SECTION I. INTRODUCTION

The avocado, *Persea americana* Mill. of the Lauraceae family) is indigenous to the subtropics of Central America and one of a few commercially significant members of the genus *Persea*. The avocado fruit is classified botanically as a berry comprising the seed and the pericarp, which is separated into the rind known as the exocarp, the fleshy edible portion or mesocarp (Plate 1), and the thin layer next to the seed coat, the endocarp (Biale and Young, 1971; Seymour and Tucker, 1993).

Internationally, the most prominent commercial varieties are Hass, which originated from a Guatemalan seedling and Fuerte, a Guatemalan/Mexican hybrid. In addition to Hass and Fuerte, there are another 10 varieties grown in Australia that provide a succession of mature fruit for about 10 months of the year. Examples are Shepard (February), Fuerte (March), Sharwil (April), Wurtz (May) and Hass (June-December) (Alexander, 1978; AHC, 1996-7). Avocado fruit are grown in widely distributed areas in Australia from Mareeba, North Queensland (17°S) to the Inland irrigation areas of Victorian, South Australia and New South Wales, as well as Pemberton, Western Australia (34°S) (Chaplin, 1984; Bolton, 1995).
There is a considerable international trade in avocado fruit. World production in 1996 was about 2,093,000 tonnes with about 50% originating from North and Central America (FAO, 1996).

Plate 1. Longitudinal section of an avocado fruit
(After Biale and Young, 1971).

For the financial year 1991-92 total Australian production was 12,000 tonnes increasing to 16,316 tonnes for the financial year 1996-97. By the year 2000, it is estimated that Australian production will be 27,290 tonnes. This will lead to oversupply of the domestic market and
growers will need to export. Since Australia is distant from overseas markets, avocados shipped by sea must tolerate an extended period in storage. The estimated shipping time is up to 7 weeks to Europe (Jessup, 1991; AHC, 1996-97).

Successful export marketing must rely on decreasing the rate of ripening sufficiently to allow for the shipping time and orderly marketing in the importing country (Bower and Cutting, 1988). Storage life of avocados stored at the recommended storage temperature 4.5 - 7°C in air (Zauberman and Jobin-Decor, 1995) is frequently not long enough to permit shipping by sea to distant markets. Previous research has shown that storage in a controlled atmosphere (CA) in the absence of ethylene at low temperatures can extend storage life (Chaplin et al., 1983). Several methods or combinations of CA are available for decreasing the rate of ripening. However, the combination of high metabolic activity and their susceptibility to chilling injury (CI) means that care is necessary in the choice of storage conditions for avocados (Bower and Cutting, 1988).

CI in the form of mesocarp discoloration has been a serious problem in avocados shipped at low temperatures (Bower and van Lelyveld, 1985). The symptoms vary from a light greying of the mesocarp tissue in the early stage of development and darkening of the vascular strands when the injury is severe (Chaplin et al., 1983).
The fruit may ripen abnormally and become more susceptible to decay (Lee and Young, 1984; Cutting and Woltensholme, 1992).

Delaying ripening and associated changes in fruit are the main benefits of controlled atmosphere (Kader et al., 1989). CA storage of avocado at low temperature has been used for the shipment of avocado fruit to overseas markets. There is evidence that CA combined with low temperatures may decrease the incidence of CI and delay ripening, thus allowing storage for a longer period (Barmore and Rouse, 1976; Salunkhe and Desai, 1984; Bower et al., 1990; Wang, 1990; Faubion and Kader, 1994; Pesis et al., 1994; Meir et al., 1995).

Several published reports have raised the possibility that CA may actually enable lower storage temperatures to be used without causing CI. This possibility has not been fully explored. Such studies need to be evaluated for each cultivar to determine their specific requirement (Hatton and Spalding, 1990). Barmore and Rouse (1976) reported successful storage of Hass for 5-9 weeks at 5 and 7°C compared to 2 weeks in air. The best results were obtained in an atmosphere of 2% oxygen and 10% carbon dioxide. Meir et al., (1995) reported satisfactory results following storage of Hass at 5°C for five to nine weeks in an atmosphere of 3% oxygen and 8% carbon dioxide compared to 2 weeks in air. They reported that this atmosphere reduced the development of CI and ethylene production, and retarded ripening changes including softening and colour changes.
During the final stages of development, fruit undergo a complex series of physiological and biochemical events involving changes in colour, flavour, aroma and texture that make them both attractive and tasty to eat. It is generally acceptable that ripening is genetically programmed and requires differential gene expression. Thus, it is important to utilize this knowledge in developing new ways to improve storage life (Gray et al., 1992). This proposition is supported by the observation that low oxygen concentration retards both the synthesis and action of ethylene (Kanellis et al., 1991). Kanellis et al., (1989) showed that transferring avocado fruits to 2.5 % O₂ suppresses the activity of immuno reactive protein and abundance of mRNA for cellulase. Furthermore, 2.5 % O₂ delayed the development of a number of polypeptides that normally appear during ripening (Kanellis et al., 1989a). Therefore, the aim of this research was to examine the effectiveness of gas mixtures containing low oxygen and raised carbon dioxide concentrations (CA) for extending storage life and reducing the incidence of CI.

A further aim was to identify possible correlations between the level of O₂ and/or CO₂, the incidence of CI, the activity of some ripening related enzymes and changes in proteins during ripening following CA storage at low temperatures.
Specific objectives of this thesis were:

1. Study the physiology of avocado fruit stored in different combinations of O₂ and CO₂ concentration at low temperature.

2. Study some biochemical changes in avocado fruit stored under CA conditions at low temperature and

3. Search for changes in the production of proteins that may be related to reduction in CI induced by CA.
SECTION II. REVIEW

2.1. Physiology of Ripening

2.1.1. Fruit development

The growth of the avocado fruit follows a single sigmoid pattern similar to other fruit (Fig. 2.1). In most fruits, the early period of fruit growth following anthesis is characterized by rapid cell division. Subsequent growth is due to cell expansion. However, in the avocado cell multiplication continues in the growing fruit and in general the fruit continue to grow while attached to the tree (Seymour et al., 1993).

Therefore, the ultimate size of the fruit is determined not only by cell division in the early growth stage and cell expansion but also by cell multiplication throughout the entire growth period (Biale and Young, 1971; Bower and Cutting, 1988). The avocado of commercial culture is a climacteric fruit that normally does not ripen while attached to the tree (Awad and Young, 1980; Zauberman et al., 1988; Bower and Cutting, 1988; Cutting and Wöstenholme, 1992a). The reasons for this phenomenon are not understood. Tingwa and Young (1975) postulated that avocado fruit (cultivar Hass and Fuerte), with a portion of stem and/or the peduncle retained, ripened more slowly than when it was removed. They concluded that the stem and peduncle may act as a sink for ripening hormones produced in the fruit. However, Eaks (1973) found that
Fig. 2.1. Compositional changes in avocado fruit associated with development (After Biale and Young, 1971).

harvesting fruit with or without the attached peduncle had no significance effect on the ripening rate of avocado. (Physiological maturity in an avocado can be defined as the stage of development when growth has been completed and the fruit is capable of ripening to a state of readiness for consumption (Lee et al., 1983).) Fruit ripening is associated with changes in colour, taste and texture (Bower and Cutting, 1988). The process involves many catabolic and anabolic changes, requiring large
amounts of energy as well as prolonged integrity of the cell membranes (Seymour et al., 1993).

2.1.2. Respiratory climacteric

The respiration rate of fruit usually declines after harvest and remains low until the onset or ripening, however, this lag period between harvest and ripening becomes progressively shorter with successive harvests. In avocado fruit the onset of ripening is accompanied by an increase in respiration called the respiratory climacteric (Bennett et al., 1987; Kader et al., 1989; Seymour et al., 1993). Production of carbon dioxide increases from 40 mg.kg$^{-1}$.h$^{-1}$ at the pre climacteric stage to about 170 mg.kg$^{-1}$.h$^{-1}$ during the peak at 21°C (Fig. 2.2). Softening of the fruit begins coincides with the respiratory peak (Biale and Young, 1971).

Avocado is one of the most metabolically active fruits (Awad and Young, 1979). The respiratory substrate in avocado is probably glucose since the respiratory quotient (RQ), the ratio of CO$_2$ produced to O$_2$ consumed, remains at 1 during the climacteric (Seymour et al., 1993). It has been shown that any treatment which suppresses this respiratory climacteric also retards softening (Biale and Young, 1971). Ethylene has been shown to be involved in the respiratory climacteric and ripening phase (section 2.3.1).
2.1.3. Changes in colour.

Colour is the most obvious change that occurs in many fruit including avocado and is often the major criterion used by consumers to determine whether the fruit are ripe. In most fruit, colour changes are primarily brought about by degradation of chlorophyll, which unmask other preformed pigments, or is accompanied by the biosynthesis of one or more pigments, usually either anthocyanins or carotenoids (Wills *et al.*,...
Fruit of the Hass cultivar change colour from green to a characteristic brown-black during normal ripening, whereas Fuerte retains its green colour even when soft (Biale and Young, 1971; Meir et al., 1995).

2.1.4. Changes in texture.

The most noticeable change in avocado fruit associated with ripening is softening due to changes in cell wall biochemistry (Brady, 1987; Nothnagel, 1987; Seymour et al., 1990). Changes in the pectic component are thought to be mainly responsible for softening and this led to investigation of the pectic enzymes (Biale and Young, 1971) and pectic polysaccharides (Seymour et al., 1990). Avocado softening is associated with an increase in the activities of hydrolytic enzymes such as cellulase and polygalacturonase (Awad and Young, 1979; Tucker and Laties, 1984; Kanellis et al., 1989; Kutsunai et al., 1993) and a specific cellulase gene family designated cel1 and cel2 (O'Donoghue and Huber, 1991; Cass et al., 1990; Tonutti et al., 1995). Electron microscopy studies of the cell walls showed that early in ripening the middle lamella begins to disappear and this is associated with pectin removal from the cell wall matrix. Progressively, a loss of organization and density in the cell wall occurs and finally, the walls almost completely disappear during the post climacteric phase (Bower and Cutting, 1988). The ripeness of avocado is directly related to their firmness and
measurements of firmness can be used to manage shelf life (Peleg et al., 1990).

2.2. Biochemistry of Ripening.

2.2.1. Ethylene biosynthesis.

The simple gas ethylene influences a diverse array of plant growth and developmental processes including, senescence, cell elongation, and fruit ripening (Hamilton et al., 1990; Mattoo and White, 1991; Davies, 1995; Wilkinson et al., 1995; Bleecker and Schaller, 1996; Kieber, 1997; McGrath and Ecker, 1998). It plays an important regulatory role in the physiology of plants, in particular postharvest physiology of fruit (Theologis, 1993, 1994; Mathooko, 1996). It is generally accepted that methionine is the common precursor of ethylene throughout diverse arrays of plants tissue where the hormone occurs and exerts its many effects (Adam and Young, 1979). Ethylene is synthesized via the following pathways (Fig. 2.3). Enzymatic activities converting methionine to AdoMet (AdoMet synthetase, AdoMet to ACC (ACC synthase), and ACC to Ethylene (ACC oxidase, formerly called ethylene forming enzyme (EFE) (Adams and Yang, 1979; Moore, 1989; Kende, 1993) as well as the genes encoding them have been demonstrated.
Fig. 2.3. The ethylene biosynthesis pathway; **Hatched block**: this is normally suppressed and is the rate-limiting step. **Large black line**: induction of synthesis of ACC synthase and ACC oxidase; **Double arrows**: inhibition of the reaction. ACC, AdoMet, AOA (Aminoxyacetic acid), AVG (aminothoxyvinylglycine), DAdoMet (decarboxylated AdoMet), MACC (1-(malonylamino)cyclopropane-1-carboxylic acid), MTA (5'-methylthiadenosine) and MTR (5'-methylthioribose), respectively (After Yang, 1985; Abeles et al., 1992; Mathooko, 1996).
in plants (Bouquin et al., 1987; Fluhr and Mattoo, 1996). AdoMet synthetase is a key enzyme that provide AdoMet for not only the syntheses of ethylene but also for transmethylation reactions in proteins (McKeon et al., 1995; Fluhr and Mattoo, 1996).

2.2.2. Metabolism of 1-aminocyclopropane-1-carboxylic acid (ACC)

ACC is considered to be the rate-limiting factor for ethylene biosynthesis in most plant tissues and it has been the focus of a considerable amount of research (Arteca, 1989; Dean and Mattoo, 1991). Adams and Yang (1979) identified ACC as an intermediate in the biosynthesis of ethylene in apple tissue. In many plant tissues the ethylene production rate was found to be directly correlated to its endogenous ACC content and markedly increased by exogenous application of ACC (Lizada and Yang, 1979; Bufler, 1986). Its activity is highly regulated and closely parallels the level of ethylene biosynthesis (Brady and Speirs, 1991; Mattoo and White, 1991; Mattoo and Suttle, 1991; Kieber and Ecker, 1993; Kende, 1993).

ACC synthase catalyses the elimination of 5'-methyl thio adenosine (MTA) from AdoMet to ACC and its capable of playing a key role in regulating ethylene production such as in the ripening process. During the pre climacteric stage enzyme activity is low but then increases several hundred fold and becomes abundant during the climacteric stage (Abeles et al., 1992; Oetiker and Yang, 1995). This enzyme shows
only slight activity in preclimacteric avocado, and inhibitor studies with fruit discs indicate that the increase in the activity of this enzyme during ripening requires RNA and protein synthesis (Sitrit et al., 1986). It is strongly inhibited by aminoethoxy vinyl glycine (AVG), a known inhibitor of pyridoxal phosphate-mediated enzyme reactions. Other ACC inhibitors are L-canaline and N-benzyloxy carbonyl-L-α-amino oxy propionic acid (Abeles et al., 1992).

Other points in the biosynthetic pathway which may be important in this case include the conversion of ACC to ethylene by ACC oxidase and malonylation of ACC to 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) (John et al., 1989). A significant level of MACC is found in preclimacteric avocados which may play an important role in regulating endogenous ACC level in the preclimacteric stage. This indicates that at least part of the low level of ACC, which is synthesized in the course of fruit growth is conjugated to MACC (Sitrit et al., 1986; Seymour and Tucker, 1993). MACC is also found as the major conjugate of ACC in tomato fruit throughout all ripening stage, from immature green through to the red ripe stage (Peiser and Yang, 1998). Low temperature stimulates autocatalytic ethylene production in mango fruit (Lederman et al., 1997) and chilling induced ethylene production in plants involves the normal pathway of ethylene biosynthesis (Yang and Hoffman, 1984; Yang, 1985).
The capacity to convert ACC into ethylene in some cultivars of apple is induced more rapidly at low temperature (0-5°C) than at 20°C (Jobling et al., 1991). In climacteric fruits both ACC synthase and ACC oxidase activity are induced during ripening and are control points in the regulation of ethylene biosynthesis (Oetiker and Yang, 1995; Gorny and Kader, 1996a). ACC oxidase activity in avocado fruit rises gradually during the pre-climacteric phase and then sharply at the onset of the ethylene climacteric (Starret and Laties, 1991).

The final step in the biosynthesis of ethylene in higher plants involves oxidation of ACC to ethylene with the release of CO₂ and cyanide. This reaction is catalysed by ACC oxidase and requires oxygen for its activity (Dean and Mattoo, 1991; Imaseki, 1991; Smith et al., 1994). It is inhibited by Co²⁺, CO₂ and salicylic acid and is feedback-regulated by CO₂ (Bufler, 1986). Like ACC synthase, ACC oxidase also undergoes inactivation (Smith et al., 1994). ACC oxidase has been purified and biochemically characterized from avocado. It requires iron, ascorbate and CO₂ for activity (McGarvey et al., 1992; McGarvey and Christoffersen, 1992; Zarembinski and Theologis, 1994). Ethylene is produced from C-2 and C-3 of ACC, that correspond to C-3 and C-4 of Methionine when the tissue was incubated in air. The carboxyl group (C-1) of methionine and ACC is released as CO₂ during ethylene biosynthesis (Imaseki, 1991). Hoffman and Yang (1980) reported that the pre climacteric Fuerte avocado contained less then 0.05
nmol g⁻¹ fresh weight of ACC, however during the climacteric it increased dramatically to about 45 nmol.g⁻¹ (Seymour and Tucker, 1993). In avocado, as in other climacteric fruit, the onset of ripening is marked by a large rise in ethylene production. Ethylene biosynthesis seems to be initiated in the distal end of the mature fruit (Adato and Gazit, 1977; Seymour and Tucker, 1993, Wang, 1990). Ethylene production in avocado is suppressed by elevated carbon dioxide concentrations (Marcellin and Chaves, 1983).

Wang (1990) concluded that high carbon dioxide concentrations inhibit ethylene action, therefore synthesis of ripening enzymes and softening are delayed. Reduction in ethylene production in Hass occurred when fruit were stored for three days at 5.5°C in 20 per cent carbon dioxide. Also internal ethylene peaked at a much lower concentration (van Eeden et al., 1990). The capacity of tissue for ethylene biosynthesis is affected by the concentration of oxygen (Wang, 1990; Lange and Kader, 1997a).

It has long been a commercial practice to store fruit in CA to retard ripening (Kader, 1986, 1995). Ethylene production is inhibited by CO₂ in climacteric fruit by inhibiting ACC synthase action (Bufler, 1984; Chavez-Franco and Kader, 1993). Mathooko (1996) proposed that the loss in ACC synthase activity during CO₂ treatment could result from depletion of ACC synthase protein due to rapid degradation and/or inhibition of synthesis, inactivation of ACC synthase in vivo or a
combination of both processes. Carbon dioxide may also regulate ethylene biosynthesis during fruit ripening by promoting the conjugation of ACC into MACC. The effect of elevated CO$_2$ on ACC oxidase activity in ripening of fruit is concentration dependent. Carbon dioxide also inhibits *in vivo* ACC oxidase activity in avocado (Chevery *et al.*, 1988; Lange and Kader, 1997). Therefore, inhibition of ethylene biosynthesis by elevated CO$_2$ concentrations at the levels of both ACC synthase and its oxidation to ethylene may, at least in part, contribute to the extension of postharvest storage life of fruit in addition to its action as an antagonist of ethylene action (Mathooko, 1996).

### 2.2.3. Polyamines

Polyamines are small, positively charged dipathic amines, that play a variety of roles in plant physiology. They are involved in cell division, embryogenesis, root formation, floral initiation and development, pollen formation and fruit development (Evans and Malmberg, 1989; Egea-Cortines and Mizrahi, 1991; Malmberg *et al.*, 1998). Putrescine, spermidine and spermine are collectively referred to as polyamines. Polyamines exist in all organisms, although the presence of these three forms is not universal, and some taxa may have other polyamines or polyamine like-compounds (Smith, 1985; Malmberg *et al.*, 1998). Winer and Apelbaum (1986) proposed that the naturally occurring
polyamines may acts as modulators of some cellular and physiological processes during development and ripening of avocado fruit.

Fig. 2.4. The polyamines biosynthetic pathway and its linkage to ethylene biosynthesis. The substrates and biosynthetic enzymes are ACC, ADC, arginase (Agn), decarboxylated S-adenosylmethionine (dAdoMet), ODC and AdoMetDC. The inhibitors are AVG, DL-α-difluoromethylarginine (DFMA), DL-α-difluoromethylornithine (DFMO), and methylglyoxal-bis(guanylylhydrazone) (MGBG) (After Walden et al., 1997).
They may affect cell division and macro molecular synthesis while also acting as potent inhibitors of many senescence related processes in many plants (Galston, 1983; Roberts et al., 1984; Kramer and Wang 1989). Ethylene and polyamines may show opposite effects in relation to fruit ripening and senescence (Winer and Apelbaum, 1986). Before onset and during senescence a switch in AdoMet metabolism toward ethylene biosynthesis occurs. During this phase accumulation of polyamines declines while extensive production of ethylene results in promotion of senescence of the plant organ (Saftner and Baldi, 1990; Fluhr and Mattoo, 1996). However, Kakkar and Rai (1983) stated that polyamines and ethylene biosynthesis pathways do not actively compete for the same substrates at any stage of avocado fruit development and ripening. They may act by inhibiting the conversion of AdoMet to ACC (Fig.2.3) thus blocking ACC dependent ethylene synthesis (Apelbaum et al., 1981; Even-Chen et al., 1982; Smith, 1985; Kushad and Dumbroff, 1991).

Polyamines are synthesised (Fig 2.4.) from either arginine or ornithine by arginine decarboxylase (ADC) and ornithine decarboxylase (ODC). ADC is widespread in higher plant tissue, and it has been purified and characterized from avocado, rice and oats (Mehta et al., 1997). An extract from the avocado mesocarp and the seed coats shows ADC activity throughout fruit growth and ripening whereas no
ODC could be detected (Winer and Apelbaum, 1986). In contrast, Kushad et al., (1988) reported that ADC and ODC were active during the early stage of avocado fruit development. Galston and Kaur-Sawhney (1995) stated that in most cases when putrescine accumulated due to stress, ADC was activated by de novo synthesis while ODC inhibitors were ineffective in blocking putrescine biosynthesis (Faust and Wang, 1992). Putrescine can be produced directly from ornithine by the action of ODC or indirectly from arginine by ADC (Slocum et al., 1989,1991; Galston and Kaur-Sawhney, 1995). AdoMet decarboxylase (AdometDC) probably is the rate-limiting step that provides the aminopropyl groups that are used by spermidine and spermine synthase, respectively to convert putrescine to spermidine and spermine (Slocum, 1991; Faust and Wang, 1992; Walden, 1997). Because AdoMet is a substrate for both ACC and polyamines, it may be questioned whether competition exists for AdoMet between the ACC - ethylene pathways and spermidine and spermine biosynthesis (Even-Chen et al., 1982). No such competition was observed in avocado during fruit development and ripening (Kushad et al., 1988), because polyamines peak earlier than ethylene (Evans and Malmberg, 1989). A correlation has also been reported between early cell division and putrescine and spermidine levels in avocado pulp (Apelbaum, 1986; Winer and Apelbaum, 1986; Evans and Malmberg, 1989).
Inhibitors of both polyamines and ethylene biosynthesis make it possible to probe the interaction of polyamines with ethylene in several ways. The most common hypothesis tested is that polyamines and ethylene may regulate each other's synthesis, either directly or by metabolic competition for AdoMet (Evans and Malmberg, 1989; Valero et al., 1997). CA storage involving low oxygen and high CO₂ concentrations is widely used to prolong the storage life of apples. Low oxygen concentration (1 %) at 3 and 3.5°C storage induced higher levels of all three polyamines and significantly inhibited the softening of apples at both temperatures compared to fruit stored in air (Kramer et al., 1989; Gorny and Kader, 1996,1997).

2.2.4. Cell Wall Enzymes

Ripening of avocado fruit is initiated upon removal from the tree and involves a series of coordinated metabolic events that alter their anatomy, biochemistry, physiology, and gene expression (Brady, 1987,1992). These alterations affect many characteristics such as colour, texture and flavour (Seymour et al., 1993), and lead to development of a soft edible fruit (Christoffersen et al., 1982). It is well established that for ripening to proceed, both expression of specific genes and subsequent syntheses of enzymes associated with normal ripening are required for normal ripening (Dominiguez-Puigjamet et al., 1992; Seymour et al., 1990; Huber and O'Donoghue, 1993). Softening is one of the most
pronounced changes associated with avocado ripening. In avocado there is a dramatic increase in cellulase enzyme activity due to de novo synthesis of its protein correlated with an increase in the steady state amount of cellulase mRNA (Cass et al., 1990; Kanellis and Kalaitzis, 1992; Zamorano and Merodio, 1993a).

The climacteric rise in ethylene production during avocado ripening is normally associated with an increase in respiration rate and flesh softening. Therefore, some studies on avocado have focused on cell wall degrading enzymes (Christoffersen et al., 1984). It is well established that oxygen levels below those in air retard the rate of ripening and softening of climacteric fruit (Kader, 1986; Kanellis et al., 1991). Metzidakis and Sfakiotakis (1993, 1995) found that the activity of the enzymes cellulase and polygalacturonase (PG) was suppressed by low O₂ concentrations (1, 2 and 5 %). The activity of these enzymes was very low in pre climacteric avocado fruit and their activity increased significantly three days after the application of propylene. Another study found that alcohol dehydrogenase (ADH) isoenzymes could be induced by exposure of avocado fruit to gas mixtures containing 2.5, 3.5 or 5.5 % O₂ (Kanellis et al., 1991, 1993). Increased activities of both pyruvate decarboxylase (PDC) and ADH were observed when pears were kept in low O₂ and high CO₂ concentrations (Ke et al., 1994, 1995).
2.3. Cool Storage

2.3.1. Chilling injury in stored avocados

Chilling injury (CI) is a physiological disorder that generally occurs at temperatures in the range 0 - 12°C. CI is the major limiting factor in the storage and transportation of avocado, the extent of which is a function of duration of storage at temperature within the chilling range (Markhat, 1984). Symptoms vary from a light grey discoloration of mesocarp tissue and the darkening of vascular stands, development of undesirable flavours and odours, pitting, failure to ripen, scald-like browning or darkening of the skin and susceptibility to decay, particularly in the distal portion commonly occurs (Sharon and Khan, 1979; Chaplin et al., 1983; Lee and Young, 1984; van Lelyveld and Bower, 1984; Bower and van Lelyveld, 1985; Zauberman et al., 1985; Cutting and Wolstenholme, 1992a; Marangoni et al., 1996). Mesocarp discoloration is a serious problem in avocado shipped at low temperatures. Bower and van Lelyveld (1985) found that restricted ventilation of shipping containers was more important than chilling, due presumably to either decreased oxygen or/and elevated carbon dioxide concentrations. Reducing oxygen content may stimulate the incidence of chilling injury, however raising carbon dioxide content might stimulate or inhibit the
incidence of chilling injury (Kader et al., 1989; Metzidakis and Sfakiotakis, 1995). It has been postulated that CI of avocado is caused by a phase transition of membrane lipids below the critical of chilling temperature (Lee and Young, 1984; Zauberman and Fuchs, 1973; Marangoni et al., 1996).

Susceptibility to CI varies among avocado cultivars. Hass avocado stored for two weeks at 0 or 5°C displayed a normal climacteric pattern and ethylene production when returned to 20°C, and developed no chilling injury symptoms, however when storage time was extended to about 4 - 6 weeks the symptoms were moderate to severe (Eaks, 1983). Chaplin et al., (1983) reported that when opposite ends of intact avocado were exposed individually to ethylene enriched air for up to 6 weeks at either 1.5 or 5°C, the severity of CI in ripened fruit was significantly greater in fruit ends exposed to low ethylene concentrations. A similar result was reported by Fuchs et al., (1989) when the symptoms were observed in stored Hass avocado stored for 22 days at 0°C.

3.2. Controlled atmospheres storage

There has been much research on the responses of fruit and vegetables to CA since the early work on apples by Kidd and West in 1927 (Laties, 1995). It has been shown that the reduction in respiration rates achieved by decreasing oxygen concentration and/or raising carbon dioxide concentration at controlled temperatures can extend
the storage life of many products (Kader et al., 1986). Generally reduction in oxygen to 1-3 % gives useful extension in storage life, but the responses to elevated carbon dioxide are much more variable (Wang, 1990). Some fruit may be injured in more than 1 % CO₂, whereas others may benefit by atmosphere of 5 to 15 % CO₂ (Weichmann, 1986; Wang, 1990).

The effect of elevated carbon dioxide upon the respiration of fruit and vegetables is of particular interest in the context of CA systems (Mathooko, 1996a). One of the major consequences of elevated carbon dioxide concentration is the reduction of respiration rates (Blanpied, 1989; Bender et al., 1993). Carbon dioxide may act both as inducer and a suppressor of respiration depending on its concentration in situ, internal concentration, duration of exposure, the commodity and temperature during and subsequent to carbon dioxide exposure (Kader, 1986; Mathooko et al., 1995).

Lowering oxygen and elevating carbon dioxide concentrations may prolong the storage life of avocado but the composition of the atmosphere must be carefully controlled to avoid anaerobic respiration. CA storage may also reduce chilling injury, because low O₂ and high CO₂ levels retard respiration and ethylene production (Wang, 1990; Ke et al., 1995).

The avocado is highly sensitive to anaerobic conditions when oxygen is excluded and unable to maintain aerobic metabolism. However, a
critical oxygen concentration was not determined (Biale and Young, 1971).

CA can extend the storage life of avocado. Some studies with CA containing 2.5 to 21% oxygen showed that the time to reach the climacteric peak was extended in proportion to the decrease in concentration of oxygen. Kader et al. (1989) suggested that a minimum oxygen concentration of 3% is required to maintain aerobic respiration, when avocado are stored in CA for several weeks.

According to Biale and Young (1971) carbon dioxide should be kept below 10% to avoid physiological disorders. In contrast, Durand et al., (1984) and Salunkhe and Desai (1984) reported that CA containing 2 - 10% oxygen and 10 - 21% carbon dioxide could double storage life of avocado. Bower et al., (1990) found that Fuerte avocado stored for 28 days at 5.5°C, in 2% O₂ and 10% CO₂ show a lower incidence of physiological disorders. Lula avocados can be stored successfully for 8 weeks in CA of 2% O₂ and 10% CO₂ at 4-7°C and 98-100% RH (Hatton and Spalding, 1990). The best CA conditions for storage of Anaheim and Fuerte avocado in Brazil are 6% O₂ and 10% CO₂ at 7°C. These avocados can be stored for 38 days in CA (Hatton and Spalding, 1990).

Intermittent exposure of unripe Hass avocado to 20% CO₂ every week for 2 days then stored in air following 3 weeks storage delayed senescence at 12°C, reduced CI at 4°C and controlled decay at both temperatures. Further decayed was controlled at both temperatures (Marcellin and Chaves, 1983). Symptoms of skin injury in Lula avocado appeared first in fruit held only 3 days in 0.5% O₂.
and 25% CO₂. Skin injury was slight in 0.5% O₂ and 0% CO₂ and absent in 21% O₂ and 25% CO₂ (Hatton and Spalding, 1990).

The following conclusions may be drawn from this review:

- Chilling Injury (CI) at temperatures below 12°C is the principal factor limiting cool storage life of pre climacteric avocado fruit. Critical chilling temperatures and symptoms of CI vary among cultivars depending upon their genetic origins.

- Storage in controlled atmosphere (CA) in the absence of ethylene can give a useful extension in storage life, although there are no consistent recommendation on the concentrations of oxygen and carbon dioxide.

- CA atmospheres containing specific combinations of low oxygen and raised carbon dioxide concentration may delay or increase resistance to the development of CI but no clear recommendations can be deduced from published information.

- CA atmospheres that may reduce CI in sensitive fruit, alter the metabolism of the fruit by altering the expression of some enzymes including those involved in ethylene and alcohol synthesis.
SECTION III.
EFFECT OF CONTROLLED ATMOSPHERE ON STORAGE LIFE

3.1. Introduction

Storage at 5 - 7°C is generally recommended for the most common commercial cultivar Hass. Storage at lower temperatures causes CI. However, storage life at 5 - 7°C in air may be too short for export by sea freight to distant markets. Chaplin et al., (1983) show that CI was more severe if low concentrations of ethylene were present at 1.5°C. Following this observation useful extension of storage life at 5°C was obtained in modified atmosphere in the presence of an ethylene absorbent (Chaplin, 1984). Much work on CA storage of avocado at 5 - 20°C has been done Softening is also delayed in Fuerte avocado stored in atmospheres of 3 - 5 % O₂ and 3 - 5 % CO₂ at 7°C (Hatton and Spalding, 1989). However, it is still an open question whether CA storage below 5°C is beneficial.

Zauberman and Jobin-Decor (1995) presented some data which showed that a useful extension of storage life could be achieved by storage at 2°C in air. However, Meir et al. (1995) have reported a delay in colouring and softening and reduced CI in Hass avocado following storage in CA (3 % O₂ and 8 % CO₂) at 5°C.

The objective of these experiments was to examine the effectiveness of a range of combinations of low oxygen and raised carbon dioxide concentrations on the skin colour, softening and the incidence of CI following storage at low temperatures.
3.2. Material and Methods

3.2.1. Source of fruit.

Mature avocado fruit (*Persea americana* Mill cv Hass) were obtained from Ambrosia Orchards at Kulnura, New South Wales.

3.2.2. Experimental plan.

The fruit were transported 120 km by road to the University of Western Sydney Hawkesbury, Richmond, New South Wales. Fruits were sorted for weight uniformity (200 - 245 grams each), dipped in 0.2 % ‘Sportak’ fungicide solution (Prochloraz-N-propyl-N- [2-(2,4,6-trichlorophenoxy) ethyl] imidazole-1-carboxamide, Schering Pty. Ltd.), dried at 20°C for about 30 minutes and then enclosed in 30 L polyethylene containers (Plate 3.1).

Plate 3.1. Controlled Atmosphere containers were ventilated with metered flows of humidified air and mixed gases.
Samples of harvested fruit were stored singly in each container at temperatures of 20°C with three replicates. The containers were ventilated with air at a flow rate of about 6 L.h⁻¹ and monitored for ethylene and respiration rate till the fruit ripened. Samples of 36 fruit were stored in each CA chamber at temperatures of 0 and 5°C with three replicates. The containers were ventilated with air (control) or CA at a flow rate of about 12 L.h⁻¹. Representative samples of 12 fruit were transferred from each CA chamber to air at 20°C after 3, 6 and 9 weeks at 0°C and 5°C respectively. The atmospheres were generated by mixing regulated flows of air, carbon dioxide and a nitrogen enriched stream (Plate 3.2 left). The nitrogen stream (99.5 % N₂) was generated with a Permea Air Separator (Model CPA - 2, Monsanto, U.S.A) or with a Pressure Swing Adsorption Apparatus (Domnick Hunter Ltd, UK). Gas streams at regulated pressures were metered with adjustable flow meters and combined to generate the required CA mixtures. CA mixtures were monitored with a Fruit Store Analyser type 770 L (David Bishop Instruments, Heathfield, East Sussex, UK) and the composition was recorded at 4 hourly intervals. Oxygen and carbon dioxide were maintained at limits of ± 0.2 % of the desired concentrations (Plate 3.2 right).

Experiment 1: In 1995 - 1996 the fruit were harvested in November then stored in CA containing 2.5, 5, 7.5 % oxygen with 5, 7.5 and 10 % carbon dioxide in all combinations (9 mixtures).
Plate 3.2. **Left:** Flow meters used to regulate the composition of the CA mixtures admitted to the storage containers illustrated in Fig 3.1. **Right:** Equipment for automatically analysing the gas streams (Bishop Oxystat 2002). The equipment included a Fruit Store Analyser type 770 L to monitor the oxygen and carbon dioxide concentrations in the CA mixtures. An Epson Printer model LX 300 was used to record the analyses at 4-hourly intervals.
Experiment 2: The fruit were harvested in February then stored singly at 20°C and ethylene production and respiration rate were monitored till the fruit ripe. In 1996 - 1997 the fruit were harvested in February then stored in CA containing 2.5, 5 % oxygen with 5, 7.5 % carbon dioxide in all combinations (4 mixtures). These mixtures were found to be the most promising in experiment 1. Fruit were transferred from the plastic containers to 20°C after 3, 6 and 9 weeks storage at 0°C and 5°C. Three fruit from each treatment were enclosed singly in polyethylene containers (one L), which were ventilated with humidified air streams (6 L.h⁻¹) at 20°C. These fruit were used for measurement of respiration rates and ethylene production. Another nine fruit from each treatment were taken for measurement of colour changes, firmness and the incidence of CI. The CA stored fruit were assessed after 0, 2, 4 and 6 days at 20°C to determine the shelf life of the fruit. Observations were made on three replications of fruit.

3.2.3. Fruit colour.

The skin of Hass changes from green to a dark brown/black colour as the fruit ripen. Colour changes in each fruit were rated on a scale of 1 - 4, where 1 = green; 2 = changing colour from green to brown; 3 = most of the peel was brown with some green colour; 4 = most of the peel was brown (Chaplin et al., 1983; Meir et al., 1995).

3.2.4. Fruit firmness.
Flesh firmness was measured on two locations on each fruit with an Effegi penetrometer mounted on a drill press (12 mm tip), following removal of small pieces of skin. Firmness was expressed as newtons (Kgf x 9,807 = Newtons (N)) (Kader, 1982).

3.2.5. Chilling injury

Visual assessments of chilling injury were performed by cutting the fruit longitudinally into halves and scoring the appearances of the flesh (pulp) using a subjective scale, where 0 = no discoloration; 1 = very light discoloration; 3 = medium discoloration; and 4 = severe discoloration (Pesis et al., 1994; Meir et al., 1995).

3.2.6. Rates of respiration and ethylene production

The respiration rates were measured in terms of carbon dioxide production (mL.kg\(^{-1}\).h\(^{-1}\)). Carbon dioxide was measured by withdrawing 1 mL samples of gas by hypodermic syringe from the outlets of the plastic respiration containers and injecting them into a gas chromatograph (Gow Mac, Model 580, USA). The chromatograph was fitted with a stainless steel column (2 m X 0.3 mm ID) packed with 80 - 100 mesh Porapak Q (Supelco, USA) and a thermal conductivity detector. The carrier gas was helium with a flow rate at 28 ml.min\(^{-1}\). The gas chromatograph was calibrated with a standard mixture containing 5 % carbon dioxide in air (BOC Gases, Australia). The peaks of the separated
gases were recorded on a strip chart recorder (Miller Graphic Controls Pty. Ltd., Australia)

The rate of respiration of avocado was calculated as follows:

\[
(1) \quad \% \text{ CO}_2 = \left[ \frac{\text{Peak height sample} \times A}{\text{Peak height standard} \times A} \right] \times \% \text{ CO}_2 \text{ in Std} - 0.035
\]

\[
(2) \quad \text{mL CO}_2 \text{.kg}^{-1} \text{.h}^{-1} = \frac{\% \text{ CO}_2 \times \text{FR} \times 10}{W}
\]

where \( A = \) attenuation

\( \text{FR} = \) flow rate (L.h\(^{-1}\))

\( W = \) weight of samples (kg).

\( \text{Std} = \) standard gas mixture

Ethylene production was expressed as \( \mu \text{L}. \text{kg}^{-1} \text{.h}^{-1} \). Ethylene concentrations were measured by withdrawing 1 mL samples of gas by hypodermic syringe from the outlets of plastic respiration containers and injecting them into a gas chromatograph (Gow Mac, Model 580, USA) fitted with a stainless steel column (2 m X 0.3 mm ID) packed with activated alumina (80-100 mesh) and a flame ionization detector.

The retention time and concentration of ethylene were calibrated against an ethylene mixture containing 1.9 \( \mu \text{L.L}^{-1} \) in nitrogen (BOC Gases, Australia). Peaks of separated gases were recorded on a strip chart recorder (Miller
Graphic Controls Pty. Ltd., Australia). Operating conditions were: column temperature 110°C, detector temperature 110°C, injection port temperature 80°C, air 300 mL.min⁻¹, hydrogen 24 mL.min⁻¹ and nitrogen carrier gas 28 mL.min⁻¹.

The rate of ethylene production by avocado was calculated as follows:

\[
\text{C}_2\text{H}_4 \ (\mu\text{L.kg}^{-1}.\text{h}^{-1}) = \frac{\text{Peak height of samples} \times A}{\text{Peak height of standard} \times A} \times \frac{C \times FR}{W}
\]

where  
A = attenuation  
C = concentration of ethylene in standard mixture (μL.L⁻¹)  
FR = flow rate (L.h⁻¹)  
W = weight of samples (kg).

3.2.7. Statistical Analysis of Data.

The data were statistically analysed using COSTAT version 2.0 (Cohort software Berkeley California U.S.A., 1990). All physical and biochemical parameters were analysed using a completely randomised analysis of variance and a test of Duncan's multiple range comparisons. A Standard Errors were used to demonstrate variability of some parameters.
3.3. RESULTS AND DISCUSSION.

3.3.1. Experiment 1 (1995-1996)

3.3.1.1. Colour changes

Hass fruit for this experiment were obtained from Kulnura, New South Wales in November 1995. Fruit were transferred from cool storage at 0°C to 20°C at intervals of 3, 6 and 9 weeks. Skin colour changes were assessed at two-day intervals (Fig. 3.1 - 3.3). All fruit held in various CA conditions were still green when transferred to air after 3 weeks storage. The data showed that fruit stored for 3 weeks in atmospheres containing 2.5% O₂ combined with 5 and 7.5% CO₂, and 5% O₂ with 5% CO₂ coloured normally (attained score 4). However, the fruit stored in CA containing 10% CO₂ only achieved colour stages 3 and 3.5. The fruit stored in an atmospheres of 5% O₂ with 7.5% CO₂ changed colour rapidly by the second day at 20°C and then remained constant at score 3 until day 6 (Fig. 3.1).

Fruit stored in CA for 6 and 9 weeks changed colour slowly during storage. They had almost reached colour stage 2 when they were transferred to air, except for the fruit stored in an atmosphere of 2.5% O₂ combined with 7.5 or 10% CO₂ which remained green. Fruit ripened at 20°C following storage in treatments 1 (2.5% O₂ + 10% CO₂), 7 (7.5% O₂ + 10% CO₂), 9 (7.5% O₂ + 7.5% CO₂) and air for 6 weeks did not attain full colour (Fig 3.2). Fruit transferred to air at 20°C for 6 days following storage in treatments 1 (2.5% O₂ + 10% CO₂) and 2 (2.5% O₂ + 5% CO₂) for 9 weeks only achieved colour stage 3 (Fig. 3.3).
Similarly, fruit stored in CA for 9 weeks changed colour slowly during storage. All CA treatments almost reached colour stage 2. Darker colour was noted in fruit stored in 2.5 % O$_2$ and 10 % CO$_2$. This fruit had reached colour stage 2.5 when transferred to air from 0°C and only achieved colour stage 3 after 6 days at 20°C.
Fig 3.1. Skin colour change of avocado fruit ripened in air for 6 days at 20°C following storage at 0°C for 3 weeks (data 1995-1996). Means for each ripening time with different letters were significantly different at p<0.05 (Duncan's multiple range comparisons).
Fig 3.2. Skin colour change of avocado fruit ripened in air for 6 days at 20°C following storage in CA at 0°C for 6 weeks (data 1995-1996). Means for each ripening time with different letters were significantly different at p<0.05 (Duncan's multiple range comparisons).
Fig 3.3. Skin colour change of avocado fruit ripened in air for 6 days at 20°C following storage at 0°C for 9 weeks (data 1995-1996). Means for each ripening time with the different letters are significantly different at p<0.05 (Duncan’s multiple range comparisons).
3.3.1.2. Fruit firmness

Samples of fruit were transferred from cool storage at 0 to 20°C at intervals of 3, 6 and 9 weeks (Fig. 3.4). Fruit firmness was measured after holding the fruit at 20°C for 6 days. All fruit held in CA for 3 weeks softened normally after transfer to air at 20°C. Fruit stored in an atmosphere containing 10 % CO₂ combined with 2.5 and 5 % O₂ for 6 weeks remained firm (78.45 N) after transfer to air for 6 days at 20°C. Fruit from the other atmospheres softened normally. Fruit stored for 9 weeks in 10 % CO₂ and 2.5 % O₂ softened after transfer to 20°C but fruit from other mixtures containing 10 % CO₂ did not soften. However, the flesh of fruit from 10 % CO₂ had broken down. Fruit from other CA mixtures and air softened normally.

These results showed that storage for more than 3 weeks in CA mixtures containing 10 % CO₂ was injurious. Normal softening were inhibited and in the case of fruit held in 10 % CO₂ and 2.5 % O₂ the tissue broke down. The scores for skin colour development appeared normal for all CA mixtures and air, however colour was abnormal (brown black purple) in the 10 % CO₂ mixtures.
Fig. 3.4. Changes in firmness of avocado fruit following transfer to air at 20°C for 6 days after storage at 0°C for 3, 6 and 9 weeks (data 1995-1996). Means for each ripening time with different letters were significantly different at p<0.05 (Duncan's multiple range comparisons).
3.3.1.3. Chilling injury.

The severity of CI in the flesh was assessed at day 6 following transfer of the fruit to air at 20°C after CA storage at 0°C for 3, 6 and 9 weeks (Fig. 3.5). CI was not detected in fruit stored in air or in fruit stored in most CA treatment after 3 weeks storage. Very light discoloration (score 1) was observed in fruit stored in 5 % O₂ + 10 % CO₂ and 7.5 % O₂ + 7.5 % CO₂. More discoloration was noted in the fruit stored in CA containing 5 % O₂ combined with 7.5 % carbon dioxide.

The severity of CI was more severe in most fruit after transfer to air at 20°C for 6 days following CA storage for 6 weeks at 0°C. Very light discoloration (score 1) was observed in fruit stored in 2.5 % oxygen and combined with 5 and 7.5 % carbon dioxide and 5 % oxygen combined with 5 % carbon dioxide, while fruit stored in air and in 7.5 %CO₂ and 7.5 %O₂ had light discoloration. These fruit attained maximum colour 4 (normal) compared to other treatments that only achieved colour score 3 (Fig. 3.2). Fruit from all other atmospheres had high levels of CI.

After 9 weeks in CA conditions only fruit held in 2.5 % oxygen combined with 5 and 7.5 % carbon dioxide, and 5 % O₂ combined with 5 % CO₂ exhibited light flesh discoloration. Overall, these treatments gave the best results in this study and the fruit ripened normally.
Fig. 3.5. Severity of CI of avocado flesh after 6 days in air at 20°C following transfer from storage at 0°C for 3, 6 and 9 weeks (data 1995-1996). Means for each ripening time with different letters were significantly different at p<0.05 (Duncan's multiple range comparisons).
CI in other CA and air treatments was very severe after ripening in air at 20°C for 6 days following 9 weeks at 0°C. It is apparent that atmospheres containing 10% CO₂ injure the fruit by interfering with softening. The symptoms of CI in these treatments were black lesions on the skin and grey black discoloration of the flesh. Similar symptoms were reported in Hass fruit stored at 0° and 2°C (Hopkirk et al., 1994).

The most promising atmospheres were 2.5% O₂ + 5% CO₂, 2.5% O₂ + 7.5% CO₂, 5% O₂ + 5% CO₂ and 5% O₂ + 7.5% CO₂. It was resolved to further test this more limited range of atmospheres at 0 and 5°C, 5°C was included as Hass has been shown to be much less chilling sensitive at this temperature.
3.3.2. Experiment 2: 1996-1997.

3.3.2.1. Colour changes

The most promising atmospheres from experiment 1 were selected for further evaluation in 1996-1997. Hass fruit for this experiment were obtained from Kulnura, NSW in February 1997. The fruit were stored in atmospheres containing 2.5 % O₂ combined with 5 and 7.5 % CO₂, and 5 % O₂ combined with 5 and 7.5 % CO₂ at 0 and 5°C for 3, 6 and 9 weeks (Fig. 3.6 - 3.8). The fruit coloured normally after storage for 3 weeks at 0°C following transfer to air at 20°C for 6 days. The rate of skin colouring of avocado stored in CA was slower after storage for 3 weeks at 5°C (Fig. 3.6). These fruit were still green in colour (score 1-2) following 6 days at 20°C and they attained a dark colour when fully softened (day 14). These results showed that skin colour changed more rapidly following storage for 3 weeks at 0°C compared to storage at 5°C. This difference was probably due to an acceleration of ripening resulting from chilling at 0°C. It was noted that in experiment 1 fruit stored in the same atmosphere coloured normally after storage for 3 weeks at 0°C following transfer to air at 20°C (Fig. 3.1).

Fruit stored for 6 weeks in CA at 0°C had almost reached colour stage 2 when they were transferred to air, but they did not attain full colour by 6 days at 20°C (Fig. 3.7). However, fruit stored for 6 weeks at 5°C in CA
Fig. 3.6. Changes in colour of avocado fruit following transfer to air at 20°C after storage at both temperatures 0°C (above) and 5°C (bottom) for 3 weeks. Means with the different letters were significantly different at p<0.05 (Duncan’s multiple range comparisons).
Fig. 3.7. Changes in colour of avocado fruit following transfer to air at 20°C after CA storage at both temperatures 0°C (above) and 5°C (bottom) for 6 weeks. Means for each ripening with different letters were significantly different at p<0.05 (Duncan's multiple range comparisons).
Fig. 3.8. Changes in colour of avocado fruit following transfer to air at 20°C after CA storage at both temperatures 0°C (above) and 5°C (bottom) for 9 weeks. Means for each ripening time with different letters were significant different at p<0.05 (Duncan’s multiple range comparisons).
coloured normally by 6 days, except for fruit stored in an atmosphere of 2.5 % \text{O}_2 combined with 5 % \text{CO}_2, which required 10 days to attain full colour. These fruit ripened normally but CI was detected in ripened fruit. Fruit stored at 0\textdegree C for 9 weeks continued to colour during CA storage. They had reached colour stage 2.5 - 3 when they were transferred to air. However, the fruit stored in CA for 9 weeks at 5\textdegree C remained green after transfer to air for 6 days (Fig. 3.8). Avocado fruit stored in air changed colour while in storage for 9 weeks at 5\textdegree C. They reached colour stage 2 and remained at this stage for 2 days then attained maximum colour after 6 days at 20\textdegree C. These results support those of Meir et al., (1995) who reported that CA storage retarded changes in skin colour of Hass following storage for 3, 6 and 9 weeks.
3.3.2.2. Fruit firmness

The rates of softening of the fruit following transfer to air at 20°C were strikingly affected by storage temperature (Figs. 3.9 and 3.10). Fruit were fully soft (< 10 N) following 3 weeks storage at 0°C and this fruit developed a normal brown black skin. The differences between the CA treatments and air storage were not significant. In contrast fruit stored 3 weeks at 5°C in all CA mixtures were firm and green on transfer to air and had not ripened fully after 6 days at 20°C. These fruit required up to 14 days to complete ripening (data not shown). Fruit stored in air at 5°C softened completely within 6 days at 20°C. All fruit stored for 6 and 9 weeks at 0°C from all atmospheres softened normally during ripening at 20°C for 6 days. Fruit stored at 5°C took progressively less time to complete softening following 6 and 9 weeks storage and by 9 weeks softening was essentially completed by 6 days in air at 20°C. Atmospheres of 2.5 % O₂ + 5 % CO₂, 2.5 % O₂ + 7.5 % CO₂, 5 % O₂ + 5 % CO₂ and 5 % O₂ + 7.5 % CO₂ retarded softening significantly.

The observations that storage at 0°C for 3 weeks can accelerate softening compared to storage at 5°C for 3 weeks has not been reported previously. CA did not affect softening in this experiment, confirming observations in the 1995-1996 experiment for atmospheres containing up to 5 % O₂ and 7.5 %CO₂. This suggest that chilling for up to 3 weeks at 0°C accelerates ripening. Softening of the fruit stored at 5°C was retarded by CA mixtures compared to air as expected since this temperature has been
recommended for Hass and is not expected to cause CI. The progressively earlier softening following storage for 6 and 9 weeks at 5°C would be expected as the fruit approached the end of their storage life.

Fig. 3.9. Changes in firmness of avocado fruit following transfer to air for 6 days after storage at 0°C for 3, 6 and 9 weeks (data 1996-1997). Means for each treatment with different letters were significantly different at p<0.05 (Duncan’s multiple range comparisons).
Fig. 3.10. Changes in firmness of avocado fruit following transfer to air for 6 days after CA storage at 5°C for 3, 6 and 9 weeks (data 1996-1997). Means for each treatment with different letters were significantly different at p<0.05 (Duncan’s multiple range comparisons).
3.3.2.3. Chilling injury

CI of the flesh was assessed after 6 days at 20°C following transfer from 0º or 5ºC storage for 3, 6 and 9 weeks (Figs. 3.11 and 3.12). No CI was detected in fruit stored in air or CA mixtures after 3 weeks at 0ºC storage but CI was detected and became progressively more severe after storage for 6 and 9 weeks (Plate 3.3 - 3.10).

Fig. 3.11. Severity of CI of avocado flesh after 6 days at 20°C following transfer to air after storage at 0°C for 6 and 9 weeks (data 1996-1997). No symptoms of CI were apparent after 3 weeks storage. Means for each treatment with different letters were significantly different at p<0.05 (Duncan's multiple range comparisons).
Contrary to expectations, CA did not reduce CI at 0°C, and instead appears to have made it worse. These results contrasts with those of Experiment 1 (1995-96) in which the same atmospheres delayed CI. Since the fruit used in these experiments came from the same trees, the poorer response of the fruit in Experiment 2 (1996-97) may have been due to the advanced physiological age of this fruit. Lee et al. (1983), reported that differences in physiological ages of the fruit resulted in differences in time to reach the respiratory climacteric.

There was no CI in all CA treatments after 3 and 6 weeks at 5°C but symptoms were detected in the air stored control after 6 weeks storage (Fig. 3.12). The symptoms of CI may be related to the development of brownish black in the peel due to low O₂ and/or high CO₂ concentrations. Slight symptoms of CI were observed after 9 weeks storage in the CA mixtures, but the air stored controls broke down after transfer to air at 20°C. Comparison of the results obtained in this experiment showed that all CA atmospheres retarded CI at 5°C, but if anything, increased injury at 0°C. Fruit stored for 9 weeks at 5°C in CA containing 2.5 % O₂ + 5 % CO₂ and 5 % O₂ + 7.5 % CO₂ developed very slight discolouration. There was no CI in fruit stored in CA comprising 2.5 % O₂ + 7.5 % CO₂, or 5 % O₂ + 5 % CO₂ and the fruit ripened normally (Plate 3.7 - 3.10). The results of CA storage at 5°C confirmed similar studies by Meir et al. (1995) who reported that Hass fruit stored in CA of 3 % O₂ and 8 % CO₂ for 9
weeks at 5°C were free of CI. Faubion and Kader (1994) reported that severe CI developed in Hass stored in air for 6 weeks at 5°C but only moderate symptoms after 10 weeks storage in 2% O₂ + 5% CO₂. Metzidakis and Sfakiotakis (1995) reported that the symptoms of physiological disorders of avocado fruit exposed to low O₂ concentrations were characterised by browned areas in the epidermis usually near the stem. Abnormal ripening was associated with generalised browning of the flesh. Pesis et al. (1994) found that pre storage treatment of Fuerte fruit with 3% O₂ and 97% N₂ for 24 h prior to cold storage for 3 weeks at 2°C resulted in light CI in 10% of the fruit, whereas more than 50% of control fruit had indicate very severe CI. Treating Fuerte avocado with 2% O₂ and 10% CO₂ or 25% CO₂ for 3 days before storage for 28 days at 5.5°C reduced the incidence of CI (Bower et al., 1990).
Fig. 3.12. Severity of chilling injury of avocado flesh after 6 days at 20°C following transfer from CA storage at 5°C for 3, 6 and 9 weeks (data 1996-1997). Means for each treatment with different letters were significantly different at p<0.05 (Duncan's multiple range comparisons).
Plate 3.3. CI symptoms of avocado fruit after 6 days in air at 20°C following CA (2.5 % O₂ + 5 % CO₂) storage at 0°C (A) and 5°C (B) for 6 weeks. The fruit stored at 0°C exhibited severe discoloration whereas the fruit stored at 5°C were not discoloured.
Plate 3.4. CI of avocado fruit following transfer to air for 6 days after CA(2.5% O₂ + 7.5% CO₂) storage for 6 weeks at 0°C (A). The fruit stored at 5°C in the same atmosphere exhibited no discolouration (B). The fruit stored at air for 6 weeks at 0°C exhibited CI and thickening of the skin (C).
Plate 3.5. Fruit exhibited very light discolouration after 6 days in air at 20°C following CA(5%O₂+5%CO₂) storage for 6 weeks at 0°C (A). The fruit stored at 5°C in the same atmosphere exhibited no discolouration (B). The fruit stored in air for 6 weeks at 5°C exhibited very light discolouration (C).
Plate 3.6. CI of avocado fruit after transfer to air for 6 days following CA (5% O₂ + 7.5% CO₂) storage for 6 weeks at 0°C (A) and 5°C (B). The fruit stored at 0°C showed very light discolouration, whereas the fruit stored at 5°C were not discoloured.
Plate 3.7. CI symptom of avocado fruit after 6 days in air at 20°C following CA (2.5 % $O_2 + 5 % O_2$) storage at both temperatures of 0°C (A) and 5°C (B) for 9 weeks. The fruit stored at 0°C exhibited severe discoulouration whereas the fruit stored at 5°C were not discoloured.
Plate 3.8. CI symptoms of avocado fruit after transfer to air at 20°C following CA (2.5 % O₂ + 7.5 % CO₂) storage at 0°C (A) and 5°C (B) for 9 weeks. The fruit stored at 0°C exhibited severe discolouration whereas the fruit stored at 5°C were not discoloured. The fruit exhibited severe discolouration after transfer to air for 6 days at 20°C following air storage at 0°C for 9 weeks (C).
Plate 3.9. CI symptoms of avocado fruit after transfer to air at 20°C following CA (5 % O₂+5 % CO₂) storage at 0°C (A) and 5°C (B) for 9 weeks. The fruit stored at 0°C exhibited severe discoloration whereas the fruit stored at 5°C were not discoloured. The fruit exhibited severe discoloration and had become very soft following transfer to air for 6 days at 20°C after air storage at 5°C for 9 weeks (C) indicating severe tissue breakdown.
Plate 3.10. CI symptoms of avocado fruit after transfer to air at 20°C following CA (5% O₂ + 7.5% CO₂) storage at 0°C (A) and 5°C (B) for 9 weeks. The fruit stored at 0°C exhibited severe discoloration whereas the fruit stored at 5°C were not discoloured.
3.4. Conclusions:

1. Skin colour developed normally (attained score 4) in both experiments 1 and 2 following CA storage for 3 weeks at 0°C. However, the fruit changed colour during storage in atmospheres containing high CO₂ (7.5 and 10 %) for 6 weeks and attained only a maximum score of 3 during ripening in air for 6 days at 20°C. Softening of fruit stored at 0°C was inhibited by CA containing 10 % CO₂. The results show that these atmospheres caused physiological damage. Atmospheres containing 10 % CO₂ were omitted from Experiment 2.

2. In experiment 2 storage of fruit at 0°C induced more rapid ripening when the fruit were returned to air. CA did not prevent this response. In contrast, CA storage suppressed development of skin colour following storage for 3 weeks at 5°C and the fruit were still green (score of 1-2) on transfer to air at 20°C. This fruit attained maximum colour after 14 days. Time to ripen decreased with longer storage and all CA stored fruit ripened normally following storage for 6 and 9 weeks. Air stored controls broke down after 9 weeks storage.

3. Fruit suffered no CI and ripened normally following transfer to air for 6 days at 20°C after CA storage for 3 weeks at both 0°C and 5°C. However, CI symptoms developed after CA storage for 6 and 9 weeks at 0°C. The symptoms were more severe following storage at 0°C in experiment 2 than in experiment 1, probably because the fruit were physiological older.
4. There was no CI in atmospheres of 2.5 % O₂ + 7.5 % CO₂, and 5 % O₂ + 5 % CO₂ following storage for 9 weeks at 5°C and only very light discolouration in atmospheres of 2.5 % O₂ + 5 % CO₂ and 5 % O₂ + 7.5 % CO₂ (score 0.5).

5. Although Hass avocado do not ripen while attached to the tree, it appears that physiological changes continue that may reduce the ability of CA mixtures containing low O₂ and raised CO₂ concentrations to delay CI.

6. Experiment 2 led to the original observation that storage for up to 3 weeks at 0°C actually stimulated ripening when the fruit were returned to 20°C whereas storage at 5°C delayed ripening. In the latter fruit CA delayed ripening and senescence compared to air storage.

7. Comparative studies of biochemical changes in fruit stored in 0° and 5°C in the most promising atmosphere may explain the observed differences in physiological responses.

8. Based on the review of literature these measurements should include respiration rate, ethylene production, changes in ACC oxidase activity, ACC and polyamines concentrations. Fruit from experiment 2 were used for this phase of the study, which is described in section IV.
SECTION IV

BIOCHEMICAL CHANGES IN AVOCADO IN CA STORAGE

4.1. Introduction

Exposure to high concentrations of carbon dioxide has been shown to slow the respiration rate of avocado (Yang et al., 1962; Biale and Young, 1971; Hatton and Spalding, 1990; Lange and Kader, 1997). Marcellin and Chaves (1983) showed that intermittent exposure of unripe Hass avocado to 20% CO₂ while stored in air delayed senescence at 12°C and reduced fruit ethylene evolution more than respiration. The rates of respiration and ethylene production were lower following pre-storage treatment of avocado fruit with an atmosphere of 3 %CO₂ and 97 %N₂ for 24 hours prior to storage at 2°C and 17°C (Pesis et al., 1994). Use of CA storage appears promising for the extension of shelf life of avocado and has numerous commercial possibilities, however the specific requirements of each cultivar should be evaluated (Hatton and Spalding, 1990). Measurements of ethylene production and the rates of respiration are important for monitoring whether the fruit remain capable of normal ripening. Storage atmospheres should be controlled to prevent anaerobic conditions. Storage in low O₂ (0.25%) and high CO₂ concentrations (80%) induced the appearance of new alcohol dehydrogenase (ADH) isoenzymes in avocado (Kanellis et al., 1991; Ke et al., 1995) and suppressed the expression of ACC synthase and ACC oxidase in
apple (Gorny and Kader, 1996a). Application of polyamines has been shown to inhibit the production of ethylene in apple tissues (Winer and Apelbaum, 1986). While there is some evidence for an inverse relationship between the concentrations of polyamines and ethylene production during avocado fruit development, their roles in avocado ripening remain unknown (Kushad et al., 1988).

In this study, biochemical responses of Hass avocado ventilated with gas mixtures containing low O₂ and raised CO₂ during storage at 0 and 5°C were examined. The rates of respiration and ethylene production, activity of ACC oxidase, and concentrations of ACC and polyamines were determined.
4.2. Materials and Methods

4.2.1. Experimental Plan.

Mature avocado fruit (*Persea americana* Mill cv Hass) were obtained from a commercial orchard at Kulnura, New South Wales. The fruits were transported 120 km to the UWSH, Richmond Campus where they were treated as described in section 3.2.


The fruit were harvested on November then sorted for mass uniformity, dipped in 0.2% ‘Sportak’ (Schering Pty. Ltd), and dried at 20°C for about 30 minutes. Samples of 12 fruits were enclosed in each of thirty polyethylene containers (10 L) and were stored at 0°C. Groups of three containers were ventilated with the following atmospheres at 12 L.h⁻¹. (Table 4.1). One sample from each atmosphere was transferred to 20°C at 3, 6 and 9 weeks of storage. Three fruit from each treatment were enclosed singly in polyethylene containers (1 L) which were ventilated with humidified air at 6 L.h⁻¹. These fruit were used for measurement of rate of respiration and ethylene production. Another three fruit from each treatment were taken for analyses of ACC oxidase activity, ACC and polyamines concentrations at two-day intervals. Fruit from this experiment were assessed for their ability to ripen, softening and the occurrence of chilling injury (Section III).
Table 4.1. Experimental CA storage plan at 0°C (1995-96).

<table>
<thead>
<tr>
<th>Atmospheres</th>
<th>At harvest</th>
<th>3 weeks</th>
<th>6 weeks</th>
<th>9 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2.5 % O₂ + 10 % CO₂</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2.5 % O₂ + 7.5 % CO₂</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2.5 % O₂ + 5 % CO₂</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5 % O₂ + 10 % CO₂</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5 % O₂ + 7.5 % CO₂</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5 % O₂ + 5 % CO₂</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7.5 % O₂ + 10 % CO₂</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7.5 % O₂ + 5 % CO₂</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*+ Indicates when samples were transferred to 20°C.*

The rates of respiration and ethylene production of freshly harvested avocado were measured daily. ACC concentration and the activity of ACC oxidase of freshly harvested avocado were measured at two-day intervals. The rates of respiration and ethylene production were measured daily at 20°C following CA storage for 3, 6 and 9 weeks at 0°C. The activity of ACC oxidase, ACC and polyamine concentrations were analysed at days 0, 2, 4 and 6 at 20°C following CA storage (Table 4.2).

The activity of ACC oxidase and ACC concentration were measured in fresh samples, polyamines concentrations were examined in tissue samples that were frozen in liquid N₂, then stored at -80°C.
Table 4.2. Fruit sampling plan following storage at 0°C.(1995-96)

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Rates of Respiration and Ethylene production</th>
<th>ACC oxidase activity, ACC and Polyamines concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+ (3)</td>
<td>+ (3)</td>
</tr>
<tr>
<td>1</td>
<td>+ (3)</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+ (3)</td>
<td>+ (3)</td>
</tr>
<tr>
<td>3</td>
<td>+ (3)</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+ (3)</td>
<td>+ (3)</td>
</tr>
<tr>
<td>5</td>
<td>+ (3)</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>+ (3)</td>
<td>+ (3)</td>
</tr>
</tbody>
</table>

+ (3) Three fruit were assessed at each time interval.

**Experiment 2: 1996-1997.**

Fruit were harvested on February then sorted for mass uniformity, dipped in 0.2 % ‘Sportak’ (Schering Pty. Ltd), dried at 20°C for about 30 minutes. Samples of 36 fruits were enclosed in each of thirty polyethylene containers (30L). Fifteen containers were stored at 0 and 5°C respectively. Groups of three containers were ventilated with the following atmospheres at 12 L.h⁻¹. (Table 4.3). One sample from each atmosphere was transferred to 20°C at 3, 6 and 9 weeks of storage. Three fruit from each treatment were enclosed singly in polyethylene containers (1 L) ventilated with humidified air at 6 L.h⁻¹. These fruit were used for measurement of rate of respiration and ethylene production. Another nine fruit from each treatment were analysed for ACC oxidase activity and ACC concentrations at two-days interval. The rates of respiration and ethylene production of freshly harvested avocado were measured daily. ACC concentration and the activity of ACC oxidase of freshly
harvested avocado were measured at two-day intervals. Other fruit from these treatments were assessed for changes in skin colour, softening and CI following transfer to 20°C (Section III). The rates of respiration and ethylene production of fruit were measured daily at 20°C following CA storage for 3, 6 and 9 weeks at 0°C. The activity of ACC oxidase and ACC concentrations were analysed at days 0, 2, 4 and 6 at 20°C following CA storage for 3, 6 and 9 weeks at 0° and 5°C.

Table 4.3. Experimental CA storage plan at 0° and 5°C (1996-97).

<table>
<thead>
<tr>
<th>Atmospheres</th>
<th>Storage times</th>
<th>At harvest</th>
<th>3 weeks</th>
<th>6 weeks</th>
<th>9 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2.5 % O₂ + 5 % CO₂</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>2.5 % O₂ + 7.5 % CO₂</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>5 % O₂ + 5 % CO₂</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>5 % O₂ + 7.5 % CO₂</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

+ Indicates when samples were transferred to 20°C

The following measurements (Table 4.4) were made after each storage interval (the number of fruit from each replication is shown in brackets).

Table 4.4. Fruit sampling plan following storage at 0 and 5°C (1996-97).

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Rates of Respiration and Ethylene production</th>
<th>ACC oxidase activity and ACC concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+ (3)</td>
<td>+ (3)</td>
</tr>
<tr>
<td>1</td>
<td>+ (3)</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+ (3)</td>
<td>+ (3)</td>
</tr>
<tr>
<td>3</td>
<td>+ (3)</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+ (3)</td>
<td>+ (3)</td>
</tr>
<tr>
<td>5</td>
<td>+ (3)</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>+ (3)</td>
<td>+ (3)</td>
</tr>
</tbody>
</table>

+ (3) Three fruit were assessed at each time interval.
4.2.2. Rates of respiration and ethylene production

As described in Section 3.2.6.

4.2.3. Concentration of 1-aminocyclopropane-1-carboxylic Acid (ACC)

ACC concentrations were determined at each sampling interval in the pulp of three individual fruit used for measurements of flesh firmness according to the procedure of Lizada and Young (1979) with the following modifications. Plugs of pulp tissues were taken from the equatorial region with a No. 8 cork borer and sliced with a razor blade to yield about 1 g fresh weight tissue. The pulp samples were placed in 25 x 180 mm test tubes and heated by microwave for 30 seconds at full power using a Panasonic Model NN-6558 microwave oven to inactivate enzyme activity. Five ml of 0.1 M HCl in 80 % methanol was then added and the tubes held at 20°C for 4 hours with occasional mixing. ACC was determined in 0.5 mL aliquots of the extract without filtration in 30 mL McCarthy bottles. The caps were modified and fitted with silicone rubber seals to enable insertion of hypodermic needles.

To determine recoveries, 1 nM ACC (Sigma) was added to one bottle of paired samples. Samples were brought to equal volume (800 µL) with water and neutralised to the phenolphthalein end point with drop-wise
addition of 10% KOH. Mercuric chloride (0.2 mL of 0.1 M) was added and the McCarthy bottles were sealed. Ten drops of 5% NaOCl in 50% NaOH (2:1) were injected via a 25-gauge needle and the solution was mixed for 20 seconds to convert ACC to ethylene. Gas samples were taken for ethylene analysis after 15 minutes using an Autosystem Gas Chromatograph (Perkin Elmer) fitted with a Photoionization Detector (PID). Operating conditions were: oven temperature 130°C, injector port temperature 90°C and detector temperature 100°C. The pore column and analytical column were each 90 cm x 2 mm ID stainless steel, packed with activated alumina (80 - 100 mesh) ACC was calculated using the percentage recovery of ACC added to the paired samples. The results were reported as nmoles/g of fruit tissue.

4.2.4. ACC oxidase activity

ACC oxidase activity was determined in pulp tissue sections according to the procedure of Butler (1986) with the following modifications. Plugs of pulp tissues were taken from the equatorial region with a No. 8 cork borer and sliced with a razor blade to yield about 1 g fresh weight tissue. The pulps samples were placed in 25 x 180 mm test tubes containing 10 mL of solution comprising 0.1 mM ACC dissolved in 0.4 M Sucrose and 0.02 M CaCl₂ in water. After 30 minutes the pulp discs were removed from the solution and they were quickly blotted dry with tissue paper and placed in a 10 mL plastic syringe. Immediately after enclosure in
the syringe, CO$_2$ was added to establish a concentration of 5% CO$_2$. After 30 minutes the accumulated ethylene was measured by removing a 1 mL sample and analysed with an Autosystem Gas Chromatography (Perkin Elmer) fitted with PID detector (Section 4.2.3). ACC oxidase activity (the ability to convert ACC to ethylene) was expressed nL g$^{-1}$ of fruit tissue. Saturating concentrations of ACC were supplied to ensure that ACC oxidase activity was not limited by substrate availability (Jobling, 1993).

4.2.5. Polyamine.

Polyamine concentrations were determined at each sampling interval in pulp sections of three individual fruit used for flesh firmness according to the procedure of Kramer et al., (1989) with the following modifications. Pulp tissues were taken in the form of cubes from the equatorial region with a knife to yield about 2 g fresh weight samples. Pulp samples were stored at -80°C for later extraction.

Extracts for polyamine analysis were prepared by homogenising 2.0 g of tissue in 15 mL of 5% perchloric acid using a Waring blender. Before homogenisation, 1,8-octanediamine (150 nmol.g$^{-1}$ fresh weight) was added as an internal standard. The homogenate was then centrifuged at 8000 x g for 20 minutes (Beckman GS-6R Centrifuge). The supernatant was saved for polyamine analysis. Dansylation was performed by mixing 400 µL of 10 mg dansyl chloride.mL$^{-1}$ (in acetone) and 150 µL of saturated sodium bicarbonate
with 200 μL of tissue extract. After incubation overnight at room temperature, 250 μL proline.mL⁻¹ was added and the incubation was continued for one hour. After centrifugation in a Beckman GS-6R Centrifuge at 8000 x g for 10 min, the pH of the supernatant was adjusted to 6.8. Samples of 100 μL of the supernatant were used for HPLC analysis (Hugo et al., 1987). HPLC was performed with a system consisting of two pumps (Waters 501 and Waters 510). Samples were injected using a Waters U6K injector onto a reverse-phase 25 cm C-18 column (Supelco). Samples were eluted from the column at a flow rate of 1.5 mL.min⁻¹ with a programmed solvent gradient of 0, 100, 0; 15, 0, 100; 18, 0,100; where the first number was the time (minutes), the second number was the percent of buffer A (60 Methanol: 40 water), and the third number was the buffer B (10 ethanol). Elution was completed in 18 min. Products were detected with a Tunable Absorbance Detector (Waters 484) using an excitation wavelength of 365 nm. The pumps were controlled and data collected and analysed using a Master 386 SX Computer system equipped with a Baseline 810 Chromatography Work Station (Dynamic Solutions). Total polyamines were quantified by the comparison of sample peak areas with those of the known standard. Each mean was the average of three independent samples from each treatment (Kramer and Wang, 1989).
4.3. Result and Discussion


4.3.1.1. Rates of respiration and ethylene production of avocado fruit at 20°C after CA storage at 0°C.

Freshly harvested fruit showed climacteric patterns of CO₂ and ethylene production, with peaks recorded on the 14th day (Fig. 4.1). The pattern of changes in respiration rates and ethylene production during ripening of avocado fruit transferred to air at 20°C were measured daily for 6 days, following CA storage for 3 weeks at 0°C (Fig. 4.2). The rates of respiration and ethylene production of fruit stored in air were higher than those of fruit stored in CA treatments. Respiration rates and ethylene production show climacteric-like peaks by days 2 - 4 for air and CA compared to harvest control that reached a peak at 14 days. The lowest rates of ethylene production were recorded in fruit stored in CA mixtures of 5 % O₂ combined with 7.5 or 10 % CO₂. Generally, the CA treatments reduced the respiratory peak and ethylene production as compared to air. In comparison to harvested fruit that reached a peak at day 14 (Fig. 4.1), these data show that ethylene production and respiration were stimulated by chilling at 0°C, peaking 2 - 3 days after transfer to 20°C and decreasing thereafter. CA treatments at
low temperature (0°C) generally reduced the rates of respiration and ethylene production.

Similar patterns of changes in respiration rates and ethylene production during ripening of avocado at 20°C were observed after CA storage for 6 weeks at 0°C (Fig. 4.3). The climacteric-like peaks occurred one or two days later than in fruit stored for 3 weeks. As noted for fruit stored for 3 weeks at 0°C, ethylene production was low in an atmosphere of 5% CO₂ combined with 5 and 7.5% O₂ after storage for 6 weeks at 0°C but in both cases respiration rates were similar to those in the other CA mixtures. The increase in CO₂ production by avocado stored at 0°C was possibly due to the increased ethylene production stimulated by chilling. However, the rates of respiration of CA fruit were remained lower than the fruit stored in air. A similar persistent suppression of CO₂ production was reported for Fuerte pre-treated in a low O₂ atmospheres (3% O₂ and 97% N₂) during storage at 2°C and 17°C (Pesis et al., 1994). An increase in respiration following chilling appears to be a common response in non-climacteric lemons, beans and potatoes (Wang, 1990).

After 9 weeks at 0°C initial respiration rates were generally higher in air stored fruit after one day at 20°C than in CA stored fruit. Peak respiration rates were similar to those for fruit stored for 3 and 6 weeks at 0°C, but ethylene production was generally lower (Fig. 4.4). The respiration rates reached a peak 2 - 3 days after transfer to 20°C. The fruit softened after 6
days in air, except those fruit stored in an atmosphere 10 % CO₂ combined with 5 and 7.5 % O₂.

The observed increase in respiration appeared to be related to development of symptoms of CI (Fig 3.5). The data reported here confirm the work of Lange and Kader (1997a, 1997b) who reported that Hass avocado stored in air had higher respiration rates than fruit stored with a high CO₂ concentration.

Despite a general reduction in respiration rates in fruit stored in CA after transfer to air at 20°C and some reduction in ethylene production by some CA mixtures there were no clear association between the CA mixtures and the incidence of CI (Fig 3.5) with the possible exception of mixtures containing 5 and 7.5 % CO₂ combined with 2.5 % O₂ and 5 % CO₂ combined with 5 % O₂.

It is proposed that CI in Hass fruit at 0°C stimulates synthesis of ethylene and increased respiration rates, Ca partly suppressed the increase in respiration following transfer to 20°C after storage at 0°C for up to 9 weeks but only had an effect on ethylene production for the first 3 weeks of storage.

As CI became more severe by 6 weeks of storage at 0°C, the increase in ethylene production after transfer to 20°C was diminished.
Fig. 4.1. Rates of respiration (A), ethylene production (B), ACC concentrations (C) and ACC oxidase activity (D) of harvested avocado fruit in November 1995 (exp 1) and February 1997 (exp 2). Results are an average of triplicate measurements.
Fig. 4.2. Rates of respiration (A+B) and ethylene production (C+D) of avocado fruit after transfer to air at 20°C following storage for 3 weeks at 0°C. Data were obtained in experiment 1. Means for each ripening time with the different letters were significantly different at p<0.05 (Duncan's multiple range comparisons).
Fig. 4.3. Rates of respiration (A+B) and ethylene production (C+D) of avocado fruit after transfer to air at 20°C following storage for 6 weeks at 0°C. Data were obtained in experiment 1. Means for each ripening time with the different letters were significantly different at p<0.05 (Duncan's multiple range comparisons).
Fig. 4.4. Rates of respiration (A+B) and ethylene production (C+D) of avocado fruit after transfer to air at 20°C following storage for 9 weeks at 0°C. Data were obtained in experiment 1. Means for each ripening time with the different letters were significantly different at p<0.05 (Duncan’s multiple range comparisons).
4.3.1.2. Changes in ACC levels in fruit at 20°C after CA storage at 0°C.

ACC was not measurable until day 6 after harvest in control fruit ripened at 20°C (Fig. 4.1). Peak concentrations occurred on day 12, two days before the ethylene climacteric peak. ACC concentrations were very low in fruit removed from storage following 3 weeks at 0°C (Fig. 4.5) but then rose to a peak on day 2 and subsequently decreased to low concentrations on day 6. The CA mixtures did not have a consistent effect on ACC concentration. ACC was measurable in most treatments upon removal from 0°C after 6 weeks storage and maximum concentrations were measured on day 4 (Fig. 4.6). Subsequently, the concentrations decreased rapidly except for the air stored control fruit. ACC was measurable on transfer to 20°C following 9 weeks storage and it then rose to a maximum concentrations on day 2 in air and in some CA treatments and day 4 in others (Fig 4.7). Maximum concentrations of ACC and ethylene production were less than in fruit stored for 3 and 6 weeks at 0°C (Fig. 4.4 C+D and Fig. 4.7). In general, the pattern of changes in ACC concentrations reflected the changes in ethylene production (Section 4.3.1.1).

An accumulation of ACC in response to chilling at 0°C has been reported for Granny Smith apples (Jobling et al., 1991), while changes in ACC concentrations have been suggested as an indicator of chilling injury (Wang, 1990). In the present study, avocado fruit ripened earlier than freshly harvested control fruit evidenced by softening and skin colour changes. Contrary to other reports, CA had no consistent effects on ACC concentrations in this study. Gorny and Kader (1996) reported that low O₂ and rose CO₂ in apple can act individually and synergistically to suppress ACC synthase and this suppression are highly correlated to inhibition of ethylene biosynthesis.
Fig. 4.5. ACC concentrations in avocado fruit after transfer to air at 20°C following storage for 3 weeks at 0°C. Means for each ripening time with different letters were significantly different at p<0.05 (Duncan's multiple range comparisons).
Fig. 4.6. ACC concentrations in avocado fruit after transfer to air at 20°C following storage for 6 weeks at 0°C. Means for each ripening time with the different letters were significantly different at p>0.05 (Duncan's multiple range comparisons). Data were obtained in experiment 1 (1995-1996).
Fig. 4.7. ACC concentrations in avocado fruit after transfer to air at 20°C following storage for 9 weeks at 0°C. Means for each ripening time with the different letters were significantly different at p>0.05 (Duncan's multiple range comparisons). Data were obtained in experiment 1 (1995-1996).
4.3.1.3. Changes of ACC oxidase activity in fruit at 20°C after CA storage at 0°C

ACC oxidase activity of freshly harvested fruit ripened at 20°C paralleled the changes in ACC concentrations (Fig 4.1). ACC oxidase activity appeared to increase from day 4 and ACC from day 6. This result agrees with the reports of Sitrit et al., (1986) and Starret and Laties (1991). ACC oxidase activity paralleled ACC concentrations in fruit transferred to air at 20°C from storage at 0°C after 3, 6 and 9 weeks (Figs. 4.8 - 4.10). After 3 weeks storage ACC oxidase activity was measurable although ACC concentration was very low (Fig. 4.8). Activity of ACC oxidase increased to a peak at 2 days at 20°C, then decreased. ACC oxidase activity on the day of removal was similar in fruit after storage for 6 and 9 weeks (Figs 4.9 & 4.10) but significantly higher than in fruit stored for 3 weeks. These data show that ACC activity increased while the fruit were stored at 0°C but subsequent increased at 20°C essentially paralleled the changes in ACC concentrations. Similar parallel changes in ACC oxidase and ACC concentrations have been reported in apple (Jobling et al., 1991). The CA mixtures in this experiment had no effect on ACC oxidase activity. This observation contradicts Gorny and Kader (1996) who showed that induction of ACC oxidase is suppressed in CA treatments of apples compared to storage in air. The data show that chilling (CA storage at 0°C) accelerated the activity of ACC oxidase (2-4 days) as compared to freshly harvested fruit ripened at 20°C (14 days).
ACC oxidase activity in freshly harvested fruit ripened at 20°C rises constantly throughout the lag period increasing substantially only at the onset of autocatalytic ethylene production (Sitrit, et al., 1986; Starrett and Laties, 1991). This study showed that storage at 0°C stimulated both ACC and ACC oxidase activity.
Fig 4.8. ACC oxidase activity in avocado fruit after transfer to air at 20°C following storage for 3 weeks at 0°C. Means for each ripening time with the different letters were significantly different at p<0.05 (Duncan's multiple range comparisons). Data were obtained in experiment 1 (1995-1996).
Fig 4.9. ACC oxidase activity in avocado fruit after transfer to air at 20°C following storage for 6 weeks at 0°C. Means for each ripening time with the different letters were significantly different at p<0.05 (Duncan's multiple range comparisons). Data were obtained in experiment 1 (1995-1996).
Fig 4.10. ACC oxidase activity in avocado fruit after transfer to air at 20°C following storage for 9 weeks at 0°C. Means for each ripening time with the different letters were significantly different at $p>0.05$ (Duncan's multiple range comparisons). Data were obtained in experiment 1 (1995-1996).
4.3.1.4. Changes in polyamines concentrations of fruit following transfer to air at 20°C after CA storage for 3, 6 and 9 week at 0°C

Polyamines Concentrations of polyamines were not measured in freshly harvested fruit during ripening at 20°C. Unripe avocado fruit have been reported to have relatively higher concentrations of polyamines than ripe fruit (Winer and Apelbaum, 1986). In the present study, the concentrations were high in all samples on the day of transfer from 0°C to 20°C, after storage for 3, 6 and 9 weeks and subsequently decreased during storage in air at 20°C (Figs. 4.11-4.13). This suggested that the initial concentrations in the fruit were high and CA mixtures had no consistent effects on polyamine concentrations or the rates of change during ripening at 20°C. In contrast, Kramer et al. (1989) reported that the concentrations of polyamines were higher in CA-stored apples than in air-stored fruit and the maximum concentrations coincided with the ethylene climacteric. Polyamine concentrations decrease during avocado fruit development (Winer and Apelbaum, 1986; Kushad et al., 1988) and between the immature and mature stages of development prior to the onset of climacteric ethylene production in tomato fruits (Kakkar and Rai, 1993). Because of lack of any changes in responses to CA it was decided not to conduct any further work on polyamines.
Fig 4.11. Changes in polyamines concentrations in avocado fruit after transfer to air at 20°C following storage for 3 weeks at 0°C. Means for each ripening time with the different letters were significantly different at p>0.05 (Duncan's multiple range comparisons). Data were obtained in experiment 1 (1995-1996).
Fig 4.12. Changes in polyamines concentrations in avocado fruit after transfer to air at 20°C following storage for 6 weeks at 0°C. Means for each ripening time with the different letters were significantly different at p>0.05 (Duncan's multiple range comparisons). Data were obtained in experiment 1 (1995-1996).
Fig 4.13. Changes in polyamines concentrations in avocado fruit after transfer to air at 20°C following storage for 9 weeks at 0°C. Means for each ripening time with the different letters were significantly different at p>0.05 (Duncan's multiple range comparisons). Data were obtained in experiment 1 (1995-1996).

4.3.2.1. Rates of Respiration and Ethylene productions of avocado fruit at 20°C after CA storage at 0 and 5°C.

The most promising atmospheres (2.5 % O₂ + 5 and 7.5 % CO₂; 5 % O₂ + 5 and 7.5 % CO₂) from experiment 1 were selected for further evaluation in 1996 - 1997. Fruit were stored at 0°C and 5°C for 3, 6 and 9 weeks.

The pattern of respiration and ethylene production of freshly harvested fruit stored in air at 20°C were similar to those found in experiment 1 (Fig. 4.1). Peak rates were recorded on about day 12. Furthermore, the patterns of respiration rates and ethylene production at 20°C following storage at 0°C were similar to those observed in experiment 1 (Figs. 4.14-4.16). CA had no effect on these patterns or rates. Thus, these data confirm the observations from experiment 1 that ripening initiation was not prevented when metabolism increased again after removal to 20°C.

Respiration rates and ethylene production of fruit stored in CA in experiment 1 were similar to these measured in experiment 2 following storage for 3 weeks at 0°C. The data showed an increase in the rates of respiration and ethylene production of CA fruit at 0°C compared to at 5°C and fruit at 5°C and harvested fruit (Fig 4.1.). There was a marked increase in the rates of respiration and ethylene production of
the fruit following transfer to 20°C after CA storage at 0°C (Fig. 4.14 A). There were no symptoms of CI in the fruit held in CA storage at 0°C for 3 weeks (Fig. 3.5). A similar result was also found in experiment 1 (Fig. 4.2 - 4.5).

The respiration rate of fruit following transfer to air at 20°C after CA storage at 0°C for 6 and 9 weeks increased after 4 to 5 days compared to 11 days in freshly harvested fruit. Similar patterns were observed in fruit stored in the same treatments in experiment 1 (Figs 4.3 - 4.4). Ethylene production of fruit following transfer to air at 20°C after CA storage at 0°C for 6 and 9 weeks was measurable after one day and continued to increase for the next 2 - 3 days whereas an increase in ethylene production by freshly harvested fruit was not detected until day 10.

The rates respiration and ethylene production of fruit stored in CA for 3 weeks at 5°C were lower than those of air and CA treatments at 0°C. Fruit from CA treatments at 5°C generated normal climacteric patterns of respiration and ethylene production with peaks after 13 - 15 days (Fig. 4.17). The fruit remained green in colour (Fig. 3.6) after 6 days at 20°C, but subsequently ripened normally as indicated by softening and development of a brown black skin colour. Respiration of fruit stored for 3 weeks in air at 5°C reached a peak after 4 days at 20°C. A peak in ethylene production was recorded on day 7. Fruit stored in CA containing 5 % CO₂
produced more CO₂ than those kept in mixtures containing 7.5 % CO₂. Avocado kept in low oxygen (2.5 %) produced less ethylene than those kept in air.

The patterns of changes in respiration rates and ethylene production during avocado ripening at 5°C after storage for 6 weeks in CA are shown in Fig 4.15 (A+B). Respiration and ethylene production rates in air-stored fruit after one day at 20°C were high and peak rates were recorded on day 5 showing that fruit ripened normally and that ripening had advanced while in storage at 5°C (Fig. 4.18). Some flesh discoloration was found after 6 days at 20°C (Plate 3.5). Similarly, fruit from the CA treatments reached peak respiration and ethylene production rates following 6 weeks storage at 5°C sooner than fruit stored for 3 weeks (Fig. 4.18). However, CA treatments delayed ripening compared to air control. After 9 weeks at 5°C peak rates of respiration and ethylene production were recorded in air stored fruit on day 4 at 20°C (Fig. 4.16). However, this fruit had indicated by severe flesh browning by 6 days indicating that it was over stored (Plate 3.9). Although respiration and ethylene production were not measured after 6 day at 20°C, all CA fruit stored at 5°C for 9 weeks ripened normally without CI. Because of variability among treatments, it was not possible to determine which CA mixture gave the best results.

The stimulation of ethylene production and respiration followed storage for 3 weeks at 0°C observed in both Experiments 1 and 2 was
evidence of chilling stress in avocado. This response was absent in fruit stored at 5°C which were successfully stored for 6 weeks in air and at least 9 weeks in the CA mixtures. Fruit stored at 0°C for 6 weeks developed CI on return to 20°C and these symptoms became progressively worse after 9 weeks storage. The data also shown clearly that under chilling condition 0°C CA mixtures did not clearly affect the chilling stress whereas at 5°C the CA mixtures effectively delayed ripening.

The response to CA at 5°C could be explained by a reduction in ethylene production as proposed by other investigators. Hatton and Spalding (1990) showed that atmospheres containing 2 - 6 % O₂ and 3 - 10 % CO₂ inhibit softening of ‘Lula’ avocado and reduced CI in CA storage at 5°C. ‘Lula’ a Western Indian cultivars is known to be more sensitive to CI than ‘Hass’ fruit used in these experiment. Marcellin and Chaves (1983), found that apples stored in 3 % O₂ produced two or three times less ethylene than those kept in air.

Similarly, a reduction in ethylene production by high CO₂ concentrations (20-40%) in ‘Hass’ avocado stored in 20°C has been reported (Lange and Kader 1996). Gorny and Kader (1996) showed that the combination of low O₂ and elevated CO₂ had a synergistic effect in suppressing ethylene biosynthesis in apples. Reducing the oxygen level below 10 % caused a reduction in the rates of banana ripening (Wang et al., 1990). Chavez-Franco and Kader (1993)
showed that elevated CO₂ (5 - 20%) inhibits ethylene production in pears.

It was expected that fruit stored in the most promising atmospheres identified in Experiment 1 would effectively inhibit ripening and prevented CI at 0°C. However, it was found that fruit stored in these atmospheres at 0°C in experiment 2 had more CI than fruit stored at 0°C in experiment 1 (Fig. 3.11). This difference may have been related to the physiological age of the fruit as indicated by difference between dry matter of the fruit harvested in November (experiment 1) and February (experiment 2). Fruit for experiment 2 were harvested from the same trees as experiment 1. The data reported here confirm the work of Meir et al (1995) who found that 5°C was suitable for storage of ‘Hass’ avocado in CA (3 % O₂ and 8 % CO₂) for 9 weeks.
Fig. 4.14. Rates of respiration (A) and ethylene production (B) of avocado fruit after transfer to air at 20°C following storage for 3 weeks at 0°C. Data were collected in experiment 2 (1996-1997). Means for each ripening time with the different letters were significantly different at p<0.05 (Duncan's multiple range comparisons).
Fig. 4.15. Rates of respiration (A) and ethylene production (B) of avocado fruit after transfer to air at 20°C following storage for 6 weeks at 0°C. Data were collected in experiment 2 (1996-1997). Means for each ripening time with the different letters were significantly different at p<0.05 (Duncan's multiple range comparisons).
Fig. 4.16. Rates of respiration and ethylene production of avocado fruit following after transfer to air at 20°C following storage for 9 weeks at 0°C (A+C) and 5°C (B+D). Data were collected in experiment 2 (1996-1997). Means for each ripening time with the different letters were significantly different at p<0.05 (Duncan's multiple range comparisons).
Fig. 4.17. Rates of respiration (A) and ethylene production (B) of avocado fruit following transfer to air at 20°C after CA storage for 3 weeks at 5°C. Data were collected in experiment 2 (1996-1997). Means for each ripening time with different letters were significantly different at p < 0.05 (Duncan's multiple range comparisons).
Fig. 4.18. Rates of respiration (A) and ethylene production (B) of avocado fruit after transfer to air at 20°C following storage for 6 weeks at 5°C. Data were collected in experiment 2 (1996-1997). Means for each ripening time with different letters were significantly different at p < 0.05 (Duncan's multiple range comparisons).
4.3.2.2. Changes in ACC concentrations in fruit at 20°C after CA storage at 0 and 5°C.

ACC was not measurable until day 6 after harvest in freshly harvested control fruit ripened at 20°C. Peak concentrations were measured on day 10, two days before the ethylene climacteric peak (Fig. 4.1). The concentrations of ACC in fruit following transfer to air at 20°C after CA storage for 3, 6 and 9 weeks at 0°C were very low (Fig. 4.19 - 4.21) then they rose to a peak on day 2 - 4 and subsequently declined to low concentrations at day 6. The earlier in all treatments after 3 weeks at 0°C increase in ACC concentration paralleled the changes in ethylene production (Section 4.3.2.1.) and occurred at least 2 days earlier than in fruit stored for 3 weeks at 5°C (Fig. 4.19). These data were similar to those recorded for the fruit stored in the same treatments in experiment 1 (Fig. 4.5). The concentrations of ACC of fruit were lowest in fruit stored in CA containing 5% O₂ combined with 7.5% CO₂ at 0°C. ACC concentrations in this treatment resulted in low ethylene production (Fig. 4.14 - 4.16).

ACC was measurable in most treatments on removal from 0°C after 6 weeks storage and maximum concentrations were measured on day 4 (Fig. 4.20). Subsequently the concentrations decreased rapidly by day 6. Similar results were obtained with the same atmospheres in experiment 1 (Fig. 4.6).

After 9 weeks at 0°C, ACC was measurable on transfer from storage and then rose to maximum concentrations on day 4 in air and CA treatments.
Maximum concentrations were generally lower than in fruit stored for 3 and 6 weeks at 0°C. The pattern of changes in ACC concentrations in fruit stored at 0°C in experiment 2 was similar to those observed in experiment 1. The maximum concentrations of ACC in 0°C fruit were similar to those measured in freshly harvested control and in fruit stored at 5°C. These similarities support the proposal that chilling stress during the first 3 weeks of storage at 0°C stimulates ACC biosynthesis and ethylene production resulting in premature ripening when the fruit are returned to 20°C. However, CI develops by 3 weeks at 0°C and becomes progressively worse with longer storage.

In keeping with the data for ethylene production that showed storage at 5°C delayed ripening (Section 4.3.2.1.), ACC did not begin to accumulate until day 4 at 20°C following storage for 3 weeks (Fig. 4.19). ACC concentrations were eight time higher in air stored fruit by day 6 compared to CA stored fruit. The patterns of ACC concentrations of fruit following transfer to air after CA storage for 3 weeks at 5°C (Fig. 4.19) were similar to the ethylene patterns (Fig. 4.17). ACC concentrations were not measurable until day 4 and showed only a small increase by day 6, except the fruit stored in air in which there was a large increase. Insufficient fruit were available to enable ACC measurement after day 6. ACC concentrations remained low in fruit stored for 6 weeks at 5°C and then increased during ripening at 20°C (Fig. 4.20). An increase in ACC was measured by day 2 in air control, 2.5 % O₂ +
7.5% CO₂ and 5% O₂ + 5% CO₂ treatments. ACC reached a maximum in air control and 5% O₂ + 5% CO₂ treatments on day 4, but no increase was found in fruit stored in 2.5% O₂ + 5% CO₂ until day 6. After 9 weeks storage at 5°C, ACC concentrations on removal from storage were low, but increased to a maximum by day 4 in all treatments (Fig. 4.21).

Although ACC concentrations increased ahead of the rises in ethylene production in all treatments stored at 5°C, the patterns were similar to normal ripening. This is supported by progressively earlier onset of the ethylene climacteric after 6 and 9 weeks storage, the absence of CI at least until 9 weeks of storage and the effectiveness of some CA mixtures in slowing the accumulation of ACC for the first 6 weeks of storage. Air control fruit accumulated ACC much sooner than CA fruit and reached a maximum concentration sooner. After 9 weeks at 5°C air control fruit became very soft and the flesh brown after transfer to 20°C.

In other species of fruit the effects of elevated CO₂ concentration on the concentrations of ACC are inconsistent. Elevated CO₂ concentration did not cause any change in ACC content in ‘Granny Smith’ apples (Chevery et al., 1988) or in Golden Delicious apple (Gorny and Kader 1996a), however Chaves and Tomas (1984) reported an increase in ACC content in ‘Granny Smith’ apple. Elevated CO₂ also inhibits accumulation of ACC in tomatoes (Mathooko et al., 1995).
Fig 4.19. ACC concentrations in avocado fruit after transfer to air at 20°C following storage for 3 weeks at 0°C (A) and 5°C (B). Means for each ripening time with the different letters were significantly different at p>0.05. (Duncan's multiple range comparisons). Data were obtained in experiment 2 (1996-1997).
Fig 4.20. ACC concentrations in avocado fruit after transfer to air at 20°C following storage for 6 weeks at 0°C (A) and 5°C (B). Means for each ripening time with the different letters were significantly different at p<0.05 (Duncan’s multiple range comparisons). Data were obtained in experiment 2 (1996-1997).
Fig 4.21. ACC concentrations in avocado fruit after transfer to air at 20°C following storage for 9 weeks at 0°C (A) and 5°C (B). Means for each ripening time with the different letters were significantly different at p>0.05. (Duncan’s multiple range comparisons). Data were obtained in experiment 2 (1996-1997).
4.3.2.3. Changes of ACC oxidase activity in fruit at 20°C after CA storage at 0 and 5°C.

ACC oxidase activity of freshly harvested fruit during ripening at 20°C paralleled the changes in ACC concentrations (Fig 4.1). ACC oxidase activity appeared to increase from day 4 and ACC from day 6 then reached a peak at day 10. ACC oxidase activity paralleled ACC concentrations of fruit transferred to air at 20°C from storage at 0°C after 3, 6 and 9 weeks (Figs. 4.22 - 4.24). After 3, 6 and 9 weeks storage at 0°C ACC oxidase activity was low (Fig. 4.22). Activity in fruit stored for 3 weeks increased to a peak at 2 days at 20°C, but the peaks was delayed until day 4 in fruit stored for 6 and 9 weeks (Figs. 4.23 and 4.24). These data show that there were some increases in ACC activity while the fruit were stored at 0°C and subsequent increase at 20°C, essentially paralleling the changes in ACC concentrations. A similar pattern was observed in the same atmospheres in experiment 1 (Figs. 4.8-4.10). A similar parallel between ACC oxidase activity and ACC concentrations has been reported in apple (Jobling et al., 1991). The CA mixtures had no effect on ACC oxidase activity at 0°C. This observation contradicts Gorny and Kader (1996) who showed that CA treatments suppressed induction of ACC oxidase activity in CA stored apples. The data show that chilling stress stimulated the activity of ACC oxidase (2 - 4 days) compared to freshly harvested control fruit. ACC oxidase activity of fruit
following transfer to air at 20°C after CA storage for 3, 6 and 9 weeks at 5°C are shown in Fig. 4.22 to Fig. 4.24.

ACC oxidase activity was low in fruit transferred from 5°C to 20°C after 3, 6 and 9 weeks storage. Activity increased slowly at 20°C in fruit stored for 3 weeks at 0°C in parallel with ACC concentrations. Peak activity was measured after 2 days at 20°C following storage for 6 weeks at 5°C in air, 2.5 % O₂ + 7.5 % CO₂, and 5 % O₂ + 5 % CO₂. An increase in activity was delayed to day 6 in 2.5 % O₂ + 5.0 % CO₂ and 5 % O₂ + 7.5 % CO₂. By 9 weeks at 5°C ACC oxidase activity reached a maximum by day 4 at 20°C in all treatments.

Overall, the patterns of change in ACC oxidase activity paralleled the changes in ACC concentrations in all treatments following storage at 0°C and 5°C. It is noteworthy that ACC oxidase activity was slightly higher in 0°C fruit on transfer to 20°C than in 5°C stored fruit thus supporting the notion that chilling stress at 0°C stimulates both ACC synthesis and ACC oxidase activity. As with ACC, CA mixture did not override the effect of chilling at 0°C but they retarded these changes at 5°C.
Fig 4.22. ACC oxidase activity in avocado fruit after transfer to air at 20°C following storage for 3 weeks at 0°C (A) and 5°C (B). Means for each ripening time with the different letters were significantly different at p>0.05. (Duncan's multiple range comparisons). Data were obtained in experiment 2 (1996-1997).
Fig 4.23. ACC oxidase activity in avocado fruit after transfer to air at 20°C following storage for 6 weeks at 0°C (A) and 5°C (B). Means for each ripening time with the different letters were significantly different at p>0.05. (Duncan's multiple range comparisons). Data were obtained in experiment 2 (1996-1997).
Fig 4.24. ACC oxidase activity in avocado fruit after transfer to air at 20°C following storage for 9 weeks at 0°C (A) and 5°C (B). Means for each ripening time with the different letters were significantly different at p>0.05. (Duncan's multiple range comparisons). Data were obtained in experiment 2 (1996-1997).
4.4. Conclusions:

1. Storage for 3 weeks at 0°C stimulated ripening compared to CA storage at 5°C. Respiration and ethylene production showed climacteric-like peaks by day 2-4 at 20°C following storage in air and CA at 0°C. Harvested control fruit a reached climacteric peak at 12-14 days. No CI developed following 3 weeks storage at 0°C, but was severe after 6 and 9 weeks storage.

2. Respiration and ethylene production of fruit stored in CA at 5°C were low upon removal from cool storage and increased to climacteric peaks accompanied by normal ripening and no CI at least for 6 weeks of storage.

3. CA mixtures at 0°C had no significant effects on the patterns or rates of respiration and ethylene production of fruit after transfer to 20°C. In contrast, all CA mixtures at 5°C retarded the increases in respiration and ethylene production following transfer to 20°C, but this response diminished progressively with longer storage.

4. ACC concentrations were very low in fruit on removal from storage at 0°C, but increased at 20°C to concentrations similar to those found in freshly harvested fruit during ripening. ACC concentrations were low in fruit stored at 5°C on transfer to 20°C and then increased slowly as the fruit began to ripen. The rate of accumulation increased progressively following 6 and 9 weeks storage.
5. The maximum concentrations of ACC were similar in all treatments and in freshly harvested control fruit during ripening.

6. The activities of ACC oxidase paralleled the changes in ACC concentrations in fruit transferred to air at 20°C from storage at 0°C and 5°C after 3, 6 and 9 weeks.

7. CA mixtures did not significantly modify the responses in fruit stored at 0°C, but retarded ripening at 5°C.

8. The polyamines concentrations were high on the day fruit were transferred from 0°C to 20°C after storage 3, 6 and 9 weeks and subsequently decreased during ripening stage at 20°C. CA mixtures had no consistent effects on polyamines concentrations during storage following transfer to 20°C.

9. Study of changes in proteins during CA storage at 0° and 5°C could explain the physiological and biochemical results obtained in these experiments. These include the stimulation of ripening following storage for 3 weeks at 0°C and why CA storage was effective at 5°C but not 0°C. Preliminary studies using 2D-PAGE are reported in Section V.
SECTION V

EFFECT OF CONTROLLED ATMOSPHERE STORAGE ON PROTEIN

5.1. Introduction

Ripening of avocado, as with other climacteric fruit, is a genetically controlled process that includes the continuous synthesis degradation and activity of both proteins and RNA. Specific genes are expressed resulting in the synthesis of enzymes associated with normal ripening (Seymour and Tucker, 1993).

During the ripening of avocado, extensive cell wall degradation leads to dramatic softening of the mesocarp tissue (Awad and Young, 1979). This softening is thought to be associated with the accumulation of hydrolytic enzymes viz. cellulase (endo-β1,4 glucanase), endo polygalacturonase (Pesis et al., 1978; Kanellis et al., 1991; Kanellis et al., 1992; Buse and Laities 1993) and other cell wall degrading enzymes. There is also a dramatic increase of total protein and mRNAs during the ripening of avocado (Pesis et al., 1978; Tucker and Laities, 1984; Christoffersen et al., 1984; Cass et al., 1990; McGarvey et al., 1992; Zamorano et al., 1994). Other studies have demonstrated that cellulase (cell1) shown to be expressed in the mesocarp during ripening may also be involved in the abscission of mature fruit (Loukalakis et al, 1994; Tonutti et al., 1995).

Kanellis et al., (1989) showed that low oxygen atmosphere interferes with the accumulation of cellulase protein, mRNA and delayed the development of a number polypeptides that appear during ripening (Kanellis et al., 1989a). Avocado fruit are known to produce several electrophoretic variants of the polygalacturonase protein
(Kanellis et al., 1991) but whether these represent different gene products or post-translational modifications of one or more primary polypeptides is unknown (Kutsunai et al., 1993). Some changes in gene expression during avocado ripening have been shown to be suppressed by storage conditions such as low oxygen concentrations (Kanellis et al., 1989b).

Conventional polyacrylamide gel electrophoresis (PAGE) or any other one-dimensional separation method do not give sufficient resolution if more than 100 proteins are present (Jansen and Ryden, 1989; Patel and Rickwood, 1996). The development of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) by O’Farrell has enabled the resolution of hundreds of individual proteins (Hames, 1990; Conradsen and Pedersen, 1992; Jungblut, 1997).

The aim of this study was to examine the efficacy of 2D-PAGE for detecting changes in the production of proteins that may be induced in avocados stored in CA mixtures (2.5 % O₂ and 5 % O₂ combined with 5 and 7.5 % CO₂ respectively), at two storage temperatures (0°C and 5°C).

5.2. Material and Methods
5.2.1. Fruit.

Fruit were transferred from CA storage to plastic containers ventilated with humidified air at 20°C after 3, 6 and 9 weeks storage at 0° and 5°C. There were 3 replicates of single fruit of ten treatments of experiment 2 (Section 3.2). The fruit were sampled after 0, 4 and 6 days at 20°C. Freshly harvested fruit were also sampled during ripening at 20°C. Mesocarp was sampled by removing cylinders of tissue (4 g) with a No. 8 cork borer and a razor blade from the equatorial region, placed in tubes, frozen in liquid N₂ and stored at -80°C for further extraction.

5.2.2. Protein extraction.

The suitability of two buffer solutions for the extraction of protein from avocado tissue were compared using the protocols detailed below.

5.2.2.1. Tris/βmercaptoethanol extraction buffer

Tissue (1 g) was sliced, frozen in liquid N₂ and ground with a mortar to generate a powder. The frozen powder was rapidly homogenised in 4 mL of ice-cold extraction buffer according to the method of Kanellis et al., 1991. The buffer contained of 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 20 mM NaHCO₃, 20 mM MgSO₄, 10 mM EDTA, 5 mM β-mercaptoethanol, 0.5 mM PMSF, 10 μM leupeptin, and 10 % v/v glycerol (Sigma). The tube was sealed and shaken vigorously for 15 min at room temperature after which the phases were separated by centrifugation at 20,000 x g for 20 min at -6°C. Precipitation was allow to occur overnight at -20°C. The following day, proteins were collected by centrifugation at 20,000 x g for 20 min at -6°C. The aliquots were enclosed in 1.5 mL Eppendorf
and stored at -80°C for further protein analysis using one dimensional polyacrylamide gel electrophoresis (Section 5.2.3).

5.2.2.2. Phenol-protein extraction buffer

This extraction medium is made for protein analysis by 2D-PAGE (Section 5.2.3.2). Individual fruit were pitted, sliced and stored at -80°C. Samples of fruit from each CA treatments were extracted with phenol-protein extraction buffer according to Barent and Elthon (1992) with the following modifications: Avocado fruit tissue (2 - 4 g) was frozen in liquid N2 and ground with a mortar to generate a powder. The frozen powder was rapidly suspended in 10 mL of phenol-protein extraction buffer in a 45 mL centrifuge tube. An equal volume of water-saturated phenol (Sigma) was then added. The tube was sealed and shaken vigorously for 5 min at room temperature after which the phases were separated by centrifugation at 7000 x g for 10 min. The phenol phase was recovered and re-extracted with an equal volume of protein extraction buffer and 1 mL of water-saturated phenol. After three or four re-extractions, the proteins were precipitated from the phenol phase by addition of five volumes of 0.1 M ammonium acetate in methanol (previously cooled at -20°C). Precipitation occurred overnight at -20°C. The following day, proteins were collected by centrifugation at 7000 x g for 10 min following which the protein pellets were washed three times with ammonium acetate in methanol and once with acetone (pre-cooled at -20°C). The pellet was dried under vacuum for 5 min and solubilised in a 350 mL of O’Farrell lysis buffer, urea (9.5 M); Tris base, pH=7.5 (35 mM); DTT 1% (w/v); Biolyte 3/10 0.7 % (v/v); and Biolyte 5/7 1.3 %(v/v). The protein solution was
clarified by centrifugation for 5 min at 7000 x g following which aliquots were either immediately subjected to electrophoresis or stored in 1.5 mL Eppendorf tubes at -80°C. Each extraction procedure was repeated twice for each sample of fruit. Three to four gels were obtained from one extract of fruit for each sample of fruit from each CA treatment.

5.2.3. Protein separation

The extracted proteins were separated using one and two dimensional gel electrophoresis described below.

5.2.3.1. One-dimensional polyacrylamide gel electrophoresis (1-D PAGE)

Gels for one dimensional electrophoresis (0.3 x 20 x 20 cm) were cast in a Multicell-II-xi-Protein chamber (BioRad). The 12 % resolving gel was degassed for 15 min with a vacuum pump and polymerisation initiated with 1.6 mL of 10 % ammonium persulfate (APS) and 0.25 mL of TEMED. Aliquots (50 mL) of this solution were poured to give gels 18 cm in depth. The resolving gels were then overlayed immediately with water-saturated 2-butanol to exclude oxygen and to level the surface of the gels. The polymerisation was generally complete after about 1 h, however, the gels were stored overnight to assure complete polymerisation. The gels were washed and then overlayed with 4 % stacking gels about 1.5 cm in depth. The gels was loaded with 25 - 40 μL samples of tissue extracts (Section 5.2.2.1) and 20 μL SDS-PAGE Standard protein (BioRad) and overload with buffer solution (Hochstrasser et al., 1986; Hochstrasser et al., 1988). Electrophoresis was conducted
using tank and chamber buffer solutions at a constant current of 40 mA/gel until the bromophenol blue tracking dye was 1 cm from the bottom of the gel. After fixing in a solution containing 40 % ethanol, 10 % acetic acid, and 50 % ddH₂O for 60 min with continuous shaking, protein bands were visualised by silver-nitrate staining solution (section 5.2.4.). The gels were then washed three times for 15 min with ddH₂O (250 mL/gel) and stained in 0.2 % (w/v) silver nitrate with continuous shaking. After the gels were washed for 5 min, developing solution (36.72 % sodium carbonate, 0.5 % formaldehyde) was added with continuous shaking until protein spots appeared. The developing solution was decanted and 15 % acetic acid was added to stop darkening of the gels. The gel were then washed for 10 min and sealed in a plastic bags with a small amount of ddH₂O and kept at 4°C for further analysis of protein patterns (Laemli, 1970).

5.2.3.2. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

5.2.3.2.1. First-Dimension; Isoelectric focusing (IEF).

Isoelectric focusing gels, utilising N,N'-methylenebisacrylamide (Bis) as the crosslinking agent, contained 10 g of urea, 6.5 mL deionized water (ddH₂O) and 3 mL of acrylamide/Bis (BioRad). After the urea was dissolved, 0.8 mL of 3/10 ampholynes and 0.2 mL 5/7 ampholynes (BioRad) were added to the solution (solution A). A second solution was prepared by mixing 0.3 % (w/v) of CHAPS and 1 % (v/v) of igepal-C 30 (Sigma, formerly Nonidet-40 detergent) in 0.9 mL ddH₂O (solution B). A final solution (solution C) was made by mixing 1 mL of solution B with the entire solution A. The mixture was degassed for 5 minutes. To initiate polymerization, 6 μL of 10% (w/v) ammonium persulfate (APS) and 3 μL of
TEMED (Sigma) were added and swirled in Vortex mixer 8 to 10 times (O’Farrell, 1975; Hochstrasser et al., 1988; Hames and Rickwood, 1990). Glass capillary tubes (1.5 mm ID, 6.0 mm OD, 180 mm) were marked 14 cm from one end and the bottom end sealed with Parafilm and placed in a Tube Gel Casting Stand (BioRad) (the remaining 20 mm was used to load the sample). Aliquots of 1 mL of solution C were gently pipetted into these glass tubes along the side wall to prevent air bubbles in the solution, and the capillary tubes were filled by drawing the liquid in each tube to the 14 cm mark. The glass tubes were carefully overlayed with water-saturated 2-butanol and allowed to sit undisturbed for 1 hour to complete polymerisation. After polymerisation was completed, the top part of the tubes were washed twice with ddH₂O. Water was removed and the tubes filled with 25 μL overlay buffer (20 % glycerol, 80 % ddH₂O and a trace of bromophenol blue). The tubes were polymerised for another 2-3 hours. The tubes were loaded with 40-80 μg samples of tissue extracts (Section 5.2.2.2), with or without 10 μL 2-D SDS PAGE standard protein (BioRad) and overlayed with buffer solution (Hochstrasser et al., 1986; Hochstrasser et al., 1988). Lower anolyte buffer (3.5 L of 0.06 % (v/v) H₃PO₄) was poured into the lower chamber of the tube gel apparatus then the tubes were loaded into the apparatus. The samples were overlayed carefully with the upper catholyte buffer (20 mM NaOH) to avoid mixing the samples with upper catholyte buffer. The upper reservoir was completely filled with the upper catholyte buffer (Barent and Elthon, 1992; Rosenberg, 1996). The lid of the electrophoresis chamber (175-IEF, BioRad) was fitted firmly and the power cables were attached to the power supply (Power Pac 3000, BioRad). Isoelectric focusing was performed at 5°C with constant voltage of
300 V for 3 h, followed by 700 V for 15 h, and then 1000 V for 24 h (O’Farrell, 1975; Janson and Ryden, 1989).

After the separation was complete the IEF gels were slowly removed from the tubes by forcing them out with a 2.5 mL syringe filled with ddH₂O (Righetti et al., 1990). The gels were frozen at -80°C until required.

5.2.3.2.2. Two-dimensional; Separation by Molecular Weight

5.2.3.2.2.1. Protocol for casting resolving gels.

The PROTEAN II xi multi-gel casting chamber (BioRad), glass plates and spacers were cleaned. The chamber was prepared by using bevelled glass plates, 1.5 mm spacers and bevelled inner glass plates. Bevelled inner plates facilitated the application of the first dimension gels. The chamber was filled to a height approximately 2.0 cm below the top of the bevelled inner (shorter) glass plates. Six slab gels (3 x 200 x 200 mm) were casting in the casting chamber.

Resolving Gels contained 130 mL acrylamide/Bis solution (37.5:1), 80 mL Tris Base (pH 8.8), 3.2 mL 10 % SDS and 110 mL ddH₂O. The solution was degassed for 20 min with a vacuum pump. To initiate polymerisation 1.6 mL 10 % APS and 145 µL TEMED were added (O’Farrell, 1975). The solution was poured simultaneously into six gels to give 180 mm in depth. Then 2-butanol was added immediately for levelling the surface and to exclude oxygen from the resolving gels. Polymerisation was allowed to occur undisturbed for at least one hour. After polymerisation was
generally complete the top part of the gel sandwiches was washed with ddH₂O. Then the gels were left in water for 24 h to elute unpolymerised monomer.

5.2.3.2.2.2. Protocol for casting gels.

The previously cast resolving gels were removed from storage and fitted with clamps so that the arrow on the clamps faced inward and the top plates met the corresponding notches in the clamps. The clamp screws were tightened. The casting gel contained 7.8 mL acrylamide/bis solution (37.5:1), 15 mL Tris Base (pH 6.8), 600 μL 10% SDS and 36.5 mL ddH₂O. The solution was degassed for 20 min. To initiate polymerisation 250 μL 10% APS and 45 μL TEMED were added (O’Farrell, 1975). The casting gel was poured into the top part of the resolving gel sandwiches and water saturated 2-butanol was used for levelling the surface and to exclude oxygen. Polymerisation was allowed to occur undisturbed for at least one hour. After washing with ddH₂O the first dimension gels (IEF gel) were ready to slide onto the second dimension gels working from one side to the other (Rickwood et al., 1990).

5.2.3.2.2.3. Equilibration of the first dimension (IEF) gels.

The IEF gels were equilibrated in a solution which contained 10 % tris base (pH 6.8), 36 % urea, 30 % (v/v) glycerol and 2 % SDS. Solution D contained 50 mL of the equilibrium solution and 2 % DTT/DTE (BioRad). Solution E contained 50 mL of the equilibrium solution and 2.5 % iodoacetamide (BioRad) and 250 μL 1 % bromophenol blue (Sigma). The IEF gels were equilibrated in solution D for 10 min and solution E for 3 min.
5.2.3.2.2.4. Loading the IEF gels onto slab gels.

The IEF gels were slid onto the stacking gels using a piece of Parafilm. Care was taken to avoid trapping air bubbles between the two gels. It was necessary to ensure that the IEF gels were in intimate contact with the stacking gels (Rickwood et al., 1990).

5.2.3.2.2.5. Running the second dimension.

The three central cooling cores were placed into a lower buffer chamber. The lower chamber contained 15 L of lower electrolyte buffer (0.6 % Tris Base + 2.88 % glycine + 0.1 % SDS). The upper electrolyte buffer (1.2 % Tris Base + 5.76 % glycine + 0.2 % SDS) was poured into each upper buffer chamber (Hochtrassser et al., 1988). The lid was fitted on top of the lower chamber to fully enclose the PROTEAN II xi 2-D multi cell and the power cables were attached to the power supply (Power Pack 3000, BioRad). The second dimension gels were developed at 5°C with constant voltage of 150 V and current of 40 mA per slab of the gel for 1 h, followed by 300 V and 40 mA for 4 h. At the end of the run the gels were removed from the glass plates for staining (Dunn, 1993).

5.2.4. Silver-nitrate staining procedure.

The 2D-gels were fixed in solution containing 40 % ethanol, 10 % acetic acid, and 50 % ddH₂O for 60 min with continuous shaking. The gels were then washed for 5 min prior to incubating for about 16 h in a solution containing 30 % ethanol (v/v), 6.8 % sodium acetate (w/v), 2 % sodium thiosulfate (w/v), and 56 %
ddH₂O (Solution F). Then 5.2 mL of 25% glutaraldehyde was added to the 1 L solution F before the gels were incubated.

The gels were then washed three times for 15 min with ddH₂O (250 mL/gel) and stained in 0.2 % (w/v) silver nitrate with continuous shaking. After the gels were washed for 5 min, developing solution (36.72 % sodium carbonate, 0.5 % formaldehyde) was added with continuous shaking until the spots appearance. The developing solution was decanted and 15 % acetic acid was added to stop darkening of the gels. The gels was the washed for 10 min and sealed in a plastic bags with a small amount of ddH₂O and kept at 4°C for further analysis of protein patterns (Jungblut, 1997).
5.3. Results and Discussion

The ripening processes in avocado begins with an increase in the rates of respiration and ethylene production. The CO₂ climacteric peak appeared about one day after the ethylene peak and then respiration subsequently declined as the fruit entered the post climacteric stage (Fig. 4.1). To determine the changes during ripening, total proteins were extracted at different stages of ripening. Samples of protein extracts were separated by SDS-PAGE analysis (Plate 5.1). As the fruit ripened, some polypeptides, for example the 23 and 75 kD bands that were noticeable in unripe fruit, decreased. Polypeptides at 16.5, 25, 36 and 56 kD present early in ripening and increased further during ripening. Whereas, other polypeptides (43, 53, 70 and 76 kD) remained fairly constant at the pre-climacteric and and post-climacteric stages of ripening.

Two-dimensional electrophoresis (IEF/PAGE) protein analysis improved the resolution of the major polypeptides. Total proteins of unripe and ripened fruit were fractionated on 2D-PAGE and stained with silver. Examples of 2D-PAGE chromatographs of proteins from unripe (Plate 5.2.) and ripe freshly (Plate 5.3) harvested avocado fruit showed that polypeptides developed during ripening that were not detected in unripe fruit. The main reports of proteins induced during ripening in avocado have focused on cell wall hydrolytic enzymes and ethylene synthesis. For example, a native enzyme with molecular weight of 50 and 55 kD has been identified as cellulase (Awad and Lewis, 1980; Christoffersen et al., 1993). Christoffersen (1984) and Christoffersen (1987) showed that cellulase increases as the avocado fruit ripened.
The appearance of three ripening related polypeptides with estimated molecular weights of 80 kD (pI 3.6), 36 kD (pI 5.8) and 16.5 kD (pI 5.7) was observed in fruit at the climacteric stage (Plate 5.3).

Total proteins from avocado fruit subjected to various CA treatments (2.5 % O₂ combined with 5 or 7.5 % CO₂, and 5 % O₂ combined with 5 or 7.5 % CO₂) were fractionated on 2-D PAGE and stained with silver and compared with harvested unripe fruit extracts. Mixtures containing low O₂ and high CO₂ caused some alterations in the polypeptides profile. Analysis of 2D-PAGE proteins from unripe avocado following storage for 3 weeks in CA conditions at 0°C showed the disappearance of some polypeptides (Plates 5.5 to 5.8), summarised in Table 5.1).

Table 6.1. Qualitative changes in abundance of polypeptides in ripening avocado following 3 weeks storage at 0°C.

<table>
<thead>
<tr>
<th>MWr(kD)/ pI</th>
<th>Qualitative changes *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air</td>
</tr>
<tr>
<td>61 (6.6)</td>
<td>p</td>
</tr>
<tr>
<td>57 (5.9)</td>
<td>p</td>
</tr>
<tr>
<td>41 (7.8)</td>
<td>p</td>
</tr>
<tr>
<td>39.5 (6.5)</td>
<td>p</td>
</tr>
<tr>
<td>36 (5.8)</td>
<td>p</td>
</tr>
<tr>
<td>34 (6.4)</td>
<td>p</td>
</tr>
<tr>
<td>33 (5.1)</td>
<td>p</td>
</tr>
<tr>
<td>16.5 (5.7)</td>
<td>nd</td>
</tr>
</tbody>
</table>

* p = present  nd = not detected

134
Three polypeptides with estimated molecular weights of 41 kD (pI 7.8), 36 kD (pI 5.8) and 33 kD (pI 5.1) were found in air stored fruit but were not detected in fruits stored in CA. These groups of polypeptides (Plate 5.4) in avocado stored in air for 3 weeks at 0°C following transfer to 20°C. (Plates 5.5 - 5.8). The disappearance of polypeptides was probably due to inhibition of protein synthesis in low O₂ and high CO₂ concentrations in storage (Plates 5.5 - 5.8). The physiological changes observed in fruit when ripened following CA storage at 0°C for 3 weeks were normal in colour change (Figs 3.1 and 3.6) and softening (Figs 3.4. and 3.9) and there was no incidence of CI after 6 days at 20°C. The decline in avocado fruit firmness coincides with the increase in both cellulase and polygalacturonase (PG) activities (Awad and Young, 1979). Thus, the rates of softening during storage should correspond to changes in the expression of both enzymes. Christoffersen et al. (1984) has reported that the in vitro translation product of cellulase is 53 - 54 kD whilst PG appear as a number of polypeptides with molecular mass of 55, 52, 49, 48 and 46 kD.

These results are consistent with the finding of Kanellis et al. (1989a) that storing ‘Hass’ avocado in low O₂ and high CO₂ suppressed the induction of polypeptides associated with ripening. A polypeptide with an estimated molecular weights of 34 kD (pI 6.4) was identified as glycerol 3-P- dehydrogenase by Patel and Rickwood (1996) appeared in fruit stored in CA treatments and in fruit stored in air.
Analysis of 2D-PAGE proteins from unripe avocado following storage for 3 weeks in CA conditions at 5°C showed the disappearance of some polypeptides (Plates 5.9 to 5.10), summarised in Table 5.2.

Table 5.2. Qualitative changes in abundance polypeptides in avocado ripening following 3 weeks storage at 5°C.

<table>
<thead>
<tr>
<th>MWr(kD)/ pI</th>
<th>Qualitative changes *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5 % O₂/ 5 % CO₂</td>
</tr>
<tr>
<td>61 (6.6)</td>
<td>p</td>
</tr>
<tr>
<td>57 (5.9)</td>
<td>p</td>
</tr>
<tr>
<td>41 (7.8)</td>
<td>nd</td>
</tr>
<tr>
<td>39.5 (6.5)</td>
<td>p</td>
</tr>
<tr>
<td>36 (5.8)</td>
<td>nd</td>
</tr>
<tr>
<td>34 (6.4)</td>
<td>p</td>
</tr>
<tr>
<td>33 (5.1)</td>
<td>p</td>
</tr>
<tr>
<td>16.5 (5.7)</td>
<td>p</td>
</tr>
</tbody>
</table>

* p = present    nd = not detected

This study indicated that presence of polypeptides of 61kD (pI 6.6), 57kD (pI 5.9), and 39.5 kD (pI 6.5) in fruit stored in both CA and air. Kanellis et al. (1989b), who separated proteins by one-dimensional SDS-PAGE and staining with silver reported that polypeptides of 74, 61, 57, 37.7 and 16.6 kD remained fairly constant during ripening in air. These protein must be abundant to show clear changes in one dimension.

Plate 5.10 showed that fruit stored in air for 9 weeks at 5°C expressed a different pattern. Some polypeptides with low molecular weights appeared clearly.
However, these polypeptides were not detected in other CA and air treatments. This fruit had broken down after 6 days at 20°C (Fig. 3.6 and Plate 3.9C).

5.4. Conclusions:

The results described in this section are preliminary. Much more work is required to improve procedures for extracting total proteins and to achieve reproducible gels. The high content of lipids in avocado interferes with phenol extraction which is an effective extractant for plums (Abdi, N personal communication UWSH). In a preliminary experiment a modification using a lipid solvent improved separation of protein. Further work using 2D-PAGE is justified since the preliminary results showed differences between air and CA treatments.
Plate 5.1. SDS-PAGE of total proteins extracted from avocado fruit. Lanes A, B and C correspond to samples at different stages of ripening (0, 12 and 14 days after harvest). SDS standard (std) used as a molecular mass relative marker. Proteins (40 μg) separated by electrophoresis were visualised by silver staining. The open arrows indicate polypeptides that increased during ripening, and closed arrows indicate polypeptides that remain constant.
Plate 6.2. Two-dimensional PAGE chromatograph of proteins extracted from unripe freshly harvested avocado fruit. Proteins (60 μg) separated by electrophoresis were visualised by silver staining. The arrows indicate locations were some polypeptides were absent in unripe fruit but were detected in ripefruit.
Plate 5.3. Two-dimensional PAGE chromatograph of proteins extracted from ripe avocado fruit. Proteins (60 μg) separated by electrophoresis were visualised by silver staining. The arrows indicated the locations of polypeptides that appeared during ripening but were not detected in unripe fruit.
Plate 5.4. Two-dimensional PAGE chromatograph of proteins extracted from avocado fruit following storage in air for 3 weeks at 0°C. Proteins (60 µg) separated by electrophoresis were visualized by silver staining. The arrows indicate some polypeptides present in air but not in CA treatments.
Plate 5.5. Two-dimensional PAGE chromatograph of proteins extracted from avocado fruit following storage in CA (2.5% O₂ + 5% CO₂) for 3 weeks at 0°C. Proteins (60 μg) separated by electrophoresis were visualised by silver staining. The arrows indicate locations of polypeptides absent in this fruit but present in fruit stored in air.
Plate 5.6. Two-dimensional PAGE chromatograph of proteins extracted from avocado fruit following storage in CA (2.5%O₂ + 7.5%CO₂) for 3 weeks at 0°C. Proteins (60 µg) separated by electrophoresis were visualised by silver staining. The arrows indicated locations of polypeptides absent from this fruit but present in fruit stored in air.
Plate 5.7. Two-dimensional PAGE chromatograph of proteins extracted from avocado fruit following storage in CA (5 %O₂ + 5 %CO₂) for 3 weeks at 0°C. Proteins (60 μg) separated by electrophoresis were visualised by silver staining. The arrows indicate locations of polypeptides were absent in CA compared to fruit stored in air.
Plate 7.8. Two-dimensional PAGE chromatograph of proteins extracted from avocado fruit following storage in CA (5%O₂ and 7.5%CO₂) for 3 weeks at 0°C. Proteins (60 μg) separated by electrophoresis were visualised by silver staining. The arrows indicate some polypeptides missing in CA compared to fruit stored in air.
Plate 5.9. Two-dimensional PAGE chromatograph of proteins extracted from avocado fruit following CA (2.5 %O₂ and 5%CO₂) storage for 3 weeks at 5°C. Proteins (60 μg) separated by electrophoresis were visualised by silver staining. The arrows indicate locations of some polypeptides missing from this fruit but detected in fruit stored in air.
Plate 5.10. Two-dimensional PAGE chromatograph of proteins extracted from avocado fruit following storage in Air for 9 weeks at 5°C. Proteins (60 µg) separated by electrophoresis were visualised by silver staining. Black arrows show polypeptides that were not detected compared to fruit stored in air for 3 weeks. Light arrows indicated polypeptides that were not detected in fruit stored for only 3 weeks.
CA can reduce or delay CI of avocado fruit stored at 2 - 5°C (Faubion and Kader 1994). The responses of avocado (Persea americana Mill cv Hass) to CA at 0°C was determined. Storage atmospheres consisted of air (control); 2.5 % O₂ combined with 5, 7.5 and 10 % CO₂; 5 % O₂ combined with 5, 7.5 and 10 % CO₂; 7.5 % O₂ combined with 5, 7.5 and 10 % CO₂. The fruit were transferred to air at 20°C after 3, 6 and 9 weeks in CA at 0°C and physiological and biochemical changes in the fruit were monitored on day 0, 2, 4 and 6. After 3 weeks storage in CA, chilling injury was not detected in most CA treatments. After 6 and 9 weeks, fruit stored CA in mixtures of 2.5 % O₂ + 5 % CO₂, 2.5 % O₂ + 7.5 % CO₂ and 5 % O₂ + 5 % CO₂ had slight symptoms of CI, but symptoms were more severe in air and other treatments. These atmospheres were tested in a second experiment with additional CA storage at 5°C. The physiological changes such as in colour and textures were similar to those observed in experiment 1, but CI was more severe in experiment 2 possibly because the fruit were physiologically older or a seasonal offer. The fruit for experiment 2 were harvested from the same tree 3 months later. Although Hass avocado fruit remain unripe while attached to the tree physiological changes occur that may make the fruit less tolerant to chilling stress. However, Zauberman and Jobin-Decor (1995) did not detect any obvious differences in fruit responses to low storage temperatures for fruit from three different harvests. Another factor may have been the temperature of the fruit during growth. Higher fruit temperature during summer may have rendered the fruit more susceptible to CI. Work in New Zealand
has shown that exposed fruit are warmer and more sensitive to CI (Hopkirk et al., 1994).

Comparison of the responses of fruit stored at 0 and 5°C led to the original observation that chilling stress at 0°C caused premature ripening. This stress initiated an early increase in ACC biosynthesis and ACC oxidase activity, that was reflected in higher rates of ethylene production and respiration. Although symptoms of CI were not apparent after 3 weeks at 0°C, fruit stored for 6 and 9 weeks at 0°C suffered severe CI. Assays of ACC and ethylene production can provide sensitive indicators of chilling stress in avocado fruit. Chaplin (1984) showed that exposure of avocado to low concentrations of ethylene in air or CA at 5°C caused rapid development of CI. It is suggested that the induction of ethylene production in fruit stored at 0°C was a factor in the high incidence of CI observed in this fruit. A reduction in ripening time of ‘Fuerte’ following cold storage was observed by Cutting and Wolstenholme (1992) who reported that the fruit transferred to 21°C after storage at 5.5°C ripened faster than fruit stored at ambient temperatures. Induction of ethylene synthesis by cold storage has been reported in pome fruit (Jobling et al., 1991). Zaubermann and Jobin-Decor (1995) found that fruit stored at 5 or 8°C started to ripen during storage, whereas fruit at 2°C did not. The data obtained in experiment 2 confirm that Hass begin to ripen at 5°C. However, contrary to the reported benefit of storage at 2°C, further cooling to 0°C induced severe stress resulting in rapid onset of ethylene synthesis and CI on returned to 20°C.

Furthermore, storage of avocado in CA was ineffective in preventing the induction of ethylene synthesis and CI in fruit stored at 0°C. The fruit used in these
experiments were not sensitive to CI at 5°C and CA produced the expected delay in ethylene production and ripening. Further work will be needed to show whether CA storage at intermediate temperature between 0 and 5°C could be used and the influence of physiological age and growing conditions on the selection of a low but non-chilling storage temperature. Zaubermann and Jobin-Decor (1995) showed that storage at 2°C in air extended storage life without CI.

Concentrations of polyamines were investigated in experiment 1 because it has been proposed that they may counter the effects of ethylene on fruit ripening and senescence (Winer and Apelbaum, 1986). The results showed that polyamines remained at high concentrations in both air and CA storage at 0°C and then decreased steadily after return to air at 20°C. There were no clear differences in polyamines concentrations among the air and CA treatments and no further investigation of three compounds was conducted.

This investigation of changes in total protein by 2-D PAGE can only be regarded as a pilot study. Given more time extraction procedures need to be refined, including an effective procedure for removing lipids which can interfere with the gel separation of proteins. Other refinements include the development of meticulous techniques including controlling the amount of protein added to each gel and the many other steps involved with 2-D PAGE. As expected from published work some proteins were found to increase during ripening, while others disappeared. CA appeared to influence changes in concentrations of some proteins in fruit stored at both 0 and 5°C. It is recommended that this phase of the work be repeated. The first experiment should concentrate on comparing ‘Hass’ fruit stored in both air
and two of the most promising CA mixtures at 0, 2 and 5°C to search for proteins associated with ethylene biosynthesis. Storage at 2oC should be included became the work of Zauberman and Jobin-Decor (1995) suggests that this is the lowest storage to temperature that may be safely used for Hass without inducing CI. Published data for avocado should enable identification of these proteins. To confirm identification these proteins could be extracted from the gels, amino acids sequenced and compared with published data. The objective of this experiment would be to test the hypothesis that chilling temperatures induce premature production of the enzymes associated with ethylene production, comparison of proteins by 2D-PAGE and other specific test could reveal whether chill induced enzymes are the same as those associated with normal ripening. As part of this experiment the influence of selected CA mixtures on proteins in fruit stored at 5°C should be examined. Delayed synthesis of protein associated with ethylene biosynthesis would be expected. In addition, more detailed examination of the separated gels could be throw light on other biochemical responses induced by CA. The availability of 2-D analysis software (Melanie II, Hochstrasser & MELANIE Geneva, Switzerland) should assist the detection and identification of polypeptides that are affected by CA storage at low temperatures. Polypeptides shown to be associated with softening (cellulase, polygalacturonase), ripening (ACC synthases and ACC oxidase activity), and low oxygen stress (ADH) should provide valuable reference points for comparing these responses.

The results of this study do not permit firm recommendations for CA storage of Hass at temperature below 5°C. However, the finding that storage at 0°C induces severe CI and early induction of ethylene synthesis when the fruit are returned to a
ripening temperature, whereas a published report that the fruit can be stored successfully in air at 2°C, suggest that further work with CA at storage temperature of 2 - 5°C are justified. CA storage at 5°C can extend storage life about 50%. If a similar response can be achieved at 2°C, sea freight will provide a reliable means of shipping the fruit to European markets.


PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES
OF AVOCADO FRUIT TO CONTROLLED
ATMOSPHERE STORAGE

Eko Basuki
M.App.Sc (UNSW)

A thesis presented in fulfilment of the requirement
for the degree of Doctor of Philosophy

Faculty of Science and Technology
University of Western Sydney, Hawkesbury
Richmond, NSW 2753
AUSTRALIA

August, 1998
PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thesis Summary</td>
<td>vi</td>
</tr>
<tr>
<td>Declaration</td>
<td>vii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>viii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td>List of Plates</td>
<td>xii</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xiv</td>
</tr>
</tbody>
</table>

**SECTION I. INTRODUCTION**

1

**SECTION II. REVIEW**

2.1. Physiology of Ripening
   2.1.1. Fruit development 7
   2.1.2. Respiratory climacteric 9
   2.1.3. Changes in colour 10
   2.1.4. Changes in texture 11

2.2. Biochemistry of Ripening
   2.2.1. Ethylene biosynthesis 12
   2.2.2. Metabolism of 1-aminocyclopropane-1-carboxylic acid (ACC) 14
   2.2.3. Polyamine 18
   2.2.4. Cell Wall Enzymes 22

2.3. Cool Storage
   2.3.1. Chilling injury in stored avocado 24
   2.3.2. Controlled atmosphere storage 25
## SECTION III. EFFECT OF CONTROLLED ATMOSPHERE ON STORAGE LIFE

### 3.1. Introduction

### 3.2. Material and Method

- **3.2.1. Source of fruit**
- **3.2.2. Experimental plan**
- **3.2.3. Fruit colour**
- **3.2.4. Fruit firmness**
- **3.2.5. Chilling injury**
- **3.2.6. Rates of respiration and ethylene production**
- **3.2.7. Statistical analysis of data**

### 3.3. Results and Discussion

- **3.3.1. Experiment 1: 1995 - 1996**
  - **3.3.1.1. Colour changes**
  - **3.3.1.2. Fruit firmness**
  - **3.3.1.3. Chilling injury**
- **3.3.2. Experiment 2: 1996 - 1997**
  - **3.3.2.1. Colour changes**
  - **3.3.2.2. Fruit firmness**
  - **3.3.2.3. Chilling injury**

### 3.4. Conclusions

## SECTION IV. BIOCHEMICAL CHANGES OF AVOCADO IN CA STORAGE

### 4.1. Introduction

### 4.2. Material and Methods

- **4.2.1. Experimental plan**
- **4.2.2. Rates of respiration and ethylene production**
- **4.2.3. Concentration of 1-aminocyclopropane-1-carboxylic Acid (ACC)**
- **4.2.4. ACC oxidase activity**
- **4.2.5. Polyamine**
4.3. Result and Discussion

4.3.1. Experiment 1: 1996-1996
4.3.1.1. Rates of respiration and ethylene production of avocado fruit at 20°C after CA storage at 0°C.
4.3.1.2. Changes in ACC levels in fruit at 20°C after CA storage at 0°C
4.3.1.3. Changes in ACC oxidase activity in fruit at 20°C after CA storage at 0°C
4.3.1.4. Changes in Polyamines concentrations in fruit following transfer to air at 20°C after CA storage for 3, 6 and 9 weeks at 0°C

4.3.2. Experiment 2: 1996 - 1997
4.3.2.1. Rates of respiration and ethylene production of avocado fruit at 20°C after CA storage at 0 and 5°C
4.3.2.2. Changes in ACC concentrations in fruit at 20°C after CA storage at 0 and 5°C.
4.3.2.3. Changes of ACC oxidase activity in fruit at 20°C after CA storage at 0 and 5°C.

4.4. Conclusions

SECTION V. EFFECT OF CONTROLLED ATMOSPHERE ON PROTEIN CHANGES

5.1. Introduction

5.2. Materials and methods
5.2.1. Fruit
5.2.2. Protein extraction
5.2.2.1. Tris/βmercaptoethanol buffer
5.2.2.2. Phenol-protein extraction buffer
5.2.3. Protein separation
5.2.3.1. One-dimensional polyacrylamide gel electrophoresis (1D-PAGE)
5.2.3.2. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)
5.2.3.2.1. First-dimension; Isoelectric focusing (IEF)
5.2.3.2.2. Two-dimension; Separation by molecular weights
5.2.3.2.2.1. Protocol for casting resolving gels
5.2.3.2.2.2. Protocol for casting gels
5.2.3.2.2.3. The equilibration of the first dimension (IEF) gels
5.2.3.2.4. Loading the IEF gels onto slab gel.
5.2.3.2.5. Running the second dimension

5.2.4. Silver-nitrate staining procedure

5.3. Results and Discussion

5.4. Conclusions

SECTION VI. GENERAL DISCUSSION AND CONCLUSIONS

SECTION VII. LITERATURE CITED
The primary objective of this research was to study the physiological and biochemical changes in Hass avocado fruit stored in different combinations of O₂ and CO₂ concentrations at both 0°C and 5°C, and to determine whether storage in controlled atmosphere (CA) can decrease the incidence of chilling injury (CI). A secondary objectives was to identify possible correlations between CA, the incidence of CI, the activity of some ripening related enzymes and changes in proteins during ripening at 20°C following storage at low temperatures.

Fruit suffered no CI and ripened normally following CA storage for 3 weeks at both 0°C and 5°C then transferred to air for 6 days at 20°C. CI symptoms did develop after CA storage for 6 and 9 weeks at 0°C. No CI occurred in atmospheres of 2.5% O₂ + 7.5% CO₂ and 5% O₂ + 5% CO₂ following storage for 9 weeks at 5°C, and only very slight discoulouration in atmospheres of 2.5% O₂ + 5% CO₂ and 5% O₂ + 7.5% CO₂. Control fruit stored in air became very soft and exhibited severe flesh browning following 9 weeks storage at 5°C. CA storage for up to 3 weeks at 0°C stimulated ripening as compared to CA storage at 5°C. Following transfer to air at 20°C, these fruit developed climacteric-like peaks in respiration and ethylene production 2 - 4 days later. In comparison, the climacteric peaks in respiration and ethylene production were not recorded until day 14 in freshly harvested fruit ripened in air. After 6 and 9 weeks storage peak rates were recorded about one day later, but still much earlier than freshly harvested fruit. Ethylene production was slightly lower in fruit after 9 weeks CA storage. CA had little effect on the pattern of respiration and ethylene production after transfer to air at 20°C.

ACC concentrations were very low in fruit upon removal from storage following 3 weeks at 0°C and 5°C. ACC was measurable in most CA treatments upon removal
from 0°C after 6 and 9 weeks storage. ACC accumulated gradually in fruit stored at 5°C following transfer to air at 20°C. The onset of ACC accumulation occurred progressively sooner following 6 and 9 weeks storage at 5°C. CA delayed the accumulation of ACC. ACC began to accumulate after 2 - 4 days in parallel with increased ethylene production in fruit stored at 0°C. This early increase in ACC appears to be a consequence of chilling stress. Storage in CA did not prevent the early increase in ACC. The change in activity ACC oxidase paralleled the change in ACC concentration in fruit transferred to air at 20°C, from storage at 0° and 5°C, after 3, 6 and 9 weeks. The polyamines concentrations were higher on the day of transfer from 0°C to 20°C after storage 3, 6 and 9 weeks, then subsequently decreased during ripening at 20°C. There were no consistent effect of CA on polyamines concentrations.

Changes in proteins during ripening were analysed by 2D-PAGE, some polypeptides, e.g. 23 and 75 kD were detected in unripe fruit but decreased with ripening. Polypeptides of 16.5, 25, 36 and 56 kD were present early in ripening and their levels further increased during ripening. The appearance of three ripening related polypeptides with estimated molecular weights 80 kD (pI 3.6), 36 kD (pI 5.8) and 16.5 kD (pI 5.7) was observed in fruit at the climacteric stage. Three polypeptides with estimated molecular weights of 41 kD (pI 7.8), 36 kD (pI 5.8) and 33 kD (pI 5.1) were found in air stored fruit but were not detected in fruit stored in CA. This research showed that CA did not ameliorate CI at 0°C, instead storage at 0°C caused a premature increase in ethylene production when the fruit were returned to air at 20°C. In contrast, CA storage at 5°C retarded ethylene production and ripening in fruit after it was returned to air at 20°C.
DECLARATION

The Research reported in this thesis has not been submitted for a higher degree at any other University or Institution.

Eko Basuki
ACKNOWLEDGMENTS

My gratitude goes to the Ministry of Education and Culture, Republic of Indonesia, for the provision of study leave which have made this research for this degree possible.

Ph.D. Supervisors,

Assoc. Prof. G. Skurray, University of Western Sydney, Hawkesbury, Richmond N.S.W.

Dr. W.B. McGlasson, University of Western Sydney, Hawkesbury, Richmond N.S.W.

I would like to thank my supervisors for all assistance, encouragement and critical analysis during this research. I have been fortunate to have been supported by such understanding and inspiring scientists.

I would like also thank Gary Morgan, Rob Sturgess and Margaret M. Ryan for their technical support, Jane Malfroy and Robin Heath for their help with the English language.

My appreciation also goes to AUSAID for a scholarship and constant support.

I thank Nasser Abdi for his guidance on 2D-PAGE methods for protein analysis.

Finally, I am indebted to my wife, son and daughter in Hobartville, NSW and my parents in Indonesia for their support and encouragement.
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Compositional changes in avocado fruit associated with development (After Biale and Young, 1970)</td>
<td>8</td>
</tr>
<tr>
<td>2.2</td>
<td>Respiration rate and ethylene production of Hass avocado during the ripening stage at 20°C.</td>
<td>10</td>
</tr>
<tr>
<td>2.3</td>
<td>The ethylene biosynthesis pathway.</td>
<td>13</td>
</tr>
<tr>
<td>2.4</td>
<td>The polyamines biosynthetic pathway and its linkage to ethylene biosynthesis.</td>
<td>19</td>
</tr>
<tr>
<td>3.1</td>
<td>Skin colour change of avocado fruit ripened in air for 6 days at 20°C following storage at 0°C for 3 weeks (data 1995-1996).</td>
<td>39</td>
</tr>
<tr>
<td>3.2</td>
<td>Skin colour change of avocado fruit ripened in air for 6 days at 20°C following storage in CA at 0°C for 6 weeks (data 1995-1996).</td>
<td>40</td>
</tr>
<tr>
<td>3.3</td>
<td>Skin colour change of avocado fruit ripened in air for 6 days at 20°C following storage at 0°C for 9 weeks (data 1995-1996).</td>
<td>41</td>
</tr>
<tr>
<td>3.4</td>
<td>Changes in firmness of avocado fruit following transfer to air at 20°C for 6 days after storage at 0°C for 3, 6 and 9 weeks (data 1995-1996).</td>
<td>43</td>
</tr>
<tr>
<td>3.5</td>
<td>Severity of chilling injury of avocado flesh after 6 days at 20°C following transfer from storage at 0°C for 3, 6 and 9 weeks (data 1995-1996).</td>
<td>45</td>
</tr>
<tr>
<td>3.6</td>
<td>Changes in colour of avocado fruit following transfer to air at 20°C after storage at both temperatures 0°C (above) and 5°C (bottom) for 3 weeks (data 1996-1997).</td>
<td>48</td>
</tr>
<tr>
<td>3.7</td>
<td>Changes in colour of avocado fruit following transfer to air at 20°C after storage at both temperatures 0°C (above) and 5°C (bottom) for 6 weeks (data 1996-1997).</td>
<td>49</td>
</tr>
</tbody>
</table>
Figure 3.8  Changes in colour of avocado fruit following transfer to air at 20°C after CA storage at both temperatures 0°C (above) and 5°C (bottom) for 9 weeks (data 1996-1997).

Figure 3.9. Changes in firmness of avocado fruit following transfer to air for 6 days after storage at 0°C for 3, 6 and 9 weeks (data 1996-1997).

Figure 3.10. Changes in firmness of avocado fruit following transfer to air for 6 days after CA storage at 5°C for 3, 6 and 9 weeks (data 1996-1997).

Figure 3.11. Severity of chilling injury of avocado flesh after 6 days at 20°C following transfer from storage at 0°C for 3, 6 and 9 weeks (data 1996-1997).

Figure 3.12. Severity of chilling injury of avocado flesh after 6 days at 20°C following transfer from CA storage at 5°C for 3, 6 and 9 weeks (data 1996-1997).

Figure 4.1. Rates of respiration (A), ethylene production (B), ACC concentrations (C) and ACC oxidase activity (D) of harvested avocado fruit in November 1995 (exp1) and in February 1997 (exp2).

Figure 4.2. Rates of respiration (A+B) and ethylene production (C+D) of avocado fruit after transfer to air at 20°C following storage for 3 weeks at 0°C.

Figure 4.3. Rates of respiration (A+B) and ethylene production (C+D) of avocado fruit after transfer to air at 20°C following storage for 6 weeks at 0°C.

Figure 4.4. Rates of respiration (A+B) and ethylene production (C+D) of avocado fruit after transfer to air at 20°C following storage for 9 weeks at 0°C.

Figure 4.5. ACC concentrations in avocado fruit after transfer to air at 20°C following storage for 3 weeks at 0°C.

Figure 4.6. ACC concentrations in avocado fruit after transfer to air at 20°C following storage for 6 weeks at 0°C.
Figure 4.7. ACC concentrations in avocado fruit after transfer to air at 20°C following storage for 9 weeks at 0°C.

Figure 4.8. ACC oxidase activity in avocado fruit after transfer to air at 20°C following storage for 3 weeks at 0°C.

Figure 4.9. ACC oxidase activity in avocado fruit after transfer to air at 20°C following storage for 6 weeks at 0°C.

Figure 4.10. ACC oxidase activity in avocado fruit after transfer to air at 20°C following storage for 9 weeks at 0°C.

Figure 4.11. Changes in polyamines in avocado fruit after transfer to air at 20°C following storage for 3 weeks at 0°C.

Figure 4.12. Changes in polyamines in avocado fruit after transfer to air at 20°C following CA storage for 6 weeks at 0°C.

Figure 4.13. Changes in polyamines in avocado fruit after transfer to air at 20°C following CA storage for 9 weeks at 0°C.

Figure 4.14. Rates of respiration (A) and ethylene productions (B) of avocado fruit after transfer to air at 20°C following storage for 3 weeks at 0°C.

Figure 4.15. Rates of respiration (A) and ethylene productions (B) of avocado fruit after transfer to air at 20°C following storage for 6 weeks at 0°C.

Figure 4.16. Rates of respiration and ethylene productions of avocado fruit after transfer to air at 20°C following storage for 9 weeks at 0°C (A+C) and 5°C (B+D).

Figure 4.17. Rates of respiration (A) and ethylene productions (B) of avocado fruit after transfer to air at 20°C following storage for 3 weeks at 5°C.

Figure 4.18. Rates of respiration (A) and ethylene productions (B) of avocado fruit after transfer to air at 20°C following storage for 6 weeks at 5°C.
Figure 4.19. ACC concentrations in avocado fruit after transfer to air at 20°C following storage for 3 weeks at 0°C (A) and 5°C (B).

Figure 4.20. ACC concentrations in avocado fruit after transfer to air at 20°C following storage for 6 weeks at 0°C (A) and 5°C (B).

Figure 4.21. ACC concentrations in avocado fruit after transfer to air at 20°C following storage for 9 weeks at 0°C (A) and 5°C (B).

Figure 4.22. ACC oxidase activity in avocado fruit after transfer to air at 20°C following storage for 3 weeks at 0°C (A) and 5°C (B).

Figure 4.23. ACC oxidase activity in avocado fruit after transfer to air at 20°C following storage for 6 weeks at 0°C (A) and 5°C (B).

Figure 4.24. ACC oxidase activity in avocado fruit after transfer to air at 20°C following storage for 9 weeks at 0°C (A) and 5°C (B).
# LIST OF PLATES

<table>
<thead>
<tr>
<th>Section 1.</th>
<th>Plate 1.1.</th>
<th>Longitudinal section of an avocado fruit</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Section 3.</th>
<th>Plate 3.1.</th>
<th>Controlled Atmosphere containers were ventilated with metered flows of humidified air and mixed gases.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plate 3.2.</th>
<th>Flow meters used to regulate the composition of the gas mixtures admitted to the storage containers illustrated in Plate 4.1.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>32</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plate 3.3.</th>
<th>CI symptoms of avocado fruit after 6 days in air at 20°C following CA (2.5 %O₂+5 %CO₂) storage at 0°C (A) and 5°C (B) for 6 weeks. The fruit stored at 0°C exhibit severe discolouration whereas the fruit stored at 5°C were not discoloured.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>59</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plate 3.4.</th>
<th>CI symptoms of avocado fruit following transfer to air at 20°C after CA (2.5 %O₂+7.5 %CO₂) storage for 6 weeks at 0°C (top). The fruit stored at 5°C in the same atmosphere exhibited no discolouration (centre). The fruit stored at air for 6 weeks at 0°C exhibited CI and thickening of the skin (bottom).</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plate 3.5.</th>
<th>Fruit exhibited very light discolouration after 6 days in air at 20°C following CA (5 %O₂+5 %CO₂) storage for 6 weeks at 0°C (A). The fruit stored at 5°C in the same atmosphere exhibited no discolouration (B). The fruit stored in air for 6 weeks at 5°C exhibited very light discolouration.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>61</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plate 3.6.</th>
<th>CI symptoms of avocado fruit after transfer to air at 20°C following CA (5 %O₂+7.5 %CO₂) storage for 6 weeks at 0°C (A) and 5°C (B). The fruit stored at 0°C showed very light discolouration, whereas the fruit stored at 5°C were not discoloured</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>62</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plate 3.7.</th>
<th>CI symptoms of avocado fruit after 6 days in air at 20°C following CA (2.5 %O₂+5 %CO₂) storage at of 0°C (A) and 5°C(B) for 9 weeks. The fruit stored at 0°C exhibited severe discolouration whereas the fruit stored at 5°C were not discoloured</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>63</td>
</tr>
</tbody>
</table>
Plate 3.8. CI symptoms of avocado fruit after transfer to air at 20°C following CA (2.5 %O₂+7.5 %CO₂) storage at 0°C (A) and 5°C (B) for 9 weeks. The fruit stored at 0°C exhibited severe discoloration whereas the fruit stored at 5°C were not discoloured. The fruit exhibited severe discoloration after transfer to air for 6 days at 20°C following air storage at 0°C for 9 weeks (C).

Plate 3.9. CI symptoms of avocado fruit after transfer to air at 20°C following CA (5 %O₂+5 %CO₂) storage at 0°C (A) and 5°C (B) for 9 weeks. The fruit stored at 0°C exhibited severe discoloration whereas the fruit stored at 5°C were not discoloured. The fruit exhibited severe discoloration and had become very soft following transfer to air for 6 days at 20°C after air storage at 5°C for 9 weeks (C), indicating severe tissue breakdown.

Plate 3.10. CI symptoms of avocado fruit after transfer to air at 20°C following CA (5 %O₂+7.5 %CO₂) storage at 0°C (A) and 5°C (B) for 9 weeks. The fruit stored at 0°C exhibited severe discoloration whereas the fruit stored at 5°C were not discoloured.

Section 5.
Plate 5.1. SDS-PAGE of total protein extracted from avocado fruit. Lanes A, B and C correspond to samples at different stage of ripening (0, 12 and 14 days after harvest). SDS standard (std) used as a molecular mass relative marker. Protein (40 μg) separated by electrophoresis were visualised by silver staining. The open arrows indicate polypeptides that increased during ripening, and closed arrows indicate polypeptides that remain constant.

Plate 5.2. Two-dimensional PAGE chromatograph of proteins extracted from unripe freshly harvested avocado fruit. Protein (60 μg) separated by electrophoresis were visualised by silver staining. The arrows indicated locations where some polypeptides were absent in unripe fruit but were detected in ripe fruit.

Plate 5.3. Two-dimensional PAGE chromatograph of proteins extracted from ripe harvested avocado fruit. Protein (60 μg) separated by electrophoresis were visualised by silver staining. The arrows indicated locations where some polypeptides were absent in unripe fruit but were detected in ripe fruit.
Plate 5.4. Two-dimensional PAGE chromatograph of proteins extracted from avocado fruit following storage in air for 3 weeks at 0°C. Protein (60 μg) separated by electrophoresis were visualised by silver staining. The arrows indicated some polypeptides present in air but not in CA treatments.

Plate 5.5. Two-dimensional PAGE chromatograph of proteins extracted from avocado fruit following storage in CA (2.5 %O₂ + 5 %CO₂) for 3 weeks at 0°C. Protein (60 μg) separated by electrophoresis were visualised by silver staining. The arrows indicated locations of polypeptides absent in this fruit but present in fruit stored in air.

Plate 5.6. Two-dimensional PAGE chromatograph of proteins extracted from avocado fruit following storage in CA (5 %O₂ + 7.5 %CO₂) for 3 weeks at 0°C. Protein (60 μg) separated by electrophoresis were visualised by silver staining. The arrows indicated locations of polypeptides absent in this fruit but present in fruit stored in air.

Plate 5.7. Two-dimensional PAGE chromatograph of proteins extracted from avocado fruit following storage in CA (5 %O₂ + 5 %CO₂) for 3 weeks at 0°C. Protein (60 μg) separated by electrophoresis were visualised by silver staining. The arrows indicated locations of polypeptides were absent in CA compared to fruit stored in air.

Plate 5.8. Two-dimensional PAGE chromatograph of proteins extracted from avocado fruit following storage in CA (5 %O₂ + 7.5 %CO₂) for 3 weeks at 0°C. Protein (60 μg) separated by electrophoresis were visualised by silver staining. The arrows indicated some polypeptides missing in CA compared to fruit stored in air.

Plate 5.9. Two-dimensional PAGE chromatograph of proteins extracted from avocado fruit following storage in CA (2.5 % O₂ + 5 %CO₂) storage for 3 weeks at 5°C. Protein (60 μg) separated by electrophoresis were visualised by silver staining. The arrows indicated some polypeptides missing from this fruit but detected in fruit stored in air.
Plate 5.10. Two-dimensional PAGE chromatograph of proteins extracted from avocado fruit following storage in air for 9 weeks at 5°C. Protein (60 μg) separated by electrophoresis were visualised by silver staining. Black arrows show polypeptides that were not detected compared to fruit stored in air for 3 weeks. Light arrows indicated polypeptides that were not detected in fruit stored for only 3 weeks.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>ACC oxidase</td>
<td>1-aminocyclopropane-1-carboxylic acid oxidase</td>
</tr>
<tr>
<td>ADC</td>
<td>arginine decarboxylase</td>
</tr>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>AdoMet</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>AdoMetDC</td>
<td>S-adenosylmethionine decarboxylase</td>
</tr>
<tr>
<td>Agn</td>
<td>arginase</td>
</tr>
<tr>
<td>AOA</td>
<td>aminooxyacetic acid</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>AVG</td>
<td>aminoethoxyvinylglycine</td>
</tr>
<tr>
<td>Bis</td>
<td>N,N'-methylenebisacrylamide</td>
</tr>
<tr>
<td>CA</td>
<td>controlled atmosphere</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>Ca(OH)₂</td>
<td>calcium hydroxide</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate</td>
</tr>
<tr>
<td>CH₃</td>
<td>methyl</td>
</tr>
<tr>
<td>CI</td>
<td>chilling injury</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>dAdoMet</td>
<td>decarboxylated S-adenosylmethionine</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>deionised water</td>
</tr>
<tr>
<td>DFMA</td>
<td>DL-α-difluoromethylarginine</td>
</tr>
<tr>
<td>DFMO</td>
<td>DL-α-difluoromethylornithine</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DTE</td>
<td>dithioerythritol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>e.g.</td>
<td><em>exempli gracia</em></td>
</tr>
</tbody>
</table>
g  gram
h  hour
HCl  hydrochloric acid
HPLC  high performance liquid chromatography
H₃PO₄  phosphoric acid
ID  internal diameter
IEF  isoelectric focusing
kD  kilo Dalton
kg  kilogram
km  kilometre
KOH  potassium hydroxide
L  litre
mA  milli ampere
MACC  1-(malonylamino)cyclopropane-1-carboxylic acid
MGBG  methylglyoxal-bis(guanylhydrazone)
MgSO₄  magnesium sulphate
MW  molecular weight
MWₙ  relative molecular weight
MTA  5’- methylthioadenosine
MTR  5’- methylthioribose
N  Newton
N₂  nitrogen
NaCl  sodium chloride
NaHCO₃  sodium hydroxy chloride
NaOCl  sodium hypochloride
NaOH  sodium hydroxide
nmol  nanno mole
N.S.W.  New South Wales
OD  outside diameter
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetra methyl ethyl diamine</td>
</tr>
<tr>
<td>UWSH</td>
<td>University of Western Sydney Hawkesbury</td>
</tr>
<tr>
<td>2D-PAGE</td>
<td>two-dimensional Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
</tbody>
</table>