did not exhibit any of these changes, while fruit that had been previously chilled exhibited a burst of ethylene production associated with high activity of ACO and ACS upon rewarming. In apples, low temperatures hasten induction of ethylene synthesizing competency and homogeneous ripening (Pech *et al.*, 1994). Storing preclimacteric Granny Smith apples in air at 0°C for more than 1 week induced them to produce ethylene earlier than fruit kept in air at 20°C (Jobling *et al.*, 1991; Lelievre *et al.*, 1995). Cold treatment was responsible for the induction of ACS and ACO activity but it stimulated ACO activity earlier than ACS (Jobling *et al.*, 1991; Larrigaudiere and Vendrell, 1993; Lelievre *et al.*, 1995). Lelievre *et al.* (1995) indicated that ACS and ACO were induced, and accumulated, during chilling of Granny Smith apples, before any transfer of the fruit to warmer temperatures. In contrast, Lara and Vendrell (1998) reported that ACS activity was inhibited by low temperature. However there is a differential effect of cold stimulation on ethylene biosynthesis among different cultivars of apples (Larrigaudiere *et al.*, 1997); Fuji apple held for 32 days at 0°C accumulated ACC and developed ACO activity. On return to 20°C these fruit had a high and sustained level of ethylene production. In contrast, Lady Williams apples chilled at 0°C accumulated ACC but ACC decreased to low levels after transfer to 20°C (Jobling and McGlasson, 1995). Sfakiotakis *et al.* (1997) using kiwifruit grown in Greece also found that chilling induced ethylene production when fruit were transferred from 0°C to room temperature (20°C). Because it is not known if softening rate of kiwifruit, and fruit sensitivity to ethylene, vary depending on season and district, it is important to determine whether such chilling temperatures affect ethylene biosynthesis during storage at 0°C or after removal to 20°C in kiwifruit grown in New Zealand.

Although ethylene biosynthesis is stimulated by different phenomenon including low temperature and infection by pathogens, or has a different pattern of production

following the same stimulation among different cultivars, the basic pathway of ethylene synthesis is probably common. It is necessary to regulate ethylene biosynthesis in order to manipulate ethylene production.

1.4 Regulation of ethylene biosynthesis

Ethylene regulates many aspects of plant growth, development and senescence in trace amounts (Lieberman, 1979). Depending upon where and when ethylene occurs, it may be beneficial or harmful to harvested horticultural crops. Therefore, the modification of ethylene biosynthesis or action and manipulation of the level of ethylene in plants by either stimulation or inhibition of its production is required for efficient postharvest technology (Yang, 1985).

Fruit have been classified as climacteric or nonclimacteric depending on their respiratory behaviour during ripening (Biale and Young, 1981). Climacteric fruit undergo a large increase in respiration accompanied by marked changes in composition and texture during ripening, whereas nonclimacteric fruit show no change in respiration. Ripening in climacteric fruit is associated with a large increase in ethylene biosynthesis (ethylene production, ACS and ACO activity) which is considered as controlling/coordinating the initiation of changes in colour, aromas, texture, flavour and other biochemical and physiological attributes (Lelievre *et al.*, 1997a). The ripening process is irreversible once autocatalytic ethylene production occurs. In contrast, ethylene biosynthesis in nonclimacteric fruit decreased or remained at constant low levels during ripening (Perkins-Veazie *et al.*, 1995). Climacteric-like respiratory increases can be induced in nonclimacteric fruit by treating them with ethylene (McGlasson, 1985). Yet this increased respiration is not accompanied by an increase in endogenous ethylene production and the respiration rate usually subsides rapidly upon removal of the exogenous ethylene. However, ethylene treatment does accelerate senescence in nonclimacteric fruit (McGlasson, 1985). It is generally accepted that there are two systems of ethylene production in higher plants (Lelievre *et al.*, 1997a; McGlasson, 1985; McMurchie *et al.*, 1972; Oetiker & Yang, 1995; Yang, 1987). System 1 ethylene is the low level of ethylene

production that is involved in basic metabolic processes in all plant tissues including fruits (both climacteric and non-climacteric fruits) and vegetative tissues, and includes wounded-induced ethylene production. System 2 ethylene is the phase of autocatalytic ethylene production with high rates accompanying the ripening process of climacteric fruit. McGlasson (1985) suggested that nonclimacteric fruit possess System 1 but not System 2 ethylene production.

Ripening of climacteric fruit occurs through preclimacteric and postclimacteric stages of fruit development. In the preclimacteric stage, fruit produce ethylene at a low rate by System 1 (Oetiker and Yang, 1995). Low levels of ethylene (System 1) produced at this stage play an essential role in determining when the climacteric will occur, despite the apparent condition of physiological stasis (Eaks, 1980; Peacock, 1972; Yang, 1985). Banana treated with 0.44 µl/l ethylene for 4 days after harvest had no immediate response but ripened on average 26.4 days after harvest while untreated fruit ripened at 38.9 days (Peacock, 1972); this indicated that preclimacteric banana fruit were responsive to ethylene but the response was slow with ethylene affecting duration of the preclimacteric stage (Oetiker and Yang, 1995). In addition, changes in the responsiveness of tissue to System 1 may be a general feature of development of competence to ripen fruit; an increase in sensitivity of preclimacteric fruits to ethylene during development has been widely recognized in climacteric fruits (McGlasson, 1985). In kiwifruit, responsiveness to low ethylene concentration (even at 0.01 µl/l) during storage at 0°C increased with advanced maturity, resulting in rapid softening rate (Retamales and Campos, 1997). However, there is no direct information available to suggest that kiwifruit softening during storage at 0°C occurs because of increasing fruit responsiveness to System 1 ethylene.

The low level of preclimacteric ethylene (System 1) production can be reduced physically by holding the fruits under hypobaric conditions, or by application of ethylene biosynthesis inhibitors such as AVG and AOA (Yang, 1985). Both AVG and AOA are well known inhibitors of ACS activity, inhibiting conversion of SAM to ACC and thus blocking ethylene biosynthesis in plant tissues (Lieberman, 1979; Yang, 1985). Response

to these inhibitors requires their application before onset of rapid ACC synthesis (Yang, 1985). Preharvest sprays of AVG to several cultivars of apples including 'Golden Delicious' (Bangerth, 1978; Bufler, 1984; Halder-Doll & Bangerth, 1987), 'Delicious (Williams, 1980), 'Cox's Orange Pippin; (Child *et al.*,1984), 'McIntosh', 'Spencer' and 'Royal Red Delicious' (Autio and Bramlage, 1982; Bramlage *et al.*, 1980) inhibited ethylene production and led to delayed softening and thus firmer fruit during storage. Therefore, AVG promises to be tool that can control preharvest drop and improve fruit quality (e.g., firmness) of apple fruit. A commercial formulation of AVG (ReTain™) has been approved for commercial application to apples in the USA. In kiwifruit, postharvest dips with AOA reduced ethylene production and delayed fruit softening during and after cool storage at 0°C (Retamales *et al.*, 1995). Hyodo and Fukasawa (1985) found that AVG strongly inhibited ethylene production in flesh discs of kiwifruit, but the effect of preharvest application of this compound to kiwifruit vines on subsequent fruit quality is not known. However, since AVG and AOA do not inhibit the conversion of ACC to ethylene (Hyodo and Fukasawa, 1985), their effectiveness will be influenced by the concentration of ACC already present in the tissue. Therefore, effectiveness of AVG and AOA will be greater when applied to plants in which ACC biosynthesis is low and/or has not been initiated (Yang and Hoffman, 1984).

It is well known that exogenous ethylene regulates its own biosynthesis in various plant systems by both positive and negative feedback regulation (Kende, 1993; Yang and Hoffman, 1984). Positive feedback regulation (autocatalysis) of ethylene biosynthesis is a characteristic feature of ripening fruits and some senescing flowers; a massive increase in ethylene production is triggered by exposure to exogenous ethylene with activation of ACS and/or ACO (Nakatsuka *et al.*, 1997). The accumulation of ACO transcripts in several cases occurs earlier than that of ACS; stimulation of ACO leads to conversion of ACC to ethylene that, in turn, induces ACS transcripts generating more ACC for ethylene production (Fluhr and Mattoo, 1996). Negative feedback (autoinhibition) has been found in a variety of fruit and vegetative tissues. In green tomato (Liu *et al.*, 1985c), citrus fruit discs and leaf tissue (Riov and Yang, 1982a, 1982b), winter squash discs (Hyodo *et al.*, 1985) and mung bean hypocotyls (Yoshii and Imaseki, 1982) exogenous ethylene

significantly inhibited endogenous ethylene production induced by ripening and wounding; this was probably due to limited availability of ACC. This autoinhibition of ethylene production was correlated with the suppression of an ACS transcript accumulation in winter squash (Nakajima *et al.*, 1990), suggesting that some of the ACS genes may be negatively regulated by ethylene. Riov and Yang (1982a, 1982b) suggested that autocatalysis involved increased synthesis of ACS and ACO, whereas autoinhibition involves suppression of the activity of theses two enzymes. Autoinhibition may also occur by promoting the malonylation of ACC to the inactive malonyl-ACC (Liu *et al.*, 1985a, 1985b). Exogenous ethylene treatment markedly promoted malonylation of ACC that is thought to be responsible for reducing ACC and consequently ethylene production rate in preclimacteric tomato fruit or in grapefruit flavedo discs (Liu *et al.*, 1985a, 1985b). Another facet to negative regulation of ethylene biosynthesis is the finding of a gene, E8 (a fruit ripening protein), whose expression inhibits ethylene production in tomato (Lincoln and Fischer, 1988a). When E8 is introduced into tomato plants, the transformed plants have reduced levels of the E8 protein and concomitantly ethylene production is markedly enhanced. One possibility is that reduction in E8 protein results in a metabolic imbalance leading to stress inducible ethylene production during fruit ripening. Alternatively E8 protein could be involved in negative feedback regulation of ethylene biosynthesis during ripening. Thus, the E8 gene product functions by suppressing ethylene production and may be an important component of a signal transduction pathway by which autoinhibition of ethylene occurs in some tissues under certain physiological conditions (Fluhr and Mattoo, 1996).

Ethylene is known to regulate fruit ripening by coordinating the expression of genes responsible for enhancing a rise in rate of respiration, autocatalytic ethylene production, carotenoid synthesis and increased activity of cell wall-degrading enzymes (Theologis, 1992). However, the induction of gene transcription by ethylene is organ specific for one gene, while for others it is not; ethylene modulates gene expression by a variety of mechanisms. The discrimination between ethylene-dependent and independent pathways and gene expression during ripening has been facilitated by the availability of transgenic plants and the molecular analysis of naturally-occurring mutant lines. While many

ripening-related processes are inhibited in fruits with reduced ethylene, some aspects of ripening remain unaffected (Lelievre *et al.*, 1997a); fruit softening is known to be one of the ripening processes that is most sensitive to ethylene. Colour changes can be either ethylene-dependent or independent according to the type of pigments and the fruit species involved; lycopene accumulation is ethylene dependent but chlorophyll loss is independent in transgenic tomato with reduced ethylene production. However, chlorophyll loss is ethylene dependent in transgenic melons. In addition, cell wall degrading enzymes can also be classified as ethylene-dependent (endopolygalacturonase, some isoforms of β-galactosidase, α-arabinosidase or galactanase) or independent (pectin methyl esterase, exopolygalacturonase) (Pech *et al.*, 1998). Ethylene obviously plays a critical role in the ripening of climacteric fruit but given the identification of ethylene independent gene expression during ripening other developmental factors must also play a role. The nature of these nonethylene components remains unknown (Lelievre *et al.*, 1997a).

Antisense RNA technology can be a useful method to control ethylene biosynthesis and ripening in fruits and vegetables (Theologis, 1994). Severe inhibition of ethylene production was observed in the transgenic lines of tomato with antisense ACS RNA (Oeller *et al.*, 1991) and antisense ACO RNA (Hamilton *et al.*, 1990). Detached, mature fruits from these lines never ripened or softened. However, the antisense phenotype could be reversed by treatment with exogenous ethylene or propylene, an ethylene analogue, with the concomitant rise in respiration (Ayub *et al.*, 1996; Fluhr and Mattoo, 1996). Hamilton *et al.* (1990) found that transformed tomato plants with antisense ACO gene (pTOM13) showed a great reduction of ethylene, a delay of ripening and an extended storage life. In these antisense tomato fruits, the colour change was initiated at about the normal time, however, the extent of reddening was reduced. Antisense fruits stored for several weeks at room temperature were more resistant to overripening and shriveling than control fruits. E8 is a fruit ripening protein that is transcriptionally activated at the onset of ripening, coincident with the increase in ethylene biosynthesis (Lincoln and Fisher, 1988b); it is related to ACO, sharing 34% amino acid sequence identity over 295 residues. Whereas a reduction in ACO activity resulted in reduced levels of ethylene

biosynthesis (Hamilton *et al.*, 1990), inactivation of E8 gene expression by antisense RNA increased ethylene evolution during ripening (Penarrubia *et al.*, 1992); E8 has a negative effect on ethylene production in tomato fruit. Thus, although E8 and ACO are structurally related, they have evolved different functions during fruit ripening (Penarrubia *et al.*, 1992).

Among nine ACS genes which have been found in tomato (Lelievre *et al.*, 1997a), LE-ACC2 and LE-ACC4 are expressed during fruit ripening (Oeller *et al.*, 1991). Expression of antisense RNA from LE-ACC2 inhibited fruit ripening in tomato plants. Antisense fruits kept in air or on plants for 90-150 days gradually developed an orange color but never turned red and soft or developed an aroma. Ethylene production was inhibited by 99.5% in antisense ACS fruits, which failed to ripen. However, treatment with exogenous ethylene or propylene, an ethylene analog, for 6 days (ethylene treatment for 1 or 2 days was not sufficient) reversed the antisense phenotype in the absence of endogenous ethylene production and induced the respiration rise and the ripening process. Oeller *et al.* (1991) suggested that the ethylene-mediated ripening process requires continuous transcription of the necessary genes for ripening and ethylene is the trigger and not the by-product of ripening. In addition, the expression of polygalacturonase (PG) and ACO genes was found to be ethylene independent (Oeller *et al.*, 1991). Therefore, Theologis (1992, 1994) suggested that antisense fruits have at least two signal transduction pathways operating during tomato fruit ripening. The ethylene-independent (developmental) pathway is responsible for the transcriptional activation of genes such as PG and ACO. The ethylene-dependent pathway is responsible for the transcriptional and posttranscriptional regulation of genes involved in respiratory metabolism and ACS gene expression.

One other approach to reduce ethylene synthesis in plants involves induction or alteration of the pathway for metabolism of ACC (Klee *et al.*, 1991). Overexpression of the enzyme ACC deaminase from *Pseudomonas* reduced endogenous levels of ACC and limited ethylene biosynthesis. After introduction into tomato plants, transgenic plants had decreased ethylene formation, perceived ethylene normally, and had a prolonged ripening phase (Klee *et al.*, 1991). Theologis (1994) suggested that the use of antisense

technology and overexpression of metabolizing enzymes such as ACC deaminase, is only the first step toward controlling fruit ripening.

1.5 Ethylene action and its inhibition

In some cases, gene transcription during ripening is primarily activated by increased sensitivity to basal ethylene levels, while in other instances it is regulated by an increase in ethylene concentration (Lincoln and Fischer, 1988b). Trewavas (1982, 1983) suggested 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. Fig. 1-4 represents a model for competence to ripen during development of climacteric fruit; two phases are considered with reference to respiration and ethylene production (Pech *et al.*, 1994). In preclimacteric fruit (during the first period), the resistance to ripening or resistance to ethylene action is high, possibly due to the existence of a high level of "ripening inhibitors" in the fruit (Yang *et al.*, 1986) or the lack of ethylene receptors (Trewavas, 1982, 1983); the chemical nature of such inhibitors is unknown. The fruit is not able to ripen and ethylene biosynthesis is very low; when harvested at this stage such fruit never develop proper flavor and aromas (Pech *et al.*, 1994). After a certain time, corresponding to the second phase, there is a progressive decrease in resistance to ethylene action or an increase in sensitivity to ethylene action by System 1 ethylene. The low level of System 1 ethylene may play an essential role by accelerating the destruction of the unknown "ripening inhibitor" or by making tissues responsive to their endogenous ethylene concentrations so that the ripening process is initiated, resulting in the autocatalytic burst of ethylene production (System 2) together with the other biochemical changes associated with ripening (Yang *et al.*, 1986). The fruit has now developed the capacity to ripen on the tree or after detachment from the tree and to produce autocatalytic ethylene; it has become capable of autonomous ripening. The transition from the premature to the mature stage may occur rather early (vertical line a in Figure 1-4) in some fruit such as apple or kiwifruit. In stone fruits including peaches and apricots, this transition happens rather late (vertical line b), while in the cantaloupe Charentais melon, it may even take place after induction of autocatalytic ethylene production (vertical line c) (Pech *et al.*, 1994). In addition, Sfakiotakis *et al.* (1997) found that low temperature (10°C) strongly

inhibited the conversion from System 1 to System 2 in kiwifruit; System 1 seemed to function in the temperature range from 1.5°C to 11°C and System 2 operated in the temperature range of 14.5°C to 40°C.

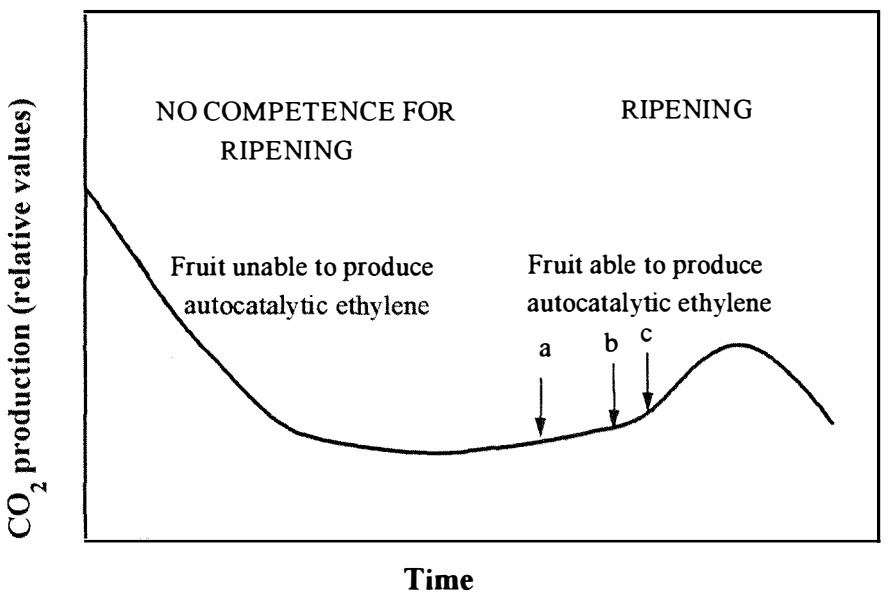

Fig. 1-4. Schematic representation of development of climacteric fruits with special reference to their competence to ripen. Vertical lines a, b and c represent the transition from the immature to the mature stage in several types of fruits (Pech *et al.*, 1994).

Ethylene perception by plant cells involves receptors that contain a protein-bound transition metal such as Cu(I) (Burg and Burg, 1967; Sisler, 1991), and binding of ethylene to these sites initiates a sequence of biochemical events leading to ripening (Veen, 1983). Variation in sensitivity may depend upon the numbers and characteristics of these ethylene receptors. There are two distinct classes of ethylene action (System 1 receptor or System 2 receptor) based on the fruit responsiveness to added ethylene (Fig. 1-5) (Yang, 1987; Oetiker and Yang, 1995). In immature (or preclimacteric) fruits, a low level of ethylene (System 1) is produced, and this plays an important role in inducing ACO activity and by accelerating the passage of the preclimacteric stage. The System 1 ethylene, in conjunction with the preexisting System 1 receptor, plays an essential role by destroying the "ripening inhibitor". At this time the tissue is not competent to express the gene needed to produce the System 2 ACS required for System 2 ethylene production nor to ripen in response to ethylene, presumably due to the lack of a System 2 receptor.

As the "ripening inhibitor" is inactivated, the System 2 receptor develops or becomes functional in mature fruits. An ethylene-System 2 receptor complex induces the development of ACS, whereas the preexisting ethylene-System 1 receptor complex coordinates the development of ACO. The development of ACS and ACO results in autocatalytic production of System 2 ethylene where the synthesis of ripening-associated enzymes (e.g. PG) begins to proceed. Both ACS and ACO gene expression are increased, and the gene family members are differentially regulated, during ripening (Lelievre et al., 1997a); three ACO genes and nine ACS genes has been found in tomato: LE-ACO1 and LE-ACO3 level are expressed at the onset of ripening and leaf senescence. Although LE-ACO1 continues to increase throughout ripening, LE-ACO3 increases transiently at the onset of each process and then declines concomitantly with the rise in ethylene production (Barry *et al.*, 1996). LE-ACO2 is not expressed during ripening or senescence but is expressed during the abscission of tomato flowers (Barry and Grierson, 1998). Among 9 ACS genes, LE-ACS2 and LE-ACS4 are induced during fruit ripening and after wounding. Further characterization of members of the ACO and ACS gene families is needed to identify individual members associated with System 1 and System 2 ethylene production or to find out what genes are regulated by different factors in preclimacteric and climacteric fruit (Lelievre *et al.*, 1997a).

The mode of ethylene action at the molecular level remains a mystery. Since ethylene is an olefin, its receptor may be either a Zn^2+- or Cu^2+-containing metalloprotein (Burg and Burg, 1967; Theologis, 1993). Ethylene action requires oxygen; this may be due to a putative oxidase hypothesized to keep the metalloprotein ethylene receptor in the oxidized form. The molecular basis for the action of ethylene has been investigated (Fig. 1-6). For a while, it was thought that ETR1 (ethylene-resistant) was the only plant ethylene receptor in *Arabidopsis* (Schaller and Bleecker, 1995), but ERS (ethylene response sensor) from *Arabidopsis* and nr (never ripe) and eTAE1 from tomato have also been isolated as ethylene receptors (Bleecker and Schaller, 1996). The identification of CTR1 (constitutive triple response) protein kinase and ETR1 ethylene receptor, provides a framework for elucidating the mechanisms by which the ethylene signal is perceived and

processed in plants. CTR1 mutations confer a constitutive ethylene response, while ETR1 mutations confer ethylene insensitivity (Theologis, 1996).

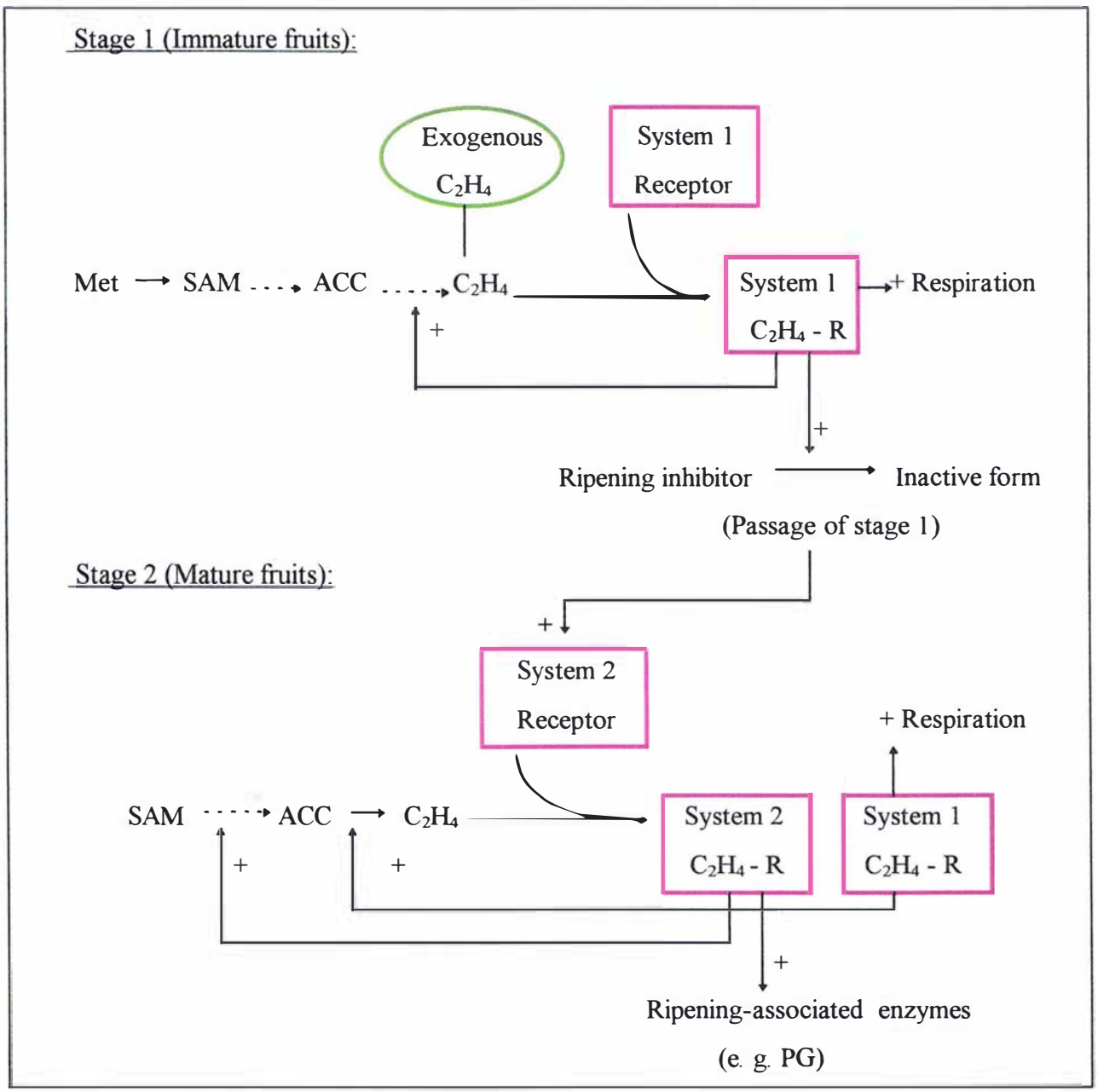

Fig. 1-5. A model showing the sequence of ethylene action on the regulation of ethylene biosynthesis in fruit maturation and ripening. + indicates the process is positively regulated. - - - - → indicates the metabolic process is not fully activated (Oetiker and Yang, 1995; Yang, 1987).

By assuming that ethylene binding to the receptor alters the activity of the His kinase domain, which in turn influences downstream components CTR1 and EIN2 (ethylene-

insensitive), two alternative models for the regulation of primary signal transduction have been proposed (Bleecker and Schaller, 1996); for model 1, ethylene is a positive regulator of His kinase activity of the receptor. The active kinase would act directly or indirectly to decrease the activity of the CTR1 protein kinase, resulting in an increase in EIN2 activity and consequently an increase in ethylene responsiveness. For model 2, ethylene is a negative regulator of the His kinase activity of the receptor. In the unbound state, the active kinase would directly or indirectly act to keep CTR1 active. Binding of ethylene to the receptor would inhibit the kinase activity, resulting in decreased activity of CTR1 and consequently an increase in EIN2 activity, leading to increases in ethylene responsiveness. These models are based on the genetic evidence that CTR1 negatively regulates EIN2 and that EIN2 may be responsible for generating some second messenger that drives ethylene responses (Ecker, 1995) (Fig. 1-6). However, more research for identification of the type of metal present in the ethylene binding site and identification of the components that connect the ethylene receptors to the protein kinases such as CTR1 is needed.

Compounds inhibiting ethylene action can be useful tools to elucidate the mode of ethylene action (Veen, 1983). The silver ion (Ag^+) is know to be a potent ethylene antagonist; Ag^+ interferes with the binding sites for ethylene (Sisler, 1982). Pre-treatment of carnation flowers with silver thiosulphate (STS), a commercial form of Ag^+, completely blocked the ethylene surge preceding wilting of petals. In addition, such a pretreatment caused the flowers to become insensitive to an ethylene treatment. However, its use is questioned as silver is a potent environmental pollutant, and many countries including Holland, propose to prohibit its use (Nell, 1992). A number of organic molecules which appear to block the ethylene receptor have been developed recently (Sisler *et al.*, 1986; Sisler and Blankenship, 1993; Sisler *et al.*, 1993; Serek *et al.*, 1994). Olefins such as transcyclooctene, trans-cyclooctadines or 2,5-norbornadiene (NBD) are effective inhibitors of ethylene action at a high concentrations but these olefins are released from receptors and diffuse from the binding site over a period of several hours. The photolytic product of diazocyclopentadiene (DACP), a competitive ethylene action inhibitor, binds irreversibly to the ethylene receptor or at least remains bound for

many days. However, DACP needs light for 'permanent' attachment to the site and is explosive in high concentrations, which limits its commercial usefulness.

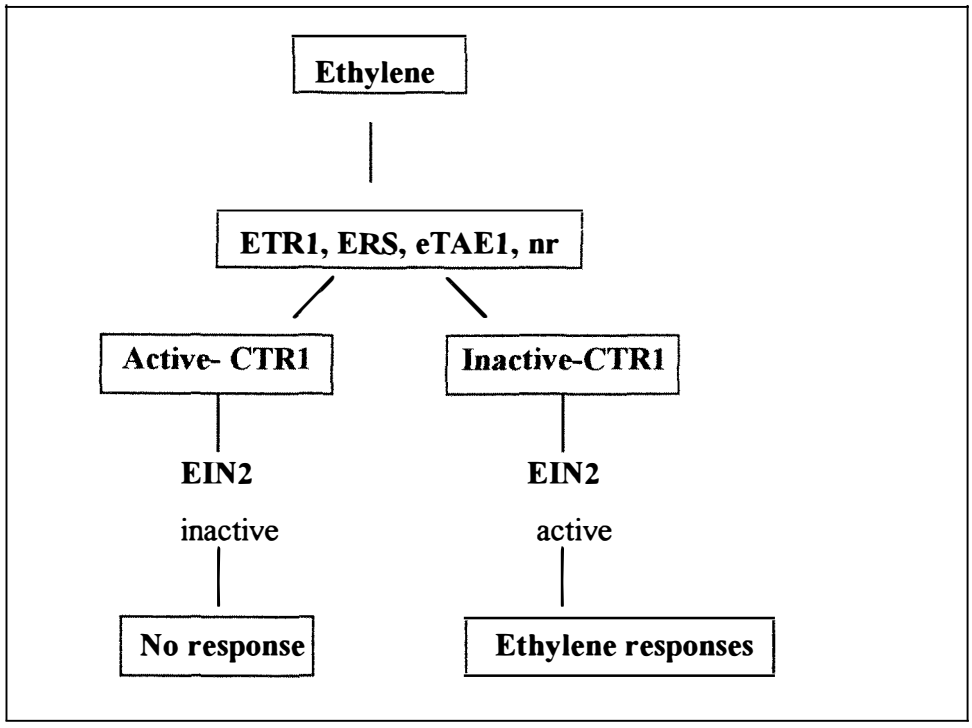

Fig. 1-6. Proposed sequences of gene action in the ethylene signal transduction pathway (Ecker, 1995).

1-methylcyclopropene (1-MCP), a cyclic olefin analogous to the photodecomposition product of DACP, is the most useful inhibitor of ethylene action developed recently. It is active at a concentration as low as 0.5 nl/l (Serek *et al.*, 1995a; Sisler *et al.*, 1996a; Sisler and Serek, 1997). 1-MCP pretreatment of ethylene sensitive flowers, including carnation and campanula, strongly inhibited ethylene effects such as bud and flower drop, leaf abscission, and accelerated flower senescence (Serek *et al.*, 1994; Serek *et al.*, 1995b; Sisler *et al.*, 1996b; Sisler and Serek, 1997). Besides flowers, 1-MCP prevented ripening of apple (Song *et al.*, 1997), banana (Golding *et al.*, 1998; Sisler and Serek, 1997) and tomatoes (Sisler *et al.*, 1996b). There is no published information available on 1-MCP effects on kiwifruit which is very sensitive to ethylene. Since most kiwifruit is stored at 0°C before export from New Zealand, and fruit soften at 0°C, any treatment that delayed softening during storage would be beneficial to growers and exporters who stand to lose many millions of dollars annually because of premature fruit softening during or after

storage. Application of 1-MCP to kiwifruit could be an effective treatment to maintain firmness by preventing kiwifruit softening that is accelerated by ethylene in ambient air.

1.6 Ethylene and kiwifruit softening

Kiwifruit softens with ethylene through 2 phases (Bonghi *et al.*, 1996; Lallu *et al.*, 1989; Ritenour *et al.*, 1999; Tonutti *et al.*, 1993); in the first phase, the largest changes in fruit firmness take place and softening occurs with no apparent increase in endogenous ethylene. The climacteric rise of ethylene began only after fruit softened to 50N (Tonutti *et al.*, 1993) or 7~10N (Bonghi *et al.*, 1996; Ritenour *et al.*, 1998). Lallu *et al.* (1989) also found that when kiwifruit was treated with ethylene firmness decreased from a harvest firmness of 78-98N to 15-20N within 3-4 days (called a rapid phase) followed by a slower phase as kiwifruit softened from 20N to an eating firmness of 7N. Fruit responsiveness to ethylene in the rapid phase increased with advanced maturity (Lallu *et al.*, 1989; MacRae *et al.*, 1989; Retamales and Campos, 1997); the rate of softening was greater in fruit of advanced maturity than in less mature fruit exposed to the same concentration of exogenous ethylene. Kiwifruit stored in an ethylene free environment at 0°C lost firmness gradually and the fruit with more advanced maturity showed increased softening in cold storage (Retamales and Campos, 1997); this might have been due to increased sensitivity to low endogenous ethylene.

Kiwifruit is very sensitive to ethylene, even at very low concentrations (Pratt and Reid, 1974; Matsumoto *et al.*, 1983; Retamales and Campos, 1997). Although kiwifruit produces low concentration of ethylene at 0°C, it is susceptible to ethylene at this temperature; even concentrations of 0.1 µl/l will increase fruit softening and reduce storage life (McDonald and Harman, 1982). Hyodo and Fukasawa (1985) and Hyodo *et al.*, (1987) suggested that the threshold concentration for initiation of autocatalytic ethylene at 21°C is 1 µl/l. Any rise in endogenous ethylene production is followed by a decrease in flesh firmness. Recently, Jeffery and Banks (1996) found that 0.01 µl/l ethylene reduced storage potential by 46% by causing advanced ripening and softening in kiwifruit (Retamales and Campos, 1997). Controlled atmosphere storage (2% O2 + 5% CO2) and ultra low oxygen (ULO) are effective methods of reducing kiwifruit softening

at 0°C (Antunes and Sfakiotakis, 1997a, 1997b; Arpaia *et al.*, 1984, 1987; Thomai and Sfakiotakis, 1997). However, the benefit of CA or ULO is minimized if ethylene and propylene are present (Antunes and Sfakiotakis, 1997a; Arpaia *et al.*, 1985, 1986); the presence of as little as 0.05 µl/l ethylene in CA accelerated softening which increased with ethylene concentration. Therefore, it is most important to scrub ethylene down to near zero for maximizing storage life of kiwifruit in both air or CA storage.

1.7 Projects investigated in this thesis

Premature softening limits storage life of kiwifruit at 0°C since kiwifruit is very sensitive to ethylene; the New Zealand kiwifruit industry has lost millions of dollars annually because of premature softening fruit. The purpose of this study was to investigate the role of ethylene in kiwifruit softening.

Therefore, the objectives of this study were to determine in kiwifruit:

(a) whether low temperature enhances ethylene biosynthesis during storage at 0°C or after removal to warmer temperatures and thus affect the softening rate.

(b) whether an ethylene synthesis inhibitor (AVG) can block ethylene biosynthesis of fruit, including sound kiwifruit and fruit infected with *B. cinerea,* and thus reduce softening rate during storage at 0°C and after removal to 20°C.

(c) whether the ethylene action inhibitor (1-MCP) can reduce softening rate during storage at 0°C and after removal to 20°C.

(d) whether kiwifruit responsiveness to ethylene changes during ripening and during storage at 0°C and 20°C by treating with ethylene and 1-MCP.

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CHAPTER TWO

MATERIALS AND METHODS

General materials and methods used for this thesis are described in this chapter.

2.1 Plant material

Kiwifruit [*Actinidia deliciosa* (A. Chev) C. F. Liang et A. R. Ferguson cv. 'Hayward'] were obtained from vines planted in 1992, spacing 5 m x 2.5 m, and trained on a winged T-bar system, growing at the Fruit Crops Unit, Massey University, Palmerston North (Lat S 40 21, Long E 175 37), New Zealand (Plate 2-1). Females vines were 'Kramer Hayward' on their own roots sourced from Te Puke, and male vines were M51, M56 and Chieftain (Ch) sourced from Kumeu. Kiwifruit were interplanted at a 4:1 female to male ratio. Fruit were harvested by snapping fruit off the stem in the morning (between 9-11 am); they were placed in a picking bag and then emptied into small plastic bins before transport to the laboratory. Fruit with defects were removed; those selected for experiments had a mean weight of 100g (range 80-120 g). To reduce incidence of *Botrytis cinerea* infection, the stem scar of each fruit was treated with fungicide ('Rovral' 0.075 %, one droplet of approximately 30 µl of solution per fruit) (O'Connor, 1994) within 2 hours after harvest. After the droplet had dried, fruit were placed in the pockets of 27 count plastic trays in one or two layer cardboard trays surrounded by a polyethylene liner. Sachets of 20 g of potassium permanganate were also enclosed. Fruit were then cured for 5 days at 15°C, 98% RH to reduce potential for *Botrytis* infection (Banos, 1995).

2.2 Gas measurements

Ethylene production and respiration rates were measured by placing individual fruit in separate air-tight plastic jars (500 ml capacity) fitted with a rubber septum (Plate 2-2). Before placing fruit inside jars, each jar was flushed with clean air. Immediately after sealing, a 1 ml gas sample was removed from each jar to determine initial carbon dioxide (CO_2) concentration. Jars were then incubated at 20°C for specific periods of time (between 30 and 90 min) depending on the rates of respiration or ethylene production of

Plate 2-1. Kiwifruit vines growing at the Fruit Crops Unit, Massey University, Palmerston North, New Zealand.

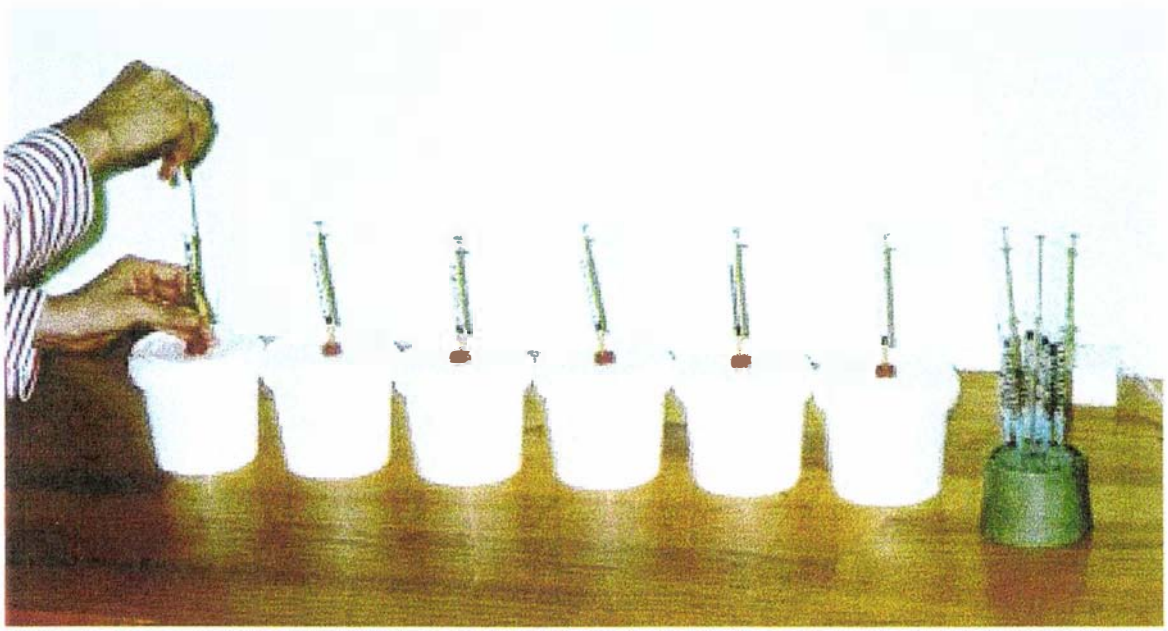

Plate 2-2. Air-tight plastic jars (500 ml capacity) fitted with a rubber septum, used for measurements of kiwifruit ethylene production and respiration rate.

fruits (CO_2 inside jars remained < 0.5%). Two 1 ml gas samples were taken from the headspace of each jar using an air tight syringe and analysed separately for CO_2 and ethylene.

Ethylene production was measured using either a Varian 3400 or Photovac 10S50 gas liquid chromatograph (GLC). The Varian GLC was fitted with a flame ionisation detector (FID)(minimum detection limit 0.01µl/l) and an activated alumina column (80-100 mesh, 1.8 m long and 0.32 cm diameter) in stainless steel. Temperatures of injector, column and detector were 110, 100 and 150°C respectively. Nitrogen was used as carrier gas with a flow rate of 30 cm^3/min., hydrogen and air for the detector (flow rates 30cm^3/min. and 300cm^3/min. respectively). The Photovac GLC was fitted with a photoionization detector (minimum detection limit 0.001µl/l), and both a pre-column (0.15m) and an analytical column (0.240m), type XE-60, made of teflon were used. Column and detector were maintained at ambient temperature (22-27°C). Clean air was used as carrier gas for both analytical and pre-columns at a flow rate of 15cm^3/min. Respiration rate was determined using an infra-red CO_2 transducer with electronic linearisation (Analytical Development Company, Hoddeston, U.K.), with O_2-free N_2 as carrier gas (flow rate 580mm^3/sec.). Output signals from the CO_2 transducer and GLC were analysed using integrators (Hewlett Packard, models 3394A, 3395 and 3396) using either peak height or peak area modes. Ethylene production and respiration rates were calculated according to the formulae in Appendix I.

2.3 Assay of ACC concentration and ACC oxidase (ACO) activity

2.3.1 ACC concentration: Flesh tissue was taken from the outer pericarp of individual kiwifruit and then stored at -74°C immediately. The samples were then freeze dried and ground to a powder. A 2 g powder sample was placed in a 50 ml tube containing 10ml water that was then centrifuged at 10,000 rpm for 20 min. at 2°C. The supernatant was assayed for ACC using a modified Lizada & Yang (1979) method. Modifications were as follows: a 0.5ml sample of supernatant was placed in a test tube containing 0.1ml 50 mM $HgCl_2$ with or without 0.1ml 100µM ACC and made up to 1.8ml with distilled water.

Reaction test tubes were sealed with a serum rubber stopper, placed in ice, and 0.2ml of a mixture of 5% NaOCl and saturated NaOH (2:1, v/v) was added by syringe through the stopper. The mixture was agitated on a Vortex mixer for 5 sec., incubated on ice for 24 min., then agitated for a further 5 sec. A 1 ml gas sample was taken from the headspace and injected into a GLC for ethylene determination. ACC concentration was calculated according to the formula in Appendix II. The efficiency of the conversion of ACC to ethylene was about 78% (Table 2-1).

Table 2-1 Efficiency of conversion of ACC to ethylene

ACC added (nmole)	C_2H_4 (nmole)	Percentage conversion to C_2H_4
0	0.1	—
5	3.9	77
10	7.8	77
20	15.3	76
40	32.5	81

2.3.2 ACC oxidase (ACO) activity: ACO activity was determined by measuring the conversion of administered ACC to ethylene. Discs (11mm diameter and 2mm thick), weighing 1.5 g, were excised with a corkborer, one disc being taken from the pericarp tissue of each of three fruits per treatment, and then placed into test tubes containing 3 ml of 10mM phosphate buffer (pH 6.1) (McKenzie and Dawson, 1969) with 0.5M mannitol and 1mM ACC. Test tubes were sealed with rubber serum caps and shaken at 100 rpm for 2 hours at 30°C. A 1 ml gas sample was withdrawn from the headspace and injected into the GLC for ethylene determination. ACO activity was calculated according to the formula in Appendix II.

2.4 Flesh firmness

2.4.1 *Destructive measurement*: using a fruit peeler, about 1 mm of skin was removed from opposite sides of the fruit at the equator. Flesh firmness (the force required to penetrate the kiwifruit in Kgf) was measured on the pared surfaces with a press-mounted (Black and Decker electric drill press) penetrometer (0-12 Kgf Effegi, fitted with a 7.9 mm diameter probe; Facchini, Alfonsine, Italy). Fruit firmness was taken as the mean of the two measurements and expressed as Newtons (N) (Kgf multiplied by 9.81) (Soule, 1985).

2.4.2 *Non-destructive measurement*:

The 'Effegi' penetrometer is the standard instrument used by the fruit industry to assess firmness but unfortunately its use is destructive. Fruit to fruit variability is a major problem in studying kiwifruit softening behaviour. A non-destructive technique for measuring kiwifruit firmness would be very useful in postharvest research by enabling repeated measurements to be made on the same fruit over time (Hopkirk *et al.*, 1996). Repeated measurements on individual fruit can reduce experimental error in research on fruit softening behaviour (Davie *et al.*, 1996).

(I) *Prototype II softness meter*: The prototype II softness meter was developed by Massey University, Palmerston North (Davie *et al.*, 1996) (Plate 2-3). It measures deformation of the fruit surface beneath a small spherical probe under constant load with time and causes negligible damage to the tested fruit. Softness was quantified as a "softness coefficient", estimated as the slope of the regression line of deformation on the natural logarithm of time. Two readings per fruit were taken (at 90°C from each other) and averaged. The scale is unitless, but a relationship between values obtained by softness coefficients and firmness (N) was obtained using the following equation 1 (Table 2-2) (Davie *et al.*, 1996):

$$Y = 137.2 \pm 6.0 / X^{0.487 \pm 0.017} \quad \text{---------- (Equ. 1)}$$

(where Y=softness coefficient value, X= penetrometer value (N) (Kgf multiplied by 9.81))

Table 2-2 The relationship between values obtained by the Penetrometer (N or Kgf) and the Prototype II softness meter (SC) derived using equation 1

N	5	10	20	30	40	50	60	70	80	90	100
Kgf	0.5	1.0	2.0	3.1	4.1	5.1	6.1	7.1	8.2	9.2	10.2
SC	62 ~ 64	44 ~ 45	32	26 ~ 27	22 ~ 23	20 ~ 21	18 ~ 19	17 ~ 18	16 ~ 17	15 ~ 16	14 ~ 15

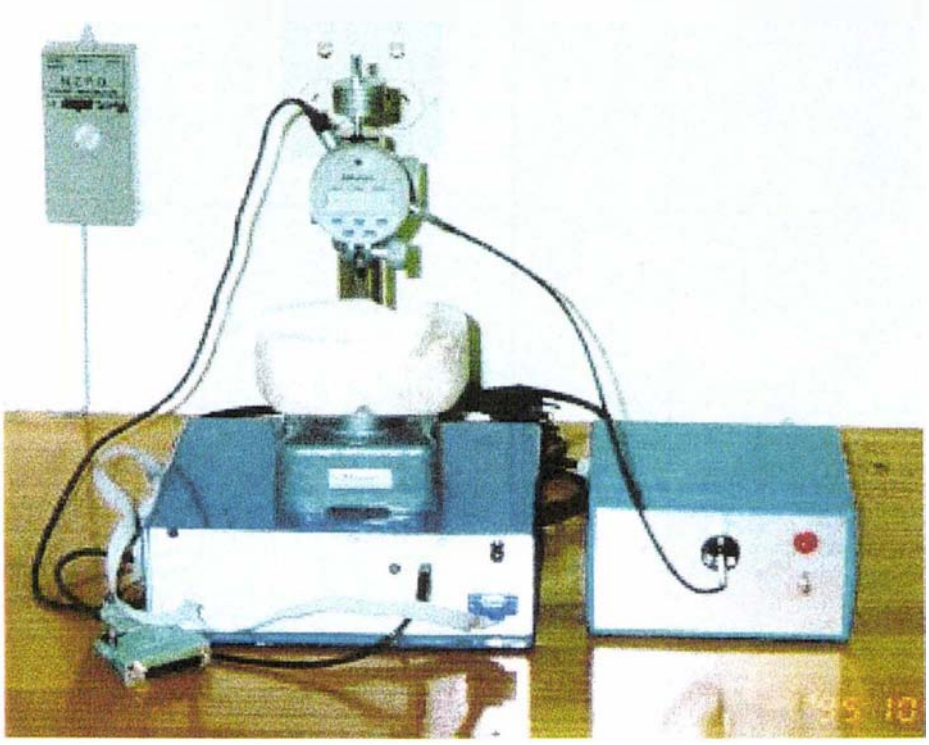

Plate 2-3. The prototype II softness meter, developed by Massey University measures deformation of the fruit surface non-destructively.

Plate 2-4. Kiwifirm developed by Industrial Research Limited, Auckland, measures fruit firmness non-destructively.

(II) *Kiwifirm*: Kiwifirm is a small hand-held device developed by Industrial Research Limited, Auckland, NZ (Plate 2-4). The device applied a known quantum of energy to the fruit surface through a small, nonpenetrating tip; it contains an in-built processor which converts characteristics of the resulting collision to display a value which ranges from 0 to 9.9 and decreases as fruit soften (Hopkirk *et al.*, 1996). Fruit firmness was obtained as the mean of four readings per fruit, taken on opposite sides of each fruit. The scale is unitless but a correlation curve between 'Kiwifirm' and penetrometer values was made from the samples taken in the experiment, with the values of 4.6, 5.7, 6.8 and 7.9 in the 'Kiwifirm' scale being approximately equal to 10, 30, 50 and 70 Newtons respectively (Appendix III).

2.5 Total soluble solids

Total soluble solids (TSS) concentration (%) of a combined juice sample obtained from slices taken from proximal and distal ends of kiwifruit was measured using a hand-held

refractometer (0-20% Atago, Atago Co., Ltd., Tokyo, Japan) calibrated at 20°C to 0% with distilled water. The prism surface and the light plate were washed and dried with clean soft tissue paper between each reading. Two readings were taken on each fruit and averaged.

2.6 Statistical analysis

Each experiment had between three to five replicates in a Randomised Complete Block Design (RCBD). The SAS System programmes (SAS 1988) were used to analyse data from each experiment for Analysis of Variance (ANOVA), means and standard errors, and a test of least significant difference (l.s.d.) and Duncan's comparison at 5% level.

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CHAPTER THREE

LOW TEMPERATURE EFFECTS ON SOFTENING AND ETHYLENE BIOSYNTHESIS OF KIWIFRUIT

3.1 INTRODUCTION

Production of ethylene in plants is induced by various environmental factors including wounding, disease, drought, cold temperature, and exposure to various chemicals. This kind of ethylene production is called stress induced ethylene production, or more simply, stress ethylene (Abeles *et al.*, 1992). Stress ethylene is one of the general phenomena observed in plant tissues subjected to various unfavorable conditions. Environmental stresses can be divided into three groups: physical stress, chemical stress and biological stress. Ethylene production is induced by many forms of physical stress. Among them are mechanical wounding, physical load or pressure, water deficit, flooding, low temperature and irradiation. Also, ethylene is induced in plant tissues by chemical stimuli such as heavy metal ions, herbicides and gases such as sulfur dioxide and ozone. Biological stresses are caused by organisms such as fungi, bacteria, viruses or insects invading and attacking the plant. Most ethylene produced in diseased tissue, as a result of a host-parasite relationship, is of host origin (Hyodo, 1991).

Postharvest temperature management is the predominant method used to maintain quality of harvested horticultural crops. Low temperature substantially reduces the velocity of many metabolic processes, including ethylene biosynthesis, that lead to natural deterioration and loss of quality of horticultural products. For certain commodities, however, the commonly accepted practice of quickly cooling freshly harvested material to low, nonfreezing temperatures can actually shorten market life because of susceptibility to a disorder called chilling injury (Saltveit and Morris, 1990). Chilling injury is responsible for substantial postharvest losses. While avoiding exposure to chilling temperature is the safest way to prevent chilling injury, most horticultural crops are perishable and deteriorate rapidly at warm temperatures (Wang, 1991).

There are two different types of response to low temperature on induction of ethylene production in plants. One is chilling stress. It is not necessarily the exposure to low temperature that induces ethylene increase. For those plants that sustain chilling injury, ethylene is produced as a result of tissue injury (called chilling injury that is eventually manifested in symptoms such as pitting, water soaking, internal breakdown, uneven or incomplete ripening, off-flavour and decay) and ethylene increase occurs only on transfer of the product to warmer temperatures after exposure to chilling temperatures. As with other forms of stress, chilling has two effects on ethylene production: it stimulates ACC synthase (ACS) and it also reduces ACC oxidase (ACO) activity, probably by disrupting membranes in which the ACO enzyme is thought to be located (Abeles *et al.*, 1992). Wang and Adams (1980, 1982) found that ACC concentration, ACS activity, and ethylene production remained low in cucumbers held at 2.5°C, a temperature which caused chilling injury, and increased rapidly only upon transfer to warmer temperatures. However ethylene production by chilled (2.5°C) cucumbers increased very little upon transfer to 25°C if the fruit were chilled for more than 4 days. Even though such fruit had increased ACC levels, they produced very little ethylene even when ACC was added. This indicated that the system which converts ACC to ethylene (ACO) was damaged by prolonged exposure to the chilling temperature, probably by severely disrupting cellular membranes.

The second type of response to low temperature is to hasten induction of ethylene synthesizing competency upon transfer from coolstore to room temperature without chilling injury symptoms developing. Since a lack of uniformity of ripening cause serious problems for the industry of several fruits such as apples, pears and kiwifruit, such cold storage is useful to synchronize ripening of those fruit (Hyodo and Fukasawa, 1985; Lallu *et al.*, 1989; Pech *et al.*, 1994; Puig *et al.*, 1996). In some cases, a cold treatment is absolutely required for the induction of autocatalytic ethylene production; for many European pear cultivars, including Bosc, Conference, Passe-Crassane and d' Anjou (Blankenship and Richardson, 1985; Knee *et al.*, 1983; Puig *et al.*, 1996; Sfakiotakis and Dilley, 1974), a period of low temperature is used commercially to induce ripening. If such fruit are kept at 15°C without cold treatment, they produce only a small amount of

ACC and do not ripen properly; cold storage at 0°C induces ACC synthesis, allows ACC accumulation and, as a consequence, the fruits start to produce ethylene and to ripen normally upon exposure to higher temperatures. The low temperatures clearly act as a trigger to stimulate increases in endogenous ethylene (Romani *et al.*, 1982). Gerasopoulos and Richardson (1997) found for pears that the longer the time at -1°C, the shorter the time required to produce more than 1 µl/l internal ethylene in an atmosphere containing 500 µl/l propylene at 20°C. Therefore, storing pears at 0°C hastened the induction of ethylene-synthesizing competency and homogeneous ripening after subsequent rewarming.

Lelievre *et al.* (1997) evaluated the role of chilling on ethylene biosynthetic gene expression in Passe-Crassane pear fruits which require a 3-month chilling treatment at 0°C for autocatalytic ethylene and ripening. The chilling treatment at 0°C stimulated ACO activity strongly, and ACS activity to a lesser extent. Therefore, fruit that had been previously chilled exhibited a burst of ethylene production associated with high activity of ACO and ACS upon rewarming, while fruit stored at 18°C immediately after harvest did not exhibit any of these changes. In other types of fruit, including some apple cultivars, low temperatures also hasten induction of ethylene synthesizing competency and homogeneous ripening (Jobling *et al.*, 1991; Lelievre *et al.*, 1995; Pech *et al.*, 1994); storing preclimacteric Granny Smith apples in air at 0°C for more than 1 week induced ethylene production earlier when returned to 20°C than in fruit maintained continuously in air at 20°C. The cold treatment induced ACS and ACO activity before transfer of the fruit to warmer temperatures, but it stimulated ACO activity earlier than ACS (Jobling *et al.*, 1991; Larrigaudiere and Vendrell, 1993; Lelievre *et al.*, 1995). However there is a differential effect of cold stimulation on ethylene biosynthesis in different cultivars of apples (Larrigaudiere *et al.*, 1997); in 'Royal Gala' and 'Starking Delicious', chilled fruit and non chilled fruit produced almost the same amount of ethylene at 20°C, while in 'Granny Smith' chilled fruit produced much higher (3 times) ethylene production than non-chilled fruit after transfer to 20°C.

Firmness is a key criterion in the assessment of quality of kiwifruit for export in New Zealand, where premature softening is typically responsible for 40-70% of fruit losses after harvest (Davie *et al.*, 1996). Postharvest softening in kiwifruit at 0°C occurs in three phases (MacRae *et al.*, 1989; MacRae *et al.*, 1990). The first phase is a lag period when very little softening occurs; this lag phase decreases as harvest maturity increases. The second phase is a rapid phase of softening that lasts for up to 6 weeks; this is where the largest reduction in fruit firmness takes place from 60-90N to about 20N. The third phase is a slow rate of softening which begins at about 20 N and continues until fruit are ripe or overripe with a firmness of less than 6~8 N regardless of harvest time. There is some evidence that a relatively sharp reduction in firmness (a phase 4 ?) occurs at the very end of this phase when final tissue disintegration is taking place and the fruit is no longer edible.

Kiwifruit are very susceptible to ethylene; even at 0°C, 0.01 µl/l ethylene increased fruit softening and reduced the storage life (Jeffery and Banks, 1996; Retamales and Campos, 1997). While it is now known that low temperature can act as a trigger to induce biosynthesis of ethylene in some cultivars of pears, apples and in kiwifruit grown in Greece (Sfakiotakis *et al.*, 1997), with the subsequent expected physiological events occurring, it is not known whether the same effect occurs during storage at 0°C or after removal to warmer temperatures for kiwifruit grown in New Zealand. Kiwifruit intended for export are harvested mature, but firm and unripe, cool-stored at 0°C for up to 4-6 months and subsequently shipped under refrigeration (0°C) to overseas markets. Although low temperature has been used to delay softening during storage in kiwifruit, it may also induce fruit to produce ethylene and to ripen rapidly upon transfer from coolstore to room temperature. This has important commercial implications as it may reduce fruit shelf life potential.

This research was conducted to characterize the effects of low temperature on ethylene biosynthesis in kiwifruit and to determine the relationship between firmness and ethylene production in this fruit.

3.2 MATERIALS AND METHODS

All kiwifruit cv. Hayward were harvested from the Fruit Crops Unit at Massey University.

In 1995, fruit were harvested on 23 May when the mean soluble solids content was 7.5 % and fruit weight was 90-140g. Fruit were cured for 5 days at 15°C, 98% RH to reduce infection by *Botrytis cinerea*. When cured, fruit were placed in the pockets of 27 count plastic trays surrounded by a polyethylene liner, put in one layer cardboard trays and then transferred to a range of temperatures (0±1, 5±1, 10±1 and 20±1°C). Fruit were removed to 20°C after 0, 7, 14, 21, 28, 42 and 64 days at these temperatures for measurements including ethylene production and firmness, the latter measured using a Prototype II softness meter which measures deformation of the fruit surface beneath a small spherical probe under constant load with time (Davie *et al.*, 1996). Three replications per treatment and 6 fruits per replicate were used for each evaluation.

In 1996, kiwifruit were harvested on 16 May when the mean soluble solids content was 7.0 % and fruit weight was 80-120g. The stem scar of each fruit was treated with the fungicide ('Rovral') and then fruit were placed in the pockets of 27 count trays surrounded by a polyethylene liner and put in two layer cardboard trays. Sachets of potassium permanganate (20g) were also enclosed. Fruit were then cured for 5 days at 15°C, 98% RH and transferred to 0°C for 0, 7, 14, 21, 42 and 64 days before removal to 20°C. On removal, half of the kiwifruit from 0°C was used for measuring firmness (measured with 'Kiwifirm') and for determining ethylene production using the same fruit at each sampling time over the entire experiment. The remaining fruit were used for measuring firmness (measured with 'Kiwifirm' and an Effegi penetrometer) and for determining ethylene production using different fruit at each sampling time; pericarp tissue from these fruit was obtained for subsequent analysis of ACC concentration and ACO activity. Three replications per treatment and 3 fruits per replicate were used for ethylene production and respiration rate, while three replications per treatment and 1 fruit per replicate were used for ACC content and ACO activity analysis (see chapter 2 for details).

In 1997, kiwifruit were harvested on 12 May when the mean soluble solids content was 7.5 % and fruit weight was 80-120g. After treating the stem scar of each fruit with fungicide ('Rovral'), fruit were placed in the pockets of 27 count trays surrounded by a polyethylene liner and put in one layer cardboard trays. Sachets of potassium permanganate (20g) were also enclosed. Fruit were then cured for 5 days at 15°C, 98% RH and transferred to 0°C for 14, 30, 52, 80, 110, 150 and 180 days before removal to 20°C. Ethylene biosynthesis (ethylene production, ACC content and ACO activity), respiration rate and firmness (using the 'Kiwifirm') were measured. Five replications per treatment and 3 fruits per replicate were used for ethylene production and respiration rate, while five replications per treatment and 1 fruit per replicate were used for analysis of ACC content and ACO activity.

All data were analysed using the SAS System programmes (SAS 1988) for Analysis of Variance (ANOVA), means and standard errors and Duncan's comparison at 5% level.

3.3 RESULTS

3.3.1 Measurements on the same fruit at each sampling time

3.3.1.1 1995 experiment

Results in 1995 were preliminary and indicative; a major problem arose during storage due to contamination of fruit by *B. cinerea*. Data from fruit stored at 0, 5 and 10°C for 21, 28, 42 and 64 days were discarded because these fruit were seriously infected with *B. cinerea*.

Firmness

When returned to 20°C, fruit stored at 0°C for 14 days softened faster than fruit stored at 0°C for 7 days which in turn softened faster than fruit maintained at 20°C continuously (Fig. 3-1A). Fruit softened to below 10N after 22, 18 and 15 days in fruit pretreated at 0°C for 0, 7 and 14 days respectively (Fig. 3-1A). Firmness of fruit stored at 5°C for 14 days and 10°C for 7 or 14 days before transfer to 20°C did not change greatly during storage at 20°C compared to fruit kept at 0°C (Fig. 3-1A and Fig. 3-2A) but fruit kept at 5°C for 7 days before transfer to 20°C softened to below 10N after 32 days (Fig. 3-2A).

Some degree of softening occurred prior to a measured increased in ethylene production in all treatments (Fig. 3-1 and Fig. 3-2).

Ethylene production

Fruit maintained at 20°C after harvest began to produce ethylene after 20 days, while fruit maintained for 7 and 14 days before removal from 0°C, produced ethylene after 13 and 11 days at 20°C respectively (Fig. 3-1B). Peak production (24, 51 and 49 µl/kg/h) was reached after 26, 20 and 14 days at 20°C for fruit that had been at 20°C or at 0°C for

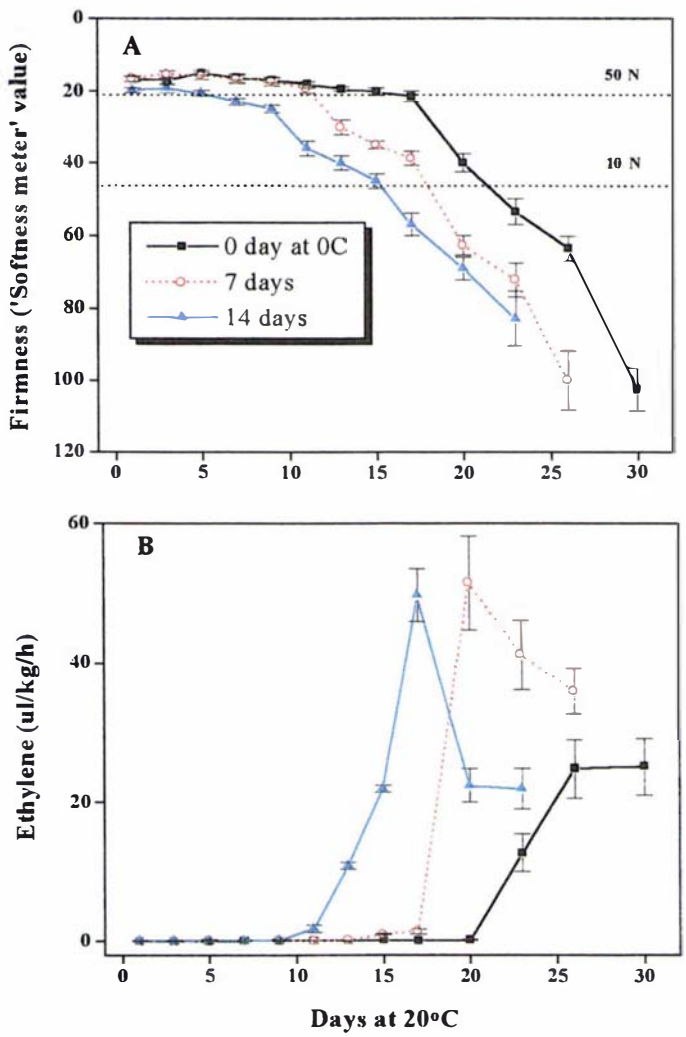

Fig. 3-1. Fimness (A) measured with 'Softness meter' and ethylene production (B) of kiwifurit maintained at 20°C following pretreatment at 0°C for 0, 7 and 14 days in 1995. Bars represent standard errors. Dotted lines show the value that is equivalent firmness to that obtained with a penetrometer.

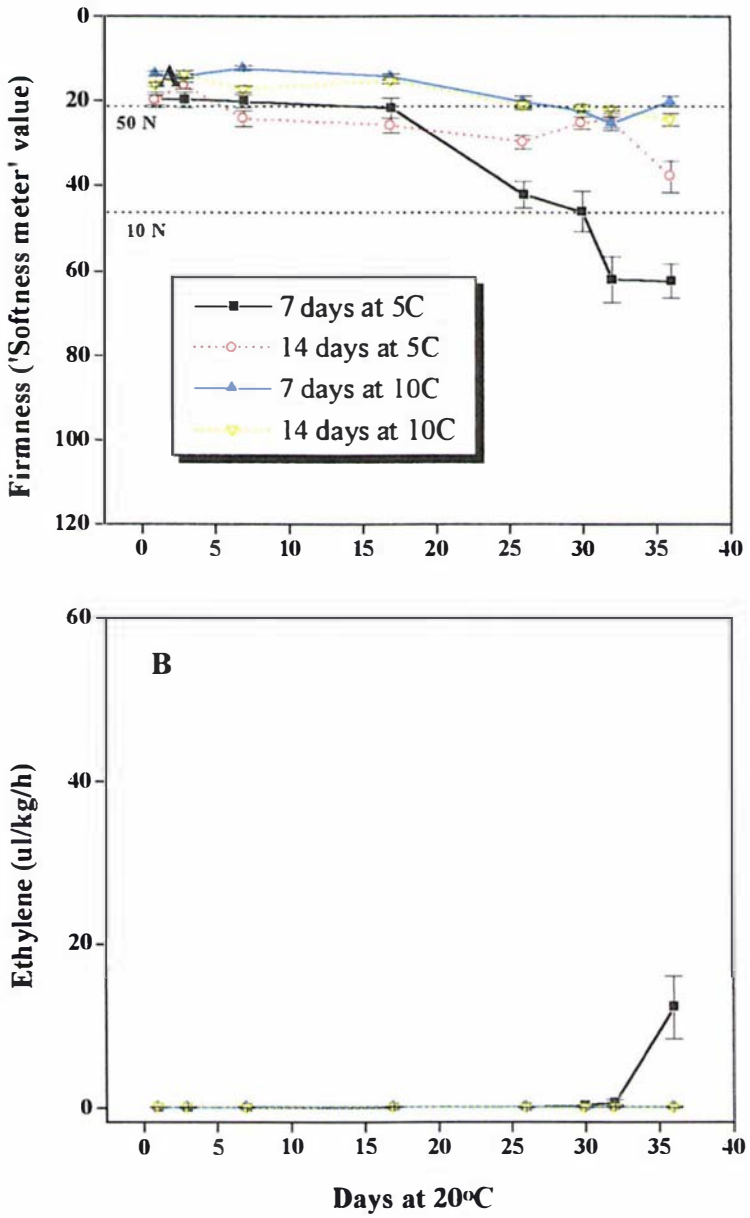

Fig. 3-2. Firmness (A) measured with 'Softness meter' and ethylene production (B) of kiwifruit maintained at 20°C following pretreatment at 5 and 10°C for 7 and 14 days in 1995. Bars represent standard errors.

7 and 14 days respectively. Peak ethylene production was similar for fruit maintained at 0°C, being about twice that produced by fruit maintained at 20°C continuously.

However, fruit stored at 5 and 10°C before transfer to 20°C maintained very low ethylene production except for fruit kept at 5°C for 7 days which began to produce ethylene 32 days after removal to 20°C reaching 12 µl/kg/h ethylene after 36 days (Fig. 3-2B).

3.3.1.2 1996 experiment

Firmness

Firmness of kiwifruit decreased with time at 0°C. Firmness of kiwifruit was 70N at harvest and after a 7 day lag at 0°C, it declined sharply to 52N after 14 days at 0°C; from 21 until 42 days at 0°C softening was slow, after which there was sharp decline in firmness to 22N at 64 days (Fig. 3-3A).

After transfer to 20°C, firmness of kiwifruit kept at 0°C for 0 and 7 days before transfer to 20°C was similar, decreasing from 70N to about 44N through 17 days. Fruit stored at 0°C for 14 days (Fig. 3-4A) had a firmness of 51N immediately after removal to 20°C, remaining at this level for 5 days, then softening to below 21N through day 17. Firmness of kiwifruit stored at 0°C for 42 days remained relatively constant (39N) for 10 days at 20°C before softening relatively rapidly. Fruit maintained at 0°C for 64 days were already softer than those in the other treatments (22N) on removal from coolstore decreasing to 10N during 17 days at 20°C.

Ethylene production

Ethylene production was low for fruit kept at 0°C for 42 days varying between 0.023 to 0.06 µl/kg/h. There was a large increase in ethylene production to 0.13 µl/kg/h by fruit stored 64 days at 0°C (Fig. 3-3B).

After removal to 20°C, there was a large variation of ethylene production between individual fruits which prevented differences from being significant; therefore data were expressed as the proportion (%) of fruit with ethylene production above a certain level (0.1 µl/kg/h ethylene) (Fig. 3-4B). No fruit stored at 0°C for 0 and 7 days produced ethylene at a rate > 0.1 µl/kg/h through 17 days at 20°C. 11% of fruit maintained at 0°C for both 14 and 42 days produced ethylene at more than 0.1 µl/kg/h after 10 days at 20°C, after which the number remained constant for fruit stored at 0°C for 14 days but increased to 33% for fruit stored at 0°C for 42 days. 33% of fruit maintained at 0°C for 64 days started to produce ethylene at a rate greater than 0.1 µl/kg/h immediately after

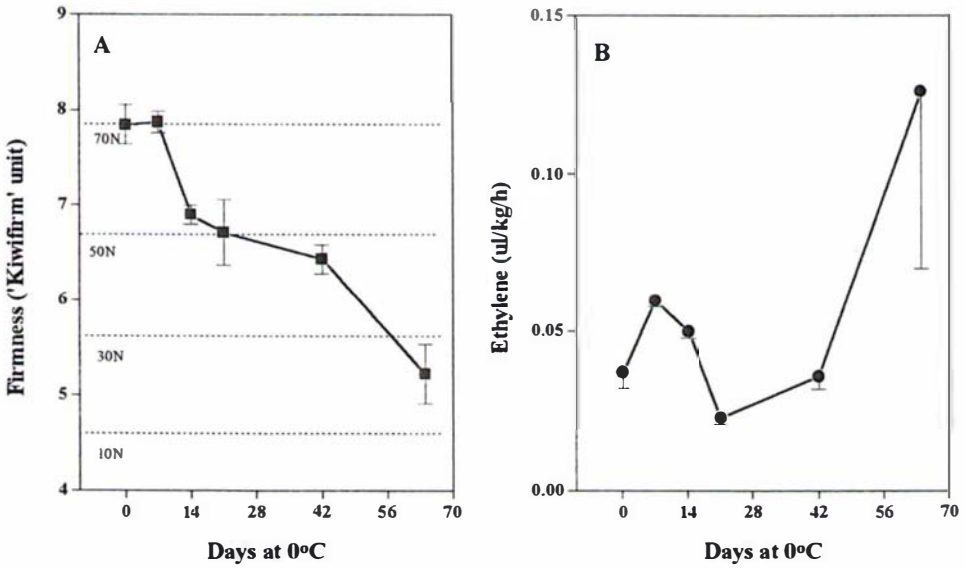

Fig. 3-3. Firmness (A) measured with 'Kiwifirm' and ethylene production (B) of kiwifruit during storage at 0°C in 1996. Bars represent standard errors.

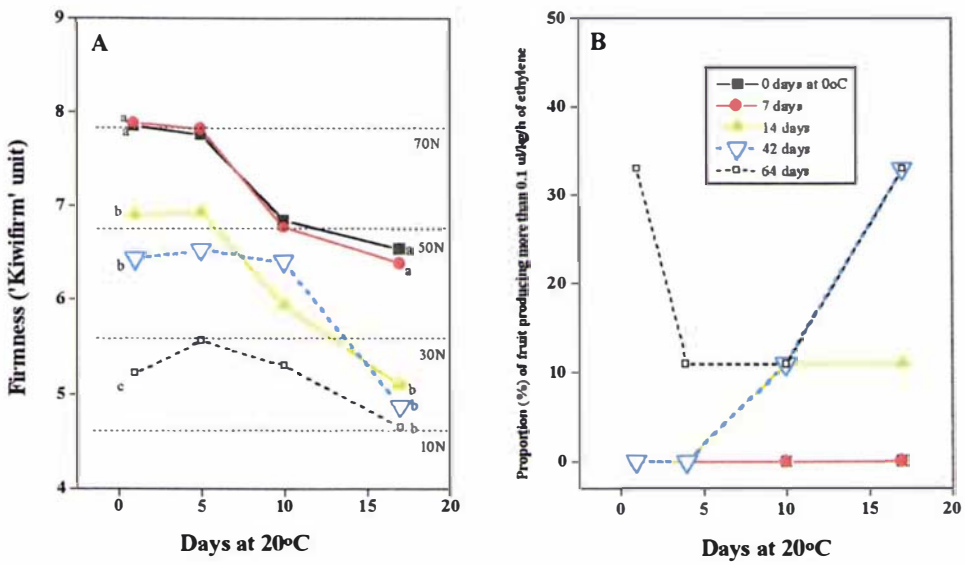

Fig. 3-4. Firmness (A) measured with 'Kiwifirm' and ethylene production (B) from kiwifruit maintained at 20°C following storage at 0°C for different times in 1996. At a given time values followed by the same letter are not significantly different by Duncan's multiple range test at the 5 % level.

removal to 20°C, after which the number decreased to 11% after 4 days and remained constant until increasing to 33% again after 17 days at 20°C.

3.3.2 Measurements on different fruit at each sampling time

Although firmness was measured non-destructively, assays for ACC concentration and ACO activity used disks taken from different fruit at each sampling time.

3.3.2.1 1996 experiment

Firmness

During storage at 0°C, firmness of kiwifruit decreased. At harvest firmness was 70N, and it decreased linearly to 49N after 21 days at 0°C; it then remained relatively constant for a further 21 days after which it softened to 24N after 64 days (Fig. 3-5A).

In general fruit from all treatments softened after removal from 0°C to 20°C, declining to 43, 32, 25, 21 and 18N after 16 days at 20°C for fruit that had been at 0°C for 0, 7, 14, 21 and 42 days respectively (Fig. 3-6A, B, C, D and E). Fruit maintained at 0°C for 64 days was relatively soft (23N) on removal to 20°C and firmness decreased slightly to 14N during 16 days (Fig. 3-6F).

Ethylene production

Ethylene production was low in kiwifruit throughout storage for 64 days at 0°C varying between 0.022 and 0.055 µl/kg/h (Fig. 3-5B). After transfer to 20°C, fruit kept for 0, 7, 14, 21 and 42 days at 0°C produced very low but detectable amounts of ethylene during 17 days at 20°C (Fig. 3-7A, B, C, D and E). In contrast, fruit kept at 0°C for 64 days began to produce ethylene after 6 days at 20°C attaining a rate of 3.31 µl/kg/h after 17 days (Fig. 3-7F).

ACC concentration

ACC was present in kiwifruit at harvest and remained at concentrations between 0.15 and 0.5 nmole/g throughout storage at 0°C despite considerable variation (Fig. 3-5C). After removal to 20°C, ACC always remained above 0.11 nmole/g in fruit from all treatments (Fig. 3-7), but no consistent pattern of ACC concentration was apparent. Fruit kept continuously at 20°C reached a peak ACC concentration after 6 days at 20°C and then decreased sharply to 0.21 nmole/g (Fig. 3-7A). Fruit stored for 7 days at 0°C before removal to 20°C had a relatively high concentration of ACC on removal from 0°C; it

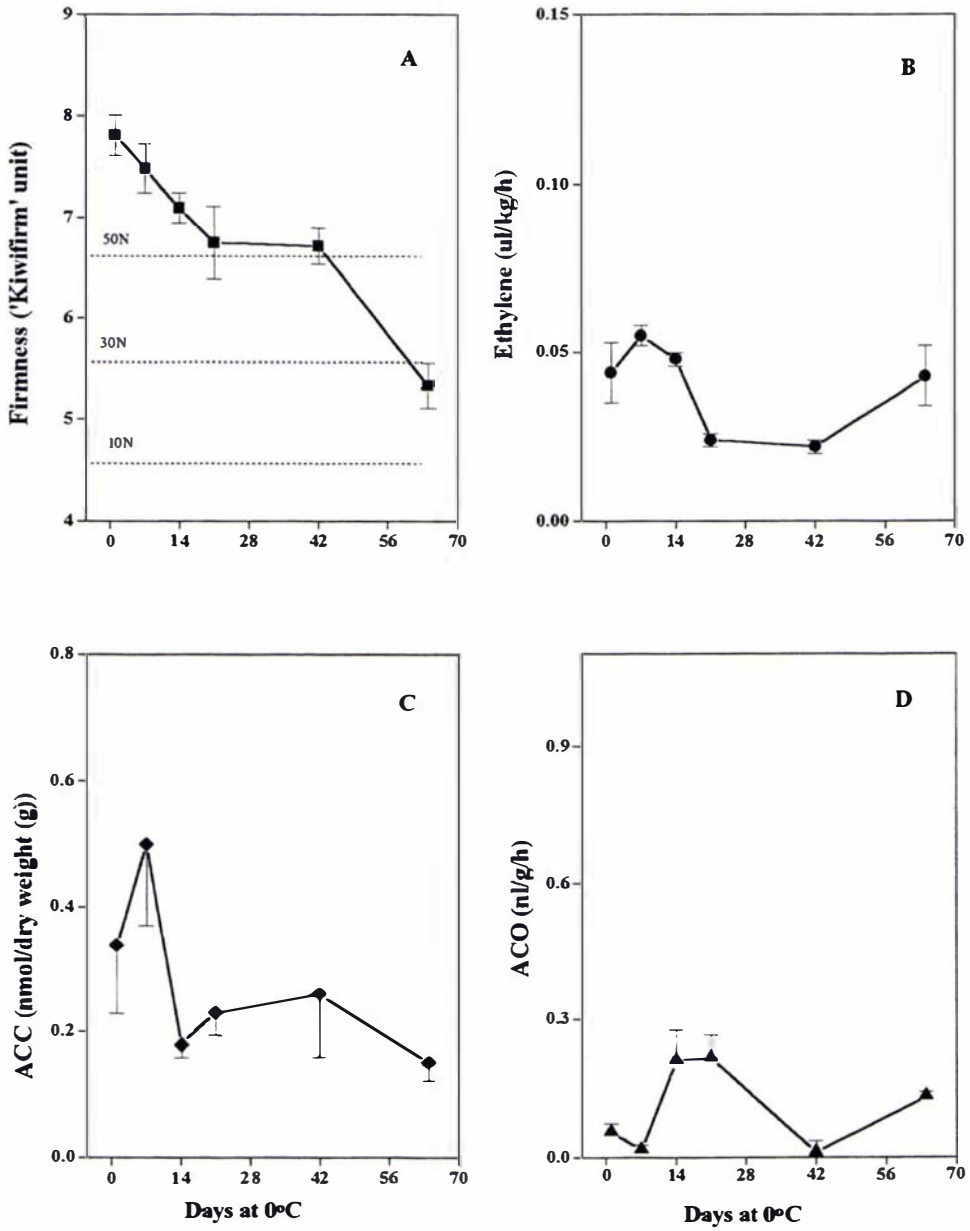

Fig. 3-5. Firmness (A) measured with 'Kiwifirm', ethylene production (B), ACC concentration (C) and ACO activity (D) of kiwifruit during storage at 0°C in 1996. Bars represent standard errors.

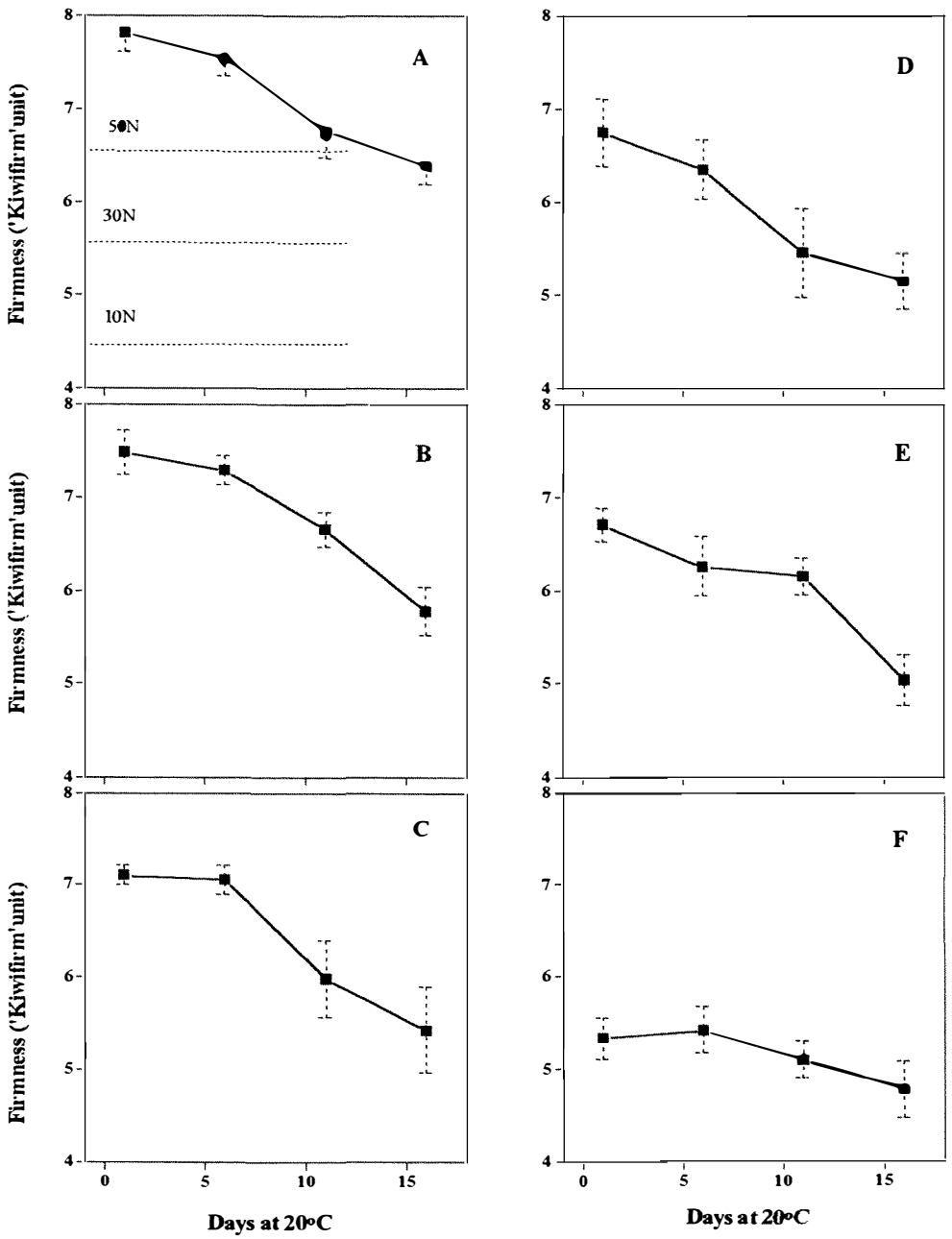

Fig. 3-6. Firmness measured with 'Kiwifirm' of kiwifruit maintained at 20°C following pretreatment at 0°C for 0 (A), 7 (B), 14 (C), 21 (D), 42 (E) and 64 days (F) in 1996. Bars represent standard errors.

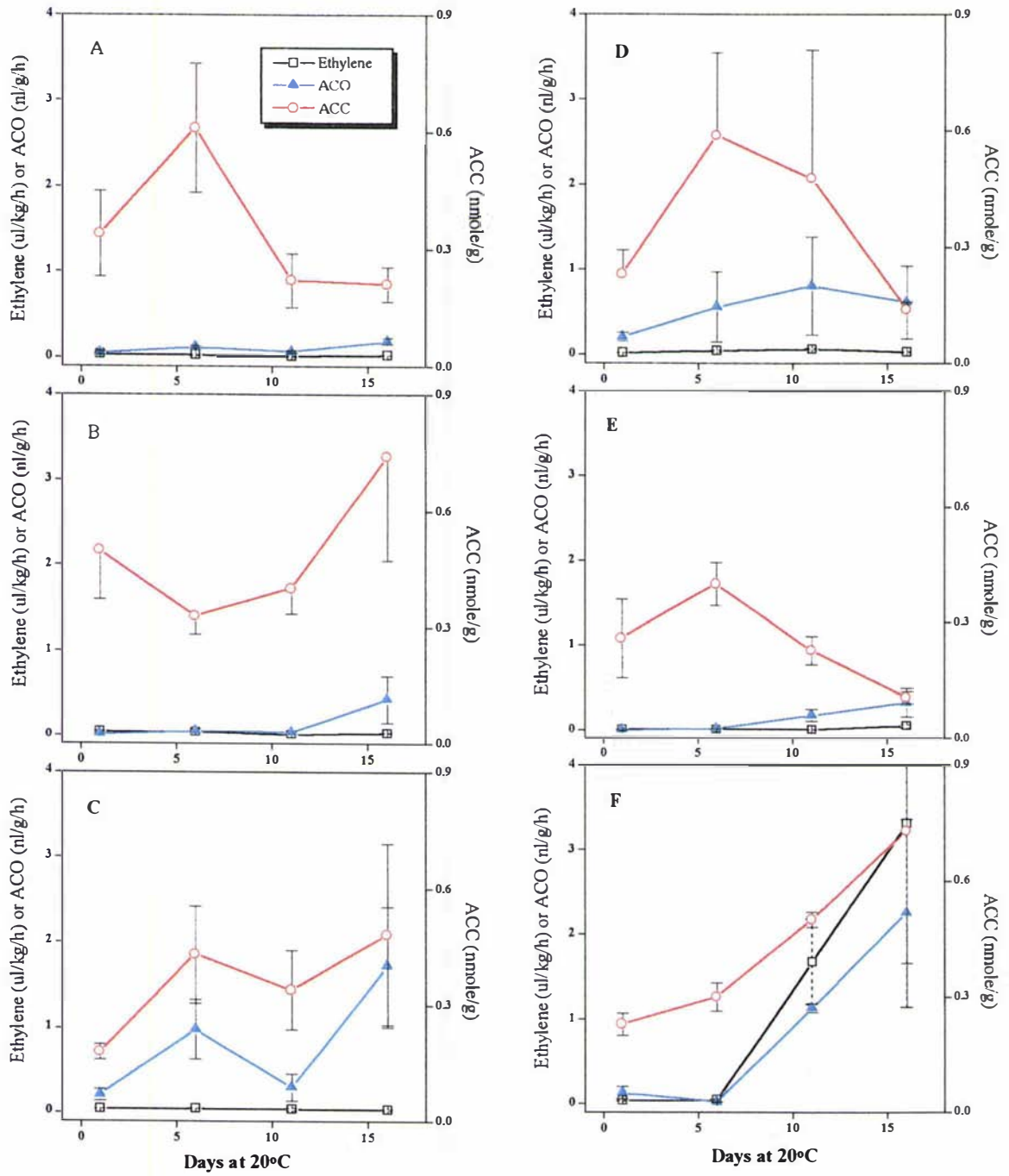

Fig. 3-7. Ethylene, ACO activity and ACC concentration of kiwifruit maintained at 20°C following pretreatment at 0°C for 0 (A), 7 (B), 14 (C), 21 (D), 42 (E) and 64 days (F) in 1996. Bars represent standard errors.

then fell slightly until 6 days at 20°C after which it increased through day 17 (Fig. 3-7B). Fruit maintained at 0°C for 14 days prior to removal to 20°C showed an increase in ACC concentration through 6 days at 20°C, after which it fluctuated (Fig. 3-7C). For kiwifruit kept at 0°C for 21 and 42 days before transfer to 20°C, fruit reached peak ACC concentrations after 6 days at 20°C (0.6 and 0.4 nmole/g respectively) and then decreased continuously to 0.14 and 0.11 nmole/g respectively (Fig. 3-7D and E). Kiwifruit kept at 0°C for 64 days before transfer to 20°C increased ACC continuously to 0.73 nmole/g through 17 days at 20°C (Fig. 3-7F).

ACC Oxidase activity

ACO activity was very low in kiwifruit throughout storage at 0°C varying between 0.01 and 0.21 nl/g/h. For fruit at harvest and after 7 and 42 days at 0°C, ACO activity was very low while fruit stored for 14, 21 and 64 days at 0°C appeared to have slightly enhanced activity (Fig. 3-5D). After removal to 20°C, fruit kept at 0°C for 0, 7, and 42 days before transfer to 20°C showed very low activity of ACO until 11 days at 20°C, after which it increased slightly (Fig. 3-7A, B and E). In fruit kept at 0°C for 14 and 21 days prior to removal to 20°C, ACO activity was 0.21 nl/g/h on removal to 20°C; it then increased after 6 days at 20°C, decreased at day 11 before increasing in fruit kept 14 days at 0°C while increasing consistently through 11 days in fruit kept at 0°C for 21 days (Fig. 3-7C and D). For fruit kept for 64 days at 0°C before transfer to 20°C, ACO activity was low until 6 days at 20°C after which it increased rapidly to 2.3 nl/g/h through day 17 (Fig. 3-7F).

3.3.2.2 1997 experiment

Firmness

During storage at 0°C, firmness of kiwifruit decreased. At harvest firmness was 73N, and it decreased linearly to 43N after 52 days at 0°C; it then remained relatively constant for a further 58 days after which it softened to 15N after 180 days (Fig. 3-8A).

In general fruit from all treatments softened after removal from 0°C to 20°C (Fig. 3-9A) declining to 56, 43, 39, 40N after 13 or 17 days at 20°C for fruit removed from 0°C after 0, 14, 30 and 52 days respectively. Fruit removed from 0°C after 80 and 110 days had an initial firmness of 42N, declining rapidly to 17 and 10N after 10 days respectively. Fruit

maintained at 0°C for 150 and 180 days was relatively soft (22 and 14N respectively) on removal to 20°C and firmness decreased to below 5N during 9 days (Fig. 3-9A).

Ethylene production

Ethylene production was low in kiwifruit throughout storage for 180 days at 0°C varying between 0.035 and 0.064 µl/kg/h (Fig. 3-8B). After transfer to 20°C, fruit kept for 0, 14, 30 and 52 days at 0°C produced very low but detectable amounts of ethylene during 17 days at 20°C (Fig. 3-9B). In contrast, fruit kept at 0°C for 80, 110, 150 and 180 days began to produce ethylene within 5 days at 20°C attaining a rate of 6.2, 6.9, 10.2 and 11.9 µl/kg/h after 9 days respectively (Fig. 3-9B).

ACC concentration

ACC was present in kiwifruit at harvest and remained at concentrations between 0.20 to 0.32 nmole/g throughout storage at 0°C despite considerable variation (Fig. 3-8C).

After transfer to 20°C, fruit kept for 0, 14, 30 and 52 days at 0°C had low but detectable amounts of ACC varying between 0.22 to 0.32 nmole/g (Fig. 3-9C). In contrast, in fruit kept at 0°C for 80, 110, 150 and 180 days, ACC concentration increased to 0.85, 0.94, 1.83 and 2.66 nmole/g after 9 days at 20°C respectively (Fig. 3-9C). However, there were no significant differences between treatments because of the large variation in ACC between individual fruit within treatments. Nonetheless for fruit kept at 0°C for 0, 14, 30 and 52 days none produced >0.5 nmole/g ACC at the last measurement at 20°C, while 20, 40, 60 and 60% of fruit kept at 0°C for 80, 110, 150 and 180 days produced >0.5 nmole/g ACC respectively.

ACC Oxidase activity

ACO activity was very low in kiwifruit throughout storage at 0°C increasing from 0.06 nl/g/h to 0.66 nl/g/h through 180 days despite considerable variation (Fig. 3-8D).

After transfer to 20°C, fruit kept for 0, 14, 30 and 52 days at 0°C had low but detectable ACO activity between 0.05 to 1.00 nl/g/h (Fig. 3-9D). In contrast, in fruit kept at 0°C for 80, 110, 150 and 180 days prior to removal to 20°C, ACO activity increased to 7.28, 10.09, 11.12 and 14.43 nl/g/h respectively after 9 days (Fig. 3-9D).

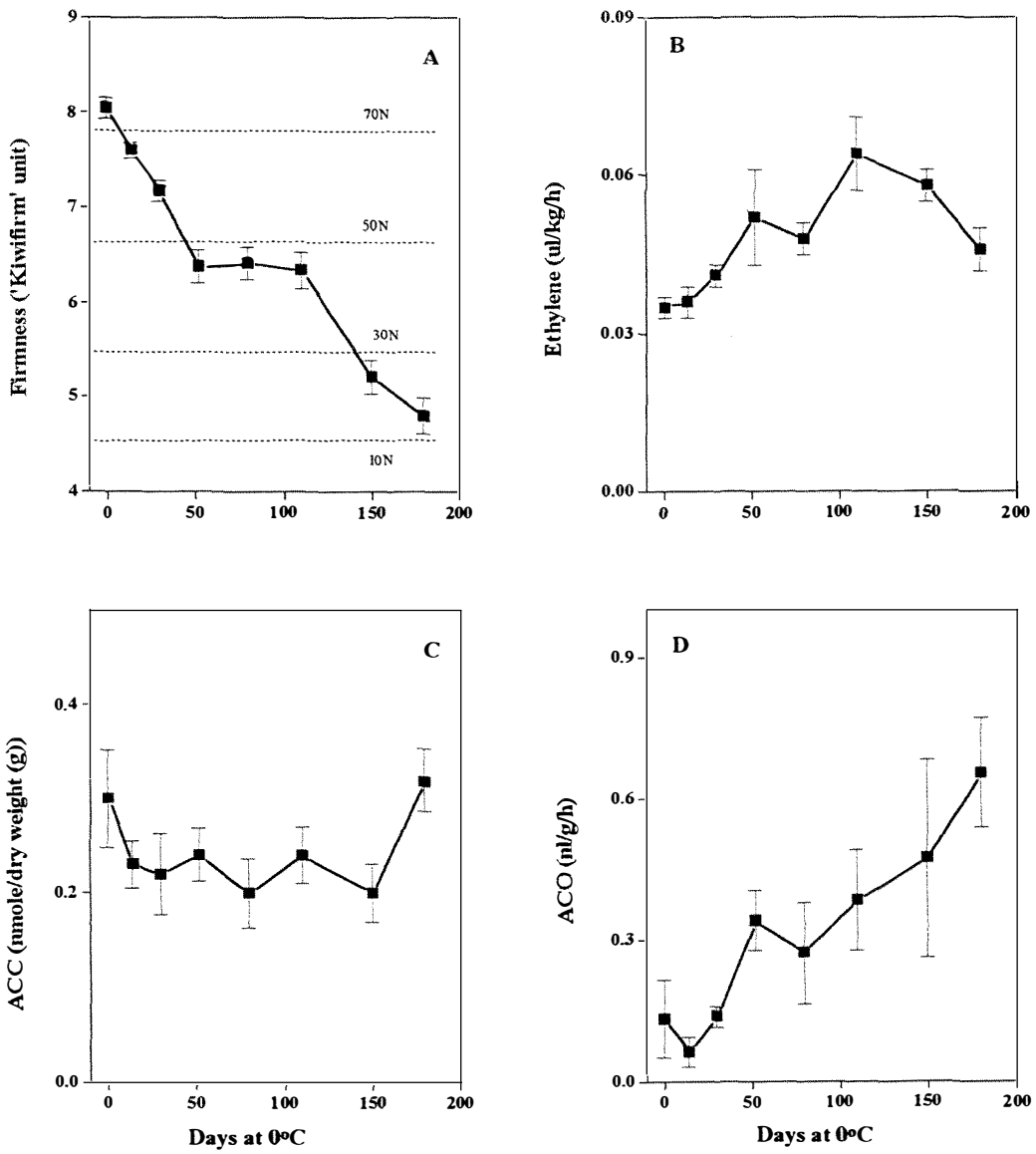

Fig. 3-8. Firmness (A) measured with 'Kiwifirm', ethylene production (B), ACC concentration (C) and ACO activity (D) of kiwifruit during storage at 0°C in 1997. Bars represent standard errors.

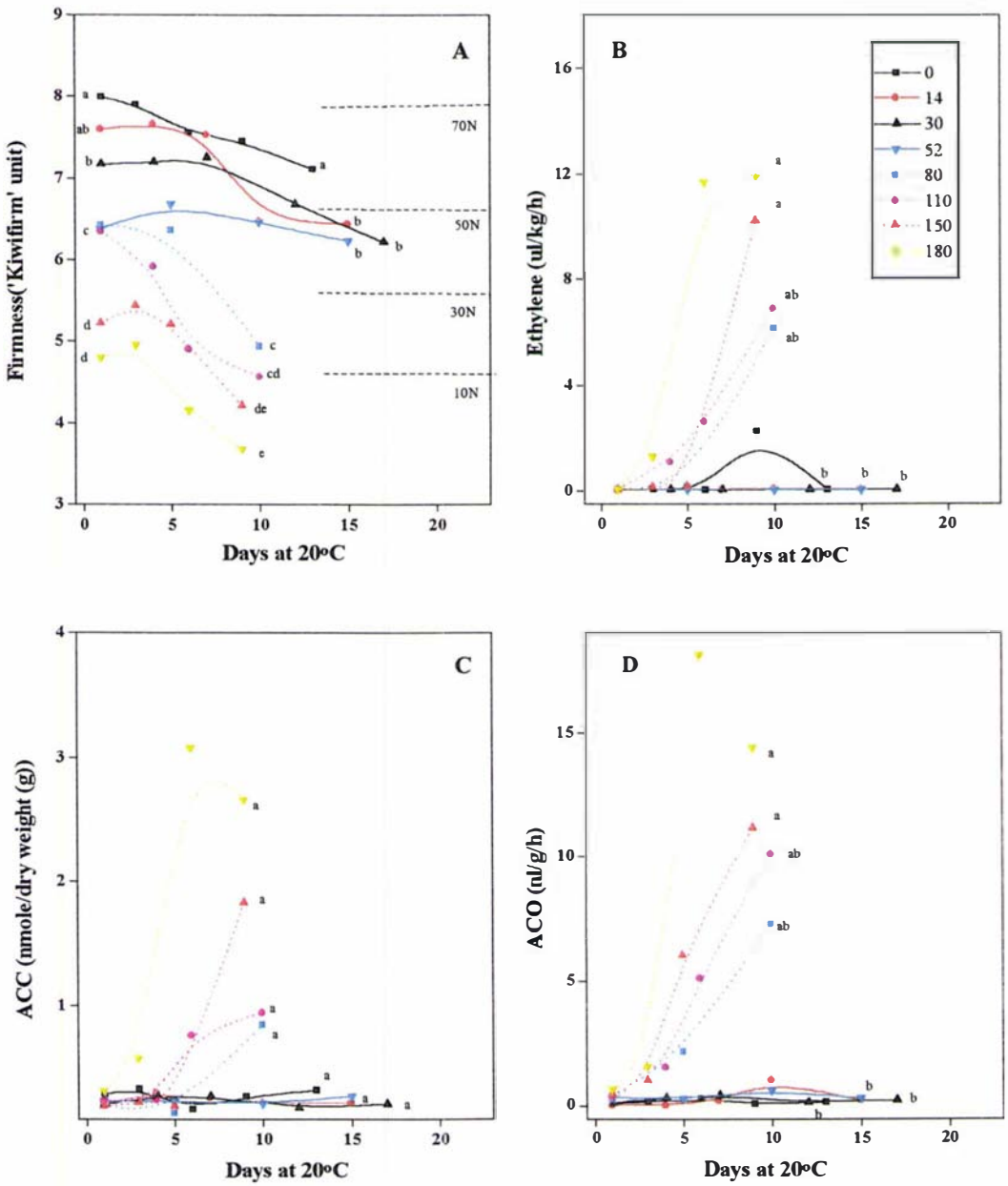

Fig. 3-9. Firmness (A), ethylene production (B), ACC concentration (C) and ACO activity (D) of kiwifruit maintained at 20°C following pretreatment at 0°C for different days in 1997. At a given time values followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level.

3.4 DISCUSSION

Kiwifruit firmness, determined using an Effegi penetrometer, decreases gradually through three phases during storage at 0°C (MacRae *et al.*, 1989; MacRae *et al.*, 1990); the first phase is a lag period when softening occurs a little (this phase disappears in more mature fruit), followed by a rapid phase and a third, prolonged phase during which fruit firmness declined more slowly. However, slightly different softening patterns were found in this study (Fig. 3-5A and Fig. 3-8A); there was no initial slow softening phase probably because fruit harvested reasonably mature (over 7.0% TSS), and initial flesh firmness decreased rapidly to 40-50N when it remained constant for 20-50 days before decreasing rapidly to 14N. This plateau of the softening curve was similar to that described as "anomalous firmness changes" by McGlone *et al.* (1997) and may be a function of the method used to measure fruit firmness. In both of these studies, a non destructive method was used to assess firmness; both methods measured impact responses following an applied force. The firmness measured by these non-destructive methods depends on whole fruit stiffness that appears to remain relatively constant for long periods during cool storage (Hopkirk *et al.*, 1996; McGlone *et al.*, 1997). However softening curves generated from penetrometer measurements on the same fruit at the same time always showed a decrease in firmness.

During storage at 0°C, ethylene production in kiwifruit was low varying between 0.02 to 0.06 µl/kg/h (Fig 3-3A, 3-3B, 3-5A, 3-5B and 3-8A, 3-8B); this approximated internal ethylene concentrations (IEC) of between 0.2 µl/l to 0.6 µl/l (Abeles *et al.*, 1992; Hyodo and Fukasawa, 1985). These low ethylene production rates throughout storage at 0°C were associated with low ACC concentrations, varying between 0.15 and 0.5 nmole/g, and ACO activity varying between 0.01 and 0.66 nl/g/h (Fig. 3-5 and Fig. 3-8). As kiwifruit continued to produce low amounts of ethylene at these ACC concentrations and ACO activities, it is unlikely that they were limiting low ethylene production that is presumed to be occurring in preclimacteric kiwifruit. Kiwifruit softened gradually throughout storage at 0°C even though ethylene production was low (Fig. 3-5 and Fig. 3-8). Since fruit softening is induced by several cell wall hydrolases including ß-galactosidase (ß-GAL), pectin methylesterase (PME) and xyloglucan

endotransglycosylase (XET) (MacRae and Redgwell, 1992), these low concentrations of ethylene are probably enough to activate these cell wall hydrolases in kiwifruit at 0°C.

Based on their respiratory behaviour during ripening, fruit have been classified as climacteric or nonclimacteric (Biale and Young, 1981; Yang and Hoffman, 1984). Climacteric fruit show a major increase in respiration and ethylene production, whereas nonclimacteric fruit have none, yet both undergo marked changes in composition and texture during ripening. Two systems of ethylene production are thought to exist (McMurchie *et al.*, 1972): System 1 is the low rate of ethylene production that seems to be associated with basic metabolic processes in all plant tissues including fruits (both climacteric and nonclimacteric fruits) and vegetative tissues (Lelievre *et al.*, 1997); rates of ethylene production by preclimacteric fruit and nonclimacteric fruit vary between 0.01 to 1.7 (Knee, 1985). System 2 represents the autocatalytic burst of ethylene production which characteristically associated with ripening in climacteric fruit. In most fruit, System 2 climacteric ethylene production is associated with the induction and/or regulation of softening (Yang *et al.*, 1986). However, this model does not appear to apply to kiwifruit since softening occurred gradually with time at 0°C despite the low level of ethylene that was present in the fruit (Fig. 3-3, Fig. 3-5 and Fig. 3-8). Softening of kiwifruit at 0°C is similar to ripening in nonclimacteric fruit where rates of ethylene production by nonclimacteric fruit were also low varying between 0.01 to 0.9 µl/kg/h during maturation and ripening (Chan *et al.*, 1998; Knee, 1985; Perkins-Veazie *et al.*, 1995). In the nonclimacteric strawberry fruit, ethylene production was low and constant (between 0.03 to 0.5 ul/kg/h) during ripening, and this was associated with low ACC concentrations varying between 1.19 and 1.95 nmole/g and ACO activity varying between 0.72 and 2.01 nl/g/h (Perkins-Veazie *et al.*, 1995). These results indicate that such low concentrations of ethylene are enough to induce softening in preclimacteric kiwifruit and ripening in nonclimacteric fruit.

After removal to 20°C, rates of ethylene production were related to duration at 0°C; the longer the time at 0°C the shorter the lag prior to ethylene production once fruit were transferred to 20°C (Fig. 3-1, Fig. 3-7 and Fig. 3-9). There was also a significant relationship between firmness and ethylene production after removal to 20°C (Fig. 3-1, 3-

4, 3-6, 3-7 and 3-9); fruit kept for the longest period at 0°C produced the earliest ethylene and softened most quickly through storage at 20°C. When the same fruit was measured at each sampling time, fruit stored for more than 14 days at 0°C prior to removal to 20°C produced more ethylene, sooner, than fruit kept at 0°C for 0 or 7 days (Fig. 3-1, Fig. 3-4). However, fruit kept at 0°C for 64 days before transfer to 20°C produced ethylene sooner than fruit kept at 0°C for less than 64 days when different fruit were measured at each sampling time (Fig. 3-7). This difference of time in inducing ethylene production may indicate that slight physical damage had occurred to the fruit following repeated measurements with the kiwifirm unit on the same fruit at each sampling time (Hopkirk *et al.*, 1996), resulted in induction of ethylene earlier than when different fruit were measured at each sampling time.

ACC concentration and ACO activity also were related to duration at 0°C before removal to 20°C (Fig. 3-7 and Fig. 3-9). Although ACC concentrations and ACO activity in kiwifruit remained low throughout storage at 0°C, they generally increased after removal to 20°C although no consistent pattern was apparent (Fig. 3-7). In 1996 after 64 days at 0°C, ACC concentration and ACO activity of fruit increased continuously after removal to 20°C (Fig. 3-7). In 1997 exposure of kiwifruit to 0°C for more than 52 days prior to removal to 20°C, induced increased ACC concentration and ACO activity with a concomitant increase in ethylene production (Fig. 3-9). This suggests that a period of 52-64 days at 0°C in kiwifruit is required to activate both ACS and ACO activity, resulting in increased ethylene production at 20°C. This contrasts with results reported by Sfakiotakis *et al.* (1997), indicating that 12 days at 0°C was enough time to induce ethylene subsequently at 20°C. This difference between 12 and 52 days possibly results from differences in responsiveness to chilling of fruit grown in different countries and climates; ethylene production in fruit from Greece, harvested at 6.9% TSS, was induced by exposure to 130µl/l propylene (Aantunes and Sfakiotakis, 1997) whereas New Zealand grown fruit, harvested at 7.9% TSS, did not produce ethylene after treatment with 100µl/l ethylene (see Chapter 6).

These results are consistent with those found in Passe-Crassane pear fruits (Lelievre *et al.*, 1997) and Granny Smith apples (Jobling *et al.*, 1991; Lelievre *et al.*, 1995); chilled

fruit exhibited a burst of ethylene production associated with high activity of ACO and ACS upon rewarming.

These results suggest that low ethylene production occurs in kiwifruit throughout storage at 0°C during which time both ACC concentration and ACO activity are low (Fig 3-5, 3-7, 3-8 and 3-9). At the preclimacteric stages of other fruit including avocado, banana and tomato, ACC concentration and ACO activity are also low due to the lack of ability to form ACC and to convert ACC to ethylene (Yang, 1980). Exposure of kiwifruit to 0°C for more than 52 days prior to removal to 20°C might somehow activate ACO and ACS activity, allowing subsequent production of ethylene (Fig. 3-7F and 3-9). Fruit kept at 0°C for 64 days softened to about 14N upon removal to 20°C at which time substantial ethylene production occurred. Although it is known that exogenous ethylene plays a key role in fruit softening, softening of kiwifruit from 70N to about 20N occurred continuously with time and apparently independently of endogenous ethylene production. Analysis of the relationship between ethylene production and firmness at 20°C of kiwifruit from all treatments in 1996 indicates that ethylene production remains at < 0.1µl/kg/h as fruit soften from 70~80N to 14N; only when fruit have softened to less than 14N does the ethylene production rate increase substantially (Fig. 3-10). This indicates that the early phase of softening (80-14N) may be induced by low endogenous ethylene concentrations varying between 0.01 to 0.1 µl/kg/h which is approximately equivalent to an IEC of between 0.1 to 1µl/l (Abeles *et al.*, 1992; Hyodo and Fukasawa, 1985). This suggests that such low ethylene concentrations may be sufficient to induce cell wall hydrolases leading to kiwifruit softening. Since System 1 is the low basal level of ethylene present in preclimacteric fruit and nonclimacteric fruit, the low ethylene production found during the early phase of kiwifruit softening can be regarded as System 1 ethylene. The final phase of softening (<14N) in kiwifruit may coincide with System 2 ethylene production (Fig. 3-10). For some reason it appears that kiwifruit must soften to at least 14N flesh firmness before the climacteric is initiated in some as yet unidentified way. Ritenour *et al.* (1999) obtained similar results with kiwifruit where ethylene production increased only after fruit firmness dropped below about 7N. Bonghi *et al.* (1996) found that the ethylene climacteric was a late event occurring when kiwifruit had softened to

about 10N, a stage where β-GAL activity and PG activity were markedly increased. Softening related changes in cell walls of kiwifruit are known to occur prior to the ethylene climacteric (Hallett *et al.*, 1992). Thus it is possible that in kiwifruit,

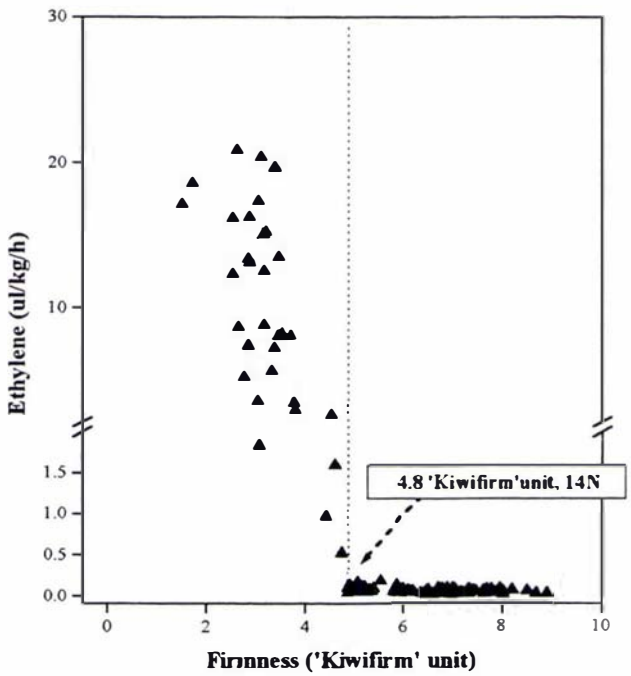

Fig. 3-10. Relationship between ethylene production and firmness of individual kiwifruit at 20°C. Data were obtained across all treatments from the 1996 low temperature experiment.

autocatalytic ethylene production does not occur until a certain stage of firmness (<14N) has been reached, coinciding with the time at which the highest activity of cell wall degrading enzymes such as PG exists (Bonghi *et al.*, 1996). It is known that some specific oligomers can act as ethylene elicitors, and it is possible that if they exist in kiwifruit they may play a part in increasing ethylene production and hence fruit softening (Aldington and Fry, 1993; Fry *et al.*, 1993).

Storing kiwifruit at 0°C for more than 52 days enhanced ethylene biosynthesis and hence rapid softening after removal to 20°C. The chilled fruit ripened more uniformly than non chilled fruit as indicated by consistency of ethylene production and firmness at 20°C; less than 7% of non-chilled fruit produced above 0.1 µl/kg/h ethylene after 10 days at 20°C, while more than 60% of the chilled fruit produced above 0.1 µl/kg/h ethylene. Since lack

of uniformity of ripening creates problems for the industry, cold storage is likely to ensure synchronisation of ripening in kiwifruit.

Kiwifruit softened gradually with time at 0°C when ethylene was low (System 1 ethylene). Since most kiwifruit are stored at 0°C before export and soft kiwifruit (below 9.8N) are rejected at condition checking prior to export, it is necessary to determine how to reduce the effects of System 1 ethylene on induction of cell wall hydrolases at 0°C. It is possible that inhibitors of ethylene biosynthesis and ethylene action could prevent such ethylene effects and increase storage potential of kiwifruit during or after storage at 0°C.

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CHAPTER FOUR

AVG EFFECTS ON ETHYLENE BIOSYNTHESIS AND QUALITY OF HAYWARD KIWIFRUIT

4.1 INTRODUCTION

Softening is one of the most significant quality alterations consistently associated with ripening of fleshy fruits. Kiwifruit texture changes dramatically during fruit ripening, firmness declining by as much as 94%, from about 60-90N at harvest to 5-8N when eating ripe (Harker and Hallet, 1994). Firmness is a key criterion in the assessment of quality of kiwifruit for export from New Zealand, but premature softening may be responsible for 40-70% of fruit losses after harvest (Davie *et al.*, 1996). Therefore, softening in kiwifruit is a common postharvest problem because it decreases the firmness of kiwifruit and reduces the fruit quality.

Ethylene gas is known as the ripening hormone and is a potent promoter of fruit softening. It is produced by many ripening fruit and induces unripe fruit to ripen rapidly (Abeles *et al.*, 1992; Puig *et al.*, 1996). Kiwifruit are very sensitive to ethylene; concentrations as low as 0.01 µl/l reduced storage potential by 46% and thus very low ethylene levels have the potential to cause the majority of loss of potential storage life of fruit (Jeffery and Banks, 1996).

Ethylene synthesis in higher plants occurs through a pathway involving, L-methionine -> S-adenosyl L- methionine (AdoMet) -> 1-aminocyclopropane-1-carboxylic acid (ACC) -> ethylene (Adams and Yang, 1979). The enzymes involved in this pathway are Ado-Met synthetase, ACC synthase (ACS) and ACC oxidase (ACO) which catalyse the conversion of L-methionine to AdoMet, AdoMet to ACC and ACC to ethylene, respectively (Yang and Hoffinan, 1984). ACS is a key rate limiting enzyme and is the main site of control of ethylene biosynthesis (Yang, 1980, 1985). Its activity is enhanced by factors that promote ethylene formation, such as auxins and stress conditions. The last step in the

ethylene biosynthesis is the conversion of ACC to ethylene, an oxygen dependent reaction catalysed by ACO (Yang, 1980).

Ethylene synthesis plays a key role in regulating fruit ripening (Abeles *et al.*, 1992). Two systems of ethylene production are believed to exist (McMurchie *et al.*, 1972; Yang, 1987). System 1 is the low level of ethylene produced by preclimacteric fruit in conjunction with the preexisting System 1 receptor before the onset of ripening; System 2 represents the autocatalytic burst with high rates of ethylene production accompanying the ripening process when the System 2 receptor develops or becomes functional in mature fruit (Oetiker & Yang, 1995).

Duration and passage of the preclimacteric stage in climacteric fruit, probably including kiwifruit, is controlled by System 1 ethylene. Reduction of ethylene production by inhibitors of ethylene biosynthesis prolongs the preclimacteric life while increased ethylene production shortens it (Oetiker & Yang, 1995). ACS requires the cofactor, pyridoxal phosphate, for maximal activity; it is strongly inhibited by vinylglycine analogs, such as aminoethoxyvinylglycine (AVG), and hydroxylamine analogs such as aminooxyacetic acid (AOA), which are well known inhibitors of pyridoxal phosphate-dependent enzymes (Yang, 1980). AVG is one of the most effective inhibitors of ACS with a Ki value of 0.2 μM (Boller *et al.*, 1979).

AVG is a synthetic plant growth regulator (Lieberman, 1979), which is well recognised as an inhibitor of ACS activity preventing the formation of ACC, the natural precursor of ethylene, thereby blocking ethylene biosynthesis in plant tissues (Boller *et al.*, 1979; Yang and Hoffman, 1984; Wenzel *et al.*, 1995). Since AVG and AOA do not inhibit the conversion of ACC to ethylene (Hyodo and Fukasawa, 1985; Yang, 1985), their effectiveness will be influenced by the concentration of ACC already present in the tissue. Earlier work indicated that in tomato and avocado fruits, AVG had little or no effect on inhibiting ethylene evolution during the climacteric because of the massive increase (several hundred times) of ACC that occurred at this time; fruit had already begun to produce ACS and hence ethylene, so application of AVG at this climacteric

stage had little effect. When applied at the preclimacteric stage, AVG inhibited ethylene production as it inhibits ACS activity; such fruit have a very low ACC content (less than 0.1nmole/g) (Hoffman and Yang, 1980). Therefore, effectiveness of AVG and AOA will be greatest when applied to plants in which ACC biosynthesis is low (Yang and Hoffman, 1984).

Inhibition of ethylene production by AVG has been tested for practical application in a number of plant organs or whole plants. Application of AVG to apple trees prior to harvest influenced a number of features affected by endogenous ethylene, indicating that ethylene biosynthesis was being inhibited. Bangerth (1978) found with 'Golden Delicious' apples that spraying trees with AVG 4 weeks before harvest reduced preharvest fruit drop, and fruit firmness in Delicious was increased following preharvest AVG application (Williams, 1980). AVG suppressed ethylene biosynthesis in 'McIntosh', 'Spencer', Royal Red Delicious, and 'Golden Delicious' apple fruit (Autio and Bramlage, 1982; Bramlage *et al.*, 1980; Curry and Patterson, 1992; Halder-Doll and Bangerth, 1987). Apple tissue contains only 2-10nmole/g of ACC even at the onset of ethylene evolution and thus is very sensitive to AVG inhibition (Yang, 1985). Therefore, AVG promises to be tool that can control preharvest drop and improve fruit quality (e.g., firmness) of apple fruit. A commercial formulation of AVG (ReTain ™) has been approved for commercial application to apples in the USA.

AVG has been applied to fruit other than apples. Romani *et al.* (1982) found that preharvest spraying of AVG (400 µl/l) to 'Bartlett' pear trees retarded postharvest ripening including the respiration and ethylene climacteric, at 20°C. Earlier work with 'Anjou', a long keeping and slow ripening winter pear, and 'Bartlett', a short lived and fast ripening pear, had shown that postharvest dipping or vacuum infiltration with AVG inhibited ethylene production in both cultivars, but after storage at -1.1°C for 2 months, softening was delayed only in 'Anjou' and not in 'Bartlett'' pears (Wang and Mellenthin, 1977). Therefore, ripening in the two pear cultivars was differentially affected by AVG.

Postharvest dipping of peaches and nectarines in AVG significantly delayed softening at 24°C, but AVG treated fruit were similar in firmness to control fruits after storage at 1.5° or 4.5°C for 12 to 18 days (Byers, 1997).

In kiwifruit, postharvest dipping with 500 μM of an experimental ACS inhibitor (LAB 181 508; BASF) reduced ethylene production and increased fruit firmness during and after cool storage, whereas postharvest dips with AOA (20μM and 200μM) had little affect (Retamales *et al.*, 1995). Ethylene production was inhibited in kiwifruit tissue discs treated with AVG, but ACO activity in the flesh tissue was not affected by AVG (Hyodo and Fukasawa, 1985). However, the effect of preharvest application of AVG to kiwifruit vines on subsequent fruit quality is not known; a knowledge of such effects might contribute to a better understanding of the role of ethylene in kiwifruit softening, as well as offer a possible management tool that could help to manipulate softening, extend storage life and improve fruit quality overall.

This study investigated the effect of preharvest applications of AVG to kiwifruit vines on ethylene biosynthesis in fruit and selected quality attributes at harvest and after storage.

4.2 MATERIALS AND METHODS

In 1996, 18 uniform vines of kiwifruit growing at the Fruit Crops Unit, Massey University, Palmerston North, New Zealand, were selected and divided into six treatments each with three replicates, in a Randomised Complete Block Design. Vines (leaves and fruits) were sprayed with a knapsack sprayer to give the following treatments:

1. Control (water + surfactant) - applied 4 weeks before harvest;
2. Water + surfactant + AVG at 250ppm - applied 4 weeks before harvest;
3. Water + surfactant + AVG at 500ppm - applied 4 weeks before harvest;
4. Control (water + surfactant) - applied 2 weeks before harvest;
5. Water + surfactant + AVG at 250ppm - applied 2 weeks before harvest;
6. Water + surfactant + AVG at 500ppm - applied 4 weeks before harvest.

Application rate was 1,000 l/ha to leaves and fruit, sprayed to run off at two application dates (4 and 2 weeks before commercial harvest, on 17 May, 1996). The surfactant used was 0.1% Freeway (1020g/L modified organosilicone compound). AVG (aminoethoxyvinylglycine) used was an experimental formulation coded 'ABG-3168', a soluble powder with 15% active ingredient, supplied by 'Nufarm Limited', New Zealand. Two AVG concentrations were used: 250 mg/l a.i. (or about 247g/ha a.i. or 100g/acre a.i.) and 500 mg/l a.i. (or about 494g/ha a.i. or 200g/ acre a.i.).
Immediately after spraying and at weekly intervals until harvest, 9 fruit per treatment were harvested, placed at 20°C for 24 h, before measuring ethylene biosynthesis, respiration rate and firmness.

Fruit (about 180-200 fruit per treatment) were harvested at commercial maturity (7 % total soluble solids (TSS) and fruit weight was 80-120g (100g mean weight)). After removing fruit with defects, the stem scar of each fruit was treated with fungicide ('Rovral' 0.075%, one droplet of approximately 30µl of solution per fruit) (O'Connor, 1994) and then fruit were placed in pockets of 27 count plastic trays surrounded with a polyethylene liner, and put in two layer cardboard cartons with 20g of sachets containing potassium permanganate. Fruit were then cured for 5 days in a 15°C, 98 % RH chamber to reduce potential for *Botrytis* infection.

After curing, half of the fruit were placed at 20°C for 24 hours to stabilise temperature then ethylene biosynthesis (ethylene production, ACC concentration and ACC oxidase activity), respiration rate and firmness were measured at weekly intervals. Fruit firmness was determined both destructively and non-destructively using an 'Effegi' penetrometer (7.9mm diameter probe) or a 'Kiwifirm' firmness tester respectively. The remaining half of the fruit were placed at 0°C for 2 months before transfer to 20°C where the same measurements as above were made at regular intervals. Detailed information on the techniques and methodology of making these measurements is contained in Chapter 2.

In 1997, 40 uniform vines of kiwifruit growing at the Fruit Crops Unit, were selected and divided into four treatments, each with five replicate vines, in a Randomised Complete

Block Design. The vines were sprayed with 1000 l/ha application rate at two application dates (6 and 4 weeks before commercial harvest, on 12 May, 1997). Two AVG concentrations used were 500mg/l a.i. (or about 494g/ha a.i. or 200g/acre a.i.) and 1000mg/l a.i. (or about 988g/ha a.i. or 400g/acre a.i.).

The treatments applied were:

1. Control (water + surfactant) - applied 4 weeks before harvest;
2. Water + surfactant + AVG at 500ppm - applied 4 weeks before harvest (500/4);
3. Water + surfactant + AVG at 1000ppm - applied 4 weeks before harvest (1000/4);
4. Water + surfactant + AVG at 500ppm - applied 6 weeks before harvest (500/6).

300 fruit were harvested (12 May, 1997) at commercial maturity (7.5 % TSS and fruit weight 80-120g (100g mean weight)). Fruit were then treated the same as for 1996 before separating five temperature treatments. Fruit were transferred to 0°C for 14, 52, 80, 110 and 180 days before removal to 20°C. After removal, 75 fruit from each treatment were placed at 20°C for 24 hours to stabilise temperature before measuring ethylene biosynthesis (ethylene production, ACC concentration and ACC oxidase (ACO) activity), respiration rate and firmness at regular intervals. Fruit firmness was determined non-destructively using a 'Kiwifirm' firmness tester.

All data were analysed using SAS System programmes (SAS 1988) for Analysis of Variance (ANOVA), means and standard errors and Duncan's comparison at 5% level.

4.3 RESULTS

4.3.1 1996 experiment

Fruit attributes were measured at weekly intervals after spraying until harvest. Firmness of fruit sprayed with 500ppm AVG 4 weeks before harvest was greater than for controls when measured both destructively, with an Effegi penetrometer, and non-destructively with a 'Kiwifirm' firmness tester (Table 4-1). Application of AVG 2 weeks before harvest had no effect on firmness compared to control fruit. There was little inhibiting effect of AVG application on ethylene production in any treatment (Table 4-1).

Neither of the AVG concentrations applied 2 weeks before harvest had any effect on firmness either immediately after harvest or after 2 months at 0°C (Table 4-2). Fruit treated with 250ppm AVG 2 weeks prior to harvest produced more ethylene and had a higher respiration rate than did controls and 500ppm AVG at harvest (Table 4-2).

Table 4-1 Ethylene and firmness (measured destructively and non-destructively at harvest) of kiwifruit treated with 250ppm or 500ppm AVG 2 or 4 weeks before harvest, between spray application until harvest. Values are means from weekly interval measurements after spraying until harvest.

Treatment	Ethylene (µl/kg/h)		Firmness (N)[1]		Firmness[2] (Kiwifirm'unit)	
	Spray time		Spray time		Spray time	
	2 wks	4 wks	2 wks	4 wks	2 wks	4 wks
Control	0.023a[3]	0.019b	73a	78ab	8.6a	8.4b
250ppm AVG	0.025a	0.025a	72a	76b	8.4a	8.5b
500ppm AVG	0.022a	0.022b	71a	82a	8.6a	8.8a

1. Firmness measured destructively with Effegi penetrometer. 2. Firmness measured non-destructively with 'Kiwifirm' firmness tester. 3. Values with the same letter within a column are not significantly different by Duncan's multiple range test at the 5 % level.

Table 4-2 Ethylene, respiration rate and firmness of kiwifruit treated with 250ppm or 500ppm AVG 2 weeks before harvest, maintained at 20°C after harvest and after 60 days at 0°C. Values are means from measurements over 4 weeks at 20°C.

Treatment	Ethylene (ul/kg/h)		CO_2 (ml/kg/h)		Firmness (Kiwifirm'unit)	
	Days at 0°C		Days at 0°C		Days at 0°C	
	0	60	0	6 0	0	60
Control	0.03b	2.52a	7.11b	10.69a	6.7a	5.1a
250ppm AVG	0.66a	4.47a	8.43a	9.89a	6.6a	5.1a
500ppm AVG	0.03b	3.39a	7.86ab	10.49a	6.6a	4.9a

Values with the same letter within a column are not significantly different by Duncan's multiple range test at the 5 % level.

Table 4-3 Ethylene, respiration rate and firmness of kiwifruit treated with 250ppm or 500ppm AVG 4 weeks before harvest, maintained at 20°C after harvest and after 60 days at 0°C. Values are means from measurements over 4 weeks at 20°C.

Treatment	Ethylene (ul/kg/h)		CO_2 (ml/kg/h)		Firmness (Kiwifirm'unit)	
	Days at 0°C		Days at 0°C		Days at 0°C	
	0	60	0	6 0	0	60
Control	1.31a	4.18a	8.00a	9.88a	5.9b	4.5b
250ppm AVG	1.06ab	4.89a	8.48a	10.60a	6.2b	4.6b
500ppm AVG	0.04b	0.17b	6.99b	9.55a	7.2a	5.4a

Values with the same letter within a column are not significantly different by Duncan's multiple range test at the 5 % level.

There was no effect of 250ppm AVG, applied 4 weeks before harvest, on any of the variables analysed, either after harvest or after 2 months at 0°C (Table 4-3). However fruit treated with 500ppm AVG 4 weeks before harvest produced less ethylene, respired less (at harvest only) and had firmer fruit than control or 250ppm AVG fruit both after harvest and after two months at 0°C.

Ethylene production : Fruit from vines treated with 500ppm AVG, 4 weeks before harvest, showed a significant reduction in endogenous ethylene production at harvest (Fig. 4-1A) and after 2 months storage at 0°C (Fig. 4-1B) compared to control and 250ppm AVG fruit. In control and 250ppm AVG fruit placed at 20°C immediately after harvest, ethylene production increased after 2 weeks but this did not occur in 500ppm AVG treated fruit during 4 weeks at 20°C.

After 2 months at 0°C, ethylene production increased slightly after 2 weeks at 20°C in 500ppm AVG treated fruit, whereas rapid ethylene production commenced after 1 week at 20°C in control and 250ppm AVG fruit (Fig. 4-1B). The 500ppm AVG treated fruit were producing only 5.5% and 3.5% and of the ethylene (0.5 µl/kg/h) from control (9.1 µl/kg/h) and 250ppm AVG fruit (14.4 µl/kg/h) respectively after 3 weeks at 20°C. When control fruit was stored at 0°C for 2 months before removal to 20°C, the rise in ethylene production from control fruit occurred earlier and reached a higher peak (9.1µl/kg/h) than control fruit placed at 20°C immediately after harvest (4 µl/kg/h) (Fig. 4-1A and B).

Respiration rate: The effect of AVG on kiwifruit respiration rate was not as consistent as that of ethylene production (Fig. 4-1C and D). Although 500ppm AVG treated fruit had a lower respiration rate than control and 250ppm AVG fruit over all, only fruit measured at week 3 after harvest and after 2 months at 0°C showed significant differences between 500ppm AVG treated and control fruit.

ACC concentration: At harvest ACC was present (0.8nmole/g) in flesh tissue of control fruit, but concentration fell after 1 week at 20°C, before increasing to a maximum of 1.6nmole/g at 3 weeks after which concentration declined (Fig. 4-2A). Although ACC concentration in 500ppm AVG treated fruit remained low (between 0.08 to 0.25nmole/g)

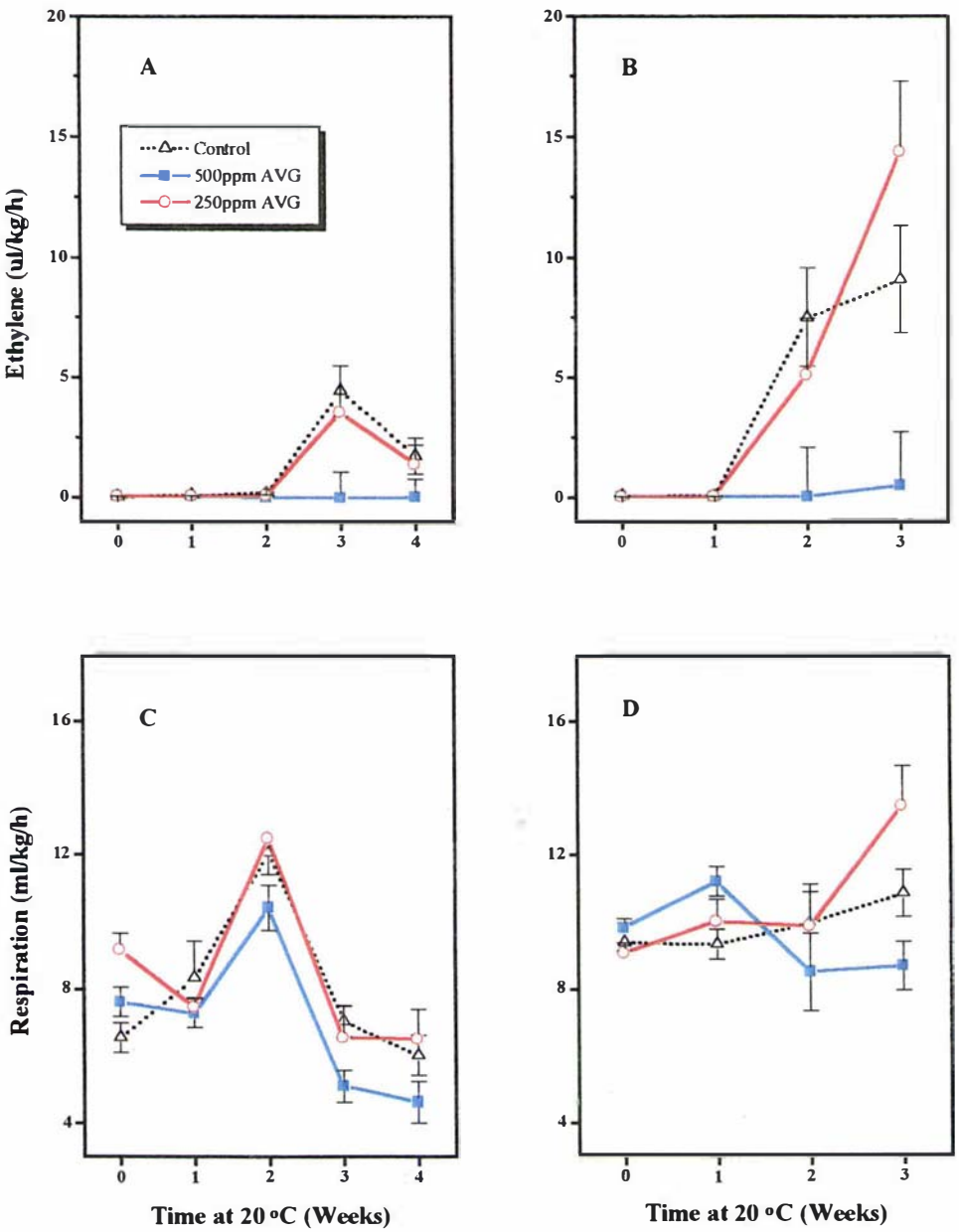

Fig. 4-1. Ethylene production (A, B) and respiration rate (C, D) of kiwifruit treated with 500ppm or 250ppm AVG 4 weeks before harvest, maintained at 20°C after harvest (A, C) and after 2 months at 0°C (B, D). Bars represent standard errors.

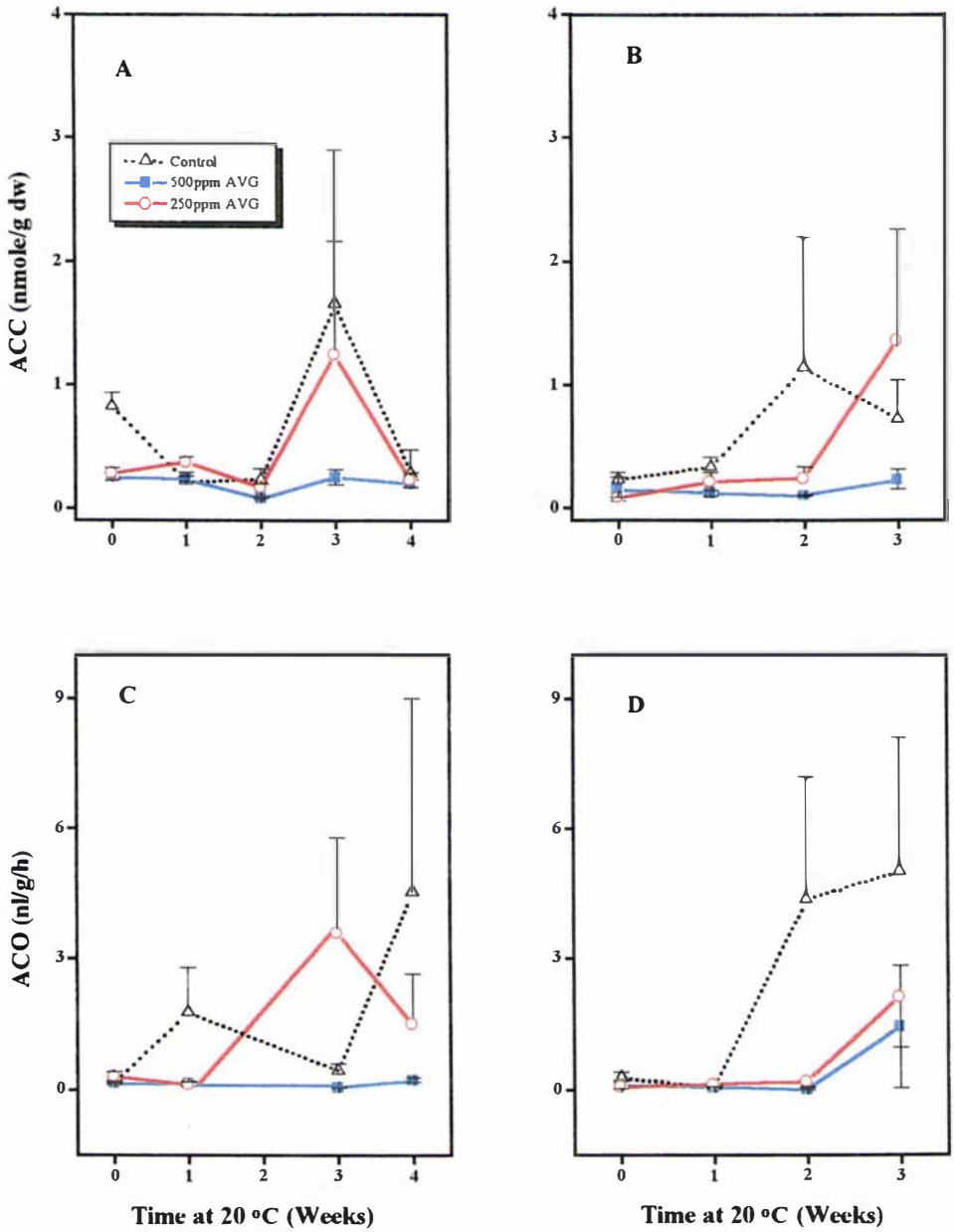

Fig. 4-2. ACC concentration (A, B) and ACO activity (C, D) of kiwifruit treated with 500ppm or 250ppm AVG 4 weeks before harvest, maintained at 20°C after harvest (A, C) and after 2 months at 0°C (B, D). Bars represent standard errors.

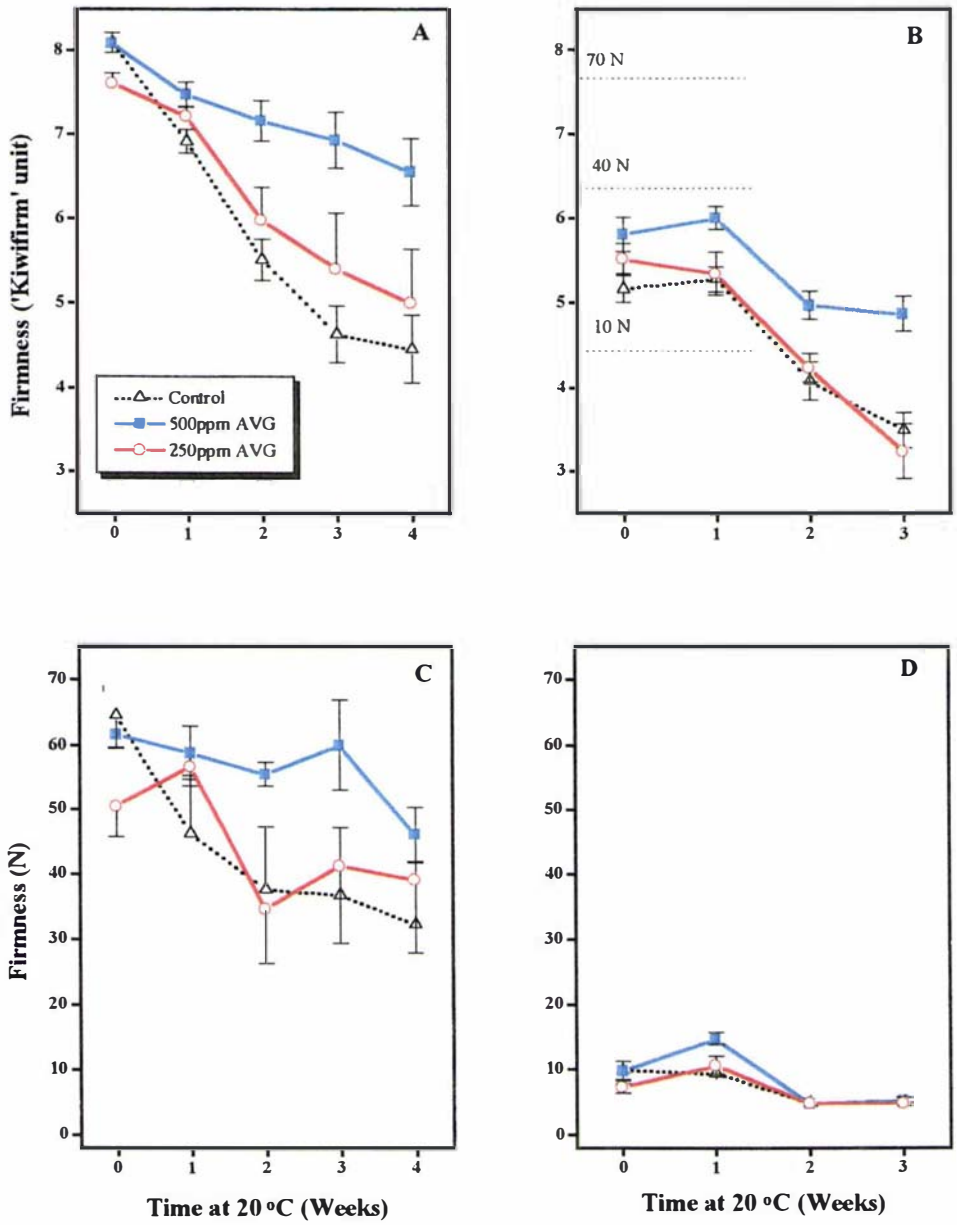

Fig. 4-3. Firmness measured with 'Kiwifirm' (A, B) and with a penetrometer (C, D) of kiwifruit treated with 500ppm or 250ppm AVG 4 weeks before harvest, held at 20°C after harvest (A, C) and after 2 months at 0°C (B, D). Bars represent standard errors.

throughout 4 weeks at 20°C, in 250ppm AVG treated fruit it increased after 2 weeks and reached a peak (1.3nmole/g) after 3 weeks at 20°C.

After 2 months at 0°C, ACC concentration (0.23nmole/g) in untreated fruit was lower than at harvest (0.82nmole/g), and increased to a maximum of 1.1nmole/g after 2 weeks at 20°C (Fig. 4-2B). ACC concentration of 500ppm AVG treated fruit remained at a constant low level (between 0.10 to 0.15nmole/g) for 2 weeks at 20°C, then increased slightly (0.23nmole/g), while 250ppm AVG treated fruit increased ACC concentration rapidly after 2 weeks, reaching 1.4nmole/g after 3 weeks at 20°C. These data indicate trends only; substantial variation in ACC concentration between individual fruit meant that overall differences were not significant.

ACO activity: Activity of ACO in both control and AVG treated fruit was low, but detectable in kiwifruit at harvest (Fig. 4-2C). There was a small increase in control fruit after 1 week (1.8nl/g/h) at 20°C, and a major increase after 4 weeks (4.5nl/g/h). Very low ACO activity (between 0.07 to 0.24nl/g/h) occurred in 500ppm AVG treated fruit through 4 weeks at 20°C, while in 250ppm AVG treated fruit it increased after 1 week, reached a peak (3.6nl/g/h) after 3 weeks at 20°C and then declined. After 2 months at 0°C, ACO activity was low in both untreated and AVG treated fruit. However, ACO activity increased in control fruit between 1 and 2 weeks at 20°C, and the increase continued through 4 weeks. ACO activity of 500ppm and 250ppm AVG treated fruit remained at almost undetectable levels until after 2 weeks at 20°C, after which there was a slight increase from 0.026 and 0.206nl/g/h to 1.4 and 2.1nl/g/h respectively. Again because of fruit to fruit variation some of these differences were not significant.

Flesh firmness: 500ppm AVG treated fruit were firmer than control and 250ppm AVG treated fruit (measured non-destructively with the 'Kiwifirm') 1 week after harvest (Fig. 4-3A) and after 2 months storage at 0°C (Fig. 4-3B). Softening of 500ppm AVG treated fruit after harvest was much slower than 250ppm AVG treated and control fruit, and after 4 weeks at 20°C, firmness of 500ppm AVG treated fruit was about 50 N compared with 18N and less than 10 N in 250ppm AVG treated and control fruit respectively (Fig. 4-3A).

After 2 months at 0°C, 500ppm AVG treated fruit were firmer than control and 250ppm AVG treated fruit and subsequently softened more slowly at 20°C (Fig. 4-3B).

These results were confirmed (especially after harvest) using the standard destructive method (with a penetrometer) (Fig. 4-3C, Fig. 4-3D). After 2 months at 0°C, firmness between control and AVG treated fruit was almost same after 2 weeks at 20°C (Fig. 4-3D).

Relationship between ethylene production and firmness of individual fruit

By plotting ethylene production of individual fruit against firmness of the same fruit (Fig. 4-4), ethylene production remained constant between 0.008 to 0.1~0.2 µl/kg/h as fruit softened from 90 to about 10N. However, when fruit reach a firmness of 10N, ethylene production increased rapidly, coinciding with the final stage of kiwifruit softening.

4.3.2 1997 experiment

The 1997 experiment was an attempt optimise rates and timing of AVG application to kiwifruit.

Table 4-4 Ethylene, respiration rate and firmness (measured non-destructively) of kiwifruit treated with 500ppm or 1000ppm AVG 4 weeks before harvest, or 500ppm AVG 6 weeks before harvest, maintained at 20°C after harvest. Values are means from measurements over 13 days at 20°C.

Treatment	Ethylene (ul/kg/h)	CO_2 (ml/kg/h)	Firmness (Kiwifirm'unit)
Control	5.52a	9.36a	7.1b
500/4	0.04b	7.19b	7.5a
500/6	3.09ab	9.07a	7.1b
1000/4	0.05b	8.07b	7.6a

Values with the same letter within a column are not significantly different by Duncan's multiple range test at the 5 % level.

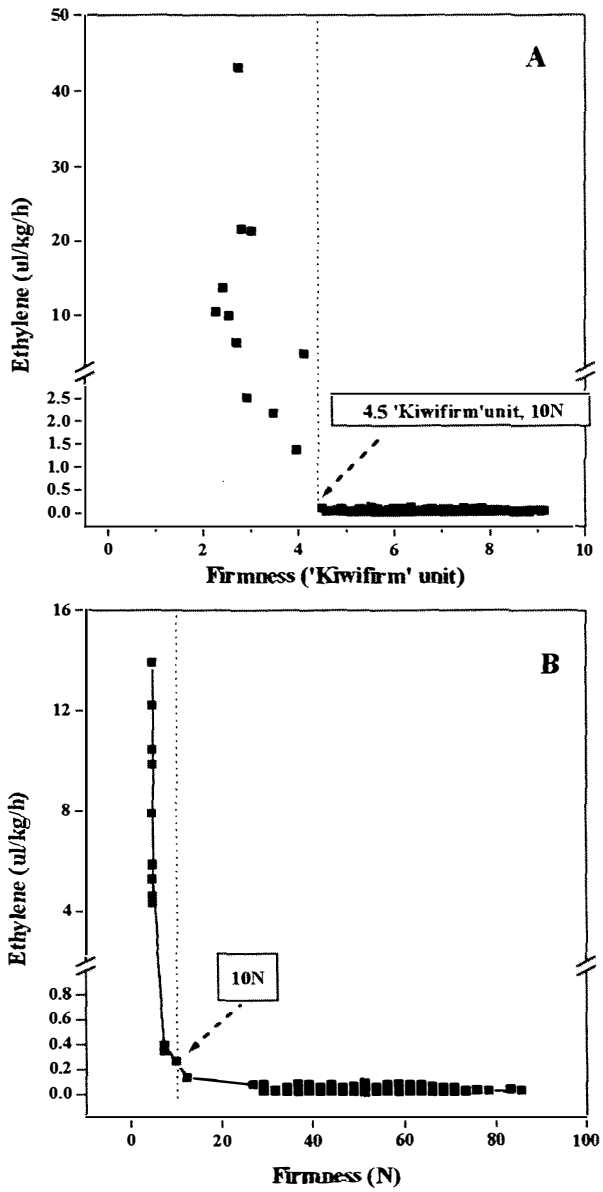

Fig. 4-4. Relationship between ethylene production and firmness of individual kiwifruit (firmness measured with the 'Kiwifirm' using the same fruit (A) and firmness measured with penetrometer using different fruit (B)) at 20°C.

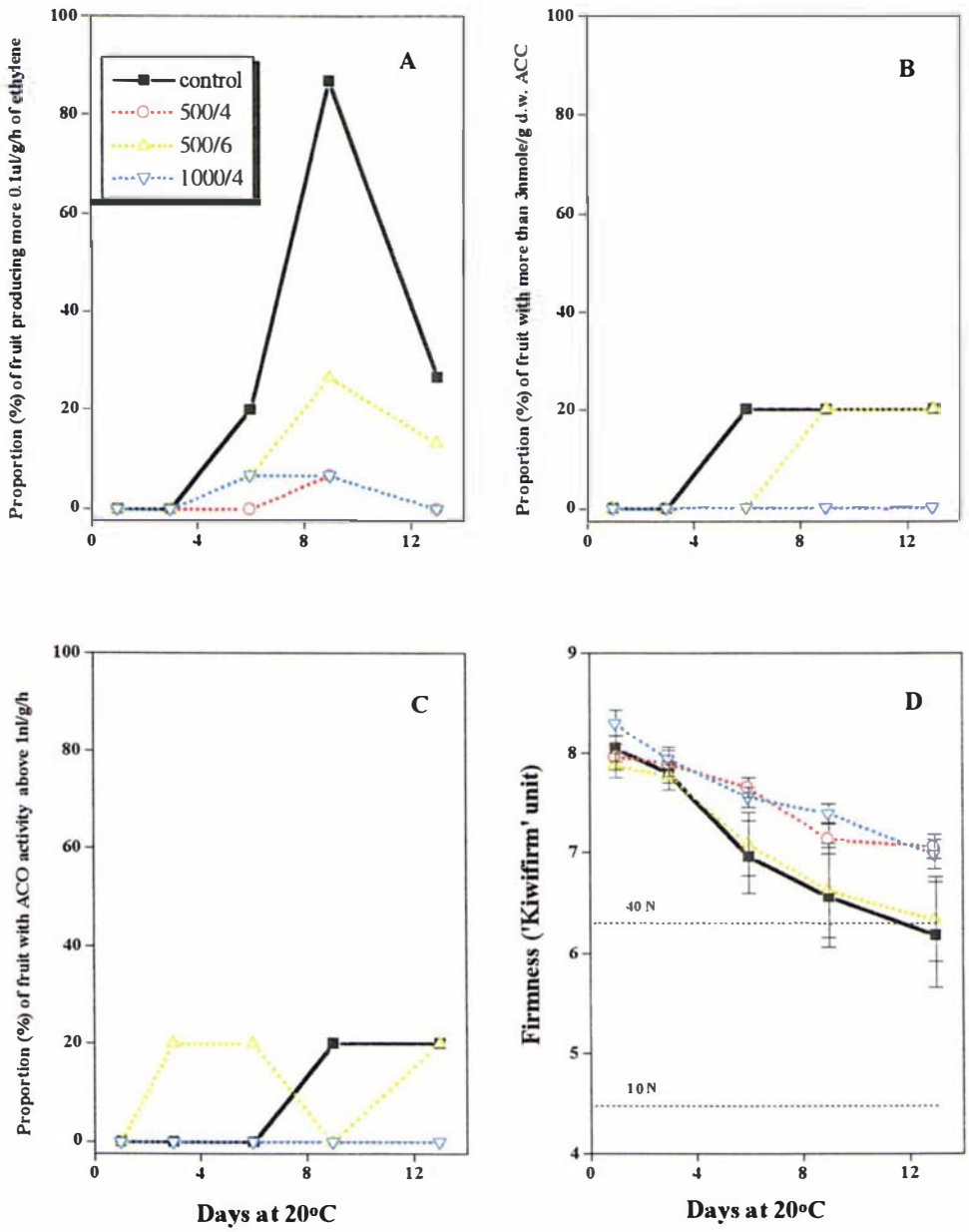

Fig. 4-5. Ethylene production (A), ACC concentration (B), ACO activity (C) and firmness (D) of kiwifruit treated with 500 or 1000ppm AVG 4 weeks before harvest or 500ppm AVG 6 weeks before harvest, maintained at 20°C after harvest. Bars represent standard errors.

After harvest

At harvest fruit from 500/4 and 1000/4 treatments produced less ethylene, had lower respiration rates and were firmer at 20°C than control and 500/6 fruit (Table 4-4).

Ethylene biosynthesis

Because there was a wide variation of ethylene biosynthesis between individual fruits, data are expressed as the proportion (%) of fruit that produced ethylene, ACC concentration and ACO activity above 0.1 µl/kg/h, 3 nmole/g and 1 nl/g/h respectively.

Most fruit from 500/4 or 1000/4 treated vines produced less ethylene after harvest than control and 500/6 fruit (Fig. 4-5A). There was no difference in ethylene production between 500/4 and 1000/4 fruit. After 9 days at 20°C, 0.1 µl/kg/h ethylene was produced by 87%, 27%, 7% and 7% of fruit from control, 500/6, 500/4 and 1000/4 treatments respectively.

ACC concentration above 3nmole/g was produced by 20% of fruit from both control and 500/6 treatments after 6 or 9 days at 20°C respectively (Fig. 4-5B). ACO activity above 1nl/g/h was produced by 20% of fruit from both control and 500/6 treatments after 9 or 3 days at 20°C respectively (Fig. 4-5C). No fruit from 500/4 or 1000/4 treatments contained ACC above 3nmole/g or ACO activity above 1nl/g/h (Fig. 4-5B and C).

Firmness

Fruit from 500/4 and 1000/4 treatments were firmer than control and 500/6 fruit 3 days after harvest and this difference increased during subsequent storage at 20°C (Fig. 4-5D). Softening rate of 500/4 and 1000/4 fruit was slower than control fruit reaching 54 and 39N respectively after 13 days at 20°C. Softening of 500/6 fruit was similar to control fruit.

After storage at 0°C

Both 500/4 and 1000/4 fruit tended to remain firmer than control and 500/6 fruit until 52 days at 0°C (Table 4-5), after which these results were reversed.

Although fruit from all treatments produced low ethylene after 14 days at 0°C, AVG treated fruit tended to produce more ethylene than control fruit after 52 days at 0°C, especially in 500/4 and 1000/4 fruit (Table 4-6).

Table 4-5 Firmness (measured non-destructively) of kiwifruit treated with 500 and 1000ppm AVG 4 weeks before harvest, or 500ppm 6 weeks before harvest, maintained at 20°C after storage at 0°C for different periods. Values are means from 5 measurements over 14 days at 20°C.

	Firmness ('Kiwifirm' units)			
Days at 0°C	Control	500/4	500/6	1000/4
14	7.1b	7.3a	7.4a	7.5a
52	6.2b	6.5a	6.2b	6.3ab
80	5.4ab	5.3b	5.7a	5.3ab
110	5.4a	5.0c	5.4ab	5.1bc
180	4.4b	4.2b	4.7a	4.4b

Values with the same letter within a row are not significantly different by Duncan's multiple range test at the 5 % level.

Table 4-6 Ethylene production (µl/kg/h) of kiwifruit treated with 500 and 1000ppm AVG 4 weeks before harvest, or 500ppm 6 weeks before harvest, maintained at 20°C after storage at 0°C for different periods. Values are means from 5 measurements over 14 days at 20°C.

	Ethylene production (µl/kg/h)			
Days at 0°C	Control	500/4	500/6	1000/4
14	0.06a	0.04a	0.04a	0.05a
52	0.83a	1.94a	2.69a	2.28a
80	7.87b	15.00a	6.78b	11.08ab
110	2.66c	10.20a	5.31bc	8.72ab
180	6.23b	11.78ab	9.16b	16.53a

Values with the same letter within a row are not significantly different by Duncan's multiple range test at the 5 % level.

4.4 DISCUSSION

Endogenous ethylene is physiologically active throughout the preclimacteric life of a fruit and it is functional in determining when the climacteric will occur (Peacock, 1972). It is known that fruit contain low concentrations of ethylene during development. Fruit become more responsive to ethylene as they advance towards the climacteric, and softening commences (Knee, 1985). By inhibiting ethylene biosynthesis, AVG delayed softening in pears and apples (Romani *et al.*, 1982; Williams, 1980). Sprays of 500ppm AVG to kiwifruit vines 4 weeks before harvest reduced ethylene production and delayed softening after harvest and after storage at 0°C (Fig. 4-1 and 4-3). Both ACC concentration and ACO activity tended to be less in 500ppm AVG treated fruit than in 250ppm AVG treated fruit and control fruit (Fig. 4-2 and Fig. 4-5). Although AVG is thought to inhibit only ACS activity and not ACO activity (Hyodo and Fukasawa, 1985; Yang, 1985), AVG did reduce ACO activity as well as ACS activity in these experiments. However, its effect on reducing softening declined or reversed after 14 days at 0°C (Table 4-5 and Table 4-6). This may be due to internal ethylene increasing to a concentration high enough to induce softening during low temperature storage (Romani *et al.*, 1982). Gerasopoulos and Richardson (1997) found that sensitivity of pear fruit to ethylene increased gradually with time at -1°C; 'Anjou' pear fruit held at 20°C in a 500 µl/l propylene atmosphere immediately after harvest softened to 10N in 14 days, while pears held at -1°C for 25 and 70 days softened to 10N after 9 and 7 days at 20°C, respectively. Therefore, this increased sensitivity to endogenous ethylene during storage at 0°C may be a reason for the declining AVG effect. In addition, research with apple plugs found that inhibition of ethylene production by AVG was temperature-dependent; AVG was less effective in controlling ethylene biosynthesis at lower than at higher temperatures (Mattoo *et al.*, 1977). AVG treated apples showed more severe low temperature breakdown (called brown core) after long storage at 0°C than control fruit, indicating that AVG possibly increased chilling sensitivity (Autio and Bramlage, 1982; Bramlage *et al.*, 1980). This may be another reason for the reduced AVG effect after long storage at 0°C in kiwifruit; it is possible that increased kiwifruit chilling sensitivity was induced by AVG resulted in more ethylene production and more rapid softening

upon removal to 20°C (Table 4-5 and 4-6) (Lallu, 1997). Since most kiwifruit is cool stored, this is an important area for further investigation.

The effectiveness of AVG varied depending on rates and timing of AVG application to kiwifruit vines. Application of 500ppm AVG, 2 weeks or 6 weeks before harvest had little or no effect on reducing ethylene production and delaying softening before or at harvest. Similar results were obtained by Williams (1980) who found no differences in 'Delicious' apple firmness before harvest when AVG (450ppm) was applied 2 weeks prior to harvest. However, some authors have found significant effects on ethylene production and firmness in apples after harvest, where AVG was applied 2 weeks before harvest (Bramlage *et al.*, 1980; Halder-Doll & Bangerth, 1987; Williams, 1980). Autio and Bramlage (1982) found that 500ppm AVG treatment 6 weeks before apple harvest delayed ripening less than did treatments applied 4 or 2 weeks before harvest. Application of 250ppm AVG to kiwifruit 2 or 4 weeks before harvest, had no effect on ethylene production, respiration and softening, either after harvest or after 2 months at 0°C (Table 4-1, 4-3). In apple, AVG concentrations of 250 and 125ppm were reported to inhibit internal ethylene concentrations (Bramlage *et al.* 1980) and delay peak ethylene production (Autio & Bramlage, 1982) in some cultivars, but most AVG experiments reported use AVG concentrations of 500ppm or higher (Bangerth, 1978; Bangerth, 1986; Bufler, 1984; Child *et al.*, 1984; Halder-Doll & Bangerth, 1987; William, 1980). Even lower concentrations of AVG (90 or 180ppm) inhibited increasing internal ethylene concentration, ACC content and softening of apple at 1°C (Kondo and Hayata, 1995). These results with kiwifruit indicate that AVG applied at 250ppm was not enough to inhibit ACC synthase to the extent of affecting ethylene production and kiwifruit softening. In kiwifruit, uptake and thus subsequent distribution of AVG may be less than that in apple; skin resistance of kiwifruit is much higher (4 times) than that in apple (Banks *et al.*, 1991) and may reduce uptake of this chemical.

The most significant results were found with 500ppm or 1000ppm AVG applied 4 weeks before harvest in kiwifruit and in apple where both concentrations inhibited ethylene

production (Fig. 4-1, Fig. 4-5) (Halder-Doll and Bangerth, 1987). AVG (500ppm) applied 4 weeks before harvest significantly inhibited and delayed endogenous kiwifruit ethylene production both after harvest and after 2 months storage at 20°C. This is consistent with work with on AOA on kiwifruit (Retamales *et al.*, 1995) and AVG on apples (Autio & Bramlage, 1982; Bufler, 1984; Child *et al.*, 1984; Halder-Doll & Bangerth, 1987). Therefore it is suggested that AVG concentrations ≥ 500ppm can penetrate the kiwifruit skin, blocking ethylene biosynthesis and thus reducing softening.

Low temperatures have been shown to hasten the induction of ethylene synthesizing competency and homogenous ripening in pears and apples (Pech *et al.*, 1994). This may be due to increased ACO activity and to a lesser extent ACS activity being induced during chilling treatment (Lelievre *et al.*, 1997). Autostimulation of ethylene synthesis is associated with high activity of ACO and ACS upon rewarming (Jobling *et al.*, 1991; Knee, 1987). When fruit was removed from cool storage and placed at a higher temperature, the sharp rise in ethylene production from control fruit was triggered one week earlier than that from control fruit placed in such conditions immediately after harvest indicating a promotive influence of low temperature on acceleration of ethylene production in kiwifruit (Fig. 4-1A, Fig. 4-1B).

Kiwifruit is very sensitive to exogenous ethylene; the rate of softening is fast when 0.01µl/l ethylene is present, even at 0°C (Retamales and Campos, 1997). This concentration of ethylene (0.01µl/l) reduced storage potential by 46% at 0°C (Jeffery and Banks, 1996). The relationship between decreased ethylene production and increased firmness in AVG treated compared to control fruit after harvest (Table 4-3, 4-4) suggests that inhibition of endogenous ethylene by AVG in treated fruit may have resulted in firmer fruit at 20°C, linking endogenous ethylene production with the softening process in kiwifruit. AVG treated fruit with 500ppm might be considered preclimacteric since the fruit produce ethylene at low rate by System 1 (Oetiker and Yang, 1995). Even though ethylene production was low varying between 0.02 to 0.07 µl/kg/h (System 1 ethylene) (Fig. 4-1A), softening occurred slowly but gradually with time (Fig. 4-3A). This appears to be similar to the process in nonclimacteric fruit where softening occurs with ripening during declining ethylene production; ethylene production

was low and constant (about 0.03 µl/kg/h) during strawberry ripening (Perkins-Veazie *et al.*, 1995). Responsiveness of pear fruit to low ethylene concentrations (even at 0.01 µl/l) increase with time at -1°C (Gerasopoulos and Richardson, 1997) and advanced maturity of kiwifruit during storage at 0°C (Retamales and Campos, 1997), resulting in rapid softening. In addition, an increase in sensitivity of preclimacteric fruits to ethylene has been widely recognized in climacteric fruits (McGlasson, 1985). Therefore, it can be suggested that softening of kiwifruit occurs with low ethylene (System 1) and it declines gradually with time because of increased sensitivity to System 1 ethylene.

When data of ethylene production from individual fruits are plotted against firmness (Fig. 4-4), it is clear that the initial phases of softening from 90N to 10N occur where ethylene production remained low (< 0.1~0.2 µl/kg/h) and constant. Only when fruit soften to less than 10N, does ethylene production (System 2) increase; this is associated with the final phase of softening to that of eating ripe fruit (6~8N) (MacRae *et al.*, 1990). Hyoda and Fukasawa (1985) indicated that the threshold ethylene concentration needed to initiate the climacteric in kiwifruit was about 0.1 µl/kg/h, similar to the rate obtained in these experiments. As ethylene is probably closely involved in kiwifruit softening (MacRae and Redgwell, 1992), this suggests that low endogenous ethylene concentrations (probably System 1 ethylene) are sufficient to induce loss of starch and solubilization of pectin in cell wall by many hydrolase enzymes including amylase, ß-galactosidase and xyloglucan endotransglycosylase early in ripening (Beever and Hopkirk, 1990; Bonghi *et al.*, 1996; MacRae and Redgwell, 1992; Redgwell and Harker, 1995). The other hydrolase enzymes, including polygalacturonase, responsible for cell wall and middle lamella dissolution are mainly involved in the later stages of kiwifruit softening (Bonghi *et al.*, 1996; Redgwell *et al.*, 1990, 1991, 1992) coinciding with the maximum concentration of ethylene occurring at the fully ripe stage in kiwifruit (Lallu *et al.*, 1989; Stavroulakis and Sfakiotakis, 1995). Gerasopoulos and Richardson (1996) found that the ethylene threshold for softening in pears was lower than that for the other ripening parameters including the climacteric rise in ethylene and respiration; 0.4 µl/l ethylene was not sufficient to induce either ethylene production or the climacteric rise in respiration but this concentration was sufficient to induce softening. Ritenour *et al.*

(1999) and Bonghi *et al.* (1996) found that ethylene production in kiwifruit increased greatly only after fruit firmness dropped below about 7N-10N and the ethylene climacteric was not related to the initial phase of kiwifruit softening (Tonutti *et al.*, 1993). Therefore, it is possible that before autocatalytic ethylene production can occur in kiwifruit, fruit must soften to < 10N.

Individual kiwifruit had distinct respiratory climacteric and ethylene peaks (Pratt and Reid, 1974), the timing of which varied markedly with time (Hyodo and Fukasawa, 1985). Even when fruit were harvested at the same chronological age, their physiological stage and internal ethylene concentration are likely to vary widely, which is probably the main reason for the considerable variation obtained among replicate samples in ACC, ACO and ethylene production rates (Yang *et al.*, 1986). Fruit harvested from different positions on kiwifruit vines have a large variation in soluble solids concentration (SSC) (Hopkirk *et al.*, 1986; Pyke *et al.*, 1996); SSC was higher in fruit from the ends of the leader than in fruit from near the centre of the vine, and fruit from near the leader had higher SSC than fruit from the ends of the canes. In the present experiments, although climacteric peaks were identified from composite samples, only a few % of the individual fruit produced ethylene, creating large variation in ethylene production, ACC concentration and ACO activity between fruit within treatments. Such variation was so great at times to prevent significant differences between treatments. One way to minimise such fruit to fruit variation would be to harvest fruit of the same physiological stage. This means that individual flowers would need to be tagged at blossom to ensure fruit of similar physiological age at harvest. Another possible reason for variation among individual fruit could be due to the AVG spray not reaching fruit in positions where large number of leaves were next to the fruit. Also little is known of the extent of AVG uptake, translocation and metabolism in plant tissue. Although kiwifruit vines were sprayed to run off, and attention was given to ensure that fruit were well treated, it is possible that the AVG solution was unevenly distribution on fruit surfaces. This could have resulted in differential uptake on and between fruit and subsequent variation in the extent of the AVG effect on ACS activity, contributing to some of the fruit to fruit variation measured. To minimise such variation in future research, more fruit need to be

included in each replicate. However, the limited time for analysing ACC concentration and ACO activity in any one day and/or the limited number of vines available for these experiments reduced the opportunity for increasing replicate number or for harvesting from selected positions on the vine.

Although AVG inhibited ethylene biosynthesis immediately after harvest, the AVG effects was minimal after 14 days at 0°C. The AVG effect on delaying kiwifruit softening was also limited after a short term coolstorage, indicating that the magnitude of the AVG treatment is unlikely to be sufficient to warrant possible commercial use to delay softening. Since kiwifruit is a very sensitive to exogenous ethylene, an alternative method for delaying softening would be to block ethylene action (Sisler and Serek, 1997); if ethylene receptor sites were blocked by a compound such as 1-methylcyclopropene (1-MCP) then ethylene binding would be reduced and fruit may remain firmer for longer during storage.

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CHAPTER FIVE

ETHYLENE BIOSYNTHESIS IN KIWIFRUIT INFECTED WITH *BOTRYTIS CINEREA* FOLLOWING AVG AND TEMPERATURE TREATMENTS

5.1 INTRODUCTION

Kiwifruit is a major export horticultural crop of New Zealand (Jenks, 1994). Because of its long storage life, it can be stored at 0°C in air for up to 6 months (McDonald, 1990). However, premature fruit softening occurs during storage and may be responsible for up to 70% of fruit losses after harvest (Banks *et al.*, 1992; Davie *et al.*, 1996). Another major problem that develops during storage is the potential for development of the postharvest pathogen *Botrytis cinerea*, causing substantial kiwifruit losses after harvest in some years (Banks *et al.*, 1992; Cheah *et al.*, 1993). At harvest and during postharvest operations, picking wounds on fruits can become contaminated by *Botrytis* spores or pieces of *Botrytis*-infected tissue. The fungus invades the fruit through the picking wound and continues to grow in fruit stored at 0°C. Stem-end *Botrytis* rot begins in the region of the picking scar and extends along the fruit (Pennycook, 1985). Therefore, premature softening and *Botrytis* infection are common problems in kiwifruit because they decrease firmness, reduce fruit quality and hence cause economic loss for growers.

Ethylene induces rapid softening in kiwifruit; concentrations as low as 0.01µl/l promote softening and thus very low ethylene concentrations will cause a major reduction of potential storage life of fruit (Jeffery and Banks, 1996; Matsumoto *et al.*, 1983; Retamales and Campos, 1997). Kiwifruit infected with *B. cinerea* soften very quickly and have a reduced storage life. In addition, this infected fruit produces ethylene. The presence of infected fruit in a tray hastened softening of other non-infected fruit in the same tray (Brook, 1991; Jeffery and Banks, 1996; Manning and Pak, 1993). Although healthy kiwifruit produce minute amounts of ethylene at low temperature (<14.8°C) where ACC production is limited, kiwifruit infected with *B. cinerea* produced considerable amounts of ethylene and accumulated ACC at 10°C (Niklis *et al.*, 1993). Generally, *Botrytis* rot does not appear in fruit until after at least 4 weeks at 0°C, after which additional fruit become infected and widespread fruit softening can occur. This

creates additional costs to the industry because of the need check trays and remove infected and soft fruit prior to export (Beever *et al.*, 1984; Pennycook, 1984).

When plants are attacked by potential pathogenic microorganisms, they generally display an active defense response, which includes rapid formation of ethylene (Takeda and Nakamura, 1990). Achilea *et al.* (1985) investigated the origin of ethylene produced during infection with *P. digitatum* in grapefruit; during initial phases of infection, the enhanced ethylene production originated from peel cells and was ACC mediated. As infection proceeded the host tissue was killed, impairing the ethylene biosynthetic pathway in fruit because of loss of membrane and cell wall integrity. Therefore the increased production of ethylene at latter stages of infection was thought to be mainly of fungal origin. Earlier work also found that 58 of 228 species of fungi examined produced ethylene (Ilag and Curtis, 1968). Recently, Qadir *et al.* (1997) found that *B. cinerea* produced ethylene in the presence of methionine. However, it was not determined whether the enhanced ethylene production in *B. cinerea* infected kiwifruit came from the pathogen, from infected kiwifruit tissue or both.

Fungal invasion of plant tissue is accompanied by secretion of a number of enzymes that degrade plant cell walls, thus facilitating penetration of host tissue by the fungus (Cooper, 1976) and breakdown of cellular integrity. Cellular disintegration is induced by cell wall degrading enzymes such as polygalacturonase (PG), pectin methyl esterase (PME), pectinase, rhamnogalacturonase (RGase) and cellulase all of which can be produced by *B. cinerea* (Gross *et al.*, 1995; Heiler *et al.*, 1993; Marcus and Schejter, 1983; Miura *et al.*, 1994; Tobias *et al.*, 1993). Such cell wall degrading enzymes depolymerise polysaccharides to glucosamine-containing oligomers which are known to be active as elicitors of ethylene biosynthesis (Basse *et al.*,1992; Fry *et al.*, 1993).

Ethylene in higher plants is synthesized through the ACC pathway; ACS is a key limiting enzyme and is the main site of control of ethylene production (Yang, 1980, 1985). The ACS enzyme is sensitive to pyridoxal phosphate inhibitors such as aminoethoxyvinylglycine (AVG) and aminooxyacetic acid (AOA). Inhibition of ethylene

production and delay of softening by AVG treatment has been found in apple, pears, peaches and nectarines and kiwifruit tissue discs (Byers, 1997; Curry and Patterson, 1992; Hyodo and Fukasawa, 1985; Romani *et al.*, 1982). It is known that ethylene induces rapid softening in kiwifruit (Jeffery and Banks, 1996) and *B. cinerea* induces ethylene and hence softening (Brook, 1991). It was thought that by treating kiwifruit with AVG, ACS would be inhibited and thus ethylene production would be reduced, resulting in a reduction of both softening and rate of hyphal growth and fungal development in fruit infected with *B. cinerea*.

This study was undertaken to determine the effects of AVG on ethylene biosynthesis in *B. cinerea* infected fruit and to determine if degree and rate of *B. cinerea* infection was influenced by AVG.

5.2 MATERIALS AND METHODS

ReTain ™ (an experimental formulation of AVG, a soluble powder with 15% active ingredient) and the surfactant (0.1% Freeway, 1020g/L modified organosilicone compound) used in this experiment were provided by Nufarm Ltd in Auckland, New Zealand.

5.2.1 AVG effects on ethylene biosynthesis in kiwifruit infected with *B. cinerea*

A total of 24 kiwifruit, cv. Hayward, vines in the Fruit Crops Unit at Massey University were selected and were sprayed (until runoff) with water (rate of 1,000l/ha) + surfactant ('Freeway' -adjuvant 0.1%) (called control vines), or water + surfactant + 500 ppm AVG (called AVG vines) 4 weeks before harvest. When the mean soluble solids content was 6.7% and fruit weight was 80-120g, kiwifruit (960) were harvested (29 April, 1997). 40 fruit from each vine were removed, ensuring the peduncles remained attached to the fruit; fruit from each vine remained separate. The following postharvest treatments were applied to each group of 40 fruit, immediately after removing the peduncle:

a) Control 1 and 2: 0.075% Rovral (14µl) was applied onto the stem scar of fruit from control vines (control 1) or AVG sprayed vines (control 2).

b) B.C : Stem scar of fruit from control vines was inoculated with *B. cinerea* (17 µl droplet containing 102,000 spores).

c) Preharvest AVG + B.C: Stem scars of fruit from AVG sprayed vines were inoculated with *B.cinerea* as for (b) above.

d) Postharvest AVG + B.C: Stem scars of fruit from control vines were inoculated with *B. cinerea* ((b) above) after allowing the applied 500µl/l AVG (14 µl droplets/fruit applied to the stem scar) to dry.

e) Pre plus Postharvest AVG + B.C: Stem scars of fruit from AVG sprayed vines were inoculated with *B. cinerea* after allowing the applied 500µl/l AVG (14µl droplets/fruit applied to the stem scar) to dry.

After allowing the applied suspensions to dry, the 160 fruit from each treatment were packed into 16 trays (10 fruit /tray), each of which was surrounded by a polyethylene liner, and put in one layer cardboard trays containing sachets of KMnO4 (20g per tray) to absorb ethylene. Four trays were immediately placed at 20°C for 12 days. The remaining trays were placed at 0°C for 4, 6 and 9 weeks. All fruit from each treatment were separated in this manner.

Fruit at 0°C were inspected weekly for symptoms of *B. cinerea* infection and the distance from the stem scar to the outer edge of the infection front was recorded. At the completion of each coolstore period, fruit were removed to 20°C where measurements for ethylene production, ACC concentration and ACC oxidase (ACO) activity were carried out at regular intervals (for details refer to Chapter 2). Two fruit from each of the four replicate trays (i.e 8 fruit per treatment) were used for these measurements at each time interval.

5.2.2 Preparation of *B. cinerea* spores

Single spore isolates: A single spore isolate of *B. cinerea* was obtained from naturally infected kiwifruit in the Massey University Plant Growth Unit coolstore. Conidial masses of *B.cinerea* were lifted from fruit using a sterilized needle, placed in deionised autoclaved water inside McCartney bottles and shaken to separate spores. A needle was

dipped into the dilute spore suspension and streaked over a plate of Water Agar that was incubated at 20°C for 18 hours. Single spore isolations were transferred to Malt Agar (MA) with a sharp needle, under a dissecting microscope and then incubated at 20°C±1 under near-UV light (Sylvania black light blue F40/BLB, 40W) using a 12 hour photo period. Distance of cultures from the lights was approximately 35cm.

Spore suspension : Spore suspensions were prepared from 14 day old colonies of *B. cinerea* by flooding colonies with 0.02% Tween-20 (polyoxyethylene sorbitan monolaurate SERVA, Feinbiochemica), rubbing with a sterile bent-glass rod and filtering through glass wool to remove hyphal fragments. Spore concentrations were measured using a haemocytometer and adjusted to the required spore concentration (102,000 spore/17ul). The spore suspension then kept at -20±2°C in a freezer until required for inoculation.

5.2.3 Kiwifruit slices

To measure ethylene production and ACO activity from different zones along the length of kiwifruit infected with *B. cinerea*, 4 fruit from preharvest and postharvest AVG treatments (see 5.2.1) were selected on the basis of lesion development (all fruit had approximately the same length of infected tissue) after 8 weeks at 0°C. After placing at 20°C for 24 hours, fruit were cut into 4 different zones and a 10mm thick transverse slice of fruit was taken from each of the following zones:

a) Infected zone - slice taken from midway between the proximal end of the fruit and the infection front
b) Invasion zone - slice taken from the infection front containing both infected tissue and sound tissue immediately ahead of the infection front
c) Adjacent zone - slice taken from sound tissue ahead of the invasion zone
d) Distal zone - slice taken from sound tissue at the distal end of the fruit

Slices were taken from equivalent zones in uninfected control fruit.

Ethylene production was measured from 4 fruit per treatment immediately before slicing at 20°C. Slices were weighed and single slices placed in 500 ml air-tight plastic jars. After incubation for 150 min., a 1 ml gas sample was taken from each jar and ethylene concentration determined by injection onto a GLC, ACO activity was determined using the same slice (See chapter 2 for details).

ACC concentration was determined at 20°C after 3 months at 0°C from 4 sound fruit and 4 infected fruit which were inoculated with *B. cinerea* before storage at 0°C.

5.2.4 Growth rate of *B. cinerea* at different temperatures

Kiwifruit (total soluble solids (7.5%), fruit weight 80-120g (100g mean weight)) were stored at 0°C for 2 months and then three treatments were applied:

a) Control, uninoculated, no wound

b) Wounded, uninoculated

c) Wounded, inoculated with *B. cinerea* (17 µl droplet containing 102,000 spores)

A 4.5mm diameter 5mm deep hole was made on the shoulder of fruit in treatments (b) and (c) and *B. cinerea* inoculated into the hole in (c) to ensure consistent infection. 12 fruit per treatment were stored at each of 4 different temperature (0, 4, 10 and 20°C). Ethylene production was measured from 6 fruit per treatment and distance from inoculation wound to the infection front was recorded from 12 fruit per treatment at regular intervals.

All data were analysed using the SAS System programmes (SAS 1988) for Analysis of Variance (ANOVA), means and standard errors and Duncan's comparison at 5% level.

5.3 RESULTS

5.3.1. AVG effects on ethylene biosynthesis in kiwifruit infected with *B. cinerea*

As fruit from both control treatments had the same ethylene production, only data from control 1 is used to compare with AVG treatments.

After harvest

Ethylene production did not increase in uninfected fruit during 12 days at 20°C (Fig. 5-1A). Kiwifruit inoculated with *B. cinerea* increased ethylene production after 6 days, reached a peak (100.5µl/kg/h) after 9 days and then declined (Fig. 5-1A). Although AVG applications did not affect the lag phase before ethylene production became apparent, both preharvest and postharvest AVG applications reduced peak ethylene production induced by *B. cinerea* by 80 and 45% decrease respectively at 20°C (Fig. 5-1A). Fruit inoculated with *B. cinerea* and treated with AVG both preharvest (as a spray) and postharvest (as droplets to the stem scar) (called pre plus postharvest AVG application) produced the same amount of ethylene as did control fruit inoculated with *B. cinerea* at 20°C.

ACC concentration remained very low varying between 0.53 to 0.76 nmole/g, in uninfected fruit during 12 days at 20°C (Fig. 5-1B). Fruit inoculated with *B. cinerea* had increased ACC concentration after 6 days, rising to a peak of 292 nmole/g after 12 days at 20°C. Preharvest, postharvest and pre plus postharvest AVG applications decreased ACC concentration relative to control fruit infected with *B. cinerea* by 95%, 73% and 65% respectively (Fig. 5-1B).

ACO activity did not increase in uninfected fruit during 12 days at 20°C but increased in inoculated fruit after 3 days at 20°C, reaching a peak of 15nl/g/h after 9 days (Fig. 5-1C). Fruit treated both postharvest and pre plus postharvest with AVG increased ACO activity after 3 days and reached peaks of 12 and 18nl/g/h respectively after 9 days at 20°C. Only in fruit treated with the preharvest AVG application was ACO activity affected significantly in *B. cinerea* infected fruit, with peak ACO activity reduced by 84% relative to control fruit infected with *B. cinerea*.

After storage at 0°C

Ethylene production remained low in uninfected fruit during 9 weeks at 0°C (Table 5-1). Fruit infected with *B. cinerea* had high ethylene production within 4 weeks and through 9 weeks. Preharvest application of AVG reduced ethylene production by 18% from *B.*

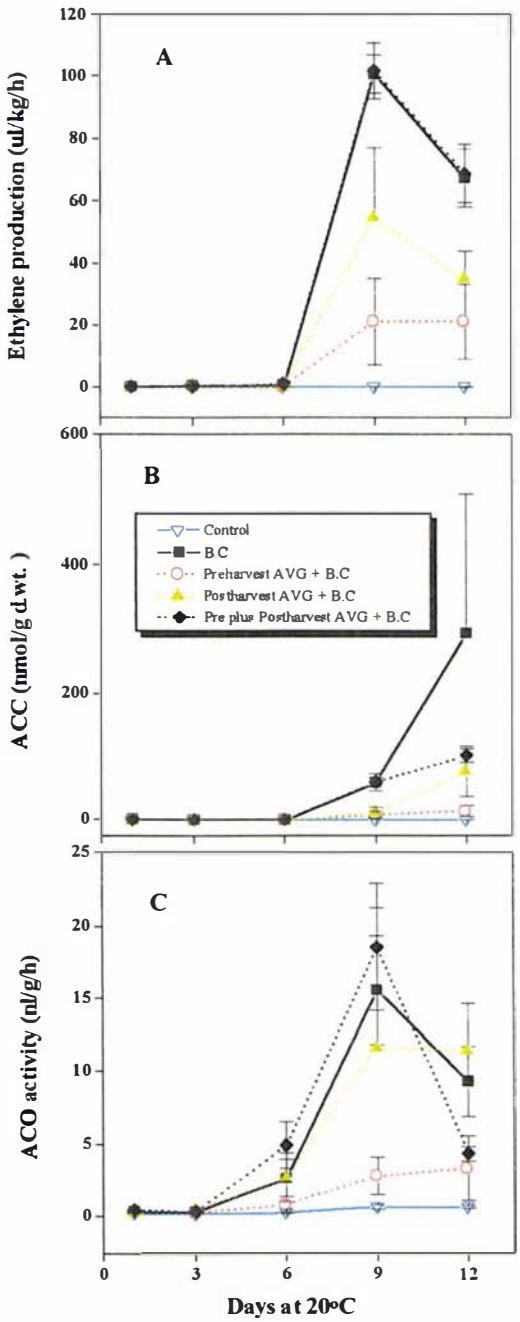

Fig. 5-1. Ethylene production (A), ACC concentration (B) and ACO activity (C) of kiwifruit infected with *B. cinerea* maintained at 20°C after harvest, following preharvest, postharvest or pre plus postharvest AVG application. Bars represent standard errors.

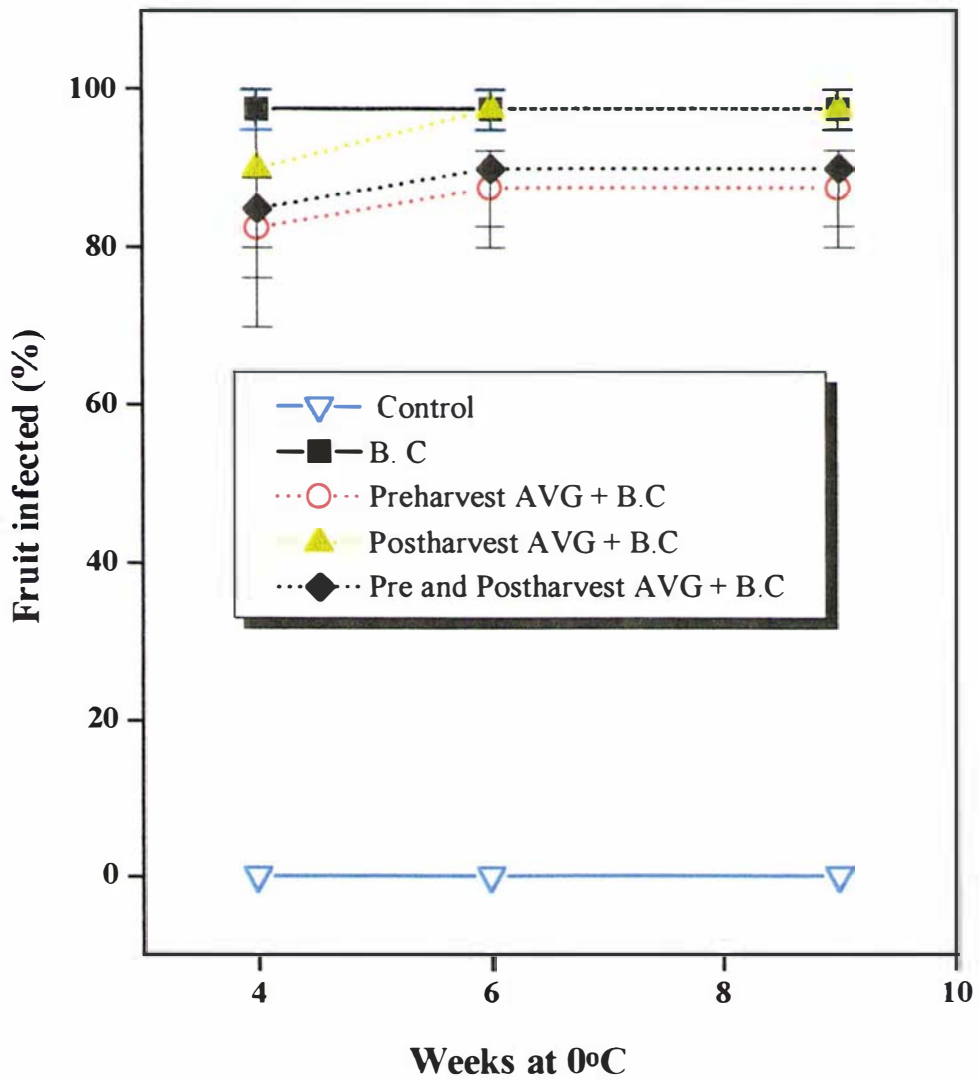

Fig. 5-2. Percent infected kiwifruit, inoculated with *B. cinerea*, following preharvest or postharvest AVG application, maintained at 0°C. Bars represent standard errors.

Table 5-1 Mean ethylene production (µl/kg/h) of kiwifruit infected with *B. cinerea* following preharvest, postharvest and pre plus postharvest AVG applications, maintained at 20°C for 7 days after 4, 6 and 9 weeks at 0°C.

Treatment	**Mean ethylene production over 7 days at 20°C**		
	Weeks at 0°C		
	4	6	9
Control	0.2c	0.1c	0.1d
B. C	62.0a	50.4a	34.0a
Preharvest AVG + B.C	49.0b	43.0b	29.1bc
Postharvest AVG + B. C	58.9a	41.8b	27.5c
Pre plus Postharvest AVG + B. C	45.2b	50.9a	32.8ab

Values with the same letter within a column are not significantly different by Duncan's multiple range test at the 5% level.

Table 5-2 Growth rate of *B. cinerea* in inoculated kiwifruit at 0°C, following preharvest, postharvest and pre plus postharvest AVG applications.

Treatments	**Growth rate of *B. cinerea* (mm/week)**
Control	0a
B. C	5.0b
Preharvest AVG + B. C	4.5b
Postharvest AVG + B. C	4.7b
Pre plus Postharvest AVG + B.C	5.3b

Values with the same letter are not significantly different by Duncan's multiple range test at the 5% level.

cinerea infected fruit through 9 weeks at 0°C but postharvest AVG application reduced ethylene only after 6 and 9 weeks and pre plus postharvest AVG applications reduced ethylene only after 4 weeks. There was no consistent AVG effect on ACC concentration or ACO activity during 9 weeks at 0°C (data not shown).

Control fruit did not become infected with *B. cinerea* throughout storage at 0°C but 98% of inoculated fruit were infected (Fig. 5-2). Preharvest or pre plus postharvest applications of AVG caused a slight (8-10%) reduction in infection after 9 weeks at 0°C in fruit inoculated with *B. cinerea*. AVG application had no effect on growth rate of *B. cinerea* in infected fruit during 9 weeks at 0°C (Table 5-2).

To determine the extent of ethylene production and ACO activity from parts of a single kiwifruit from the same treatments (5.2.1), fruit which had approximately the same amount of infected tissue were selected and divided into separate zones, representing the infected zone, invasion zone, adjacent zone and distal zone after 8 weeks at 0°C. Since there were no AVG effects in *B. cinerea* infected fruit, only fruit infected with *B. cinerea* was compared to control fruit (Fig. 5-3). Ethylene production by whole fruit infected with *B. cinerea* was much higher (5.61µl/kg/h) than that in control fruit (0.04µl/kg/h) before slicing these fruit into the above zones (Fig. 5-3A and B). All tissue zones of control fruit produced approximately the same amount ethylene (between 0.156 to 0.190µl/kg/h) (Fig. 5-3A). In infected fruit, maximum ethylene production (10.55µl/kg/h) occurred in tissue from the invasion and adjacent zones, while infected and distal zone tissue produced less ethylene but still more than equivalent zones in control fruit (Fig. 5-3B).

ACO activity was approximately the same (between 0.525 to 0.651nl/g/h) from all four zones of control fruit (Fig. 5-3C). In infected fruit, infected zone tissue had only 25% of the ACO activity of the equivalent zone in control fruit. Invasion zone tissues maintained the same ACO activity as equivalent zones in control, while adjacent and distal zone tissues had more than 4 times the ACO activity present in equivalent zones from control fruit (Fig. 5-3D).

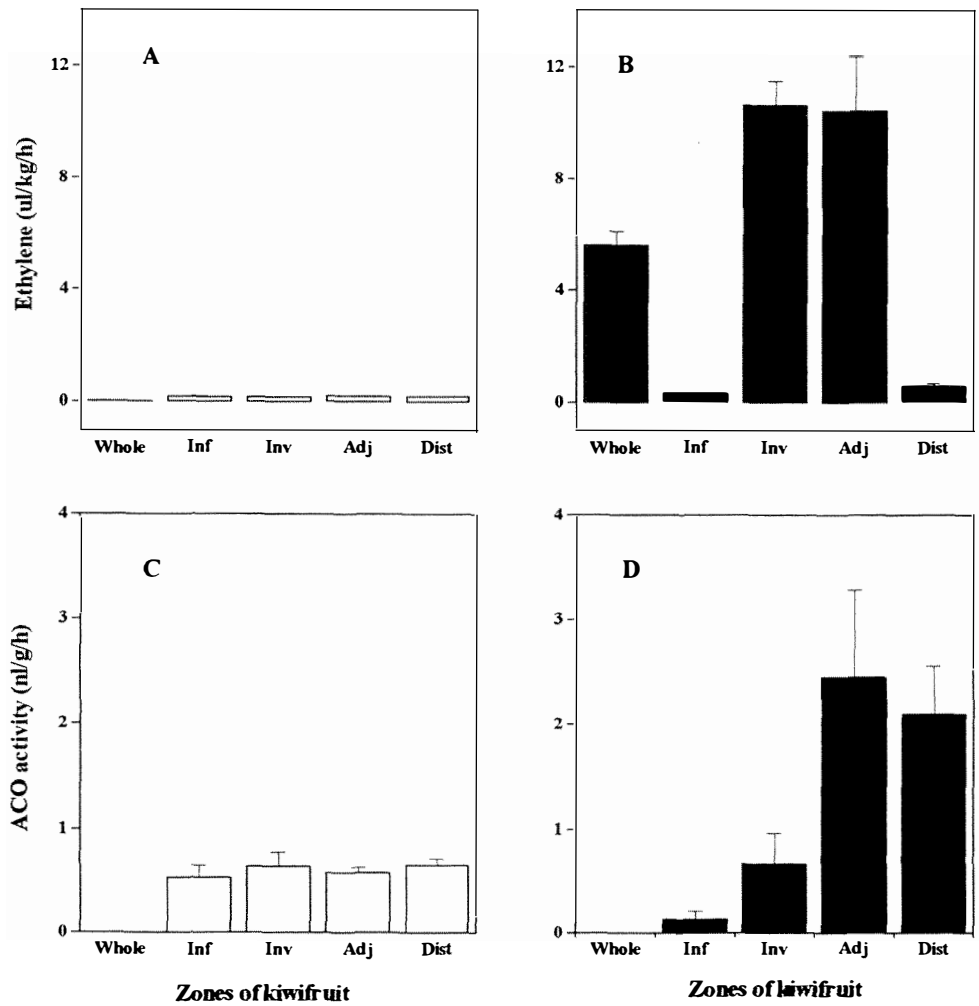

Fig. 5-3. Ethylene production (A, B) and ACO activity (C, D) from intact and different zones of control (A, C) and B. cinerea infected kiwifruit (B, D) after 8 weeks at 0°C. Bars represent standard errors.

To compare ACC concentrations in sound and infected tissue in fruit infected with *B. cinerea*, fruit infected with *B. cinerea* were removed from 0°C after 8 weeks. ACC concentration of sound tissue was approximately 20 times less than in tissue from fruit infected with *B. cinerea* (Table 5-3).

Table 5-3 ACC concentration at 20°C after 3 months at 0°C from sound and infected zones of kiwifruit infected with *B. cinerea* before storage at 0°C.

Zones of kiwifruit	ACC (nmole/g d.wt)
Sound	0.4a
Infected	7.6b

Values with the same letter are not significantly different by Duncan's multiple range test at the 5% level.

5.3.2 Ethylene production and growth rate of *B. cinerea* at different temperatures

Although wounded fruit were not inoculated with *B. cinerea*, 58%, 42%, 8% and 8% of fruit in stored at 20°, 10°, 4° and 0°C became infected with the fungus after 7, 19, 19 and 55 days at 20°, 10°, 4° and 0°C respectively; this was probably due to contamination with fungus spores that spread from fruit with stem-end rot even though wounded fruit and inoculated fruit were stored in different trays.

Ethylene production was low from control fruit (uninoculated, no wound), varying between 0.013 to 0.028µl/kg/h, and wounded fruit varying between 0.013 to 0.033µl/kg/h through storage at 0°C, while wounded fruit, inoculated with *B. cinerea*, increased ethylene production proportionally to lesion development after 30 days, reaching a peak of 0.183µl/kg/h at 70 days (Fig. 5-4A and E). At 4 and 10°C, control fruit produced very little ethylene throughout storage, while ethylene in wounded fruit began to increase after 17 days, 9 days before lesion development was apparent (Fig. 5-4B, C, F and G). Wounded fruit inoculated with *B. cinerea* increased ethylene production concomitantly with lesion development, reaching peaks at 2.59 and 4.93 µl/kg/h after 26 and 19 days at 4 and 10°C respectively. Although ethylene production in control fruit remained very low through storage at 20°C, both wounded and wounded plus inoculated fruit produced ethylene rapidly, reaching peaks of 50.0 and 57.2µl/kg/h after 9 and 7 days respectively (Fig. 5-4D). Generally. lesion development was apparent after the onset of

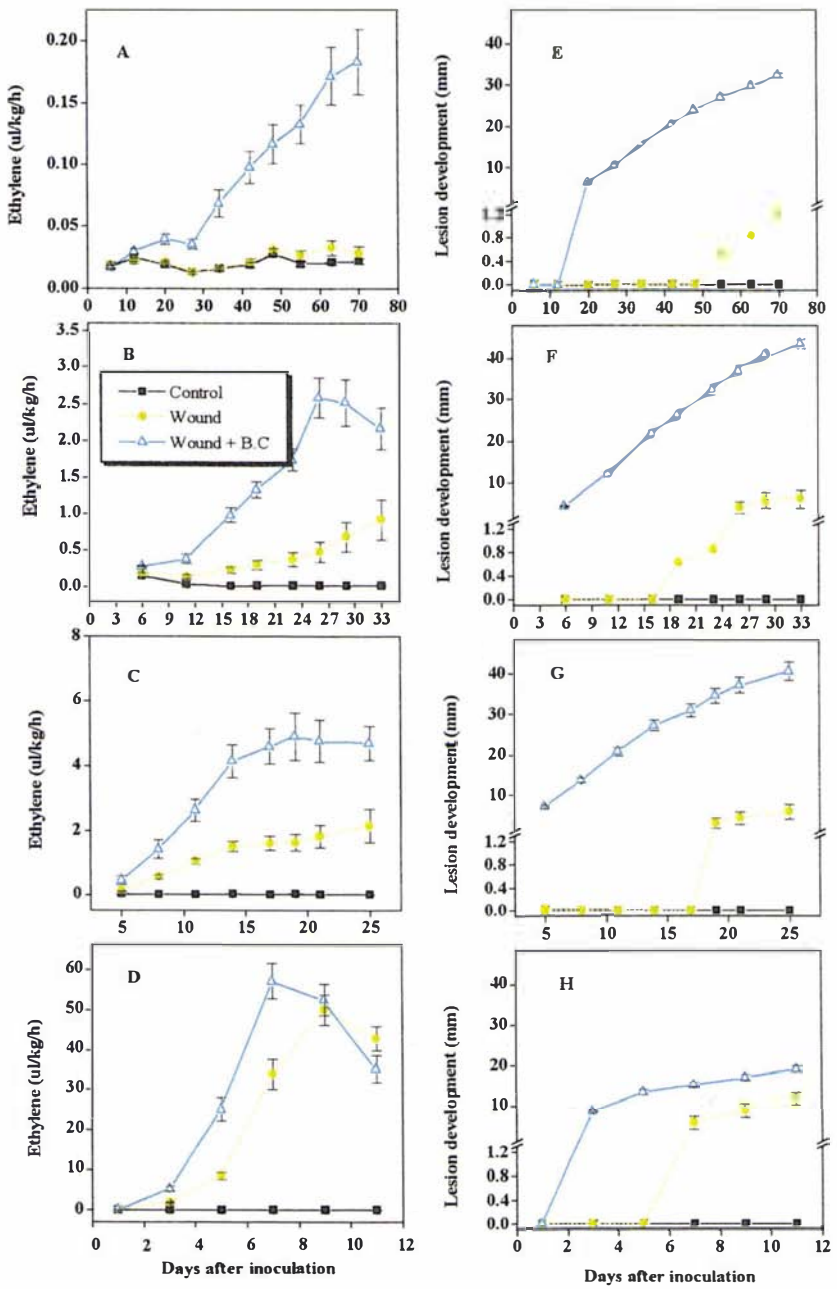

Fig. 5-4. Ethylene production (A,B,C,D) and lesion development (E,F,G,H) of fruit from control (uninoculated, no wound), wound (wounded, uninoculated) and wound + B.C (wounded, inoculated) treatments after inoculation with *B. cinerea* maintained at 0° (A,E), 4° (B,F), 10° (C,G) and 20°C (D,H). Bars represent standard errors. NB: different values on both X and Y axes for ethylene production and time.

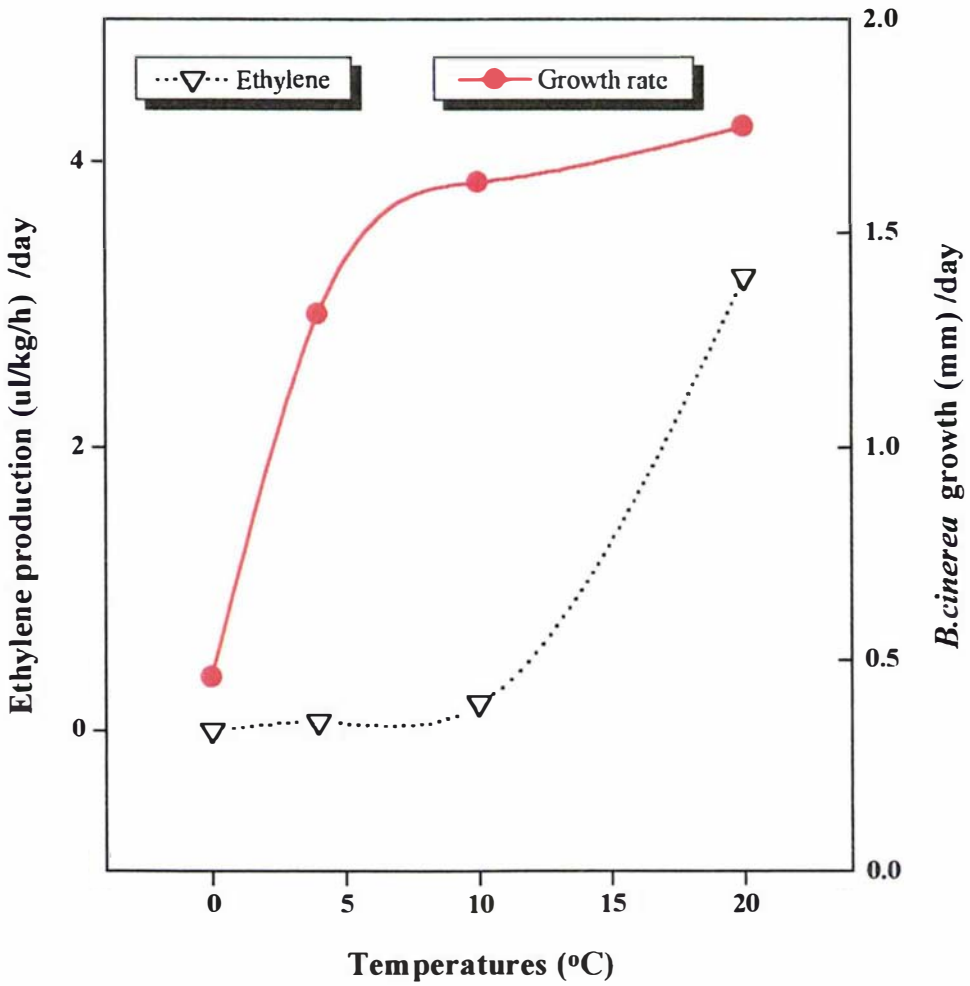

Fig. 5-5. Ethylene production rate and *B. cinerea* growth rate at a range of temperatures in inoculated kiwifruit.

ethylene production in both wounded fruit and wounded fruit inoculated with *B. cinerea* (Fig. 5-4).

Table 5-4 Percent infected fruit with *B. cinerea* in different treatments at different temperatures.

	Days at 20°C				
Treatment	1	3	7	9	11
Control	-	-	-	-	-
Wound	-	-	58%	75%	92%
Wound + BC	-	100%	-	-	-
	Days at 10°C				
Treatment	5	17	19	21	25
Control	-	-	-	-	-
Wound	-	-	42%	50%	58%
Wound + BC	100%	-	-	-	-
	Days at 4°C				
Treatment	6	19	23	26	29
Control	-	-	-	-	-
Wound	-	8%	17%	50%	67%
Wound + BC	100%	-	-	-	-
	Days at 0°C				
Treatment	6	12	20	27	55
Control	-	-	-	-	-
Wound	-	-	-	-	8%
Wound + BC	-	-	100%	-	-

In all inoculated fruit, visible signs of *B. cinerea* infection were obvious after 3, 5, 6 and 20 days at 20°, 10°, 4° and 0°C, respectively (Table 5-4). In wounded fruit, infection was first apparent after 7, 19, 19, 55 days with 58%, 42%, 8% and 8% infected at 20°, 10°, 4°and 0°C respectively, while no infection occurred in control fruit .

Ethylene production rate of fruit infected with *B. cinerea* was below 0.19µl/kg/h per day at 0, 4 and 10°C, while at 20°C the ethylene production rate was 3.2 µl/kg/h per day (Fig. 5-5). The growth rate of *B. cinerea* at 20°C, 10°C, 4°C and 0°C was 1.75, 1.62, 1.31 and 0.46 mm/day respectively (Fig. 5-5). The inoculated fruit at 20°C only developed 19mm and the growth rate in the fruit was only slightly higher than that at 10°C. It is possible that dry conditions in the laboratory may have inhibited hyphal growth since *B. cinerea* requires abundant moisture for spore production and infection (Beever, 1979). At 0°, 4°, and 10°C where moisture control was maintained, lesions developed to almost 40mm (Fig. 5-4E, F and G).

5.4 DISCUSSION

Botrytis cinerea is the major pathogen affecting cool stored kiwifruit. Storage rot caused by *B. cinerea* is the one of the major causes of postharvest losses of kiwifruit in New Zealand (Pak and Manning, 1994). Fruit infected with *B. cinerea* produced more ethylene than healthy fruit (Fig. 5-1 and Fig. 5-3) (Niklis *et al.*, 1993; Pennycook, 1986). Since kiwifruit is very sensitive to ethylene (Jeffery and Banks, 1996; Retamales and Campos, 1997), any increase in ethylene produced by infected fruit will hasten softening of other healthy fruit in a tray thus reducing storage life (Manning and Pak, 1993). The increased ethylene production is a result of the physiological stress that the invading fungal hyphae imposes on the plant tissue (Achilea *et al.*, 1985; Elad, 1990; Takeda and Nakamura, 1990); even at 4° and 10°C where kiwifruit was unable to trigger autocatalytic ethylene production because of limited ACC production (Niklis *et al.*, 1993, 1997; Stavroulakis and Sfakiotakis, 1993), fruit inoculated with *B. cinerea* produced high amounts of ethylene which was found to be associated with the rate of lesion development (Fig. 5-4).

Invasion and adjacent zone tissue in fruit infected with *B. cinerea* had enhanced ethylene production compared with comparable tissue zones in healthy fruit (Fig. 5-3). Cell wall degrading enzymes are known to break down polysaccharides into oligomers that act as 'secondary messengers' to trigger active responses in invasion and adjacent healthy zones (Basse *et al.*, 1992; Fry *et al.*, 1993). In infected fruit, *B. cinerea* probably produces cell

wall hydrolases including polygalacturonase, pectin methyl esterase, rhamnogalacturonase and cellulase (Gross *et al.*, 1995; Heiler *et al.*, 1993; Marcus and Schejter, 1983; Tobias *et al.*, 1993), which can break down cell wall polysaccharides into short chain oligomers some of which have the potential to induce ethylene production (Aldington and Fry, 1993; Basse *et al.*, 1992). In particular, specific oligomers elicit ethylene production in otherwise healthy tissue (Basse *et al.*, 1992; Fry *et al.*, 1993). It is possible that the signal sent by hyphae growing in the infection zone induces such oligomers which migrate in some as yet unknown manner to the uninfected tissue distal to this zone eliciting enhanced ACO activity and ethylene production.

AVG has been used to reduce ethylene production and delay ripening in apple and pears (Williams, 1980; Harder-Doll and Bangerth, 1987; Romani *et al.*, 1982). It has now been shown to reduce ethylene production induced by *B. cinerea* in kiwifruit (Fig. 5-1). This reduced ethylene production following AVG applications was due to reduced ACC concentration and ACO activity. Although AVG is known to be an inhibitor of ACS activity, it has not been shown to affect ACO activity previously (Hyodo and Fukasawa, 1985; Yang, 1985). Preharvest application of AVG seemed to inhibit both ACS and ACO activity in kiwifruit infected with *B. cinerea* (Fig. 5-1). However, postharvest and pre plus postharvest applications of AVG were less effective in reducing ethylene production than preharvest applications; in these treatments, ACO activity was not inhibited by AVG. Postharvest application of AVG was made at the same time as inoculation by *B. cinerea*; this combination treatment may have reduced the AVG effect. Earlier work with apple indicated that a high concentration of AVG (1000µl/l) increased % fruit set but prevented expansion of leaves (Williams, 1980). It was suggested that such small leaves could have been due to a phytotoxic effect of AVG. Pre plus postharvest AVG application did not reduce ethylene biosynthesis induced by *B. cinerea*; a phytotoxic effect of high AVG concentration may have resulted from the double application, rendering the AVG effect reduced.

Although preharvest and postharvest AVG applications reduced ethylene biosynthesis in fruit infected with*B. cinerea* at 20°C, the magnitude of this AVG effect declined with

increasing time at 0°C (Table 5-1); this may be due to increased internal ethylene production (Romani *et al.*, 1982), increased chilling sensitivity (Lallu, 1997) or increased fruit sensitivity to ethylene (Gerasopoulos and Richardson, 1997). Thus, although AVG was applied at harvest, fruit infected with *B. cinerea* produced enough ethylene to induce cell wall breakdown, resulting in easier infection after storage at 0°C for 4-8 weeks; growth rates of *B. cinerea* and % infection of fruit were similar in fruit with and without AVG application.

Once cells are killed as a result of fungal infection they cease to produce ethylene (Elad, 1990); the apparent decrease of ethylene during 9 weeks at 0°C may have resulted from the reduced proportion of healthy tissue existing in infected kiwifruit (Table 5-1). Infected zone tissue in fruit infected with *B. cinerea* produced little ethylene and ACO activity (Fig. 5-3) but ACC concentration in this zone tissue was much higher than in sound tissue (Table 5-3). The infected zone tissue was regarded as dead (Qadir, 1994) and thus ACO activity, which requires membrane integrity for its activity (John *et al.*, 1989) was reduced, resulting in low ethylene production. Although ACO activity in infected zone tissue was lower than that in control tissue, ethylene production in infected zone tissue was higher than that in control tissue; this increased ethylene may be of fungal origin (Achilea *et al.*, 1985; Ilag and Curtis, 1968; Qadir *et al.*, 1997). Similar results were found by Niklis *et al.* (1993) who showed that ACC accumulated in *B. cinerea* infected kiwifruit tissue but ethylene production was decreased.

In summary, kiwifruit infected with *B. cinerea* showed enhanced ethylene biosynthesis including ethylene production, ACC concentration and ACO activity and preharvest AVG inhibited ethylene biosynthesis immediately after harvest. However, the magnitude of the AVG effects on inhibiting ethylene biosynthesis was minimal after 4-8 weeks at 0°C, resulting in no effect on rate of *B. cinerea* infection at 0°C. Therefore AVG can not be recommended as a treatment to reduce *B. cinerea* infection in kiwifruit.

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CHAPTER SIX

1-MCP EFFECTS ON ETHYLENE ACTION OF KIWIFRUIT

6.1 INTRODUCTION

Ethylene is a plant hormone regulating fruit ripening by coordinating the expression of genes that are responsible for a variety of processes, including a rise in respiration, autocatalytic ethylene production and changes in color, texture, aroma and flavor (Oetiker and Yang, 1995). It is biologically active in trace amounts and its effects are commercially important in agriculture and horticulture (Abeles *et al.*, 1992; Yang and Oetiker, 1998). Ethylene is biosynthesized from S-adenosylmethionine via 1-aminocyclopropane-1-carboxylic acid (ACC), and catalysed by ACC synthase (ACS) and ACC oxidase (ACO) (Adams and Yang, 1979). Both enzymes are limiting in preclimacteric fruits but are induced greatly during ripening (Oetiker and Yang, 1995).

Ethylene synthesis plays a key role in regulating fruit maturation and ripening (Abeles *et al.*, 1992). It is thought that two systems of ethylene production exist in fruit (McMurchie *et al.*, 1972; Yang, 1987). System 1 is the low level of ethylene present in preclimacteric fruit before the onset of ripening (most ethylene produced by vegetative plant tissue and non-climacteric fruit can be classified as System 1); System 2 is the high rate of ethylene production occurring during the climacteric, being autocatalytic and accompanying the ripening process.

Although fruit normally produce low concentrations of ethylene during development, climacteric fruit become responsive to ethylene from a certain stage of development, or in some instances after removal from the parent plant (Knee, 1985). Changes in the responsiveness of tissue to plant growth regulators such as ethylene, rather than the endogenous concentrations of these compounds *per se*, is probably a general feature of growth regulation and developmental changes in plants; sensitivity to growth regulators

will change depending on the number of active receptors in responsive cells (Trewavas, 1982, 1983).

Ethylene can have beneficial or detrimental effects on postharvest life and quality of fruit depending on where and when it occurs. Efficient postharvest technology aims to control ethylene effects; the ability to prevent ethylene action, or manipulate fruit sensitivity to ethylene, would provide a useful tool to extend the fruit's postharvest storage life (Yang, 1985). Yang (1985) suggested four levels of manipulation for regulating ethylene responses: (a) by addition or removal of ethylene using ethephon or $KMnO_4$; (b) by stimulating or inhibiting ethylene biosynthesis; fruit ripening, flower senescence, physical wounding and chilling injury induce ethylene biosynthesis, while treatment with aminoethoxyvinylglycine (AVG) or aminooxyacetic acid (AOA) inhibit it; (c) by modifying ethylene action by changing characteristics of ethylene receptors or the number of the receptors using antagonists of ethylene action such as silver (Ag^+), norbornadiene and 1-methylcyclopropene (1-MCP); (d) by manipulating ethylene-dependent gene expression.

Ethylene is thought to bind to a receptor molecule such as ETR1 (ethylene-resistant) (Bleecker and Schaller, 1996) that is activated as a result of the interaction between ethylene and receptor (Schaller and Bleecker, 1995). This receptor is connected to the CTR1 (constitutive triple response) protein kinase that increases EIN2 (ethylene-insensitive) activity and in turn initiates a chain of reactions leading to physiological responses (Bleecker and Schaller, 1996; Ecker, 1995; Theologis, 1993). One of these primary responses in ripening fruits is the autocatalytic production of ethylene. This autocatalysis involves increased synthesis of ACS and ACO (Hoffman and Yang, 1982). Inhibitors of ethylene binding will prevent reactions (Veen, 1983) leading to this autocatalytic increase in ethylene and the accompanying rise in ACS and ACO, resulting in delayed ripening and senescence.

Certain analogues of ethylene inhibit ethylene binding (Blankenship and Sisler, 1993; Sisler *et al.*, 1986; Sisler and Blankenship, 1993; Sisler *et al.*, 1993). A cyclic di-olefin, 2,5-norbornadiene, strongly inhibits binding, and prevents the effects of ethylene but it is released from receptors and diffuses from the binding site over a period of several hours. A diazo derivative, diazocyclopentadiene (DACP), binds irreversibly to the ethylene receptor or at least remains bound for many days. However, DACP needs light for 'permanent' attachment to the site and is explosive in high concentrations, which limits its commercial usefulness. 1-methylcyclopropene (1-MCP) is the most useful compound among recently developed inhibitors of ethylene binding. 1-MCP is a relatively simple non-toxic organic compound that binds irreversibly to ethylene receptor sites and inhibits ethylene action at concentrations as low as 0.5nl/l in some systems (Serek *et al.*, 1994b; Sisler *et al.*, 1996a). 1-MCP prevented the climacteric rise of ethylene production in apple (Song *et al.*, 1997), strongly inhibited ethylene-induced ripening in banana and tomato (Serek *et al.*, 1995a) and extended vase life in cut flowers (Serek *et al.*, 1994a, 1994b). However, timing and concentration of 1-MCP application is crucial; application of 1-MCP at a preclimacteric stage of banana development delayed onset of the ethylene and respiratory climacteric, while application after autocatalytic ethylene production had commenced did not (Golding *et al.*, 1998). For younger carnations (Stage II), 2.5 nl/l of 1-MCP was sufficient to afford protection against ethylene induced senescence but 5 nl/l was required for protection for older carnations (Stage III) (Sisler *et al.*, 1996a). Although chilling treatment induced ACS and ACO mRNA transcripts in Passe-Crassane pears that require a 3-month chilling treatment at 0° to produce ethylene and ripen autonomously after subsequent rewarming, 1-MCP strongly reduced the chilling-induced accumulation of ACS and ACO transcripts, resulting in elimination of the climacteric response (Lelievre *et al.*, 1997).

Softening is one of the most significant quality alterations consistently associated with the ripening of fruits. Kiwifruit firmness can decline by as much as 94%, from about 60-90N at harvest to 5-8N when ripe (Harker & Hallett, 1994). The rate of softening is much faster when ethylene is present; at 0°C, storage potential was reduced by 46% even at the

very low ethylene concentration of 0.01 µl/l (Jeffery and Banks, 1996). Although softening in kiwifruit was stimulated by ethylene (Retamales and Campos, 1997), softening occurred steadily in fruit stored at 0°C even though ethylene concentration was low (see Chapter 3 results). One possible reason for this may be a changing sensitivity of kiwifruit to low concentrations of ethylene as maturation progresses.

The purpose of this study was to evaluate the effect of 1-MCP application to kiwifruit at different stages of maturation and ripening, on ethylene synthesis and rate of softening after harvest.

6.2 MATERIALS AND METHODS

Uniform vines of kiwifruit growing at the Fruit Crops Unit, Massey University, Palmerston North, New Zealand, were selected and fruit, average weight 100g (range 80-120g), were harvested at different maturities. After harvest, Rovral (0.075%, one droplet of approximately 30µl of solution per fruit) was applied directly to the stem scar and then each gas, 1-MCP (as EthylBloc™, a wettable powder that when dissolved in 0.9% KOH and 0.9% NaOH buffer solution (a ratio of 1 gram of powder for every 20mls of buffer solution), released the active gas (1-methylcyclopropene)), or ethylene, was applied by injection via a rubber port into sealed 24L plastic containers that contained fruit and one sachet (20g) of soda lime (Plate 6-1). After 16 hours at 20°C, the containers were ventilated with air for 6 hours. Fruit were then placed into the pockets of 27 count plastic trays in one layer cardboard trays surrounded by a polyethylene liner. One sachet (20g) of potassium permanganate per tray was enclosed. Nine individual fruit per treatment were used for measurements including firmness (both destructively and non-destructively), ethylene biosynthesis (ethylene production, ACC concentration and ACO activity) and respiration rate. Each treatment had three replicates.

Plate 6-1. Application of the gases ethylene and 1-MCP, into plastic containers (24 L capacity) that contained fruit and one sachet (20g) of soda lime.

6.2.1 Effects of ethylene on kiwifruit harvested at different maturities at 20°C

Three uniform vines of kiwifruit were selected and fruit were harvested at different maturities, 4.7, 5.3 and 7.9% TSS. 180 fruit per maturity (60 fruit from each of 3 vines) were harvested and treated with 4 concentrations of ethylene (0, 1, 10 and 100µl/l) for 16 hours before ventilating with air for 6 hours. Fruit were then maintained at 20°C and firmness (both destructive and non-destructive), ethylene production and respiration rate were measured at regular intervals. For details of measurements refer to Chapter 2.

6.2.2 Response of kiwifruit to 1-MCP concentration

Six uniform vines of kiwifruit were selected and fruit were harvested at 8.5% TSS. 60 fruit from each vine were treated with 4 concentrations of 1-MCP (0, 1, 10 and 100µl/l) for 16 hours before ventilating with air for 6 hours. Half of the fruit were then maintained at 20°C. The remaining fruit were placed at 0°C for 1 month before removal to 20°C. Firmness (both destructively and non-destructively), ethylene production, and respiration rate were measured at regular intervals.

6.2.3 Response of kiwifruit treated with 1-MCP after harvest to ethylene during storage at 20°C

Fruit were harvested from 3 uniform kiwifruit vines at 6.4% TSS. Seventy two fruit were then treated with 10µl/l 1-MCP for 16 hours before ventilating with air for 6 hours. Kiwifruit were then maintained at 20°C for 0, 1, 2, 4, 8 and 16 days before treatment with 100µl/l ethylene for 16 hours. Non-destructive firmness measurements were made at 2 day intervals.

6.2.4 Response of kiwifruit treated with 1-MCP at different storage times at 0°C to ethylene

Six uniform vines of kiwifruit were selected and 72 fruit per vine were harvested at 6.6% TSS. The fruit were divided into two groups. The first group were treated with 10µl/l 1-MCP for 16 hours at 20°C prior to storage at 0°C for 0, 8, 16, 32, 64 and 128 days before removal to 20°C. The second group were treated with 10µl/l 1-MCP for 16 hours at 20°C after storage at 0°C for 0, 8, 16, 32, 64 and 128 days. At each removal time, kiwifruit were

treated with 10µl/l ethylene for 16 hours and then non-destructive firmness measurements were made at 2 day intervals.

All data were analysed using the SAS System programmes (SAS 1988) for Analysis of Variance (ANOVA), means and standard errors and Duncan's comparison at 5% level.

6.3 RESULTS

6.3.1 Effects of ethylene on kiwifruit harvested at different maturities at 20°C

Respiration rate

There were different patterns of respiration at 20°C among fruit harvested at different maturities and treated with different concentrations of ethylene after harvest (Fig. 6-1A, B and C). Regardless of maturity at harvest, respiration of control fruit declined steadily from 8-9ml CO_2/kg/h to about 4-5ml CO_2/kg/h at day 10 (Fig. 6-1A, B and C). In fruit treated with 1µl/l ethylene, only those harvested at 7.9% TSS responded, with respiration increasing to 19mlCO_2/kg/h 1 day following treatment (Fig. 6-1C). In less mature fruit harvested at 4.7% and 5.3% TSS, respiration rate remained near base levels, albeit significantly higher than control fruit (Fig. 6-1A and B). However, in fruit harvested at 4.7 TSS, respiration rate increased sharply to about 20ml CO_2/kg/h 1 day after treatment with 10µl/l or 100µl/l ethylene, after which it declined steadily to base levels by day 10, regardless of maturity and with no difference between treatments (Fig. 6-1A). Fruit harvested at both 5.3 and 7.9% TSS, and treated with 10µl/l and 100µl/l of ethylene, had respiration rates of about 24 and 27ml CO_2/kg/h 1 day following treatment respectively, regardless of maturity, after which rates declined to base levels through day 10 (Fig. 6-1B, Fig. 6-1C).

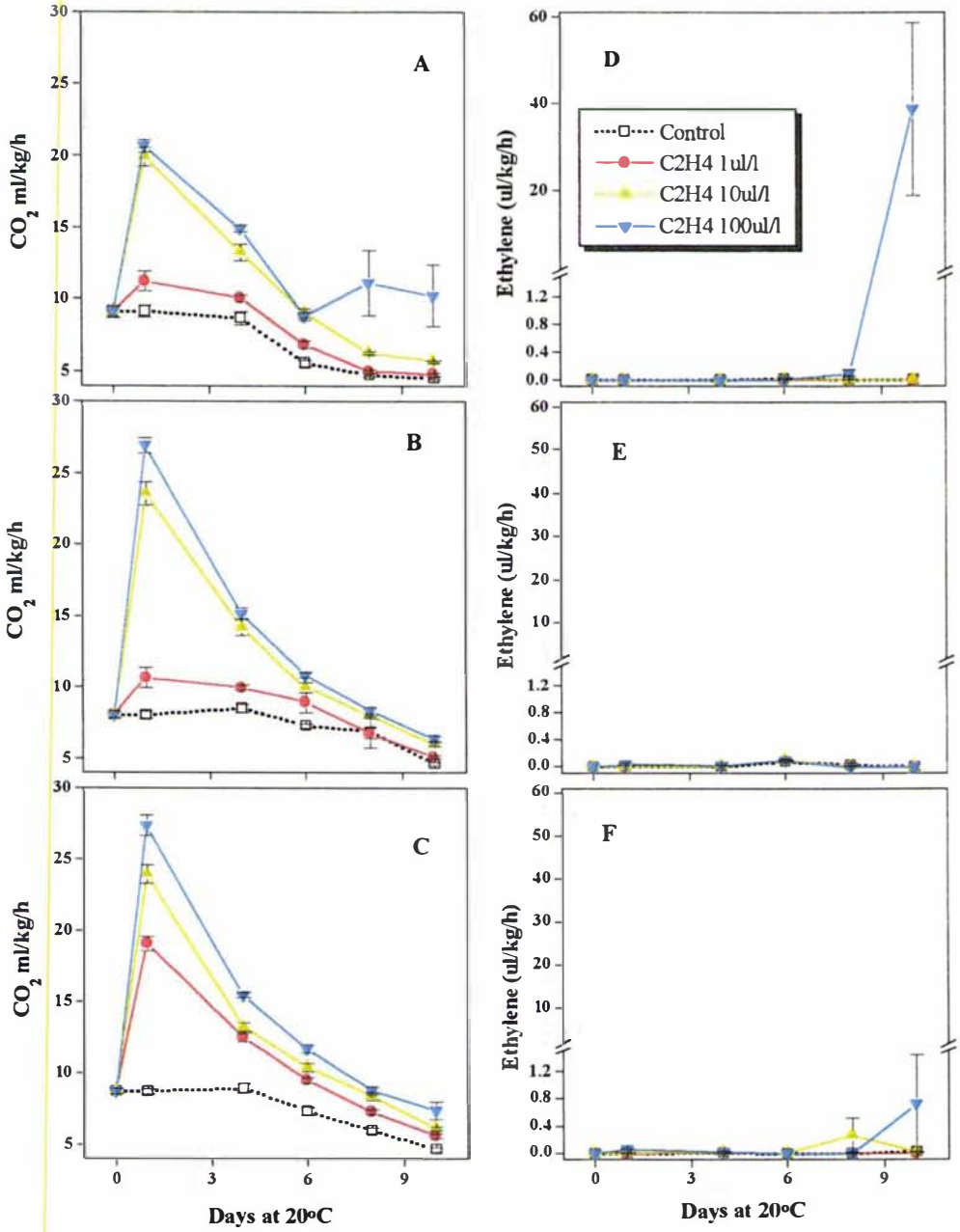

Fig. 6-1. Respiration rate (A, B, C) and ethylene production (D, E, F) of kiwifruit harvested at 4.7% (A, D), 5.3 (B, E) and 7.9% (C, F) TSS, maintained at 20°C, following treatment with ethylene immediately after harvest. Bars represent standard errors.

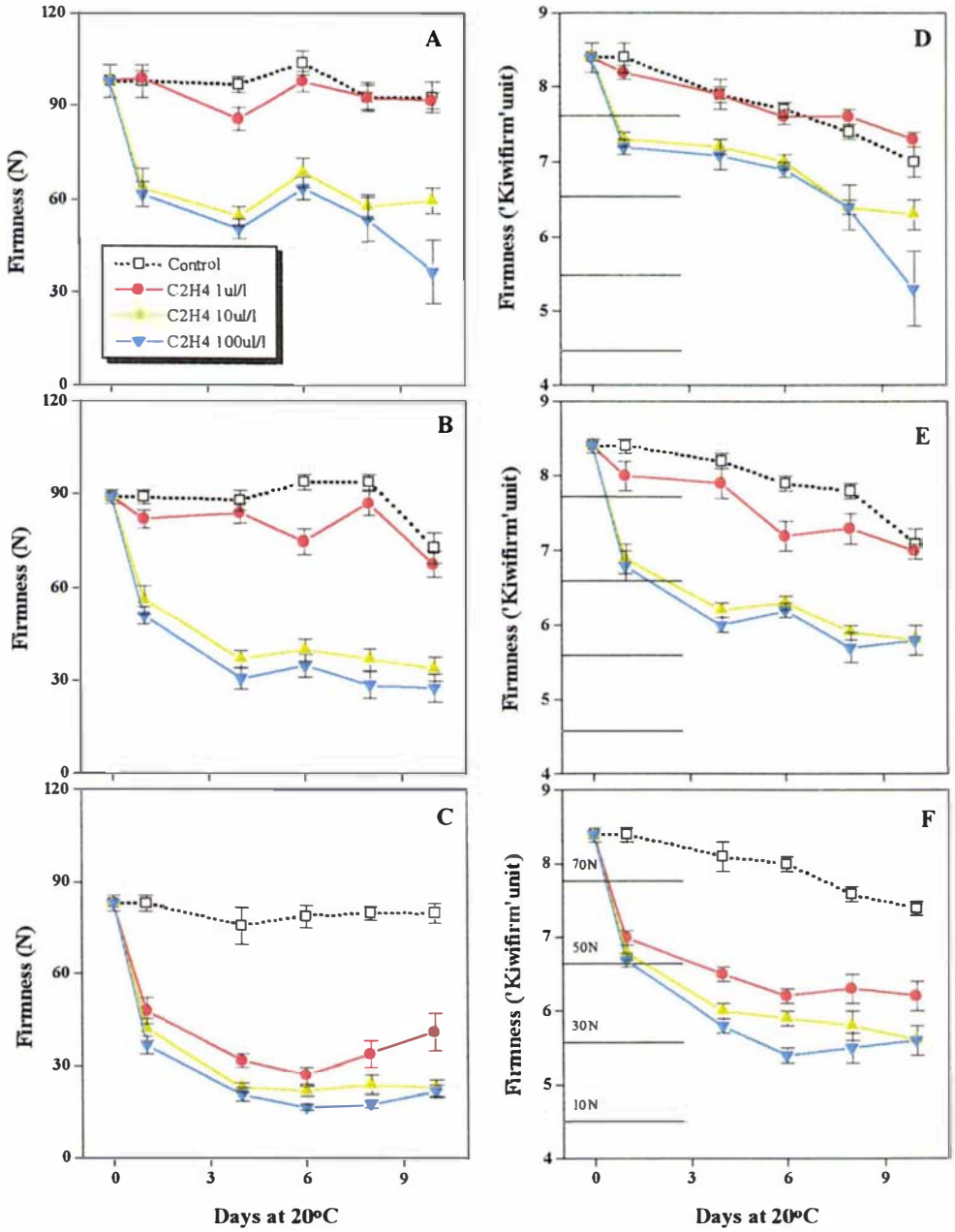

Fig. 6-2. Firmness measured destructively with a penetrometer (A, B, C) and non-destructively with 'Kiwifirm' (D, E, F) of kiwifruit harvested at 4.7% (A, D), 5.3% (B, E) and 7.9% (C, F) TSS, maintained at 20°C, following treatment with ethylene immediately after harvest. Bars represent standard errors. Lines show the value that is equivalent firmness to that obtained with a penetrometer.

Ethylene production

During 10 days at 20°C immediately after harvest, fruit produced very little ethylene regardless of maturity and ethylene treatment (Fig. 6-1D, E and F). However, for fruit harvested at 4.7% or 7.9% TSS and treated with 100µl/l ethylene, ethylene increased after 10 days at 20°C; in these treatments 3 and 1 fruit from a total of 9 fruit produced high amounts of ethylene when harvested at 4.7% and 7.9% TSS, respectively.

Firmness

Flesh firmness (measured destructively with a penetrometer) in kiwifruit declined with maturity; firmness of fruit harvested at 4.7%, 5.3% and 7.9% TSS was 98, 89 and 83 N respectively immediately after harvest (Fig. 6-2A, B and C). Control fruit maintained harvest firmness during 10 days at 20°C regardless of maturity. When treated with 1µl/l ethylene after harvest, only those fruit harvested at 7.9% TSS softened rapidly from 83 to 48N 1 day following treatment and then declined to a minimum of 27N after 6 days (Fig. 6-2C). Fruit harvested at 4.7% and 5.3% TSS and treated with 1µl/l ethylene had a firmness of 99N and 82N 1 day following treatment, and 92N and 69N respectively, the same or slightly less than control fruit (93N and 74N respectively) after 10 days at 20°C (Fig. 6-2A and B). Firmness of fruit exposed to 10µl/l and 100µl/l ethylene decreased sharply within 24 hours of treatment, after which it declined slightly, regardless of maturity. Final firmness of ethylene treated fruit after 10 days at 20°C varied with harvest maturity. Following treatment with 10µl/l and 100µl/l ethylene, fruit harvested at 4.7% of TSS softened to 60 and 37N respectively after 10 days (Fig. 6-2A) while those harvested at 5.3 and 7.9% TSS softened to about 29 and 25 N respectively (Fig. 6-2B and C), with very little effect of ethylene concentration.

These results were confirmed using the 'Kiwifirm' which measured firmness non-destructively (Fig. 6-2D, E and F). At each sampling day, different fruit were used to measure firmness throughout storage at 20°C and the same pattern of softening was obtained as that measured by penetrometer.

Relationship between ethylene production and firmness of individual fruit

By plotting data of ethylene production from individual fruit against firmness of the same fruit (Fig. 6-3), it can be seen that there was no change in ethylene production as fruit softened from 118N to about 19N. As fruit softened below 19N there was a very rapid increase in ethylene production.

6.3.2 Response of kiwifruit to different 1-MCP concentrations

Ethylene production

Untreated fruit harvested at 8.5% TSS, showed a significant increase in ethylene production 20 days after harvest, reaching 30 µl/kg/h after 30 days at 20°C (Fig. 6-4A). In fruit treated with 1 and 10µl/l 1-MCP, ethylene began to increase after 20 days at 20°C and reached 13 and 5 µl/kg/h respectively at day 30 (Fig. 6-4A). Ethylene production of fruit treated with 100µl/l 1-MCP remained low (below 0.008 µl/kg/h) through 30 days.

After 1 month at 0°C, fruit from all treatments began to produce ethylene after 16 days at 20°C, reaching 13, 15, 3 and 0.4 µl/kg/h after 26 days for fruit treated with 0, 1, 10 and 100µl/l 1-MCP respectively (Fig. 6-4B). Although the difference between treatments was not significant, 56, 33, 11 and 11% of fruit produced more than 0.1 µl/kg/h ethylene after 26 days at 20°C in fruit treated with 0, 1, 10 and 100µl/l 1-MCP respectively (data not shown).

Respiration rate

Respiration rate of fruit from all treatments declined rapidly after 10 days at 20°C, after which it increased in control fruit and fruit treated with 1 and 10µl/l 1-MCP, reaching 10.9, 8.2 and 8.4ml CO_2/kg/h respectively after 30 days at 20°C (Fig. 6-4C). 1-MCP at these concentrations reduced respiration after 20 days but there was no difference from control at 30 days. Respiration of fruit treated with 100µl/l 1-MCP remained low (5.8ml CO_2/kg/h) through 30 days (Fig. 6-4C).

After 1 month at 0°C, respiration rate of fruit from all treatments declined rapidly through 16 days at 20°C, after which it increased and reached 8.6, 7.3, 6.4 and 6.0ml CO_2/kg/h for control fruit and fruit treated with 1, 10 and 100µl/l 1-MCP respectively after 26 days (Fig.

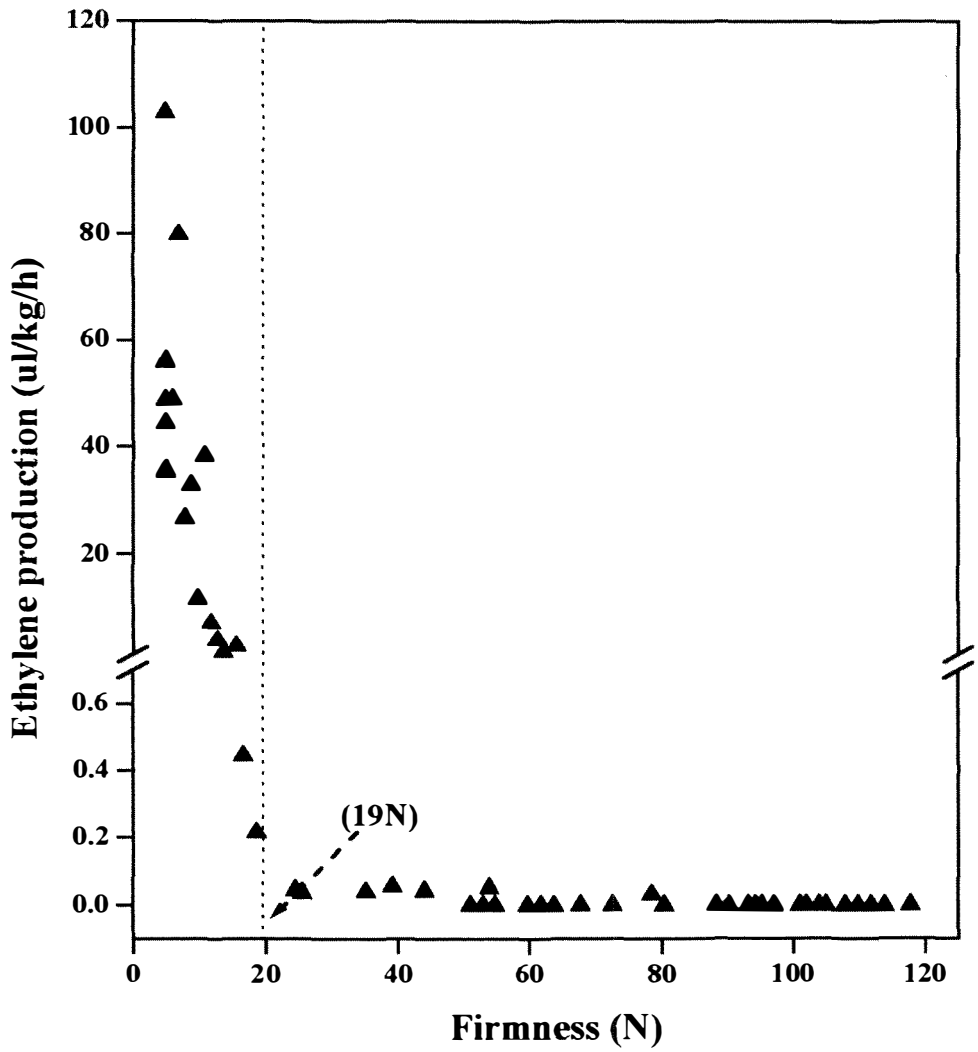

Fig. 6-3. Relationship between ethylene production and firmness of individual kiwifruit at 20°C. Data from both control and ethylene treated fruit are included.

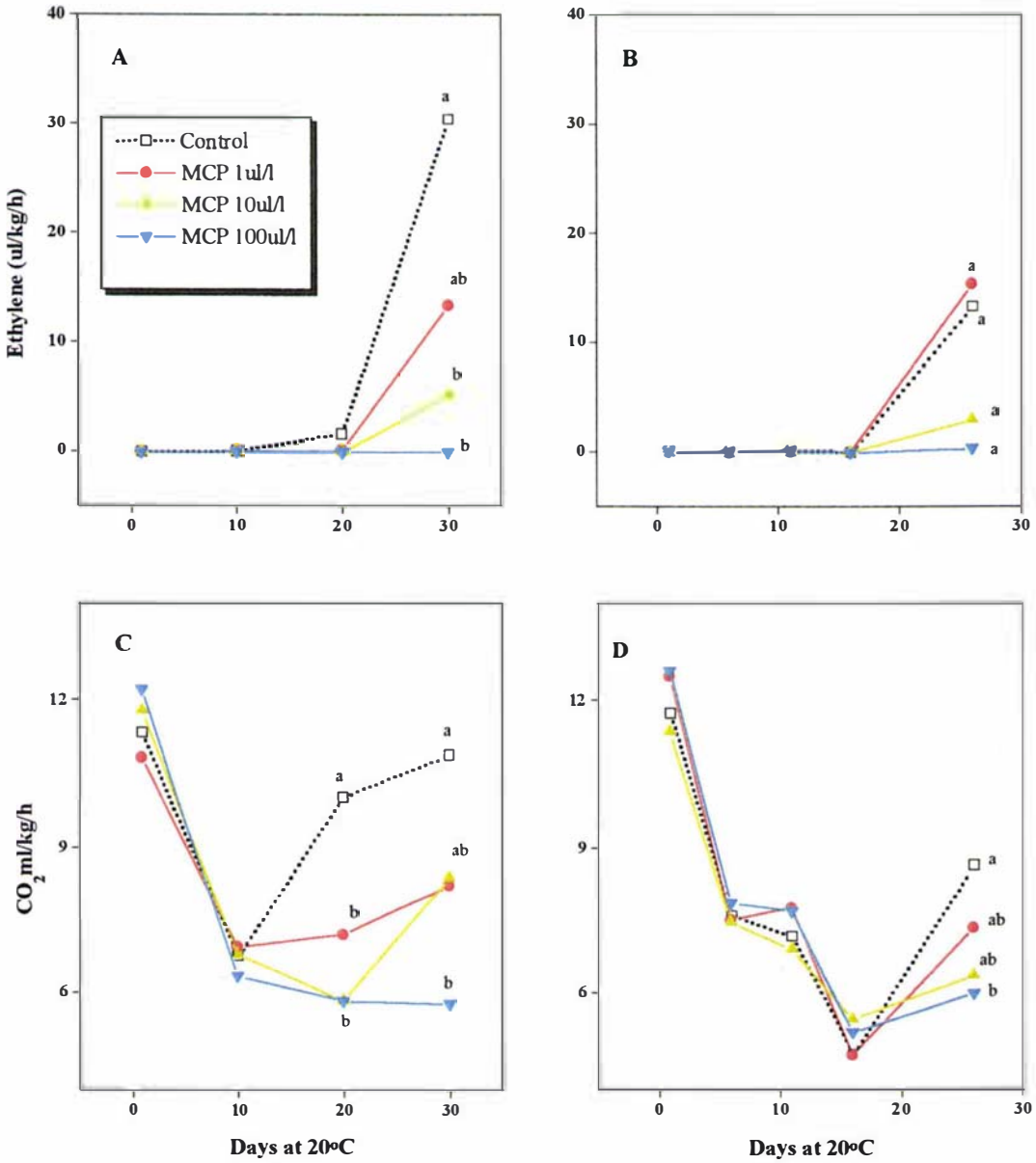

Fig. 6-4. Ethylene production (A, B) and respiration rate (C, D) of kiwifruit treated with different concentrations of 1-MCP after harvest (8.5% TSS), maintained at 20°C after harvest (A, C) and after 1 month at 0°C (B, D). At a given time values followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level.

6-4D). However, only fruit treated with 100µl/l 1-MCP respired significantly less than control fruit.

Firmness

Kiwifruit treated with 100µl/l 1-MCP were firmer than control fruit (measured destructively with a penetrometer) 20 days after harvest (Fig. 6-5A). Softening of 1-MCP treated fruit was generally slower than control fruit and after 30 days at 20°C, firmness of fruit treated with 0, 1, 10 and 100µl /l 1-MCP was 23, 45, 44 and 65N respectively.

After 1 month at 0°C, only fruit treated with 1µl/l 1-MCP were firmer than fruit from other treatments after 16 days at 20°C (Fig. 6-5B). There was no difference in firmness between treatments after 26 days at 20 °C.

Similar results were obtained using the non-destructive 'Kiwifirm' method of measuring firmness (Fig. 6-5C and D), although this method appeared less sensitive than the destructive method (Fig. 6-5D). This may due to skin resistance experienced when using the 'Kiwifirm' equipment as the skin is present in this system and may reduce sensitivity even though the flesh may be soft.

6.3.3 Response of kiwifruit pretreated with 1-MCP after harvest to ethylene during storage at 20°C

Firmness of fruit kept at 20°C for 9 days varied following treatment with 1-MCP at harvest and then treatment with ethylene after different times at 20°C (Fig. 6-6). Firmness of control fruit declined from 74, 65, 65, 65 and 32N to 61, 41, 43, 50 and 21N during 9 days at 20°C after 0, 1, 2, 4 and 8 days at 20°C respectively (Fig. 6-6). However, 1-MCP treated fruit was generally firmer than control fruit especially after 8 days at 20°C where initial firmness was 57N after which it declined to 39N through 9 days (Fig. 6-6E). 1-MCP applied to fruit immediately after harvest reduced the rate of softening in fruit subsequently treated with ethylene compared to fruit treated with ethylene alone (Fig. 6-6A, B, C, D). However, after 8 days at 20°C, softening rate of fruit treated with 1-MCP and ethylene was similar to fruit treated with ethylene alone (Fig. 6-6E). The %

reduction of softening in fruit treated with 1-MCP + ethylene, compared to fruit treated with ethylene alone after 9 days at 20°C, was much less when treated after 8 days at 20°C than it was when treated after 1 to 4 days at 20°C (Table 6-1).

Table 6-1 Reduction in softening (%) of fruit treated with MCP + ethylene compared to fruit treated with ethylene alone after 9 days at 20°C. Ethylene treatment was applied after 0, 1, 2, 4 and 8 days at 20°C, while MCP was applied at harvest (before storage at 20°C).

Days at 20°C	0	1	2	4	8
% of softening reduction	28%	52%	53%	49%	13%

6.3.4 Response of kiwifruit treated with 1-MCP at different storage times at 0°C to ethylene

Fruit treated with 10µl/l 1-MCP immediately after harvest softened at a slower rate than control fruit until 16 days at 0°C, after which the softening rate appeared similar for both control and 1-MCP treated fruit (Fig. 6-7). The fruit treated with 10µl/l 1-MCP remained firmer than control fruit throughout subsequent storage at 0°C; firmness after 128 days at 0°C was 10N and 19N in control and 1-MCP treated fruit respectively (Fig. 6-7).

Firmness (Fig. 6-8) and softening rate (Fig. 6-9) were measured during 8 days at 20°C on kiwifruit treated with 1-MCP (10µl/l) after harvest and then treated with ethylene (10µl/l) at 20°C following storage at 0°C for 0, 8, 16, 32, 64 and 128 days. Firmness of control fruit declined from 77N to 13N through 128 days at 0°C. 1-MCP treated fruit generally maintained firmness at a higher level than control fruit after transfer to 20°C through 128 days. 1-MCP applied to fruit immediately after harvest reduced softening in fruit subsequently treated with ethylene after 0 and 8 days at 0°C compared to fruit treated with ethylene alone (Fig. 6-8A and B). However, after 16 days at 0°C, ethylene and 1-MCP + ethylene treatments induced a similar response, resulting in fruit with the same firmness. Ethylene treated fruit softened rapidly between 1 and 4 days at 20°C, after

which the firmness only decreased slightly. Softening rate of fruit during 8 days at 20°C from both control and 1-MCP treatment

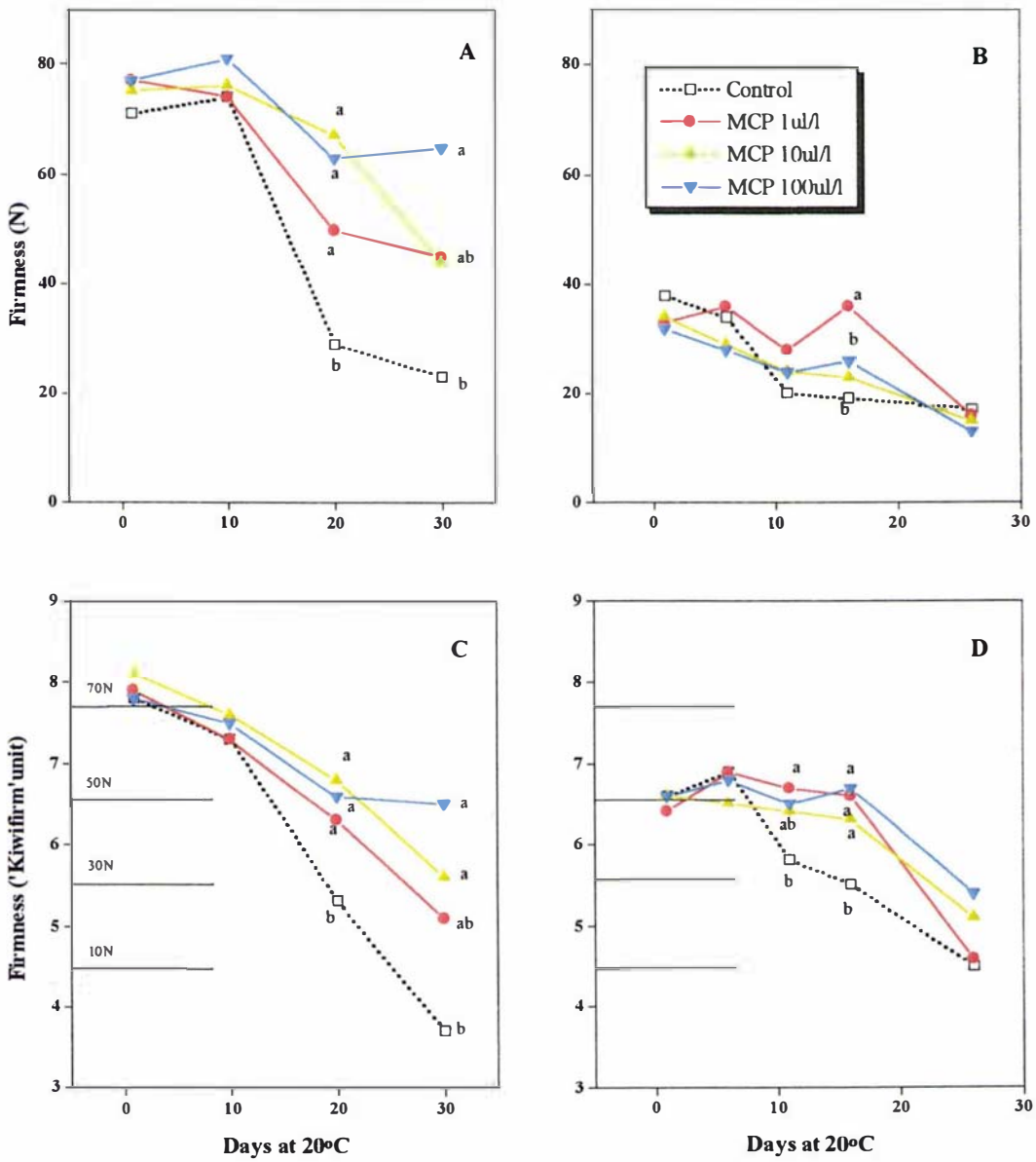

Fig. 6-5. Firmness measured with a penetrometer (A, B) and with 'Kiwifirm' (C, D) of kiwifruit treated with different concentrations of 1-MCP after harvest (8.5% TSS), maintained at 20°C after harvest (A, C) and after 1 month at 0°C (B, D). At a given time values followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level.

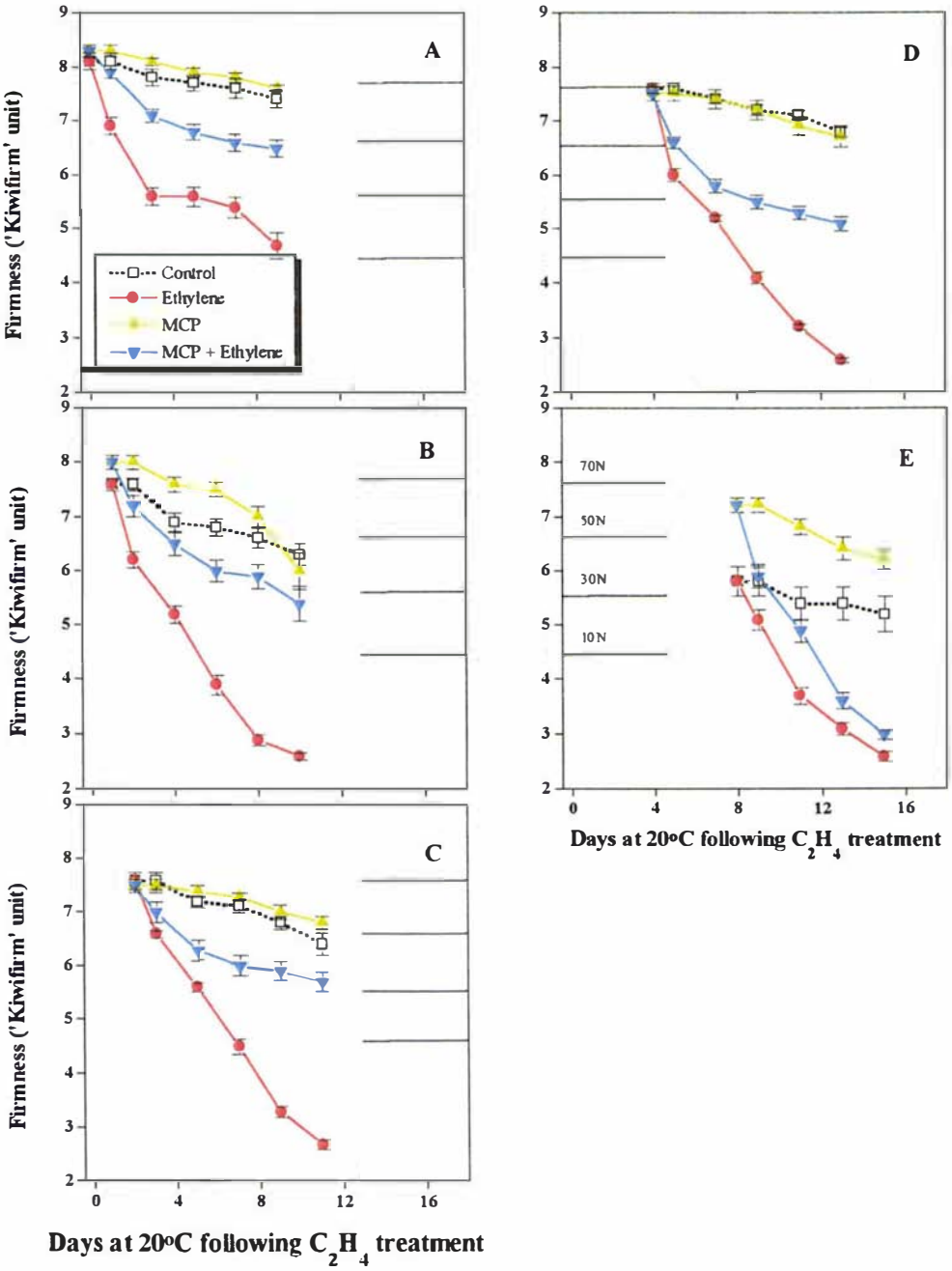

Fig. 6-6. Firmness of kiwifruit treated with 1-MCP (10µl/l) after harvest (6.4% TSS) then treated with ethylene (100µl/l) after 0 (A), 1 (B), 2 (C), 4 (D) and 8 (E) days at 20°C. Bars represent standard errors.

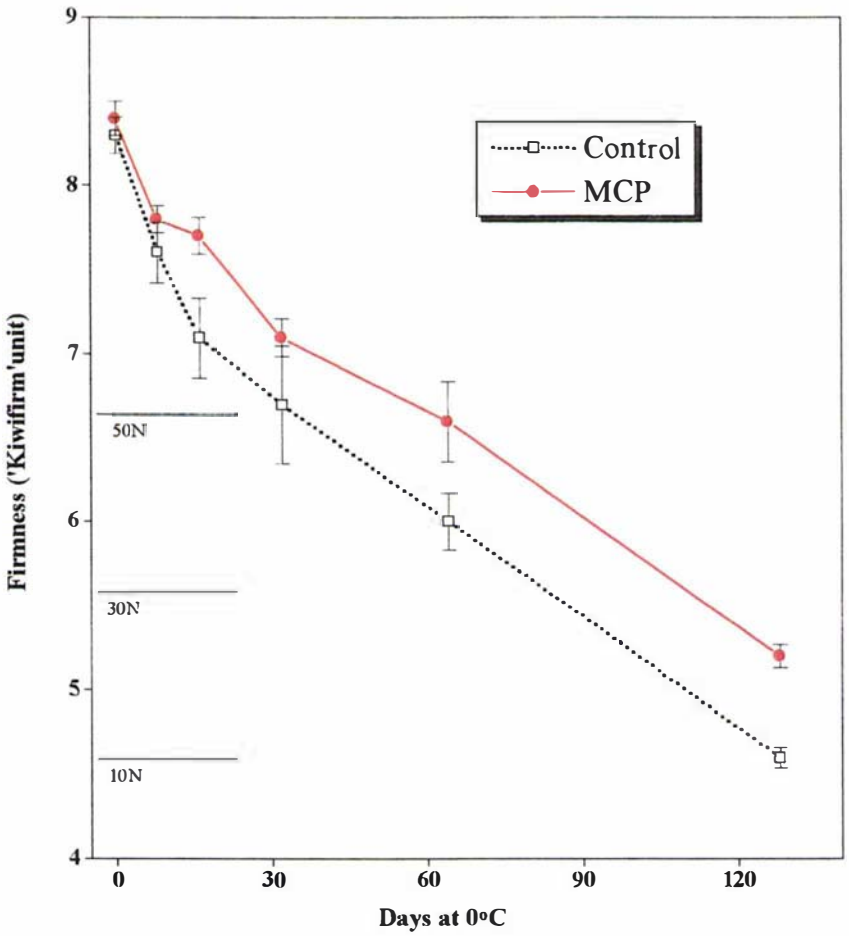

Fig. 6-7. Firmness of kiwifruit treated with 1-MCP (10µl/l) at harvest (6.6% TSS) then maintained at 0°C for 128 days. Bars represent standard errors.

remained low between 0.014 to 0.114 ('Kiwifirm' unit) / day (Fig. 6-9), but was similar for fruit treated with 1-MCP + ethylene and with ethylene alone (Fig. 6-9).

6.3.5 Response of kiwifruit to 1-MCP and ethylene after different times at 0°C

Firmness (Fig. 6-10) and softening rate (Fig. 6-11) were measured through 8 days at 20°C for kiwifruit treated with 1-MCP (10µl/l) and ethylene(10µl/l) after storage at 0°C for 0, 8, 16, 32, 64 and 128 days. Firmness of control fruit declined from 76N to 18N through 128

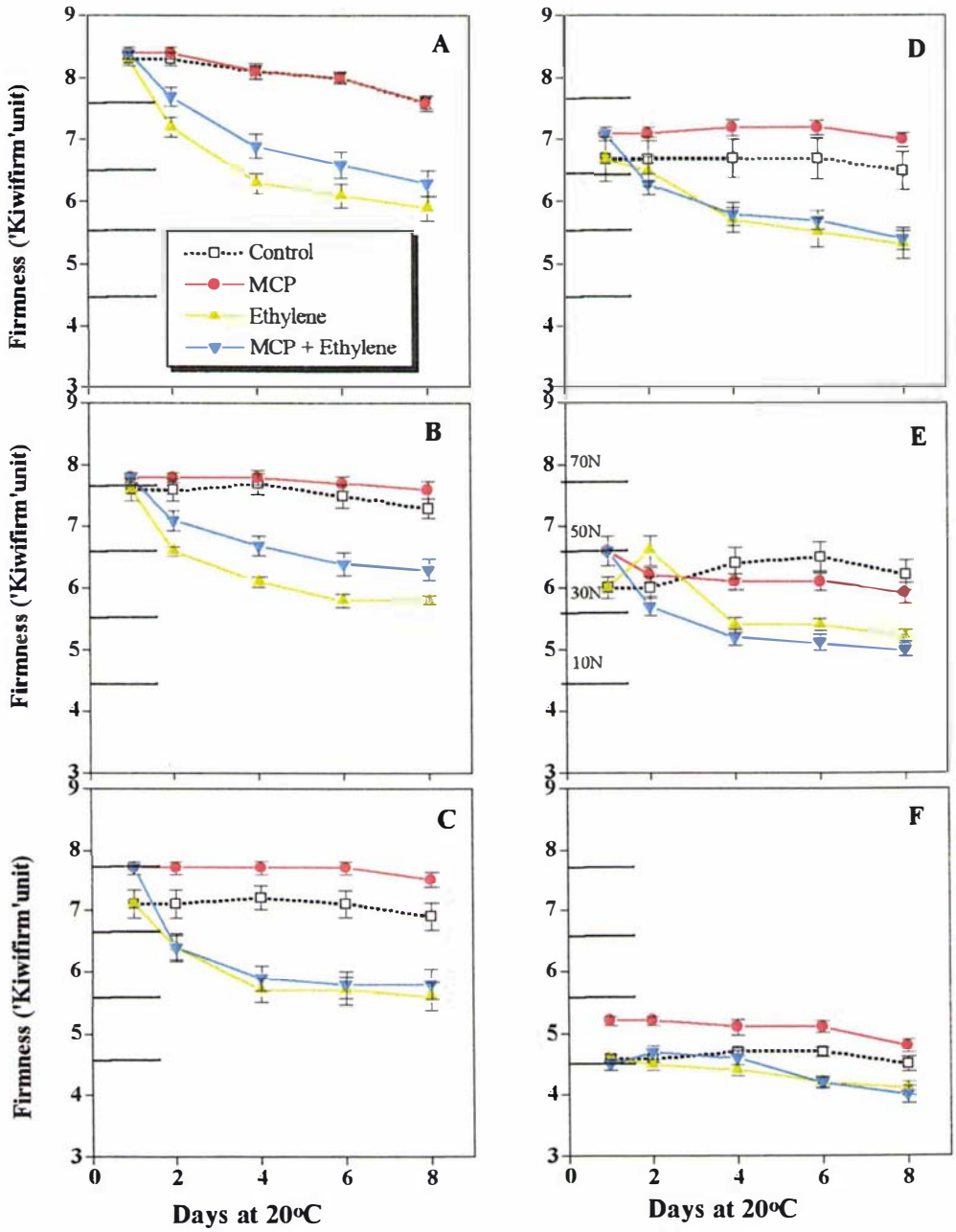

Fig. 6-8. Firmness of kiwifruit during 8 days at 20°C following treatment with 1-MCP (10µl/l) at harvest (6.6% TSS) and then treatment with ethylene (10µl/l) after 0 (A), 8 (B), 16 (C), 32 (D), 64 (E) and 128 (F) days at 0°C. Bars represent standard errors.

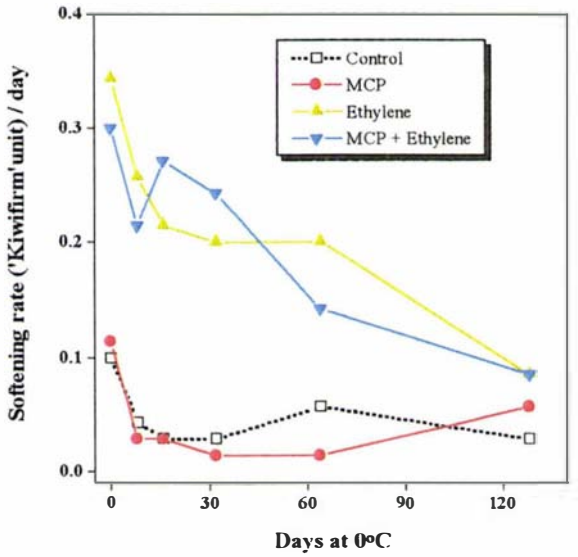

Fig. 6-9. Softening rate of kiwifruit during 8 days at 20°C following treatment with 1-MCP (10 µl/l) at harvest (6.6% TSS) and then treatment with ethylene (10µl/l) after 0, 8, 16, 32, 64 and 128 days at 0°C. The softening rate was obtained following the equation: (firmness at 8 days after ethylene treatment at 20°C - firmness after 1 day at 20°C) / 7.

days at 0°C. Firmness of control fruit and 1-MCP treated fruit were similar during 8 days at 20°C regardless of time at 0°C, although 1-MCP treated fruit were generally slightly firmer than controls; softening rate of fruit from control and 1-MCP treatments remained low between 0.014 to 0.1 'Kiwifirm'units / day (Fig. 6-11). Ethylene treatment induced rapid softening after treatment during 8 days at 20°C after storage at 0°C; ethylene treated fruit had the highest softening rate at more than 0.24 'Kiwifirm'units / day (Fig. 6-11). Firmness of fruit treated with 1-MCP + ethylene remained the same as control fruit during 8 days at 20°C; softening rate of fruit treated with 1-MCP + ethylene was the same as fruit from control and 1-MCP treatments (Fig. 6-11). In fruit treated with 1-MCP + ethylene at harvest and maintained at 20°C, firmness declined at a rate between that of control fruit and that of fruit treated with ethylene alone (Fig. 6-10A); the reason for this is not known, but it is possible that kiwifruit were stressed either because of harvest or as a result of treatment with ethylene and 1-MCP on the same day.

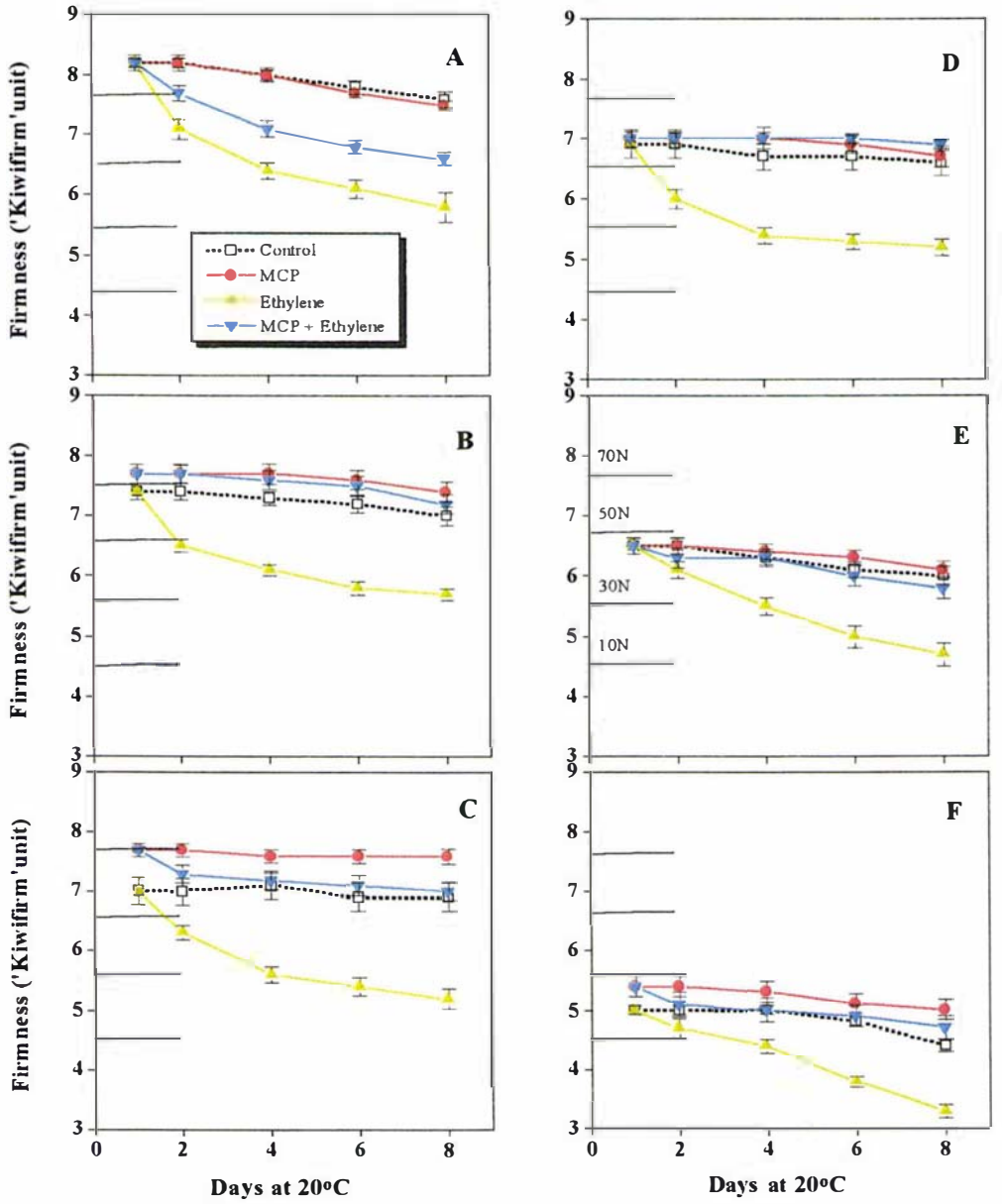

Fig. 6-10. Firmness of kiwifruit harvested at 6.6% TSS, during 8 days at 20°C treated with 1-MCP (10µl/l) and ethylene (10µl/l) after storage at 0°C for 0 (A), 8 (B), 16 (C), 32 (D), 64 (E) and 128 (F) days. Bars represent standard errors.

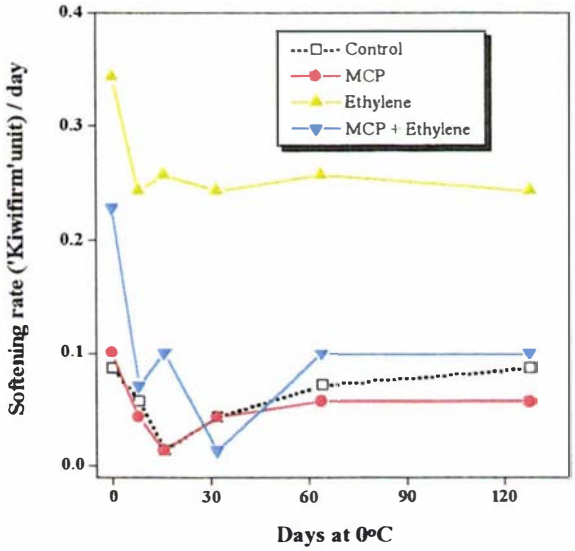

Fig. 6-11. Softening rate of kiwifruit harvested at 6.6% TSS during 8 days at 20°C treated with 1-MCP (10µl/l) and ethylene (10µl/l) after storage at 0°C for 0 , 8, 16, 32, 64 and 128 days. The softening rate was obtained following equation: (firmness at 8 days after ethylene treatment at 20°C - firmness after 1 day at 20°C) / 7.

6.4 DISCUSSION

Softening rate in kiwifruit is much increased in the presence of ethylene, even at 0°C; concentrations as low as 0.01 µl/l ethylene increase softening rate (Retamales and Campos, 1997) and thus reduce coolstorage potential considerably (Jeffery and Banks,1996). By plotting individual fruit data of ethylene production against firmness, it is clear that softening (from 118N to 19N) occurred in kiwifruit when ethylene production was very low (< 0.1 µl/kg/h), which is approximately equivalent to 1µl/l internal ethylene concentration (IEC) (Abeles *et al.*, 1992; Hyodo and Fukasawa, 1985); increased ethylene production did not occur until fruit reached a firmness of about 19N when maintained at 20°C (Fig. 6-3). It appears that the early phase of softening (118N to 19N) occurs during production of non-autocatalytic System 1 ethylene (below 0.1µl/kg/h), with the final more rapid phase of softening (<19N) coinciding with autocatalytic System 2 ethylene production. MacRae and Redgwell (1992) indicated that starch degradation by amylase and pectin solubilization by ß-galactosidase (ß-GAL)

and xyloglucan endotransglycosylase (XET) occurred during the early softening phase. This result indicates that System 1 ethylene alone is enough to induce or activate these enzymes resulting in early softening in kiwifruit. Similar results were observed by Ritenour *et al.* (1999) and Bonghi *et al.* (1996) where ethylene production in kiwifruit increased greatly only after fruit firmness dropped below about 7-10N; the ethylene climacteric was not related to the initial phase of kiwifruit softening (Tonutti *et al.*, 1993). Maximal cell wall swelling, depolymerization of the solubilized pectin by polygalacturonase (PG) and breakdown of the middle lamella occurred during the late softening phase (MacRae and Redgwell, 1992), which is associated with System 2 ethylene (Fig. 6-3).

Although most fruit contain low concentrations of ethylene during development and maturation, fruit only becomes responsive to ethylene from a certain stage of development (Knee, 1985). The rate of change of this responsiveness is probably influenced by endogenous or exogenous ethylene concentrations or by changing sensitivity of fruit tissues to ethylene (Yang, 1987). Ethylene perception by plant cells involves receptors and thus the nature and concentration of such ethylene receptors is crucial to the change in sensitivity (Trewavas, 1982; Yang, 1987); an increased number of ethylene receptors in the responsive cells may change sensitivity (Firn, 1986; Trewavas, 1982, 1983). In kiwifruit, it is possible that changing fruit sensitivity to the base level of ethylene, or System 1 ethylene production, occurs during early phases of fruit softening. Ethylene receptors may become more numerous during storage (Fig. 6-6, Fig. 6-8). Thus even though ethylene production remains low and constant, this increased sensitivity of fruit would allow ethylene to be bound to the receptors and initiate subsequent ripening related processes, one of which would be stimulation of cell wall dissolution and hence softening.

Early harvested kiwifruit were less responsive to exogenous ethylene than later harvested fruit (Fig. 6-1, Fig. 6-2); mature fruit (7.9% TSS at harvest) treated with 1µl/l ethylene softened faster to a lower firmness than early harvested fruit (4.7% and 5.3% TSS) during

10 days at 20°C. Fruit responsiveness to ethylene increased with advanced maturity, confirming results of MacRae *et al.* (1989) and Retamales and Campos (1997). Analysis of respiration rates, softening curves and ethylene production rates of kiwifruit indicates that kiwifruit responded like nonclimacteric fruit when exposed to different concentrations of ethylene (Biale and Young, 1981). Respiration rate was ethylene dependent, as was softening rate with the threshold concentration needed for these effects decreasing with increased maturity.

Kiwifruit sensitivity to ethylene was reduced by using an inhibitor of ethylene action; application of 1-MCP after harvest delayed onset of the ethylene induced climacteric and reduced respiration rate during ripening when compared to control kiwifruit (Fig. 6-4). This effect resulted in a slower fruit softening rate and firmer fruit during storage at 0°C and 20°C (Fig. 6-5, 6-6 and 6-7). Softening rate of kiwifruit decreased with increasing 1-MCP concentrations from 1 to 100µl/l (Fig. 6-5). This is consistent with results of Abdi *et al.* (1998) and Serek *et al.* (1995a, 1995b) who indicated that higher 1-MCP concentrations delayed ripening in plum and increased longevity in cut flowers more effectively than lower 1-MCP concentrations. Although 1-MCP concentrations required for inhibiting ethylene action in flowers such as cut carnations was low between 0.5 to 2.5 nl/l (Sisler *et al.*, 1996a), the concentration required to obtain meaningful responses was much higher (15 to 45 µl/l) for fruit such as banana and apple (Golding *et al.*, 1998; Song *et al.*, 1997); this may be due to easier uptake 1-MCP through the skin in flower than in fruit or the much higher surface: mass ratio that exists in flowers than fruit. These results suggest that fruit ripening in kiwifruit can be delayed by reducing responsiveness to ethylene (Sisler and Lallu, 1994; Song *et al.*, 1997).

Although 1-MCP blocked ethylene action in kiwifruit, this effect was reduced after storage at 0°C and 20°C as time between 1-MCP application and ethylene treatment increased (Fig. 6-4 , Fig. 6-5). When 1-MCP was applied to fruit at harvest, ethylene induced softening rate was reduced compared with ethylene alone before 8 and 4 days at 0°C and 20°C respectively (Fig. 6-6, 6-8, 6-9). However the ethylene induced softening

was not reduced by 1-MCP application at harvest when ethylene was applied after 16 and 8 days at 0°C and 20°C respectively (Fig. 6-6, 6-8, 6-9). This indicates that sensitivity of kiwifruit to ethylene changed with time at 0°C and 20°C, as fruit treated with 1-MCP before storage became increasingly sensitive to exogenously applied ethylene during storage (especially after 16 and 8 days at 0°C and 20°C respectively), resulting in an increasing softening rate (Fig. 6-6 and 6-8, 6-9). Gerasopoulos and Richardson (1997) showed that sensitivity of pear fruit to exogenous propylene (an analog of ethylene) increased progressively with storage time at -1°C.

Since 1-MCP binds irreversibly to the ethylene receptor sites (Serek *et al.*, 1994b) then the declining influence of 1-MCP to exogenous ethylene in kiwifruit is probably due to the synthesis of new ethylene receptors during maturation and ripening (Trewavas and Malho, 1997). It is suggested that at the time of 1-MCP application, all ethylene receptors were blocked with this compound, but that new receptors were created during storage, becoming increasingly available to bind ethylene, allowing the induction of subsequent metabolic processes that lead to known ethylene induced ripening responses such as softening. This hypothesis was confirmed by treating fruit with both 1-MCP and ethylene after storage at 0°C. This treatment reduced ethylene induced softening during ripening at 20°C (Fig. 6-10, Fig. 6-11). Treating fruit with 1-MCP after storage at 0°C, probably blocked active sites of any ethylene receptors that were formed during storage, thus preventing ethylene action and reducing softening rate during subsequent shelf life at 20°C.

Similar results were found by Sisler *et al.* (1996b) and Sisler and Serek (1997) in tomato and banana; 1-MCP treatment protected fruit against endogenous ethylene for 8 days or more in tomato and for 11-12 days in banana at 25°C after which ripening occurred at the same rate as controls. Thus, timing of application of 1-MCP will be an important factor in the magnitude of the effect of this chemical on delaying ethylene induced process such as delaying the onset of ripening; in banana and tomato fruit, Golding *et al.* (1998) and Nakatsuka *et al.* (1997) found that application of 1-MCP to preclimacteric

banana fruit or turning stage tomato fruit had a much greater effect than when applied to climacteric banana fruit or pink stage tomato fruit.

A number of techniques have been developed to protect sensitive commodities from the effects of ethylene. In the cut flower industry, many growers and retailers rely on silver thiosulfate solution, an inhibitor of ethylene action, to reduce ethylene-related problems. However, increasing concern over the long-term environmental effects of releasing heavy metals, such as silver, into soil or ground water have been raised (Nell, 1992). An environmentally safe alternative to STS is 1-MCP, a non-toxic organic compound that binds irreversibly to ethylene receptor sites and inhibits ethylene action. Therefore, 1-MCP may be a suitable compound to treat fruit and vegetables and thus represent a new way to extend the life of edible foods (Sisler *et al.*, 1996a). 1-MCP reduced softening in kiwifruit. Time of 1-MCP application may be crucial for reducing rate of softening as sensitivity to ethylene increased with advancing physiological age and during storage at 0°C and 20°C. The present results indicate that if 1-MCP is applied to kiwifruit after storage at 0°C for up to 4 months, softening rate will be reduced during shelf life at 20°C; the 1-MCP treated fruit maintained firmness above 9.8N which is the minimum firmness acceptable for export, even when ethylene was applied. However, further research is required to determine optimum concentrations and application times of 1-MCP to kiwifruit harvested at different maturities to minimize softening rate during storage at 0°C and subsequent shelf life.

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CHAPTER SEVEN

FINAL DISCUSSION

7.1 The role of ethylene in kiwifruit softening

Kiwifruit, a very important crop for export from New Zealand, can be stored for 4-6 months under recommended conditions of 0°C ± 0.5°C temperature, 98% RH and <0.03 μl/l ethylene around the fruit (McDonald, 1990). However, the NZ kiwifruit industry has lost, in past years, millions of dollars annually because of premature softening which can be responsible for 40-70% of fruit losses after harvest (Davie *et al.*, 1996). When whole fruit firmness is below 9.8N or when there are localised areas below 9.8N, such fruit is rejected at 'condition checking', the process used by industry to check on firmness and *Botrytis* infection and to remove soft or diseased fruit prior to export (Banks *et al.*, 1992).

Ethylene is a simple hydrocarbon gas produced by many ripening fruits including kiwifruit. Low concentrations of ethylene cause fruits to ripen, a property which is used commercially in the ripening of bananas, tomatoes, avocado and kiwifruit (Crisosto *et al.*, 1997a; Lallu *et al.*, 1989; Parmentier, 1998). Although kiwifruit at harvest produce negligible amounts of ethylene, the fruit is very sensitive to exogenous ethylene (Pratt and Reid, 1974; Retamales and Campos, 1997). Both the onset of ripening and the respiratory climacteric are advanced by treatment of climacteric fruit with ethylene (McGlasson *et al.*, 1978). Concentrations of ethylene as low as 0.01μl/l cause more rapid fruit softening than when ethylene is absent and storage potential can be substantially reduced even at such low concentrations (Jeffery and Banks, 1996; Retamales and Campos, 1997).

Fruit have been classified as climacteric or nonclimacteric depending on their respiratory behaviour and more recently their response to ethylene during ripening (Biale and Young, 1981; Yang and Hoffman, 1984). It is known that the onset of ripening in climacteric fruit is accompanied by a dramatic increase in ethylene production and an increase in respiration (Biale and Young, 1981). Two systems of ethylene production have been proposed in fruit (McMurchie *et al.*, 1972): System 1 is the low basal level of ethylene

present in preclimacteric fruit and nonclimacteric fruit where production rates vary between 0.01 to 1.7μl/kg/h, whereas System 2 ethylene production is the autocatalytic burst of production associated with ripening in climacteric fruit with rates varying greatly depending on the fruit (Table 7-1).

System 1 ethylene is physiologically active throughout the preclimacteric life of a fruit and is functional in determining when the climacteric will occur (Peacock, 1972). Most if not all ethylene produced by nonclimacteric fruit can be classified as System 1; nonclimacteric fruit possess System 1 but not System 2 ethylene production (McGlasson, 1985; Oetiker and Yang, 1995). Since the application of ACC to preclimacteric fruits such as apple results in only a slight increase in ethylene, fruit at the preclimacteric stage must lack the capability not only of synthesizing ACC but also of oxidizing ACC to ethylene (Yang and Oetiker, 1998). Kiwifruit kept at 0°C had low ethylene production (probably System 1) varying between 0.02 to 0.06μl/kg/h; this corresponds to an internal ethylene concentration (IEC) between 0.2 to 0.6μl/l (Abeles *et al.*, 1992; Hyodo and Fukasawa, 1985). Such kiwifruit had a low ACC concentration, varying between 0.15 and 0.5nmole/g and a low ACO activity, varying between 0.01 and 0.66nl/g/h (Fig. 3-5 and Fig. 3-8). It is assumed that the low ACC concentration and ACO activity did not limit System 1 ethylene production in kiwifruit. Preclimacteric kiwifruit at 20°C produced constant and low ethylene varying between 0.01 to 0.2 μl/kg/h, corresponding to IECs between 0.1 to 2μl/l (Abeles *et al.*, 1992; Hyodo and Fukasawa, 1985). These concentrations were present as fruit softened from 90N to 10~19N (Fig. 4-4 and Fig. 6-3) (Table 7-1).

Since ethylene is closely involved in kiwifruit softening (MacRae and Redgwell, 1992), this result suggests that such low concentrations of ethylene (System 1 ethylene) produced by preclimacteric kiwifruit are sufficient to induce cell wall hydrolase activity, resulting in softening of kiwifruit. Like preclimacteric kiwifruit, ethylene biosynthesis in nonclimacteric fruit decreases or remains at a constant low level during maturation and ripening (Chan *et al.*, 1998; Perkins-Veazie *et al.*, 1995) (Table 7-1). Although young strawberry fruit (green fruit) had the highest ethylene production rate (0.5μl/kg/h) and

ACO activity (2.01nl/g/h), more mature fruit (white, pink and red fruit) had lower ethylene production rates varying between 0.03 to 0.04µl/kg/h and ACO activity varying

Table 7-1 Ethylene production rates, ACC concentrations and ACO activities at different stages of climacteric and nonclimacteric fruit (from Biale and Young, 1981; Chan *et al.*, 1998; Hoffman and Yang, 1980; Knee, 1985; Perkins-Veazie *et al.*, 1995; Wang and Adams, 1982; Yang *et al.*, 1986).

	Rate of production					
	Preclimacteric			Climacteric		
	Ethylene (µl/kg/h)	ACC (nmole/g)	ACO (nl/g/h)	Ethylene (µl/kg/h)	ACC (nmole/g)	ACO (nl/g/h)
(Climacteric fruit)						
Kiwifruit	0.01-0.2	0.1-0.6	0.01-0.66	50	17	43
Apple	0.1	0.2	0.01	100	4	100
Tomato	0.2	0.1	-	120	10	-
Avocado	0.07-1.7	0.05	-	100-120	45	-
(Nonclimacteric fruit)						
Strawberry	0.03-0.5	1.19-1.95	0.72-2.01			
Lychee	0.1-0.9	-	-			
Cucumber	0.02-0.16	0.2-0.23	-			

- No data available

between 0.72 to 1.01nl/g/h. The low ethylene production in strawberry fruit was associated with low ACC concentration varying between 1.19 and 1.95nmole/g (Perkins-Veazie *et al.*, 1995). Although ACO activity increased in developing strawberry fruit when ACC was applied, no autocatalytic ethylene production occurred because ACS activity remained very low and/or was not detected (Perkins-Veazie *et al.*, 1995). In addition, strawberry fruit had a trend of increasing ACC levels during ripening, which is probably due to a loss of ACO activity (Perkins-Veazie *et al.*, 1995) or the inefficiency of transport between the sites of ACC formation and utilization (Brecht and Kader, 1984). Ripening processes in nonclimacteric fruit such as softening and colour change, occurred when ethylene production was low (Chan *et al.*, 1998; Perkins-Veazie *et al.*, 1995); ethylene production of lychee, a nonclimacteric fruit, declined as fruit ripened from green to red stage (Chan *et al.*, 1998). These results suggest that factors influencing softening in preclimacteric kiwifruit may be the same as those taking place in ripening of

nonclimacteric fruit, both of which occur while ethylene production is low. Such low concentrations of ethylene (System 1 ethylene) clearly play an important role in inducing some processes involved in ripening, such as softening, in preclimacteric kiwifruit and nonclimacteric fruit.

When ethylene is applied to mature, preclimacteric fruits such as apple, the ripening process is triggered and is accompanied by a massive synthesis of ethylene. This increase of ethylene production (System 2 ethylene) occurs following rapid enhancement of both ACC synthase and ACC oxidase activities (Liu *et al.*, 1985; Yang *et al.*, 1986; Yang and Oetiker, 1998). The System 2 is associated with increased ACO and ACS activity, and autocatalytic ethylene production, all accompanying the ripening process. It is known that System 2 ethylene production is involved with the induction or regulation of softening (Yang *et al.*, 1986); in tomato, the process of cell wall degradation was closely linked to the climacteric since it began as the fruit started to produce ethylene (Crookes and Grierson, 1983). However, in kiwifruit maintained at both 0°C or 20°C, softening begins before ethylene production shows any increase (Fig. 3-5, Fig. 4-4, 6-3). These results are similar to those found in several fruit such as peaches and kiwifruit, where softening occurred when ethylene production was relatively low (Ritenour *et al.*, 1999; Sfakiotakis *et al.*, 1989; Tonutti *et al.*, 1996). At 20°C, ethylene production remained below 0.1~0.2 μl/kg/h as firmness decreased from 90N to 10~19N, then increased markedly only when kiwifruit firmness decreased to below 10~19N. Both Ritenour *et al.* (1999) and Bonghi *et al.* (1996) showed that ethylene production in kiwifruit increased greatly only when fruit firmness dropped below about 7~10N. In peaches, the initial phase of softening (82N to about 55~50N) took place when ethylene production was below about 1μl/kg/h; the climacteric was a late event occurring when the fruit had softened to between 10 to 20N (Tonutti *et al.*, 1996). It is probable that the ethylene produced at this time is autocatalytic System 2 ethylene, as Sfakiotakis *et al.* (1989) have shown that autocatalytic ethylene production only commenced in kiwifruit when endogenous ethylene (IEC) reached about 0.4μl/l which is the almost same value found in this research. Therefore it is suggested that in some fruit including kiwifruit and peaches, System 2 ethylene only occurs following major changes in cell wall integrity presumably

through the action of hydrolases that sequentially attack components of the cell wall. Only when the fruit is soft (7~19N for kiwifruit, 10~20N for peaches) does ethylene production increase to high rates.

It is thought that low endogenous ethylene plays a role influencing the transition from the preclimacteric to climacteric stage in climacteric fruit (Peacock, 1972). It is known that storage in controlled atmospheres reduced ethylene production, which may be associated with either reduced IEC due to inhibition of ethylene synthesis, or decreased fruit sensitivity to ethylene, both of which would have prolonged the preclimacteric development phase of kiwifruit (Arpaia *et al.*, 1984, 1985, 1987). This suggested that regulation/manipulation of IEC by using an ethylene synthesis inhibitor such as aminoethoxyvinylglycine (AVG), which inhibits the conversion of SAM to ACC in ethylene biosynthesis, may slow down processes involved in kiwifruit ripening and in particular fruit softening. It was found that applied preharvest AVG delayed softening in pears and apples (Byers, 1997; Romani *et al.*, 1982; Williams, 1980) and reduced production of aroma volatiles in apple (Bangerth and Streif, 1987; Fan *et al.*, 1998). In kiwifruit, spraying 500ppm AVG onto vines 4 weeks before harvest did reduce ethylene production, undoubtedly due to both the reduced ACC concentration and ACO activity that occurred in this fruit (Fig. 4-1, 4-2); AVG treated fruit produced only 5.5% of the ethylene produced by control fruit after 3 weeks at 20°C (Fig. 4-1). This reduction in ACC indicates that AVG inhibited ACS activity in kiwifruit, a well recognized phenomenon in other fruit (Hyodo and Fukasawa, 1985; Yang, 1985). However in these experiments, AVG was shown also to reduce ACO activity to about 6% of that in control fruit at 20°C (Fig. 4-2). This is the first time that such an inhibitory effect of AVG on ACO activity has been reported. It is possible that lack of the substrate ACC led to a reduced ACO activity and hence low ethylene production. These results indicate that both ACS and ACO are key enzymes regulating ethylene biosynthesis in kiwifruit.

Although AVG reduced ACC concentration by inhibiting ACS activity, it did not inhibit ethylene completely. Low concentrations of ethylene were produced and softening continued through storage at 0°C and 20°C (Fig. 4-1, 4-2), suggesting that System 1

ethylene may be produced through a biosynthetic pathway not involving ACC. In lower plants (Osborne, 1989; Osborne *et al.*, 1996) and fungi (Hyodo and Uritani, 1984; Qadir, 1994; Qadir *et al.*, 1997), ACC was not an ethylene precursor. In some higher plants including Norway spruce, a terrestrial plant (*Nicotiana*) and an aquatic plant (*Spirodela*), exposed to stress conditions involving treatments with acid or copper (Chen and Wellburn, 1989; Mattoo *et al.*, 1992), ethylene was also produced without involving ACC. As System 1 ethylene appears to play an important role in kiwifruit softening (Fig. 3-10, 4-4, 6-3), it would be desirable to determine if System 1 ethylene is ACC dependent or not. If ACC is not a precursor for System 1 ethylene, then determination of the alternate ethylene biosynthesis pathway and the enzymes involved, may lead to the development of other inhibitors that when used together with AVG would inhibit ethylene production and hence softening completely.

The effectiveness of AVG varied depending on concentration and timing of application to kiwifruit vines (Tables 4-1 to 4-4). Application of 250ppm AVG had no effect on reducing ethylene production, respiration or softening either after harvest or after 2 months at 0°C. It is possible that 250ppm was not a high enough concentration to allow adequate penetration of the chemical to appropriate sites in the cell, and thus did not inhibit ACC synthase enough to affect ethylene production and consequent softening of kiwifruit. Application of 500ppm AVG, 2 weeks or 6 weeks before harvest had little or no effect on reducing ethylene production and delaying softening; the reason for this is not known, but it may be due to different rates of AVG metabolism at different stages of fruit development. Since there is no available information about AVG metabolism, further research in this area is needed.

Application of AVG (500ppm) to kiwifruit vines 4 weeks before harvest significantly inhibited ethylene production and delayed softening, resulting in an extended early phase of softening (phase 1 and 2) at 20°C with AVG treated fruit being 15N firmer than control fruit after 4 weeks at 20°C. However, the AVG effect disappeared after 2 weeks at 0°C, resulting in almost same firmness between AVG treated fruit and control fruit, which may be due to increased kiwifruit sensitivity to ethylene after 2 weeks at 0°C (Fig.

6-8, 6-9) even in the AVG treated fruit. Thus it is unlikely that AVG application will have any widespread commercial use because its effect in delaying softening is temporary.

It is known that ethylene induces kiwifruit softening at 0°C (Arpaia *et al.*, 1987; Retamales and Campos, 1997) and 20°C (Parmentier, 1998; Sfakiotakis *et al.*, 1989). Pectin solubilization and degradation in cell walls occurred when kiwifruit were treated with ethylene, suggesting a causative relationship between ethylene and kiwifruit softening (MacRae and Redgwell, 1992). The softening curve for kiwifruit at 0°C has three distinctive phases (Fig. 1-2) (MacRae *et al.*, 1990). Firmness in phase 1 falls relatively slowly, and is dependent partly on maturity at harvest (Lallu *et al.*, 1989; MacRae *et al.*, 1989). This is followed by a rapid phase of softening (phase 2) where the largest changes in fruit firmness take place (from 60-90N to about 20N). The final softening phase (phase 3) is a slow decrease in fruit firmness continuing from about 20N until fruit are overripe. There is some evidence that there is a final reduction in firmness (a phase 4 ?) at the very end of phase 3, when tissue disintegration is taking place and the fruit is no longer edible (MacRae *et al.*, 1989, 1990). Duration of the initial decrease in firmness in phase 1, the rate of softening in phase 2, and the point at which the rapid fall in phase 2 gives way to the last stage of gradual decrease in firmness, appear to differ depending on maturity at harvest, temperature, ethylene, storage atmosphere and season (Arpaia *et al.*, 1985; Lallu *et al.*, 1989; MacRae *et al.*, 1989; McDonald, 1990; Sfakiotakis *et al.*, 1989; Thomai and Sfakiotakis, 1997).

Softening of fruit during ripening has been associated with alterations in the middle lamella and cell wall structure, resulting in cell separation and softening of tissue. Such changes result from the activity of enzymes such as ß-galactosidase (ß-GAL), pectin methylesterase (PME), xyloglucan endotransglycosylase (XET) and polygalacturonase (PG). ß-GAL catalyzes the loss of galactose residues from the cell wall. PME is known to de-esterify pectic compounds of the cell wall. XET cleaves hydrogen bonded xyloglucan, resulting in a loosening of cellulose fibrils. Two types of PG have been identified (endo and exo); endo-PG randomly hydrolyzes glycosidic bonds while exo-PG

cleaves the terminal end of pectin molecules (Carpita and Gibeaut, 1993; Hallett *et al.*, 1992; Redgwell *et al.*, 1991; Salisbury and Ross, 1992).

The first decline of kiwifruit firmness in phase 1 is probably associated with starch hydrolysis by amylase; since starch grains form a physical barrier to a penetrometer, their disappearance probably results in some kiwifruit softening (Arpaia *et al.*, 1987; MacRae, 1988; MacRae *et al.*, 1989). The first change in cell walls measured during phase 1 and 2 was galactose loss from pectic side chains caused by ß-GAL (Redgwell *et al.*, 1990, 1992; Redgwell and Percy, 1992); 80% of galactose loss occurred as firmness decreased from 69N to 56N and the remaining 20% loss occurred between 56N and 34N (Redgwell and Percy, 1992). De-esterification of cell wall galacturonans by PME also occurred during this phase. Pectin solubilization was marked and the first signs of cell wall swelling was detected when fruit firmness was 56N, the first process being induced by ß-GAL and PME and the latter process by these enzymes as well as by XET (Percy *et al.*, 1996; Redgwell and Fry, 1993; Rose *et al.*, 1998; Ross *et al.*, 1993).

During phase 3 (and 4 ?) softening, PG activity increased significantly (Bonghi *et al.*, 1996; Tonutti *et al.*, 1994; Wang *et al.*, 1995). The PG is involved in depolymerising the solubilised pectin (MacRae and Redgwell, 1992). The activity of endo- and exo-PG generally increased during ripening, when pectic material in cell walls and the middle lamella was hydrolyzed. Although endo-PG and exo-PG was present at the preclimacteric stage (probably softening phases 1 and 2) of peach fruit in 2 molecular forms (pI 5.2 and 8.4) and a molecular form (pI 4.9) respectively, at the postclimacteric stage (probably softening phases 3 and 4 ?) the acidic forms (pI 5.2, pI 4.9) disappeared and activities were due exclusively to the pI 8.4 form and pI 8.8 form of endo-PG and exo-PG respectively (Bonghi *et al.*, 1994; Tonutti *et al.*, 1994); this suggests that different molecular forms of endo-PG or exo-PG become activated at different phase of kiwifruit softening. Depolymerisation continues as fruit become eating ripe (Redgwell *et al.*, 1992).

In this work, kiwifruit softening (from 90N to 10~19N) occurred during both phase 1 and 2 of the softening curve, when ethylene production was low (0.01 to 0.2 µl/kg/h) and constant (Fig. 4-4 and Fig. 6-3). It is proposed that System 1 ethylene is able to generate sufficient endogenous ethylene early in the ripening process to induce amylase enzymes and cell wall-degrading enzymes including ß-GAL, PME and XET. Increased ethylene production (System 2) only occurred as kiwifruit softened from 10N-19N to eating ripe (6-7N) (Fig. 4-4 and Fig. 6-3) during phases 3 (and 4 ?) of the softening curve (MacRae *et al.*, 1990). Maximal cell wall and middle lamella dissolution and increased plasticity of the cell wall occurred in this later stage of kiwifruit softening, being induced mainly by the ethylene-dependent cell wall degrading enzymes including PG (endo-) and galactanase (Bonghi *et al.*, 1994, 1996; Harker and Hallett, 1994; Pech *et al.*, 1998; Redgwell *et al.*, 1990, 1991, 1992). It is proposed that the major proportion of kiwifruit softening is not dependent on the ethylene climacteric as high ethylene production only occurs when cell wall softening is almost complete.

Trewavas (1982) suggested that it is sensitivity to growth substances, not concentration, that is the limiting factor in plant development. In kiwifruit responsiveness, estimated from the magnitude of the effect caused by ethylene, and sensitivity, determined as the concentration of ethylene needed to achieve 50% of maximum response (Knee *et al.*, 1985) of fruit to ethylene, increased with maturation (Fig. 6-1 and Fig. 6-2). It was found that softening occurred steadily in kiwifruit stored at 0°C, even though ethylene concentration was low. This could only occur if fruit became more sensitive to low concentrations of ethylene with time. When treated with 1µl/l ethylene after harvest, kiwifruit harvested at 7.9% TSS softened rapidly to 27N after 6 days at 20°C, while firmness of fruit harvested at 4.7% and 5.3% TSS remained at 98N and 75N respectively (Fig. 6-2). Similar results were observed in kiwifruit (Lallu *et al.*, 1989; Retamales and Campos, 1997), apple (Sfakiotakis and Dilley, 1973) and passion fruit (Shiomi *et al.*, 1996) where fruit of advanced maturity were more responsive to exogenous ethylene than less mature fruit. In pears, sensitivity to exogenous propylene increased progressively with time at -1°C (Gerasopoulos and Richardson, 1997); fruit held at -1°C softened faster to 10N than freshly harvested fruit when treated with 500µl/l propylene.

The response of kiwifruit varied depending on ethylene concentration (Fig. 6-1). Although 10 and 100µl/l ethylene induced the same response in stimulating respiration regardless of maturity at harvest, 1µl/l ethylene stimulated respiration rate only in fruit harvested at 7.9% TSS but not in less mature fruit harvested at 4.7% and 5.3% TSS. This suggests that the threshold ethylene concentration necessary for stimulating respiration rate is between 1 to 10µl/l for less mature kiwifruit (4.7% and 5.3% TSS), and decreases to between 0 to 1µl/l for more mature kiwifruit (7.9% TSS), indicating that more mature kiwifruit is more responsive at lower concentration of ethylene than less mature fruit.

Although the effectiveness of exogenous ethylene or propylene in stimulating fruit to produce ethylene is known to increase progressively during maturation in a number of fruits including apple (Sfakiotakis and Dilley, 1973) and passion fruit (Shiomi *et al.*, 1996), ethylene applied in concentrations of 1, 10 and 100µl/l did not induce early onset of ethylene production in kiwifruit harvested at 7.9% TSS within 8 days at 20°C even though respiration rate was increased with application of the ethylene (Fig. 6-1). In contrast, Sfakiotakis *et al.* (1997) found that autocatalytic ethylene in kiwifruit, was induced by 130 µl/l propylene (equivalent to 1 µl/l ethylene) after 5-6 days at 20°C. In addition, Sfakiotakis *et al.* (1997) exposed fruit to propylene continuously, whereas the present experiments used 16 hours exposure to ethylene and fruit were subsequently stored in the presence of $KMnO_4$ ethylene absorbents. This probably reduced progress of ethylene biosynthesis and hence delayed onset of autocatalytic ethylene production. It is possible this difference in response was due to differential sensitivity to ethylene or propylene between fruit grown at different areas; fruit grown in Greece may be more sensitive to ethylene or propylene than fruit grown in New Zealand.

The response to ethylene in kiwifruit may differ from that found in other traditional climacteric fruit, since when climacteric fruits are more mature, ethylene production is itself stimulated at the same time as respiration by ethylene treatment (Knee, 1985). Thus, the nature of ethylene response in kiwifruit indicates that this fruit behaves like a nonclimacteric fruit or preclimacteric fruit in that ethylene can trigger respiration rate increases but not ethylene in an autocatalytic manner (Oetiker and Yang, 1995).

By using an inhibitor of ethylene action, 1-MCP, and studying its interaction when applied with or without ethylene, it should be possible to determine the sensitivity of fruit to ethylene changes during maturation and storage. 1-MCP binds strongly to the ethylene receptor in plant tissues (Sisler and Serek, 1997). Application of this compound to bananas, apples and tomatoes delayed or inhibited ethylene production and/or ethylene induced ripening (Golding *et al.*, 1998; Serek *et al.*, 1995; Song *et al.*, 1997). In the present study, kiwifruit treated with 1-MCP immediately after harvest, had reduced ethylene production and respiration rates, resulting in slower fruit softening and firmer fruit than controls during storage at both 0°C and 20°C (Fig. 6-4, 6-5, 6-8 and Fig. 6-10). For example, rates of softening were 0.75N/day and 1.3N/day for 1-MCP treated fruit and control fruit respectively during storage at 0°C for 16 days. This 1-MCP effect was more marked at higher 1-MCP concentration (100µl/l) than at lower concentrations (1 and 10µl/l); rates of softening were 1.6N/day, 1.1N/day, 1.0N/day and 0.4N/day for fruit treated with 0, 1, 10 or 100µl/l 1-MCP respectively within 30 days at 20°C (Fig. 6-5). This indicated that softening in kiwifruit can be delayed by application of 1-MCP immediately after harvest, probably as a result of ethylene receptors becoming blocked.

Although 1-MCP (10µl/l) application at harvest reduced ethylene (10µl/l) induced softening rate at 20°C compared with softening caused by ethylene alone after 8 and 4 days at 0°C and 20°C respectively (Fig. 6-6, 6-8, 6-9 and Table 6-1), this 1-MCP effect had disappeared after 16 and 8 days at 0°C and 20°C respectively (Fig. 6-6, 6-8, 6-9 and Table 6-1). For example, fruit treated with both 1-MCP (10µl/l) and ethylene (10µl/l) at harvest and then stored at 0°C for 8 days, was 10N firmer than fruit exposed to ethylene alone after 2 days at 20°C, but after 16 days at 0°C fruit from both these treatments had the same firmness (Fig. 6-8). Similar results were found in tomato and banana (Sisler *et al.*, 1996; Sisler and Serek, 1997) where application of 1-MCP delayed ethylene-induced ripening rate of these fruit for 8~12 days at 25°C, but after this time rate of fruit ripening was the same as controls. Golding *et al.*, (1998) found that application of 45µl/l 1-MCP at a preclimacteric stage in banana fruit delayed onset of the ethylene and respiratory climacterics, but when applied after autocatalytic ethylene had commenced it had no inhibiting effect on these processes. These results suggest that 1-MCP binds irreversibly

to the ethylene receptor in kiwifruit as it appears to do in other fruit (Serek *et al.*, 1994). Application of 1-MCP at harvest would be expected to block any available ethylene receptors. If new receptors were formed during kiwifruit storage they would become increasingly available to bind ethylene, resulting in increasing ability of the fruit to induce subsequent ethylene-dependent metabolic processes including softening. Therefore, these results suggest that apparent decreasing effectiveness of 1-MCP applied at harvest in reducing the ability of exogenous ethylene to induce softening as time in storage increased, may be due to the synthesis of new ethylene receptors. It is possible that the increasing sensitivity of kiwifruit to ethylene during storage developed because ethylene receptors became more numerous. This may explain why kiwifruit softening occurs gradually at 0°C without any apparent change in ACC concentration, ACO activity and ethylene production. The low constant ethylene concentration is able to bind to new receptors as they are synthesized, or become available, and consequently are able to induce ethylene-dependent processes such as softening. For 1-MCP to be of potential commercial use, it may be necessary to make repeated applications of 1-MCP or use higher concentrations to block ethylene receptors being formed during storage and maturation.

The ethylene receptor (s) is known to be encoded by a multigene family in *Arabidopsis* (Hua *et al.*, 1995; Sakai *et al.*, 1998), in muskmelon (Sato-Nara *et al.*, 1999) and in tomato (Paynton *et al.*, 1996; Zhou *et al.*, 1996). The presence of different forms of such receptors have been used to explain changes in ethylene sensitivity of tissue (Barends and Peeters, 1995). For example, the tomato ETR gene family, including LeETR1, LeETR2 and NR, are differentially regulated throughout plant development. LeETR1 was expressed constitutively in all tissues during a range of processes including seed germination, shoot elongation, leaf and flower senescence, floral abscission, fruit set and fruit ripening, while NR expression was developmentally regulated in floral ovaries and ripening fruit, and LeETR2 expression was expressed at low levels throughout the plant but was induced in imbibing tomato seeds prior to germination (Lashbrook *et al.*, 1998). In kiwifruit, cDNA of 5 genes were differentially expressed during fruit development (Ledger and Gardner, 1994), but it is not known whether they are related to ethylene

receptors or not. pKIWI503 had reduced expression in young fruit but was highly expressed during fruit ripening, while the other four genes (pKIWI501, 502, 504, 505) were highly expressed in young fruit but had reduced expression in the later stages of fruit development. Although no ethylene receptors have been identified from kiwifruit, it is possible that like tomato, kiwifruit has several receptors which are regulated differentially for System 1 ethylene and System 2 ethylene.

If the number of ethylene receptors or formation rate of ethylene receptors vary among cultivars, different cultivars of kiwifruit may have differential sensitivity to ethylene and thus soften at different rates. Although Hayward is the predominant kiwifruit cultivar grown commercially throughout the world, several other cultivars of kiwifruit have been cultivated in New Zealand. Cultivars such as Abbott, Bruno and Monty are known to produce more ethylene and have higher respiration rates, soften faster and hence have a shorter storage life than Hayward (Cotter *et al.*, 1991; Manolopoulou and Papadopoulou, 1997; Papadopoulou and Manolopoulou, 1997). Since Hayward produces less ethylene than other cultivars so far examined, it is possible that such less ethylene production in Hayward could be a result of a slower rate of formation of new receptors, lower sensitivity to ethylene, and hence reduced ethylene-induced softening. If this is so then cultivars that show fast softening, may be more sensitive to ethylene (forming new receptors at a faster rate) than Hayward and possibly could be induced to soften at even lower endogenous ethylene concentrations.

7.2 Stress ethylene in kiwifruit

Stress ethylene is one of the general phenomena observed in plant tissues subjected to various unfavorable conditions (Hyodo, 1991). Ethylene production and ACC content are low before stress treatment but rapidly increase following stress. Conversion of SAM to ACC is a key reaction controlling the production of stress ethylene (Abeles *et al.*, 1992). Members of the ACS gene family are differentially expressed under different developmental or stress conditions (Fluhr and Mattoo, 1996); in tomato, among 9 ACS genes, LE-ACS2 and LE-ACS4 are induced after wounding. In kiwifruit two ACS genes including KWACC1 and KWACC2 were found (Ikoma *et al.*, 1995, 1999) and the

transcription of KWACC1 was induced by wounding stress. In the present study, two different stress conditions were imposed on kiwifruit and subsequent ethylene biosynthesis by fruit were determined. One stress condition was low temperature and the other was *B. cinerea* infection.

Low temperatures induce chilling stress in cucumber (Wang and Adams, 1980, 1982) and lychee (Chan *et al.*, 1998) with enhanced ethylene production occurring in chilled fruit upon transfer to warmer temperatures. This increased ethylene is associated with tissue injury such as pitting and water soaking. In contrast, the enhanced ethylene production that occurs when apples and pears are transferred to warmer temperatures after low temperature storage takes place without development of chilling injury symptoms (Blankenship and Richardson, 1985; Jobling *et al.*, 1991). Such cold storage is used to synchronize ethylene production and hence ripening in several fruits such as apples, pears and kiwifruit (Hyodo and Fukasawa, 1985; Pech *et al.*, 1994; Puig *et al.*, 1996; Parmentier, 1998) where a lack of uniformity of ripening can cause problems for the industry. The stimulation of ethylene production in pears is due to increased availability of ACC resulting from a cold-stimulation of ACS (Blankenship and Richardson, 1985). In kiwifruit ACC concentration and ACO activity remained low throughout storage at 0°C, resulting in low ethylene production varying between 0.02 to 0.06 µl/kg/h (Fig. 3-5, Fig. 3-8) probably System 1 ethylene. On transfer to 20°C after 52 days at 0°C both ACC concentration and ACO activity were stimulated, resulting in high ethylene production which is probably System 2 ethylene (Fig. 3-7, Fig. 3-9). In preclimacteric Granny Smith apples, ACO activity was stimulated during cold treatment, increasing before transfer of fruit to a warmer temperature (Jobling *et al.*, 1991; Larrigaudiere and Vendrell, 1993). The cold treatment was responsible for the induction of both ACS and ACO activity in Granny Smith apples with ACO activity being stimulated earlier than ACS (Jobling *et al.*, 1991; Larrigaudiere and Vendrell, 1993; Lelievre *et al.*, 1995). Although ACO activity and ACC concentration were low for up to 180 days at 0°C, and kiwifruit produced only small amounts of ethylene during this time (Fig. 3-5 and Fig. 3-8), when transferred to 20°C, a high rate of ethylene production occurred accompanying the increased ACC concentration and ACO activity (Fig. 3-7 and Fig. 3-9). This indicates that high

temperatures are required for an increase of ACO activity, ACC concentration and hence ethylene production to occur in kiwifruit. Sfakiotakis *et al.* (1997) have indicated that low temperatures (< 11~14.8°C) inhibited the switch from System 1 to System 2 ethylene probably because of its inhibitory effect on ACS, that in turn limited ACC production, while high temperatures (14.8~34°C) allowed autocatalytic System 2 ethylene production to proceed (Niklis *et al.*, 1993; Sfakiotakis *et al.*, 1997). Together these results support the contention that in kiwifruit System 1 can operate during storage at low temperature, but high temperatures are required for the induction and operation of System 2.

Another stress studied was that induced by *B. cinerea*, a major postharvest pathogen that has the potential to cause significant losses in kiwifruit during storage at 0°C (Manning and Pak, 1993). Kiwifruit infected with *B. cinerea* soften very quickly and hence have a reduced storage life (Niklis *et al.*, 1997). In addition, infected fruit produce ethylene which hastens softening of other non-infected fruit in the same tray (Niklis *et al.*, 1997). Kiwifruit infected with *B. cinerea* produced much more ethylene than did noninfected fruit (Fig. 5-1). Ethylene production varied in different zones along the length of infected fruit. Little detectable ethylene production, and low ACO activity, were found in infected tissue, indicating that this infected zone tissue was dead, with cells presumably having lost membrane integrity. Ethylene production and ACO activity were enhanced at, and immediately ahead of the infection front. The reason for the increase in ethylene production in tissue ahead of the infection front is not known, but may be due to a 'signal' emanating from hyphae growing in the infection zone (Aldington and Fry, 1993; Fry *et al.*, 1993). It is known that *B. cinerea* produces a range of hydrolytic enzymes within infected tissue, including PG, PME, cellulase and rhamnogalacturonase (Gross *et al.*, 1995; Heiler *et al.*, 1993; Marcus and Schejter, 1983; Tobias *et al.*, 1993). Such cell wall degrading enzymes, or their products, are known to stimulate ethylene biosynthesis (Aldington and Fry, 1993; Campbell and Labavitch, 1991a, 1991b; Fry *et al.*, 1993; Gross *et al.*, 1995); cell wall degrading enzymes break cell wall polysaccharides into short chain oligomers during maturation and ripening (Aldington and Fry, 1993). A few select oligomers are known to exert "signalling" effects including wound signal activity

and induction of ethylene synthesis, on plant tissues at very low concentrations (Aldington and Fry, 1993; Fry *et al.*, 1993; Melotto and Labavitch, 1994). After partial digestion with cellulase (ß-(1->4)-D-glucanase) specific oligomers from xyloglucan, which is composed of the sugar residues D-glucose, D-xylose, D-galactose, L-fucose and L-arabinose, can act as ethylene elicitors (Basse *et al.*, 1992; Fry *et al.*, 1993). In addition, pectic oligomers elicit ethylene production (Baldwin and Pressey, 1988; Brecht and Huber, 1988; Campbell and Labavitch, 1991a, 1991b). Pectin oligomers (homooligomers of α-1,4-D-galacturonic acid) released from the cell wall during fruit ripening increased climacteric ethylene biosynthesis and tissue reddening in whole tomato fruit and tomato pericarp discs (Brecht and Huber, 1988; Campbell and Labavitch, 1991a, 1991b). If such compounds exist in *B. cinerea* infected kiwifruit, then it is possible that they might move distal to the infection zone and induce enhanced ethylene production in non-infected tissue. In addition, it is possible that if such oligomers are produced from pectin and xyloglucan during kiwifruit softening and they act as ethylene elicitors, then they may be responsible at least in part for inducing enough ethylene to regulate phase 1 and 2 of kiwifruit softening, and that other pectin oligomers, formed following activity of PG, may elicit ethylene production in phases 3 and 4 (?) of kiwifruit softening. Further research is clearly necessary to test these hypotheses.

It is known that exogenous ethylene accelerates cell wall breakdown (MacRae and Redgwell, 1992) and this probably facilitates infection by *B. cinerea* (Kepczynska, 1993; Menniti and Maccaferri, 1998). An inhibitor of ethylene synthesis, AVG, reduced ethylene production and delayed softening in apple and pears (Halder-Doll and Bangerth, 1987; Romani *et al.*, 1982; Williams, 1980). When applied 4 weeks before harvest AVG (500ppm) reduced ethylene production induced by *B. cinerea* in kiwifruit (Fig. 5-1); this was associated with reduced ACC concentration and ACO activity. However, this effect of AVG on reducing ethylene biosynthesis in infected fruit was relatively small after 4 weeks at 0°C; AVG reduced ethylene production induced by *B. cinerea* by 80% and 21% at harvest and after 4 weeks at 0°C respectively (Fig. 5-1 and Table 5-1). Regardless of whether AVG was applied before or immediately after harvest, *B. cinerea* infected fruit produced sufficient ethylene to induce cell wall breakdown during storage at 0°C,

overwhelming any AVG effect on reducing infection rate (Table 5-2 and Fig. 5-2). Because of the relatively minor effects of AVG on ethylene production in storage and lack of any inhibitory effect on infection, it is unlikely that this compound will have any commercial application in kiwifruit for reducing *B. cinerea* infection.

7.3 A possible model for ethylene effects in kiwifruit softening

Ethylene is widely thought to have a coordinating role in regulating ripening of fruit (Oetiker and Yang, 1995; Theologis, 1993, 1994; Yang and Oetiker, 1998). Competence to ripen is thought to involve the gradual development of the fruits ability to synthesize ethylene through the increased production of the enzymes ACS and ACO and/or the increased availability of ethylene receptors (Oetiker and Yang, 1995; Pech *et al.*, 1994). Since different forms of the receptors are regulated differentially throughout plant development (Lashbrook *et al.*, 1998), it is possible that System 2 receptors are different from System 1 receptors. Oetiker and Yang (1995) have proposed that in preclimacteric fruit, only System 1 ethylene is produced; in conjunction with the preexisiting System 1 receptor, it plays an essential role by destroying the "ripening inhibitor". As the "ripening inhibitor" is inactivated, System 2 receptors develop or becomes functional in mature fruits. An ethylene-System 2 receptor complex induces the development of System 2 ACS, resulting in production of System 2 ethylene that in turn enhances the synthesis of ripening-associated enzymes involved in cell wall breakdown and autocatalytic ethylene biosynthesis (Oetiker and Yang, 1995; Pech *et al.*, 1994). To date such a 'ripening inhibitor' has not been isolated and/or identified from any fruit. It is possible that events that appear to be explained by such a concept may simply occur because new ethylene receptors are formed during maturation and ripening or following the trauma associated with infection or harvest.

A model for ethylene action during ripening of kiwifruit can be suggested based upon the preceding results and discussion (Fig. 7-1). During the early phase (phase 1 and 2) of softening of preclimacteric kiwifruit, starch degradation and cell wall solubilization occur as a result of the activity of many hydrolytic enzymes, including amylase, PME, ß-GAL and XET that are induced in kiwifruit during these initial softening phases (MacRae and

Redgwell, 1992). Oligomers derived from this initial cell wall breakdown may act as ethylene elicitors inducing low concentrations of ethylene (System 1 ethylene), being associated with low ACS and ACO activity. To date, such oligomer elicitors have not been shown to exist in kiwifruit. This System 1 ethylene may bind to ethylene receptors (probably System 1 ethylene receptors). Low temperatures (<14.8°C) allows production of System 1 ethylene during these early softening phases. Application of saturating concentrations of AVG and 1-MCP, inhibitors of ethylene production and ethylene action respectively, reduced ethylene biosynthesis and presumably blocked ethylene receptors, thus preventing subsequent steps of ethylene action and hence delaying softening. Kiwifruit soften to 10N~19N continuously because of increased fruit sensitivity to System 1 ethylene. This increased sensitivity is due to new ethylene receptors being formed (Fig. 6-8 and Fig. 6-9). As kiwifruit soften to below 10N~19N (probably phase 3 of softening), oligomers are formed following depolymerisation of both xyloglucans and pectins; these may act as ethylene elicitors somehow inducing autocatalytic System 2 ethylene. However, it is not known if oligomers derived from different polysaccharides act as different ethylene elicitors for ethylene derived through System 1 or System 2. This autocatalytic ethylene is associated with high ACS and ACO activity and a climacteric respiratory rise. In this period, high temperatures (14.8~34°C) increase ACS and ACO activity, and fruit sensitivity to System 2 ethylene. The System 2 ethylene binds to System 2 ethylene receptors, which then initiate ethylene dependent physiological responses including endo-PG activation that leads to depolymerization of the solubilized pectin, maximal cell wall swelling, breakdown of the middle lamella and final softening. The result is ready-to-eat fruit with a firmness of 6~8N.

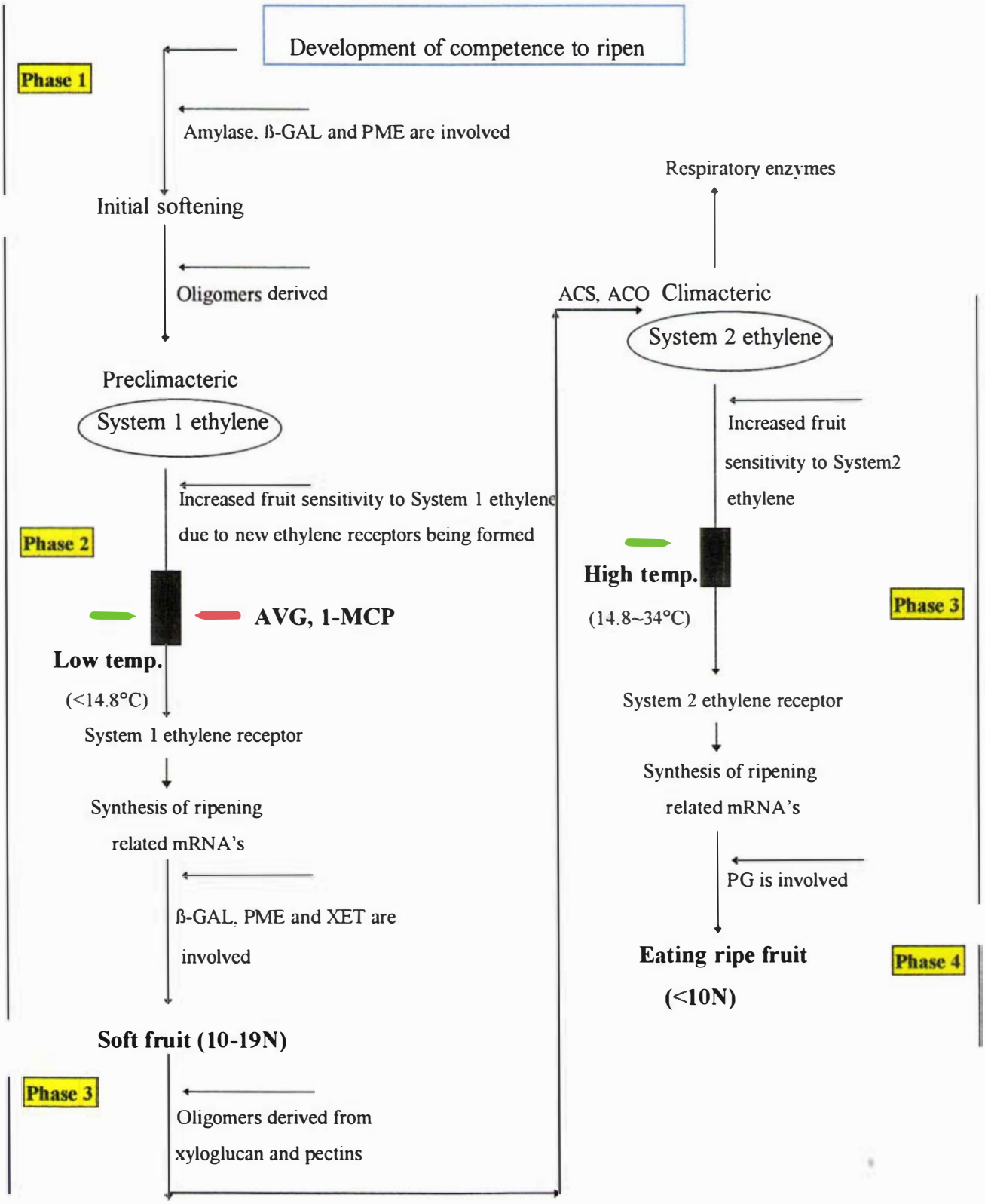

Fig. 7-1. A possible model for ethylene effects on softening of kiwifruit during storage and ripening. PME: pectin methylesterase; XET: xyloglucan endotransglycosylase; PG: polygalacturonase. Stimulation of the reaction; inhibition of the reaction.

7.4 Large variance between individual kiwifruit

Even for kiwifruit harvested at the same time, the time required for individual fruit to reach their respiratory and ethylene climacteric peaks at 20°C varies by as much as several months and it is not predictable (Pratt and Reid, 1974); in California only 13 to 50% of individual fruit began their climacteric rise during a 10 day ripening period at 20°C (Ritenour *et al.*, 1999). In the present experiments, individual kiwifruit harvested at the same date showed different patterns of softening, ethylene production, ACC concentration and ACO activity within a range of treatments including low temperature exposure, AVG application and *B. cinerea* inoculation. For example, only 11% of fruit stored at 0°C for 64 days began to produce ethylene at rates greater than 1µl/kg/h after 9 days at 20°C. Such large variation within a replicated experiment prevented significant differences being found between treatments for several experiments. This inherent physiological variation between fruit at harvest created considerable difficulties in obtaining meaningful statistical results from several experiments.

It is known that variation between individual fruit arises in part from the different physiological age at harvest. In apples, considerable variation among replicate samples was found in ACC concentration, ACO activity and ethylene production rates in fruit harvested at the same time (Yang *et al.*, 1986). Such variation may be due to different physiological ages of fruit. There is variation of kiwifruit quality in relation to position of fruit on the vine; size, firmness, soluble solids, disease incidence and taste might be affected by such positional effects (Hopkirk *et al.*, 1986; Pyke *et al.*, 1996; Smith *et al.*, 1994, 1997). Large variations in total soluble solids concentration (TSS) occurred in fruit harvested from different positions on kiwifruit vines (Hopkirk *et al.*, 1986; Pyke *et al.*, 1996); TSS tended to be higher in fruit from the ends of leaders than in fruit from the centre of the vine, and fruit near the leader tended to have higher TSS than fruit from the ends of canes. This variation in total soluble solids concentration was responsible for 29-46% of variance in postharvest attributes of the kiwifruit (Smith *et al.*, 1994). In addition, fruit size affected rate of softening in kiwifruit (Crisosto *et al.*, 1997b) and onset of softening in grapes (Trought and Tannock, 1997); large kiwifruit (about 101g) had a slower rate of softening than medium (about 93g) and small fruit (about 81g). In

Cabernet Sauvignon grapes, the large berries generally took less time to reach the onset of softening than the small berries (Trought and Tannock, 1997).

It is likely that at least some of the variation that occurred in fruit used in the present experiments was because experimental fruit was obtained from 'once over harvests' (as used in commercial orchards); limitations in fruit supply did not allow fruit to be taken from specific positions on the vine or to use exactly the same size of fruit in each treatment. The mean TSS of fruit harvested in the current experiments was 6.7% but ranged from 5.7 to 8% TSS and mean fruit weight was 100g ranging from 82g to 120g; this resulted in a large variation between individual fruit because of different physiological ages (Trought and Tannock, 1997; Yang *et al.*, 1986). Thus, it is important that fruit of the same physiological age are selected and harvested for critical postharvest physiological experiments; to know fruit age, newly opened flowers need to be tagged.

Another way to minimise variation in future studies is to use more replicates within treatments. Based on variance calculated from some experiments reported in this thesis, it has been estimated that a minimum of 280 single fruit replicates would be required to obtain significance for an ethylene difference of 0.1 µl/kg/h between treatments in a experiment investigating low temperature effects on ethylene biosynthesis. It would be physically impossible, and hence impractical, to undertake all the measurements required of 280 fruit which must be at similar developmental stages during or after any treatments. Thus other ways must be developed in order to minimize and/or cope with such fruit to fruit variation and yet achieve meaningful results. An alternative way to present data that has a wide variation between individual fruits is to express results as a proportion (%) of fruit that exceeds or is less than a certain threshold value of a particular variable. For instance data was expressed as the proportion (%) of fruit that produced ethylene production, ACC concentration and ACO activity above 0.1 µl/kg/h, 3nmole/g and 1nl/g/h respectively. This data expression can show the trend of response when kiwifruit is exposed to different treatments. However, data expressed in such a way needs to be evaluated carefully, as problems exist in analysing those data using standard statistical techniques.

7.5 Final conclusion

The main focus of this study was to attempt to elucidate the role of ethylene in initiating kiwifruit softening. It has been found that kiwifruit softening from 90N to 10~19N occurs at both 0°C and 20°C when ethylene production is low and constant, and is probably System 1 ethylene; in this time, kiwifruit behave like nonclimacteric fruit. It is possible that this ethylene may bind to the System 1 ethylene receptor. Application of an inhibitor of ethylene synthesis, AVG, did inhibit ethylene production and hence delay softening at 20°C, but this effect was minor and did not persist for more than 14 days. Storage of kiwifruit at 0°C appeared to increase fruit sensitivity to System 1 ethylene, which may have reduced the inhibitory effect of AVG on ethylene production. This increased sensitivity was probably due to new ethylene receptors being formed during storage at 0°C. Application of the ethylene action inhibitor, 1-MCP to kiwifruit, probably prevented ethylene binding to receptors making fruit less responsive to System 1 ethylene, leading to a consequent reduction in the rate of early phase softening (phase 1 and 2). When fruit softened to about 10N~19N, autocatalytic System 2 ethylene production occurred; at this time, kiwifruit behave like climacteric fruit. In this late phase of softening (phase 3 and 4 ?), it is System 2 ethylene that induced physiological processes causing final softening resulting in eating ripe fruit.

7.6 Practical implications and future direction

7.6.1 Practical implications

1-MCP, an inhibitor of ethylene action, binds strongly to the ethylene receptor and thus reduces ethylene induced softening. The 1-MCP treated fruit remained about 13N firmer than untreated fruit throughout storage at 0°C for 4 months. If such a firmness difference (13N) could be attained consistently, such 1-MCP treatment could extend storage life at 0°C by up to 27 days (from 128 to 155 days) when using minimum firmness acceptable for export (9.8N) as a commercially realistic endpoint (Fig. 7-2).

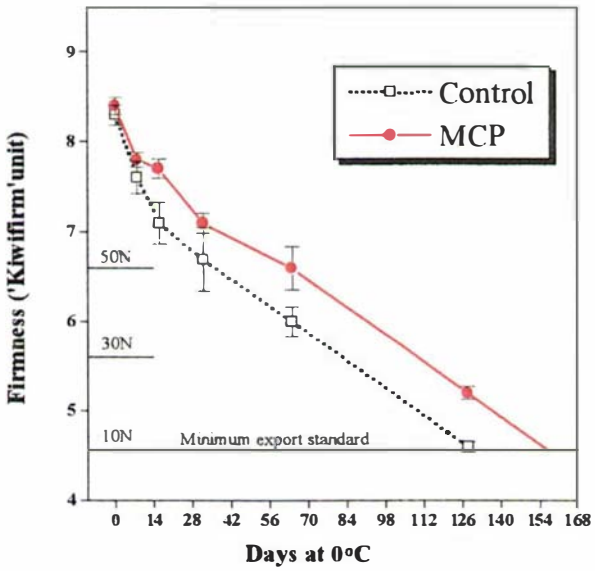

Fig. 7-2. Firmness of kiwifruit treated with 1-MCP (10µl/l) at harvest (6.6% TSS) then maintained at 0°C for 128 days, after which days at 0°C to reach to 10N was estimated using the following equation: Firmness ('Kiwifirm' unit) = -0.0223 * (days at 0°C) + 8.0459 (R^2=0.99).

It appears that kiwifruit develop new ethylene receptors during storage at 0°C. Thus, it is possible that repeated applications of 1-MCP at approximately 2 weekly intervals during storage at 0°C could reduce fruit softening rate especially in environments contaminated with ethylene. However, further research is required to determine the effectiveness of 1-MCP application rates at 0°C and other temperatures, as results to date have been achieved at 20°C. 1-MCP is a non-toxic gas, which should facilitate multiple application and/or distribution in gas tight coolstores such as those used for CA storage.

To date 1-MCP has been cleared for use on flowers and ornamental plants, but it has not yet been approved for use on food products. The inhibitory effects of 1-MCP on ethylene production and fruit softening indicate that it may have commercial potential. There is no doubt that 1-MCP will play an important role in further research to elucidate the effect of ethylene as a means to manipulate softening and other ethylene dependent processes occurring during maturation and ripening of kiwifruit and other horticultural crops.

Storage of kiwifruit at 0°C for more than 52 days enhanced ethylene production and accelerated softening when fruit were subsequently transferred to 20°C. This chilling of kiwifruit also unified ethylene induction and thus improved uniformity of firmness. For example more than 60% of fruit stored at 0°C for more than 52 days produced >0.1~0.2µl/kg/h of ethylene after 10 days at 20°C, but in fruit stored for less than 52 days at 0°C only 7% of fruit produced >0.1~0.2µl/kg/h of ethylene. It is known that ethylene can be used to synchronise ripening kiwifruit (Lallu *et al.*, 1989; Parmentier, 1998). Thus storing kiwifruit at 0°C for more than 52 days is likely to be necessary to achieve complete unification of ripening.

An inhibitor of ethylene biosynthesis, AVG, inhibited ethylene production and delayed softening in kiwifruit; fruit treated with 500ppm AVG, 4 weeks before harvest, had significantly less ethylene production, a slower softening rate and firmer fruit after harvest than control fruit. The AVG treated fruit was 9N firmer than untreated fruit after 14 days at 0°C. However, this effect was transitory; when fruit were stored at 0°C for more than 14 days at 0°C, this difference in firmness between AVG treated fruit and control fruit disappeared. Thus AVG application would appear to have little commercial potential in maintaining firmness for long periods in which if any kiwifruit is stored at 0°C for up to 6 months.

7.6.2 Future direction

The inhibitor of ethylene action, 1-MCP, delayed ethylene-induced ripening and senescence in cut flowers and fruit. However, each crop including banana, plum, tomato and cut flowers has used 1-MCP at different concentrations and application times to block ethylene actions. It is known that kiwifruit treated with 1-MCP softened more slowly than control fruit during storage. Since softening rate of kiwifruit varies depending on maturity at harvest, it will be is necessary to determine optimum concentration and application times of 1-MCP to fruit harvested at different maturities in order to optimize conditions for minimizing fruit softening at 0°C and at 20°C. Future research with 1-MCP should include assessing: (a) the effect of multiple applications of 1-MCP; ethylene receptors of kiwifruit increase with time at 0°C and 20°C and thus one time application of

1-MCP will not be enough to block ethylene receptors; (b) effectiveness of 1-MCP when applied at 0°C or at other temperatures < 20°C; it is known that 1-MCP application to kiwifruit at 20°C reduced ethylene-induced softening because it blocked ethylene receptors but it is not known whether 1-MCP application to kiwifruit at 0°C will reduce ethylene-induced softening as much as at 20°C.

It has been suggested that kiwifruit sensitivity to ethylene increased with time because of the continuous formation of ethylene receptors. A multigene family of ethylene receptors is encoded in *Arabidopsis* (Hua *et al.*, 1995; Sakai *et al.*, 1998) and tomato (Paynton *et al.*, 1996; Zhou *et al.*, 1996), and is regulated differentially throughout plant development. Like tomato and *Arabidopsis*, kiwifruit probably has several ethylene receptors that are regulated differentially during maturation and ripening. Research is needed to understand fully ethylene action during maturation and ripening in kiwifruit. Comparison of gene expression of ethylene receptors between preclimacteric kiwifruit which produce System 1 ethylene, and climacteric fruit which produce System 2 ethylene, could provide information about the multigene family of receptors which may be involved in differentially regulating System 1 and System 2 ethylene production and action.

Although applications of 1-MCP and AVG inhibited ethylene action and ethylene production in kiwifruit respectively, treated fruit continued to produce low concentrations of ethylene (System 1 ethylene) and softened gradually with time. It is likely that most softening in kiwifruit can be induced by System 1 ethylene. To determine the System 1 ethylene effect on softening, it will be necessary to measure softening in fruit from which endogenous ethylene has been removed and then which are kept in an ethylene free environment. Comparison of softening between control and ethylene free fruit will give information whether System 1 ethylene affect kiwifruit softening or not.

It is known that cell wall degradation occurs during kiwifruit softening; different cell wall hydrolase enzymes appear to be involved in different kiwifruit softening phases. Although some specific oligomers from both hemicellulose and pectin act as ethylene elicitors in fruit discs from tomato and pear, it is not known whether they exist in kiwifruit. Research

is needed to find out if and/or which oligomers exist in kiwifruit during phases 1, 2 and 3 of softening, and whether such oligomers act as ethylene elicitors in kiwifruit.

Until very recently, Hayward has been the only commercial cultivar of kiwifruit available in the market. This monoculture is of concern because this cultivar could succumb to a major pest or disease outbreak (Cotter *et al.*, 1991). Other cultivars that were grown in New Zealand, such as Abbott, Bruno and Monty, have been discarded because they soften faster and hence have a shorter storage life than Hayward (Cotter *et al.*, 1991; Manolopoulou and Papadopoulou, 1997; Papadopoulou and Manolopoulou, 1997). It is now known that 1-MCP can delay softening in Hayward kiwifruit. If 1-MCP was applied to these cultivars it may inhibit ethylene action, resulting in delayed softening and allowing a diversity of cultivars of kiwifruit to be marketed.

It is possible that Hayward is a slow ripening mutant because all of these cultivars originated from the same seed lot introduced into New Zealand. Hayward was selected from a large seedling population because it had a long storage/shelf life. Tomato mutants have contributed greatly to an understanding of the factors involved in the ripening process of this fruit. Physiological, biochemical and molecular comparison between Hayward and the other kiwifruit cultivars could provide insights into the mechanisms by which kiwifruit soften during storage. Such information would be invaluable to plant breeders for creating new cultivars with desired storage and quality attributes, which may be useful to diversify commercial cultivars for kiwifruit market.

7.7 Reference

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APPENDIX I

Ethylene production and respiration rate were calculated using the following formulae:

Ethylene production:

$rC_2H_4 = (cC_2H_4) / 1000 \times (Vjar - Vfruit) \times 1000 / Wfruit \times 60 / T$

Carbon dioxide:

$rCO_2 = [(CO_2)_f - (CO_2)_i] / 100 \times (Vjar - Vfruit) \times 1000 / Wfruit \times 60 / T$

Where:

rC_2H_4 = rate of ethylene production (µl/kg/h)

cC_2H_4 = ethylene concentration (µl/l)

rCO_2 = rate of carbon dioxide production (ml CO2/kg/h)

$(CO_2)_f$ = final carbon dioxide concentration (%)

$(CO_2)_i$ = initial carbon dioxide concentration (%)

Vjar = jar volume (ml)

Vfruit = fruit volume (ml)

Wfruit = fruit weight (g)

T = time (min.)

APPENDIX II

ACC concentration was calculated using the following formula:

c ACC = [10 nmol × E1 / (E2-E1)] × (V1 / 0.5 ml) / W

Where:

c ACC = ACC content (nmole/g (dry weight))

E1 = ethylene production without ACC (ppm)

E2 = ethylene production with ACC (ppm)

V1 = Total liquid volume (ml)

W = weight of fruit powder (g)

ACO activity was calculated using the following the formula:

a ACO = E × [(V1 - (V2 + W)] / W / T

Where:

a ACO = ACO activity (µl /g f.wt/h)

E = ethylene production (µl/l)

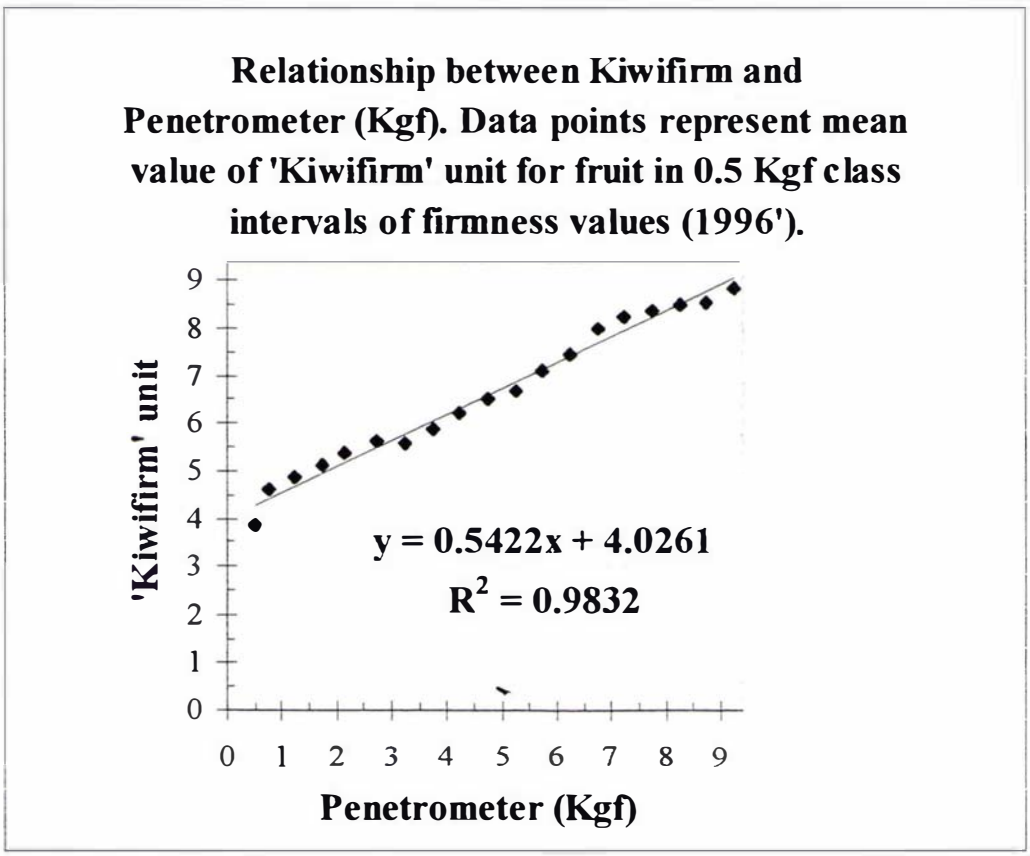
V1= vial volume (ml)

V2 = buffer volume (ml)

W = sample fresh weight (g)

T = incubation time (h)

APPENDIX III



The relationship between values obtained by the Penetrometer and Kiwifirm is obtained using the following equation:

Y=0.5422X + 4.0261 (where Y = Kiwifirm value, X=penetrometer value (kgf))

Penetrometer (Kgf)	0.5	1	2	3	4	5	6	7	8	9
Newtons (N)	4.9	9.8	19.6	29.4	39.2	49.1	58.9	68.7	78.5	88.3
Kiwifirm	4.30	4.57	5.11	5.65	6.19	6.74	7.28	7.82	8.36	8.91