MANAGEMENT OF AVOCADO POSTHARVEST PHYSIOLOGY

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Abstract

Avocados are an important horticultural crop in South Africa, especially in the provinces of KwaZulu-Natal, Mpumalanga and Limpopo. The distance to traditional export markets, phytosanitary restrictions to lucrative markets such as China, the USA and Japan and increased competition in the European market have challenged the South African avocado industry. The industry has responded with improved logistics and shipping, a co-ordinated market access program and a global system to co-ordinate exports of avocados to the European market. To remain competitive on the global market, further improvements and innovations are required to improve the efficiency of postharvest operations. These improvements and innovations should be guided by a greater understanding of postharvest physiology. Avocados are a relatively new export crop, so there is still much to be learnt about avocado postharvest physiology and the optimisation of postharvest management. In this regard, reduced temperature storage (1°C) and modified humidity packaging (MHP) were investigated for their effect on fruit physiology and quality, the effect of a water- and ABA-infusion on ripening was examined and the effect of a cold chain break on fruit physiology and quality determined; near-infrared spectroscopy was also examined for its potential for its use in the avocado industry.

As an initial study, the relationships between individual sugars, protein and oil were studied to understand the changes in avocado fruit during ripening. It was found that mannoheptulose and perseitol were the predominant sugars at harvest, but declined to very low levels during the first 10 days postharvest. The concentrations of glucose and fructose increased, while sucrose declined slightly during ripening. The concentration of protein increased sigmoidally during ripening, reflecting the increase in the ripening enzymes, particularly cellulase and polygalacturonase. The oil content fluctuated slightly during ripening. It is suggested that mannoheptulose and perseitol are important carbon and energy sources during ripening. Glucose concentration was also found to increase earlier in fast ripening fruit compared to slow ripening fruit, which is related to increased cellulase activity and may be related to the ABA functioning.

Thereafter, storage and ripening trials in two consecutive seasons showed that 1°C storage and the use of MHP for 28 days reduced mass loss, water loss, ethylene production, respiration, softening and heptose consumption, without appreciably affecting fungal rots, physiological disorders or external chilling injury, compared to fruit stored at 5.5°C and

regular atmosphere respectively. Also, the storage of fruit in MHP delayed the rise in the activity of cellulase during ripening, compared to fruit not stored in MHP, but there was no significant difference in the peak activity of cellulase, polygalacturonase or pectin methylesterase.

In a separate experiment, fruit ripening was significantly affected by the infusion of ABA in an aqueous solution. Water slightly reduced the variation in ripening while ABA reduced the time to ripening and the variation; it is suggested that water stress and ABA are intrinsically involved in the ripening processes and may act as a ripening trigger. The water concentration in fruit was measured non-destructively using reflectance NIR; this model was used to determine the maturity of fruit and the loss of water during cold storage.

In the cold chain break experiment, it was found that although fruit recovered after a cold chain break, in terms of ethylene production and respiration, there was a loss in quality because of severe shrivelling as a result of increased water loss. Fruit that were stored at 1°C were generally of a better quality at ripeness, if the cold chain was broken, compared to fruit stored at 5.5°C. In a follow-up experiment, it was found that significant changes occurred in avocado physiology over a 6h period. The respiration rate of fruit significantly increased after 4h at room temperature and mannoheptulose declined by 32% in control fruit and by 16% in ethephon-treated fruit after 6h. This demonstrates the potential for quality loss in a short amount of time.

Furthermore, a model of avocado ripening is proposed, outlining the role of water, ABA, ethylene, respiration, ripening enzymes and individual sugars. This study has contributed to the understanding of avocado postharvest physiology and should aid in better management of avocados for improved fruit quality and consumer satisfaction.

Declaration

I, Robert John Blakey, declare that the research reported in this thesis, except where otherwise indicated, is my original work. This thesis has not been submitted for any degree or examination at any other university.

Robert J. Blakey

March 2011

We certify that the above statement is correct.

Prof John P. Bower

Supervisor

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Dr Isa Bertling

Co-supervisor

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The message of the cross is foolishness to those who are perishing, but to us who are being saved it is the power of God. For it is written:

"I will destroy the wisdom of the wise;
the intelligence of the intelligent I will frustrate."

Where is the wise man? Where is the scholar? Where is the philosopher of this age? Has not God made foolish the wisdom of the world? For since in the wisdom of God the world through its wisdom did not know him, God was pleased through the foolishness of what was preached to save those who believe. Jews demand miraculous signs and Greeks look for wisdom, but we preach Christ crucified: a stumbling block to Jews and foolishness to Gentiles, but to those whom God has called, both Jews and Greeks, Christ the power of God and the wisdom of God. For the foolishness of God is wiser than man's wisdom, and the weakness of God is stronger than man's strength. Brothers, think of what you were when you were called. Not many of you were wise by human standards; not many were influential; not many were of noble birth. But God chose the foolish things of the world to shame the wise; God chose the weak things of the world to shame the strong. He chose the lowly things of this world and the despised things – and the things that are not – to nullify the things that are, so that no one may boast before him. It is because of him that you are in Christ Jesus, who has become for us wisdom from God – that is, our righteousness, holiness and redemption. Therefore, as it is written:

"Let him who boasts boast in the Lord".

1 Corinthians 1:18-31

Table of Contents

Hypotheses

The main hypothesis of this study was:

The reduction in metabolic activity and water stress during cold storage, by the reduction of the storage temperature, use of modified humidity packaging and minimisation of cold chain breaks, will result in improved fruit quality of South African 'Hass' relative to storage at 5.5°C in regular atmosphere.

Subordinate to this main hypothesis were the following:

1. The heptose sugars, mannoheptulose and perseitol, are important in avocado physiology as sources of carbon and energy in avocado ripening.
2. The storage of South African 'Hass' avocados at 1°C for 28 days will reduce metabolic activity during cold storage and will not significantly worsen external fruit quality compared to fruit stored at 5.5°C.
3. The use of modified humidity packaging will reduce the rate of water loss during cold storage of fruit stored at 1°C and 5.5°C. This, in turn, will reduce the ethylene production rate and respiration rate of fruit stored in modified humidity packaging.
4. Near infrared spectroscopy can be used to measure the moisture content of avocado fruit.
5. Avocado ripening is stimulated by water stress and an increase in abscisic acid.
6. A break in the cold chain will cause a transient increase in the metabolic activity of avocados and result in premature softening and increased internal disorders.
7. Exposure to exogenous ethylene, in the form of ethephon, will increase the risk of internal disorders during or after cold storage.

Aims

Determine the effects of 1°C cold storage, modified humidity packaging and cold chain breaks on the ripening physiology and quality of South African 'Hass' avocados to better understand the postharvest physiology of avocados and develop an integrated model of ripening. This is in relation to the desire of the South African avocado industry to export fruit to markets with phytosanitary requirements.

Objectives

The objective of the study was to analyse the critical compounds and enzymes in avocado ripening physiology and how these compounds are affected by 1°C storage and the use of modified humidity packaging, two potential commercial cold storage treatments for South African 'Hass' avocados. Specifically the objectives were:

1. Identify the role of soluble sugars and their relation to protein synthesis and oil metabolism in South African 'Hass' avocado ripening physiology.
2. Determine the effects of storage at 1°C on the physiology of South African 'Hass' avocados, particularly respiration rate, ethylene production rate, soluble sugars, total protein, cellulase, polygalacturonase and pectin methylesterase, oil and fruit quality.
3. Determine the ability of near infrared spectroscopy to measure moisture content of whole avocados.
4. Determine the effect of water and ABA infusion on the ripening pattern of avocados.
5. Determine the effects of cold chain breaks on ripening and quality during simulated export of South African 'Hass' avocados to Europe.
6. Determine the short term effects of ethephon on stress physiology.

Chapter 1
Literature review

Table of Contents

1. Introduction

Avocados are still a relatively new crop outside of Central America and the Caribbean, although crop physiology has been studied extensively, problems such as variable ripening and poor internal and external quality abound in the global avocado industry. These problems are due, in part, to the oleaginous nature of the fruit, the low concentration of sugars in the mesocarp, a protracted flowering period and chilling sensitivity. The solutions to these problems depend on adequately understanding fruit physiology and applying mitigating treatments and management practices. It may be impossible to minimise fruit variability, but it may be possible to sort fruit accordingly to minimise variation within cartons and pallets using near-infrared spectroscopy. Avocado ripening physiology, the effects of cold storage on physiology and mitigating postharvest treatments during cold storage to maintain fruit quality are reviewed. A section on near-infrared spectroscopy is included as this branch of spectroscopy offers the opportunity to measure internal quality attributes, such as fruit maturity, of avocados.

Plants are designed to function holistically, in an integrated manner, so the concepts of holism and systems biology have been used as much as possible, but this is challenging because of the linearity of writing and the multi-dimensionality of physiology.

2. Ripening Physiology

During ripening major biochemical, organoleptic and rheological changes occur that make fruit palatable. Fruit have four major physiological stages of development: growth, maturation, ripening and senescence (Figure 1). Cell division and enlargement are part of growth and determine the final size of the fruit. In avocado fruit, cell division and enlargement continue until harvest, although at a slower rate than immediately after anthesis (Cowan *et al.*, 1997). Growth and maturation, collectively termed the development stage, only occur preharvest. Fruit is deemed mature when it is capable of ripening; if immature fruit are harvested they will merely senesce and decay. In avocados, the maturation stage can be extended by delaying harvest and allowing the fruit to accumulate more oil. Ripening is the result of a number of complex physiological and physical changes with distinct anabolic and catabolic processes that require large amounts of energy and prolonged membrane integrity (Bower and Cutting, 1988; Wills *et al.*, 1998). Senescence is the period when catabolic

processes exceed anabolic processes, resulting in aging and necrosis (Wills *et al.*, 1998). Harvesting places stress on a fruit as the fruit are detached from the mother plant and must rely on water and energy reserves acquired during preharvest development. These resources are depleted during storage and ripening and the management of these resources determines postharvest fruit quality. Ripening is controlled by many physiological factors: water movement, phytohormones, mineral nutrition, carbohydrates and ripening enzymes all contribute to ripening rate and fruit quality (Awad and Young, 1979; Cutting *et al.*, 1986; Lieberman *et al.*, 1977; Tingwa and Young, 1974; van Rooyen, 2006).

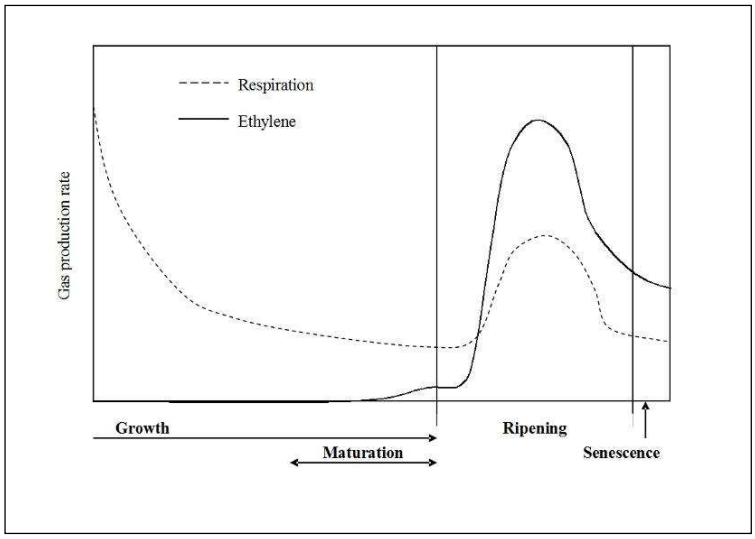

Figure 1: The four stages of avocado fruit ontogeny: growth, maturation, ripening and senescence.

2.1. The Climacteric

The climacteric phase is part of the ripening process of certain fruits during which a series of biochemical changes is initiated and accompanied by the autocatalytic production of ethylene and an increase in respiration (Rhodes, 1981). Softening, with the concomitant climacteric, is the most evident change in avocados and has been extensively and intensively studied over the years (Pesis *et al.*, 1978a). For fruit to ripen, it is necessary for fruit to accumulate sufficient energy and carbon reserves from the mother plant to supply the postharvest respiratory and metabolic requirements. Ripening requires large amounts of energy because of the numerous metabolic processes that are required to achieve softness. An adequate carbon source is also required for anabolic processes, particularly the synthesis of cell wall modifying enzymes. The management of the climacteric is critical for maintaining fruit quality. Fruit must remain in the pre-climacteric stage during cold storage to ensure

internal quality is not compromised. Fruit quality changes rapidly once fruit enter the climacteric and commercially fruit need to be monitored carefully during ripening to prevent spoilage. These points are discussed in the relevant sections.

2.2. Carbohydrates

Carbohydrates are the carbon source of all biological compounds and major energy source in respiration (Mann, 1987). Carbon dioxide is converted to glucose during the Calvin Cycle and from there various pathways, such as the shikimic acid and pentose phosphate pathways, are used for the synthesis of other organic compounds. Carbohydrates are also a major energy store in plants and are involved in cellular signalling. Sugars are therefore crucial multi-functional compounds in plant physiology. A detailed review of carbohydrates in avocado was made by Tesfay (2009).

2.2.1. Monosaccharides

Avocados contain approximately twenty sugars and sugar alcohols (Bean, 1958), including relatively high concentrations the naturally rare heptose mannoheptulose (D-manno-2-ketoheptose) and its sugar-alcohol perseitol (D-glycero-D-galacto-heptitol) (Liu *et al.*, 1999b). Although their presence in avocado has been long known (Avequin, 1831; Fischer and Passmore, 1890; La Forge, 1917; Muntz and Marcano, 1884), the functions of these sugars have not previously been clearly defined, nor their prevalence over hexoses fully explained, but their importance in avocado ripening has been suggested (Liu *et al.*, 1999a; Liu *et al.*, 2002; Meyer and Terry, 2010). Liu *et al*. (2002) proposed that mannoheptulose may be a ripening inhibitor, but the heptoses may also be involved in avocado physiology as carbon and energy sources (Liu *et al.*, 1999a; Liu *et al.*, 2002; Meyer and Terry, 2010; Tesfay, 2009; Tesfay *et al.*, 2010) and the decline in heptoses may be due to their utilisation in respiration and metabolism during ripening. Sugar alcohols are thought to function as osmo-protectants during water stress conditions (Popp and Smirnoff, 1995). Perseitol may fulfil this function, particularly in the seed and mesocarp where the water content is relatively low (Tesfay, 2009).

Monosaccharides are used as the carbon source during anti-oxidant synthesis, particularly the synthesis of ascorbic acid and glutathione from glucose (Noctor and Foyer, 1998; Smirnoff *et al.*, 2001). Monosaccharides are also the building blocks of the cell wall. Glucose is the monomer of cellulose and galacturonic acid and xylose are the predominant

monomers in pectin and hemicellulose, respectively. The low concentration of hexose sugars in the mesocarp may be due to the unusually high concentration of lipids, because fructose is a precursor in triglyceride synthesis (Basciano *et al.*, 2005; Zavaroni *et al.*, 1982). Fruit from the Lowland (West Indian) race have a higher concentration of sugars, and have a lower concentration of oil, compared to fruit from Mexican and Guatemalan races (Shaw *et al.*, 1980). This is further discussed in Section 2.4.

Besides being used as carbon and energy sources, sugars are also signalling molecules (León and Sheen, 2003) and each sugar may exert different effects (Campbell *et al.*, 2000). Glucose and ethylene are antagonistic and glucose contributes to the regulation of abscisic acid (ABA) biosynthesis and signalling (Campbell *et al.*, 2000; León and Sheen, 2003).

2.2.2. Polysaccharides

Carbohydrate polymers are used extensively in the plant for energy storage and cell wall synthesis.

2.2.2.1. Starch

The exo- and mesocarp contain very little starch, 9-11 mg.g DM^{-1} (Liu *et al.*, 1999a; Liu *et al.*, 2002; Pesis *et al.*, 1978b). Pesis *et al.* (1978b) found that the concentration of starch declined and the activity of amylase (EC 3.2.1.1 or 3.2.1.2) increased in the mesocarp during ripening; at ripeness starch was not detectable. Liu *et al.* (2002), however, found that the starch was not completely solubilised during ripening. Starch probably contributes to the energy supply during ripening (Pesis *et al.*, 1978b), but is present in lower concentrations than the heptoses.

Most plants store carbohydrates as starch, but sugar alcohols and oligosaccharides can also fulfil this function (Heldt and Heldt, 2005). Perseitol is thought to be a storage compound in avocado (Tesfay, 2009). Oligosaccharides are quite rare in avocados, but their importance should not be neglected. Further research on starch and oligosaccharides is warranted.

2.2.2.2. The Cell Wall

The cell wall is a complex structure that provides support and rigidity to plant cells and the plant as a whole. The cell wall of fruit consists of cellulose, hemicellulose and pectin (Colinas-Leon and Young, 1981). Cellulose is composed of long chains of β-1,4-glucan (Fischer

and Bennett, 1991). Hemicellulose is predominantly composed of xyloglucan with numerous side chains (Colinas-Leon and Young, 1981; Fischer and Bennett, 1991). Cellulose and hemicellulose are intrinsically associated in the cell wall microfibrils (Cosgrove, 2001; Fischer and Bennett, 1991). Pectin is a heteropolysaccharide and may consist of homogalacturonan or rhamnogalacturonan with numerous side chains (Willats *et al.*, 2001). The strength of the cell wall depends on the mechanical properties and linkages between the wall constituents (Jarvis, 1984), but the construction of the cell wall and how the constituents interact to form a strong yet tractable network is not entirely certain and a number of models have been proposed (Cosgrove, 2001; Fischer and Bennett, 1991). The substrate for the ripening enzymes, *i.e.* the cell wall, is not adequately characterised, highlighting the lack of understanding of fruit ripening.

Pectin may consist solely of α-D-1,4-galactopyranuronic acid (Gal*p*A), forming homogalacturonan, or include rhamnopyranose (Rha*p*), when the disaccharide α-D-Gal*p*A(1→2)αL-Rha*p* forms rhamnogalacturonan (Highley, 1997; Willats *et al.*, 2001). The galacturonic acid residues may be methyl-esterified, acetylated and/or substituted with xylose or apiose. Rhamnogalacturonan can have oligosaccharide side chains attached that increase the resistance to degradation by pectinases (Fischer and Bennett, 1991; Willats *et al.*, 2001). The two most common side chains are galactan and (1→5)-α-L-arabinan (Fischer and Bennett, 1991; Geshi *et al.*, 2002). The galactan side chain provides additional strength and rigidity to the wall (McCartney *et al.*, 2000). Boron and calcium are both necessary for the cross-linkage of pectin chains (Hu *et al.*, 1996; Jarvis, 1984; Matoh and Kobayashi, 1998). Pectin chains are joined when non-esterified galacturonan segments are bonded together non-covalently by calcium ions (Jarvis, 1984).

Homogalacturonan is highly methylated when exported into the cell wall and is de-esterified by the action of pectin methylesterase (PME) (Willats *et al.*, 2001). Different PME isozymes determine the methyl-esterification distribution pattern in the pectin chains, which in turn affects the physical properties of pectin gels by affecting calcium binding. The compressive strength, elasticity, water holding capacity and porosity of homogalacturonan, and therefore the cell wall, is significantly influenced by the pattern and degree of methyl-esterification in the primary cell wall (Willats *et al.*, 2001).

The degradation of the cell wall during ripening is shown in Figure 2. In an unripe fruit the fibrils and matrix of the cell wall are compacted (Figure 2A). At the climacteric the fibrils have been loosened and there is a loss of electron density; the endoplasmic reticulum is also swollen and vesiculate (Figure 2B). At ripeness, the cell wall is almost completely degraded.

The vesicles in the wall are thought to be the remnants of the plasmodesmata (Figure 2C) (Platt-Aloia, 1980). Comparisons in the cell wall at different maturity stages were not made, but may provide insight into ripening physiology as more mature fruit ripen more rapidly than less mature fruit, and stored avocados ripen more rapidly than unstored fruit (Cutting *et al.*, 1986; Cutting and Wolstenholme, 1991; Pesis *et al.*, 1978a). This is likely due to alterations in the cell wall during maturation and cold storage that has an effect on the ripening rate (Cosgrove, 2001). PME has an inverse relationship to fruit maturity, *i.e.* declining through the season and less mature fruit showed a rapid decline in PME activity postharvest while the PME activity in more mature fruit declined only after 3-5 days (Zauberman and Schiffmann-Nadel, 1972). These alterations would make the middle lamella more susceptible to enzyme action later in the season. Also, the concentration of alcohol insoluble solids (polysaccharides such as cellulose, hemicellulose, starch and pectin) declined from 30 to 20% DM as 'Hass' fruit matured from 23 to 36% DM (Eaks and Sinclair, 1978). It is thought that continued enzyme activity during cold storage results in fruit ripening sooner after removal from cold storage.

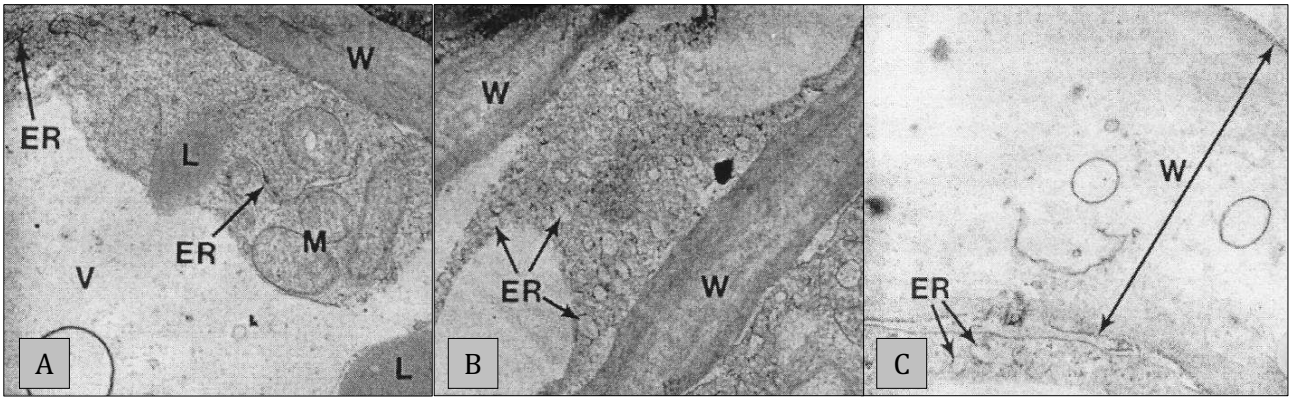

Figure 2: Electron micrographs of mesocarp cell wall of an (A) unripe, (B) climacteric and (C) ripe fruit, taken from Platt-Aloia (1980).

* ER = endoplasmic reticulum, L = lipid, M= mitochondria, V = vacuole, W = cell wall

2.3. Ripening Enzymes

The changes in the cell wall are the most important changes that occur during ripening because this brings about softening and palatability. Softening is achieved by the solubilisation and depolymerisation of the cell wall by a number of enzymes. The enzymes operating during ripening function co-operatively, synergistically and in a prescribed manner to break down the cell wall (Ali *et al.*, 2004; Awad and Young, 1979; Brummell *et al.*, 2004; Hatfield and Nevins, 1986; Hofman *et al.*, 2002a; Ronen *et al.*, 1991; Tateishi *et al.*, 2007). Therefore, looking at one enzyme in isolation will not provide adequate answers about avocado ripening (Ali *et al.*,

2004).

The cell wall is not completely solubilised; rather it is disrupted and weakened by the combined action of the ripening enzymes (Colinas-Leon and Young, 1981; Hatfield and Nevins, 1986; Pesis *et al.*, 1978a; Platt-Aloia, 1980). The cell wall has to be modified before most depolymerising enzymes can act on the cell wall because the native cell wall is not a suitable substrate. Each component of the cell wall (cellulose, pectin and hemicellulose) has an enzyme system (cellulases, pectinases and hemicellulases) composed of different individual enzymes to prepare and then depolymerise each cell wall component (Karr and Albersheim, 1970). The ability of an enzyme to depolymerise a model substrate cannot be directly correlated to its ability to act *in vivo* because its exact substrate may not be available *in vitro*; the activity of other enzymes may improve – or be a requirement for – the activity of the enzyme of interest (Goulao *et al.*, 2008).

The model crops for studying fruit ripening enzymes for climacteric and non-climacteric fruit are tomato (*Lycopersicon esculentum*) and strawberry (*Fragaria* × *ananassa*), but different crops, and even cultivars, show different patterns of enzyme expression during ripening, so inferences cannot be made from the model crops (Ali *et al.*, 2004; Brummell, 2006; Goulao *et al.*, 2008; Goulao and Oliveira, 2008). Avocado enzymology has been studied in a fair amount of detail since the late 1970s, but far more research in this area is still required. Cellulase, PG, PME, β-galactosidase, xylanase and xylosidase are all involved in avocado ripening, but only the former three enzymes have been studied in detail.

Besides being involved in fruit ripening, the cell wall modifying enzymes are also involved in response to fungal pathogens so they are critical components of postharvest quality (Matteo *et al.*, 2005; Ronen *et al.*, 1991).

2.3.1. Cellulase

The correlation between cellulase activity and fruit softening has been known for over thirty years, when it was also found that ethylene plays a role in controlling cellulase activity (Pesis *et al.,* 1978). Cellulase is critical in avocado ripening, because of its relationship with the ethylene climacteric and its activity is required for functioning of other cell wall hydrolases (Hatfield and Nevins, 1986). Avocado cellulase has been extensively studied because of its involvement in softening and sensitivity to ethylene. Cellulase activity was linked with avocado softening in the late 1970s (Awad and Young, 1979; Pesis *et al.*, 1978a). Later studies analysed cellulase activity in relation to the ethylene action inhibitor 1-methylcyclopropene

(1-MCP) and it was found that cellulase activity is inhibited by 1-MCP (Feng *et al.*, 2000; Jeong and Huber, 2004).

Measuring cellulase activity, because of the heterogeneous nature of cellulose *in vivo*, is difficult (Highley, 1997). Furthermore, the cellulase complex is also comprised of endo-β-1,4-glucanases (EC 3.2.1.4), exo-1,4-glucanases (EC 3.2.1.91) and β-1,4-glucosidases (EC 3.2.1.21). These enzymes also have isozymes with specific roles in abscission and ripening (Bonghi *et al.*, 1992; Ferrarese *et al.*, 1995; Highley, 1997; Kanellis and Kalaitzis, 1992) and induced by different phytohormones (ethylene or auxin) (Durbin and Lewis, 1988). The analysis of total enzymatic activity is often masked by the presence of multiple isozymes with different patterns of expression under different modes of regulation (Goulao *et al.*, 2008; Ronen *et al.*, 1991). It is therefore most useful to study each isozyme separately but this is seldom done.

2.3.2. Pectinases

The pectinases depolymerise and solubilise native pectin (pectinic acid) to bring about final softening in avocado fruit. Before pectin can be depolymerised pectinic acid must be demethylesterified to pectic acid (polygalacturonic acid) by pectin methylesterase (PME). This allows polygalacturonase (PG) to act on the pectic acid and produce oligogalactronic acid and galacturonic acid. β-Galactosidase (Gal) further breaks down the oligogalacturonic acid to galacturonic acid (Figure 3). It is apparent that PG activity *in vivo* is extremely limited without the action of PME.

Pathogenic pectinases have been studied extensively and intensively because pectinases are responsible for the symptoms of soft-rot diseases and because pectinases and pectin fragments induce a number of physiological responses in plants (Highley, 1997).

$$\text{Pectinic acid} \xrightarrow{\text{PME}} \text{Pectic acid} \xrightarrow{\text{PG}} \begin{matrix}\text{Oligogalacturonic acid} \\ \text{Galacturonic acid}\end{matrix} \xrightarrow{\text{Gal}} \text{Galacturonic acid}$$

Figure 3: Enzymatic break down of pectinic acid to galacturonic acid during ripening, via the action of pectin methylesterase (PME), polygalacturonase (PG) and β-Galactosidase (Gal).

2.3.2.1. Pectin Methylesterase

PME (EC 3.1.1.11) activity has been found to fluctuate considerably during avocado ripening, but is thought to decline during ripening (Awad and Young, 1979; Awad and Young, 1980; Fuchs and Zauberman, 1987; Kaiser *et al.*, 1996; Rouse and Barmore, 1974; Zauberman and Jobin-Décor, 1995). Besides preparing pectin for solubilisation by PG, the enzyme also has other roles in plant physiology. PME generates free carboxylic groups during demethylesterification which modifies the pH and cation exchange properties of the cell wall, affecting a range of ripening enzymes (Matteo *et al.*, 2005; Micheli, 2001). PME activity is modulated by pH and ion concentration, with isoform expression being sensitive to pH and the degree of esterification of the substrate, indicating a possible feedback mechanism (Bordenave *et al.*, 1996; Catoire *et al.*, 1998; Willats *et al.*, 2001). Complicating the matter further, a specific proteinaceous inhibitor of PME has been identified in kiwifruit (*Actinidia deliciosa*) and *Arabidopsis thaliana* (Matteo *et al.*, 2005). While a PME inhibitor has not been identified in avocado as yet, the possibility of its existence makes studies in pectinase activity potentially very complex.

2.3.2.2. Polygalacturonase

Polygalacturonase (EC 3.2.1.15) activity increases sigmoidally during ripening, with very little activity during the first week postharvest (Awad and Young, 1979). It has been proposed that cellulase disrupts the cell wall matrix, allowing PG access to the pectin in the wall matrix; early softening is controlled by cellulase, mediated by ethylene, with polygalacturonase responsible for final softening (Jeong *et al*., 2002). It was found that cellulose was only depolymerised when PG activity increased (Colinas-Leon and Young, 1981), lending weight to the theory that cellulase initially alters the bonding of the fibrils and the cell wall is only depolymerised later in ripening (Platt-Aloia, 1980).

PG activity is increased in wounded fruit (Zauberman and Fuchs, 1981). PG synthesis is decreased in low oxygen conditions below about 7.5% O_2 (Kanellis *et al*., 1991); PG activity is stimulated by treatment with ethylene and inhibited by treatment with 1-MCP (Buse and Laties, 1993; Feng *et al.*, 2000; Jeong *et al.*, 2002).

2.3.2.3. Galactosidase

β-Galactosidase (EC 3.2.1.23) is characterised by its ability to hydrolyse terminal, non-reducing β-D-galactosyl residues from a variety of substrates. Numerous isozymes have been identified in various species. Three isoforms have been identified in avocado: AV-GAL I, AV-GAL II and AV-GAL III (De Veau *et al.*, 1993; Tateishi *et al.*, 2001, 2002; Tateishi *et al.*, 2007). AV-GAL I is indirectly up-regulated by ethylene; the trend in activity tracks the climacteric. AV-GAL II activity increases slightly postharvest. AV-GAL III is also ethylene-dependent, but does not decline at softness as AV-GAL I does, and is the most active against native cell wall polysaccharides (Tateishi *et al.*, 2007). The activity of α- and β-galactosidase declined postharvest (Jeong *et al.*, 2002). The authors explained this decline as being a net effect and isozymes will show different responses during ripening, but the same method was used in both investigations. The decline in α-galactosidase was slowed by the application of 1-MCP, indicating that this enzyme is negatively regulated by ethylene.

2.3.2.4. Arabinanase and Galactanase

Arabinanase and galactanase degrade arabinan- and galactan-type side chains of rhamnogalacturonan (Yashoda *et al.*, 2007). Arabinanase and galactanase had the second and third highest glycanase activity, after mannanase, in ripe mango fruit - which in some ways is similar to avocado, as a climacteric subtropical fruit (Yashoda *et al.*, 2007). These enzymes have not been assayed in avocados and provide an opportunity for study. EC numbers are not provided because the enzymes have not been characterised in avocados.

2.3.3. Hemicellulases

Hemicellulose comprises about 30% of the cell wall so the hemicellulases are an important category of cell wall hydrolases.

2.3.3.1. Xylanase & Xylosidase

β-1,4-xylan is the most abundant hemicellulose and is the second most abundant polysaccharide after cellulose (Highley, 1997). Xylanase (endo-β-1,4-D-xylanase, EC 3.2.1.8) and xylosidase (exo-β-1,4-D-xylosidase, EC 3.2.1.37) are responsible for the hydrolysis of xylan. Xylanase hydrolyses xylan to xylopentose and xylose is produced from this product by xylosidase (Kar *et al.*, 2006; Ronen *et al.*, 1991) (Figure 4). The concentration of xylose

increased during ripening, indicating the activity of these hemicellulases (Hatfield and Nevins, 1986). A later study showed that, preharvest, xylanase decreased early in the season and then stabilised; xylosidase showed an opposite trend, with the ratio between the two changing from 1:1 to 1:3 through the season (Ronen *et al.*, 1991). In early season fruit, xylanase declined slightly from harvest to day 3 and then increased to ripeness. Xylosidase showed a similar trend to cellulase, with a peak at the climacteric peak, six days after harvest (Ronen *et al.*, 1991).

$$\text{xylan} \xrightarrow{\text{xylanase}} \text{xylopentose} \xrightarrow{\text{xylosidase}} \text{xylose}$$

Figure 4: Enzymatic decomposition of xylan to xylose

2.3.3.2. Mannanase and Mannosidase

β-1,4-Mannan is the an abundant hemicellulose comprised of mannose (Highley, 1997). The trends for mannanase (endo-1,4-β-D-mannanase, EC 3.2.1.78) and mannosidase (exo-β-1,4-D-mannosidase, EC 3.2.1.25) in avocados have not been published, but mannanase and mannosidase were the glycanase and glycosidase with the highest specific activities in mango (Yashoda *et al.*, 2007). It is suggested that these enzymes will similarly increase during ripening.

2.3.4. Expansins

Expansins play an important role in the fruit softening phenomenon (Giovannoni, 2001; Yashoda *et al.*, 2007). They are a group of extra-cellular proteins that regulate cell wall extension and dissolution by disrupting hydrogen bonds between cellulose microfibrils and xyloglucans (Goulao *et al.*, 2008; Rose and Bennett, 1999; Rose *et al.*, 2003). Expansins play a role in finger drop and ripening of bananas (Mbéguié-A-Mbéguié *et al.*, 2009), apple softening (Goulao *et al.*, 2008) and tomato ripening (Giovannoni, 2001; Rose *et al.*, 2000). The only published information on expansins in avocado found that expansins do cause cell wall extension in ripe avocado fruit (Rose *et al*., 2000).

2.3.5. Isozymes

The study of ripening enzymes is complicated by the number of isozymes for each enzyme (Goulao *et al.*, 2008). The relative concentrations of isozymes and their activity levels

appear to be important in regulating the ripening rate (Ali *et al.*, 2004; Soh, 2002). Cellulase has at least eleven isoforms in avocado (Kanellis and Kalaitzis, 1992). PG has a number of isozymes, because of post-translational modifications (Buse and Laties, 1993). Three isoforms of β-galactosidase have been identified in avocado (Tateishi *et al.*, 2007).

2.4. Lipids

Being an oleaginous fruit, the oil content of avocado is one of the fruit's primary characteristics. A high proportion of avocado oil is monounsaturated and there is much interest because monounsaturated fatty acids reduce blood cholesterol and thereby the risk of coronary heart disease and strokes (Requejo-Tapia, 1999). Coronary heart disease and strokes, caused by atherosclerosis, are increasingly common worldwide because of the increased consumption of saturated fats (Enser, 1995).

Oil concentration is the best measure of avocado maturity and contributes to eating quality (Hofman *et al*., 2002a). Oil content increases during maturation from about 10% to over 50% DM in late season fruit. Lipid production is energy intensive and has been identified as a yield limiting factor in avocados, compared to carbohydrate-rich fruit (Kaiser *et al.*, 1992; Wolstenholme, 1986).

2.4.1. Anatomy

Avocado mesocarp predominantly contains isodiametric parenchyma cells penetrated by vascular strands and a small percent (2%) of scattered idioblasts – specialised oil storage cells (Platt-Aloia, 1980). Idioblasts have a triple-layer cell wall comprised of a cellulose primary wall, a suberin layer and an esterified pectin tertiary wall. These cells accumulate oil to such an extent that the cytoplasm is compressed and virtually non-existent at maturity and hence are metabolically inactive (Platt-Aloia, 1980). Oil continues to accumulate until harvest so that late harvest fruit have a much higher concentration of oil than early season fruit, with the associated decrease in water content.

2.4.2. Physiology

Plants principally utilise lipids in cell structures and for energy storage. The structural lipids form the cell membrane as phospho- and glycolipids, while the storage lipids are triglycerides in the idioblasts (Requejo-Tapia, 1999). The role of oil in avocado physiology is

not well understood although there is evidence that lipid metabolism continues postharvest (Erickson and Kikuta, 1964; Kikuta and Erickson, 1968).

2.4.2.1. Fatty Acids

Avocados contain five triglycerides in significant amounts: palmitic (16:0), palmitoleic (16:1), oleic (18:1), linoleic (18:2) and linolenic (18:3) acids. Oleic acid is the most abundant fatty acid (FA) in mature fruit, followed by palmitic, linoleic, palmitoleic and linolenic (Table 1), but the relative concentrations depend on production location and cultivar (Luza *et al.*, 1990; Requejo-Tapia, 1999). The FA profile is an adaptation to environment, where fruit grown in cooler climates have a higher proportion of unsaturated to saturated fatty acids. Saturated fatty acids exist in a *trans* configuration and can therefore pack closer together to remain in a liquid crystal (fluid) state at higher temperatures. When the temperature increases, the molecules increase their kinetic movement and carbon-carbon rotation results in *cis* arrangements to increase fluidity while maintaining membrane integrity (Stryer, 1988). Unsaturated fatty acids invariably exist in the *cis* configuration, which is unsuitable for close packing of the triglycerides, so the liquid crystal state occurs at relatively lower temperatures. Micro-organisms grown at lower temperatures have increased unsaturated fatty acids to maintain the liquid crystal state of the membrane (Moreton, 1988; Stryer, 1988). Avocados grown in cooler climates also had a higher ratio of monounsaturated to saturated fatty acids (Kaiser and Wolstenholme, 1994; Requejo-Tapia, 1999). Increased FA unsaturation may also improve the ability of fruit to withstand cold storage because the cell membrane will remain liquid at lower temperatures. Minimal change in the fatty acid composition was found to occur postharvest (Luza *et al*., 1990).

Table 1: Approximate concentration of fatty acids in unripe Spanish and South African 'Hass' avocado mesocarp.

		Concentration (% total FA)	
Fatty Acid	Shorthand	Malaga[1]	Howick[2]
Oleic	18:1	55.6 - 57.7	61 - 64
Palmitic	16:0	19.8 - 20.5	20
Linoleic	18:2	12.4 - 12.9	10
Palmitoleic	16:1	8.8 - 9.7	6 - 9
Linolenic	18:2	1.2 - 1.3	1

[1] Early season fruit from Malaga, Spain with a warm Mediterranean climate (Meyer and Terry, 2008; Meyer and Terry, 2010).

[2] Mid season fruit (August) from cool subtropical KwaZulu-Natal midlands, South Africa (Kaiser *et al.*, 1992).

2.4.2.2. Preharvest

Oil synthesis requires more than twice the energy input compared to carbohydrate synthesis; this may explain the relatively low yields of avocado when compared to crops that use carbohydrates as an energy store (Wolstenholme, 1986). Kaiser *et al.* (1992) compared the oil accumulation pattern pre-harvest of two production locations and found that there was a slight decline in oil concentration in fruit from the warmer location concomitant with the spring flush (November in the southern hemisphere). They hypothesised that this could be because the oil was used as an energy source for the vegetative flush, the oil was used as an energy source for the fruit and respiration increased as the ambient temperature increased, or oil synthesis ceased. The third option seems most likely because the idioblasts are metabolically inactive once the suberin layer is laid down and there was no evidence of plasmodesmata penetrating beyond the primary cell wall into the suberin layer, preventing movement of the oil out the idioblasts (Platt-Aloia, 1980). Further, as Kaiser *et al.* (1992) noted, cell division continues until harvest and the concentration of oil may have declined relative to fruit size.

2.4.2.3. Postharvest

Early reports found that oil concentration fluctuated postharvest but increased during storage and declined during ripening; however these results were expressed on a fresh mass basis (Erickson and Kikuta, 1964). It was later concluded that this increase during storage was due to continued dehydration postharvest (Requejo-Tapia, 1999). The increase in the concentration of oil during ripening was attributed to an increased recovery of lipids due to cell wall break down (Meyer and Terry, 2008; Mostert *et al.*, 2007). This may be partly responsible, but idioblast cell walls are not degraded to the same extent as parenchyma cells and idioblasts are only 2% of the mesocarp (Platt-Aloia and Thomson, 1992). Lipid metabolism in avocados is not sufficiently understood to be able to comment without much speculation, but lipids are not inert and postharvest handling can affect the lipids in avocado fruit.

2.4.3. Health Benefits

The consumption of saturated fatty acids increases the low density lipoproteins (LDL) levels in blood. LDL, which contains cholesterol, can lead to atherosclerosis, causing coronary

heart disease and strokes (Requejo-Tapia, 1999). Monounsaturated fats have been noted for their beneficial effect reducing LDL in the blood, while polyunsaturated fatty acids reduce LDL, but in excess can reduce beneficial high density lipoproteins (HDL). Monounsaturated fatty acids, on the other hand, lower LDL and protect HDL and the consumption of monounsaturated fatty acids reduced blood cholesterol more than a fat-free diet (Grundy, 1986, 1988). Avocado is an ideal food to reduce blood cholesterol because it contains predominantly monounsaturated fatty acids with small amounts of polyunsaturated and saturated fatty acids. Avocado compares favourably to olive oil, which is noted as being a cholesterol-reducing food (Requejo-Tapia, 1999).

2.4.4. Methods to Measure Oil Concentration

Oil concentration is the best measure of maturity, but its quantification is time-consuming and specialised (Hofman *et al.*, 2002a). A number of methods have been investigated to provide an accurate, safe method to measure oil content, such as the Soxhlet, Halowax and nuclear magnetic resonance (NMR), but these methods require specialised equipment, are expensive and slow. Therefore oil content of fruit is estimated using dry matter or moisture content assuming a moisture content and oil sum to a constant value for each cultivar (Kosenthal *et al.*, 1985; Lee, 1981). This method is simple and useful as an estimation of a large batch of fruit, but is inaccurate on an individual fruit basis (Kaiser *et al.*, 1992; Kruger *et al.*, 1995). A quick method, capable of handling large sample numbers, using hexane as an extraction solvent, was recently described by Meyer and Terry (2008). This method was used to track the changes in oil content during storage and ripening and was found most useful for handling large amounts of samples. It was noted that the cold hexane extraction technique resulted in a slightly reduced extraction efficiency compared to a Soxhlet apparatus, but this method permits the quantification of fatty acids, specifically triglycerides because the extraction solution is not heated. Hexane is a more suitable extraction solvent for triglycerides because the Soxhlet method extracts more non-target compounds such as sterols, pigments and hydrocarbons, while cold extraction is well-suited for the extraction of triglycerides (Meyer and Terry, 2008). For example, Kaiser and Wolstenholme (1994) found lipid concentrations of up to 75% DM using a Soxhlet apparatus and petroleum ether as the extraction solvent. The reduced extraction time of the cold extraction method resulted in the reduced total lipid extraction efficiency, but did permit many more samples to be assayed. It also provides a more accurate measure of avocado fruit maturity because of its greater

specificity for triglycerides. Hexane also increases the oil recovery compared to super critical CO_2 and lyophilisation increases the recovery of oil, compared to oven-drying (Mostert *et al*., 2007).

2.5. Water

Fruit are typically more than 90% water and can be regarded as colourfully packaged water. Even small amounts of water loss significantly reduce product mass, reduce the consumer appeal and affect many physiological processes. Water can be regarded as the primary regulator of plant growth because of its copious effects on plant growth and physiology.

2.5.1. Maturity measurement

Water content, or dry matter, is used as a maturity marker in the avocado industry because of the lack of a suitable method to measure oil concentration, but the water content or dry matter of fruit fluctuates pre- and postharvest and is open to manipulation. This can be done to obtain better market prices for early season fruit. Fruit samples can be taken from stressed trees or sun-exposed fruit to ensure the sample water content is lower than the orchard mean to imply that the orchard is suitable for harvest earlier that is actually the case. While oil concentration could be tracked during the season using a suitable wet chemistry technique to provide a more reliable measure of fruit maturity, but it would be preferable to measure oil concentration using near-infrared spectroscopy because this method is rapid, non-destructive and non-toxic.

2.5.2. Water Stress

Pre- and postharvest water stress significantly affect fruit ripening and reduce shelf-life (Wills *et al.*, 1998) and water management is critical in determining yield and fruit quality (Bower, 1985). Although water loss is required during ripening, it must be minimised pre-harvest and during cold storage and managed during ripening to reduce physiological and quality problems at consumption.

2.5.2.1. Preharvest Water Stress

Water management is a major factor in tree growth, yield and fruit quality (Lahav and Whiley, 2002). Shoot growth is directly related to water availability by reducing the number of flushes per season, as well as trunk circumference and tree height (Lahav and Kalmar, 1977). For crop management, there are two critical periods where water stress can result in significant crop losses. These are flowering, due to increased water loss from the flowers (Whiley *et al.*, 1988) and the initial fruit growth phase (Lahav and Whiley, 2002). Water stress during flowering and initial fruit growth results in the production of ABA and ethylene and can cause the abscission of young fruit (Adato and Gazit, 1977), which results in crop losses in the current season and has affects crop management the following season. Water stress later in the growing season will reduce final fruit size. Fruit size is an important economic consideration in avocado production. Fruit size is increased by reduced intervals between irrigation and increased volumes of water applied (Lahav and Kalmar, 1977; Meyer *et al.*, 1990). For 'Hass' this increase was 9% when irrigation was shortened from an internal of 28 days to 7 days (Lahav and Kalmar, 1977). From the same study it was found that an increase in the frequency of irrigation advanced fruit maturity, measured using the mesocarp oil concentration. This is thought to be because avocado fruit are energy-expensive because of the high concentration of oil (Wolstenholme, 1986) and photosynthesis, and therefore oil accumulation, is reduced during periods of water stress.

In terms of fruit quality, fruit from trees that were water stressed (insufficient or excess water) showed more browning potential than fruit from trees which were irrigated optimally (Bower, 1985). Pre-harvest water stress is thought to increase postharvest disorders partly due to a lack of calcium in the fruit during the first 17 weeks after fruit set (Bower and Cutting, 1988). Long term water stress, particularly during the first three months of fruit development, alters the ethylene evolution pattern during ripening. If fruit are water stressed, ethylene production is initiated earlier, but the magnitude of the ethylene climacteric peak is much reduced. This early water stress affects the ethylene synthesis pathway and hence ripening physiology, causing uneven ripening and poor fruit quality (Bower, 1985), possibly because long-term water stress impairs membrane functioning (Bower, 1984).

2.5.2.2. Postharvest Water Stress

Postharvest water loss in fruit is particularly problematic because the water cannot be replaced. Even a slight loss of water from a piece of fruit causes a significant loss in income by

reducing its consumer appeal and by the loss of product mass. Physiologically, fruit with a lower water potential at harvest tend to ripen faster after storage (Bower, 1985; Dixon *et al.*, 2003; Dixon *et al.*, 2004b), and the incidence of fungal rots is higher (Dixon *et al.*, 2005). This is probably because of increased ethylene production due to stress (Lallu *et al.*, 2004). Postharvest treatments should perhaps focus more on limiting water loss to prevent the initiation of the climacteric response, because ABA and ethylene synthesis are stimulated by water stress.

2.6. Phytohormones

Phytohormones regulate and co-ordinate plant growth and development. Ethylene is the most studied phytohormone in relation to avocado fruit quality, but it must be borne in mind that phytohormones interact among themselves and other compounds to regulate physiology (Tian and Lu, 2006). There is a paucity of information on the effect of the growth phytohormones (auxins, cytokinins and gibberellins) on avocado postharvest physiology. This is probably because the stress phytohormones (ethylene, abscisic acid, jasmonic acid and salicylic acid) have a greater effect during ripening and senescence. Studies on phytohormones in seedlings abound, but it would be incorrect to draw conclusions from these studies because the plant response depends on many other factors, including tissue, maturity and concentration of the exogenous phytohormones (León and Sheen, 2003). A prolonged study was done on hormonal homeostasis in relation to the 'Hass' small fruit phenotype. It was found that elevated concentrations of ABA and reduced concentrations of indole-3-acetic acid (IAA) in the seed contributed to the cell division and therefore fruit size (Cowan *et al.*, 2005) and would presumably have a postharvest effect too.

2.6.1. Auxins

Auxins have received the much attention because of their relationship with calcium accumulation and therefore avocado fruit quality (Bower and Cutting, 1988). The export of IAA from a tissue regulates the accumulation of calcium and hence has an effect of fruit quality (Cutting and Bower, 1989). Fruit are poor IAA exporters compared to leaves and excessive competition between these tissues can reduce calcium accumulation in fruit, especially if transpiration is low (Cutting and Bower, 1989). The export of IAA is more important than transpirational flow for calcium accumulation in the fruit (Witney *et al.*, 1990b). Calcium

accumulation, and therefore auxin movement, is affected by orchard management practices, for example nitrogen fertilisation results in increased vegetative growth, reduced relative IAA export from the fruit and reduced calcium accumulation in the fruit which can cause physiological disorders like mesocarp discolouration (van Rooyen, 2006). Additionally, auxin transport is strongly inhibited by phenolic compounds (Jacobs and Rubery, 1988), and avocado fruit have high concentrations of phenolic compounds, which reduce auxin export from fruit (Cutting and Bower, 1989).

When IAA (0.1 mM) was applied to pre-climacteric tissue, ethylene production increased by 15% after 10 h, but suppressed ethylene production by 10% when applied post-climacteric (Lieberman *et al.*, 1977). The authors acknowledged that the increased ethylene production was probably due to the super-physiological concentration of IAA. A lower concentration of auxins, applied pre-climacteric, delayed ripening and when applied post-climacteric delayed senescence (Tingwa and Young, 1975).

2.6.2. Cytokinins

Cytokinins - specifically isopentenyl adenosine (IPA) - are antagonistic to ABA, whereby cytokinins promote the oxidative catabolism of ABA (Cowan *et al*., 1999), and ethylene (Lieberman *et al.*, 1977). The application of IPA (0.1 mM) suppressed ethylene production by 40% after 10 h and the application of kinetin and IPA post-climacteric reduced ethylene production by 47 and 57%, respectively (Lieberman *et al.*, 1977). However, the infiltration of cytokinin into pre-climacteric fruit had no effect on ethylene production (Tingwa and Young, 1975).

2.6.3. Gibberellins

The role of gibberellins in ripening physiology has not been studied extensively, but it is known that they decline with increasing maturity and during ripening and may be more involved in fruit growth than quality (Hofman and Husband, 1987). In apple slices, gibberellic acid-3 did not affect ethylene production and ripening (Lieberman *et al.*, 1977). The application of gibberellic acid (10 or 20 ppm) did not have a consistent effect on external chilling injury, although it has been shown to reduce chilling sensitivity in citrus (Bower and Papli, 2006).

2.6.4. Ethylene

Ethylene (C_2H_4) is a gaseous phytohormone that has extensive effects on plant physiology. It has been estimated that ethylene controls 7% of the genes in *Arabidopsis thaliana* (Zhong and Burns, 2003). Ethylene synthesis is stimulated by biotic and abiotic stresses. The biotic stresses, *i.e.* disease, will not be discussed here because that is the realm of plant pathology. Reviews on ethylene biochemistry abound (Burg, 1968; Lelievre *et al.*, 1997; Tian and Lu, 2006; Wang *et al.*, 2002). Rather than repeating these findings, an interpretation on these findings in relation to avocados are discussed.

2.6.4.1. Biosynthesis

In the 1950s it was hypothesised that ethylene is the terminal product of a chain of metabolic processes in fruit and that ethylene synthesis is connected with organic acid metabolism (Buhler *et al.*, 1957; Hall, 1951). Later, the amino acid methionine was found to be the precursor in ethylene biosynthesis. The first stage in ethylene biosynthesis is the conversion of methionine to S-adenosyl-L-methionine (AdoMet) by AdoMet synthetase (EC 2.5.1.6). AdoMet is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (EC 4.4.14). The final reaction is the formation of ethylene from ACC by ACC oxidase (Figure 5). CO_2 and hydrogen cyanide (HCN) are produced as by-products of ACC oxidation. The HCN is detoxified to β-cyanoalanine by β-cyanoalanine synthase (EC 4.4.1.9). Methionine is recycled by using a modified methionine cycle, at the cost of one molecule of ATP so ethylene synthesis does not require an increasing pool of methionine (Bleecker and Kende, 2000; Wang *et al.*, 2002). Ethylene biosynthesis is controlled by ACC synthase, which limits the conversion of AdoMet to ACC because it is present in low concentrations. The enzyme is also labile and the different isoforms contribute control (Wang *et al.*, 2002; Wills *et al.*, 1998). ACC synthase production is increased in response to abiotic stress, increasing ACC formation (Wang *et al.*, 2002). Avocado fruit only contain trace amounts of ACC at harvest, but the concentration rises before the climacteric, driven by an increase in ACC synthase activity (Blumenfeld *et al.*, 1986). These authors hypothesised that the ACC concentration in the fruit is the limiting factor preventing on-tree ripening and that the ACC synthase activity would increase postharvest leading to an increase in ACC and subsequently ethylene, but did not discuss what may cause the increase in ACC synthase activity. This may simply be the increase in stress associated with harvest. Ethylene production is autocatalytic, and is very difficult to limit production once initiated, as well as being adverse to fruit quality if stimulated before

ripening is required.

2.6.4.2. Commercial Use of Ethylene

Ethylene is used commercially to ripen fruit (e.g. citrus, bananas and avocados) uniformly and predictably to provide ripe fruit to consumers. A dose of 100 $\mu L.L^{-1}$ every few hours at 20-22°C for 24 h is used to ripen fruit in four to six days - depending on cultivar and maturity (Hofman *et al.*, 2002a); a continual stream of 10 $\mu L.L^{-1}$ is also used commercially[1]. Adverse effects of using exogenous ethylene, especially an increase in fungal rots, have been noted. This may be because the high concentration of ethylene stimulated the growth of anthracnose complex and the stem end rot complex (Flaishman and Kolattukudy, 1994), especially if exogenous ethylene is present before or during cold storage (Pesis *et al.*, 2002).

2.6.4.3. Ethylene Inhibition

1-Methylcyclopropene (1-MCP) is used commercially to block ethylene receptors (Section 4.2.5) but is also a useful tool for studying ripening physiology (Ochoa-Ascencio *et al.*, 2009). The application of 1-MCP at advanced stages of ripening affected gene expression and ripening physiology indicating that ethylene is required throughout the ripening process (Hoeberichts *et al.*, 2002; Jeong and Huber, 2004; McGarvey *et al.*, 1992). Ethylene inhibitors (*i.e.* silver ions and 1-MCP) suppressed the synthesis of cellulase mRNA and PG mRNA (Buse and Laties, 1993). Treatment with 1-MCP (450 $nL.L^{-1}$) for 12 or 24 h completely suppressed PG activity, delayed cellulase activity by four days, delayed the climacteric and reduced the ethylene production rate and respiration rate by 50 and 40% respectively. Exogenous ethylene cannot overcome 1-MCP inhibition until new receptors are synthesised and indeed there was no hastening of PG activity with the application of ethylene after 1-MCP exposure, although the exogenous ethylene did increase the PG activity very late in ripening - once new ethylene receptors had been synthesised - and the cellulase activity in 1-MCP treated fruit, subsequently treated with ethylene, was hastened by four days and increased compared to fruit that were only treated with 1-MCP (Jeong and Huber, 2004).

[1] Pers. comm. P.J. Hofman, Department of Employment, Economic Development and Innovation, Agri-Sciences Queensland, Australia.

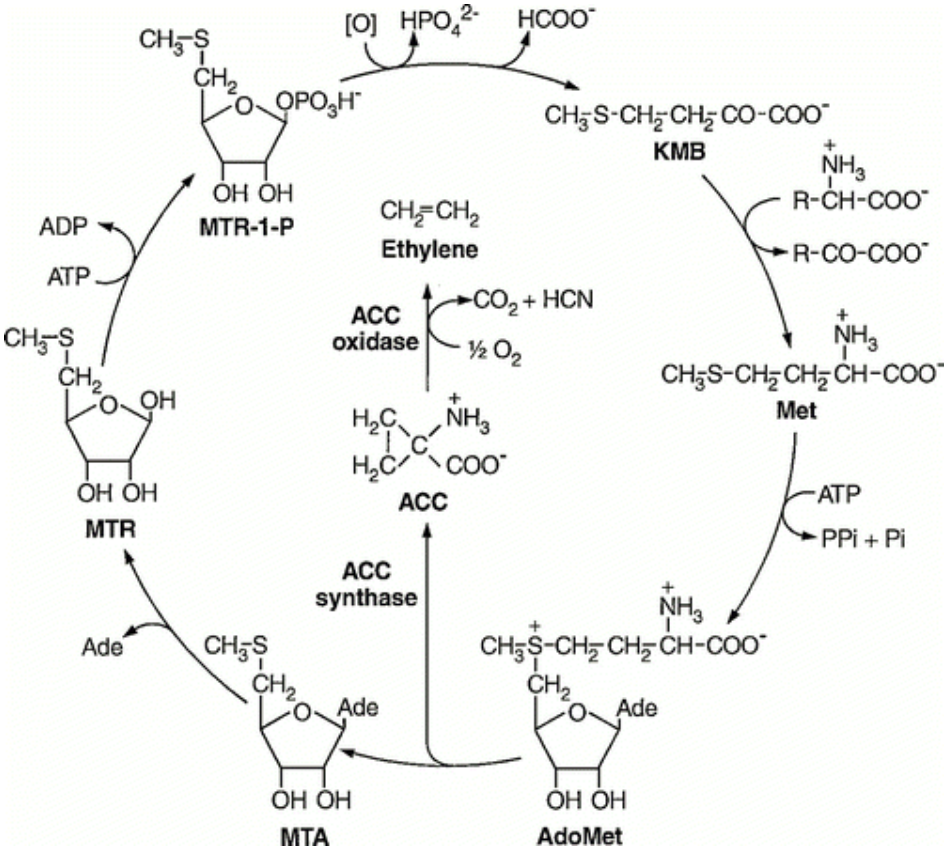

Figure 5: Ethylene biosynthetic pathway and the methionine cycle, taken from Bleecker and Kende (2000).

ACC, 1-aminocyclopropane-1-carboxylic acid;

Ade, adenine;

AdoMet, S-adenosyl-L-methionine;

KMB, 2-keto-4-methylthiobutyric acid;

Met, L-methionine;

MTA, 50-methylthioadenosine;

MTR, 50-methylthioribose;

MTR-1-P, 50-methylthioribose-1-phosphate.

2.6.5. Abscisic Acid

Water stress, ABA and ethylene are all important in the initial stages of ripening (Bower and Cutting, 1988). ABA is a ripening promoter and is closely associated with the ethylene climacteric (Adato, 1974; Adato *et al.*, 1976; Bower and Cutting, 1988; Bruinsma, 1981; Cutting *et al.*, 1986; Cutting and Bower, 1987). ABA is both synthesised and catabolised during ripening (Cutting *et al.*, 1989). Water stress resulted in an increased ABA concentration (Bower, 1985; Bower and Cutting, 1988). More mature fruit have less water and are more vulnerable to water stress, and in South Africa more mature fruit are harvested in the dry

winter months and subject to water stress (Cutting and Bower, 1987). It was found that ABA concentration correlated well with polyphenol oxidase (PPO, EC 1.10.3.1 and EC 1.14.18.1) activity and mesocarp browning (Cutting *et al.*, 1989). Delayed harvest and stress were found to increase ABA and PPO activity; the infusion of ABA resulted in increased PPO activity in late season fruit (Cutting and Bower, 1987). However, in another study ABA was found to not correlate with mesocarp disorders (Hofman and Husband, 1987).

Avocados are thought to have an endogenous ripening inhibitor because fruit will not ripen while still actively attached to the tree (Bower and Cutting, 1988; Schroeder, 1953). This ripening inhibitor has never been identified, although there has been considerable speculation in the literature (Adato and Gazit, 1974; Burg and Burg, 1964, 1965; Gazit and Blumenfeld, 1970; Liu *et al.*, 2002). The heptose sugars have been suggested (Liu *et al.*, 2002), but may just be used as a source of carbon and energy (Section 2.2.1). As mentioned previously, water relations are critical for fruit quality, because water has extensive effects on physiology. There is evidence that water relations influence the rate of ripening (Bower, 1985; Dixon *et al.*, 2003; Dixon *et al.*, 2004b; Lallu *et al.*, 2004).

2.6.6. Jasmonic Acid

Jasmonic acid (JA) is a phytohormone involved in the stress and disease response, and is linked to ABA and ethylene. The genetic and biochemical mechanisms of these interactions are currently not well understood (Tian and Lu, 2006; Wang *et al.*, 2002). What is known at present is that the ABA and the JA-ethylene signalling pathways interact antagonistically to modulate defence and stress responsive gene expression in response to biotic and abiotic stresses (Anderson *et al.*, 2004). The application of ABA reduced the basal and JA-ethylene-activated transcription of defence genes while ABA-deficient mutants showed an up-regulation of these genes (Tian and Lu, 2006). The application of methyl jasmonate reduced chilling injury severity and incidence in ‘Fuerte’, ‘Ettinger’ and ‘Hass’, at concentrations between 1 and 25 μM (Meir *et al.*, 1996). JA may also be useful in reducing sugar consumption and postharvest decay during cold storage (Ding *et al.*, 2002; Wang and Buta, 1999).

2.6.7. Salicylic Acid

Salicylic acid (2-hydroxybenzoic acid) is a ubiquitous phenolic compound with many regulatory functions, especially in defence via systemic acquired resistance (SAR). SAR is defined as a resistance to subsequent pathogen attack that develops in the uninfected,

pathogen-free parts of the plant after the initial inoculation (Raskin, 1992; Ross, 1961). This is achieved by the inhibition of catalase (EC 1.11.1.6) which results in the increase of H_2O_2 and eventual cell death (Srivastava and Dwivedi, 2000) (Section 2.7.1). This response confers resistance throughout the plant after infection and also between plants – and fruit – when converted to gaseous methyl salicylate (Taiz and Zeiger, 2002). Salicylic acid interacts with ethylene, JA and ABA (Tian and Lu, 2006). The application of salicylic acid (500-1000 μM) reduced the ripening rate of bananas, but lower doses are known to stimulate ethylene production (Taiz and Zeiger, 2002). The role of this phytohormone in avocado requires research.

2.6.8. Roles of Phytohormones in the Stress Response

Ethylene was originally regarded as a stress phytohormone because it was induced by a variety of stress signals: mechanical injury, chilling injury, heat shock, pathogen infection and exposure to chemicals and metals. It is now known that ethylene has many more roles in physiology, but its role in stress is irrefutable. Reactive oxygen species (ROS) cause cellular damage by lipid peroxidation and oxidation of cellular constituents. Anti-oxidants reduce ROS to less reactive species, preventing cellular damage (Section 2.7.). Phytohormones interact to modulate responses to ROS. The exact mechanisms are not fully understood, but the phytohormones function synergistically or antagonistically, depending on the type of stress. The salicylic acid signalling pathway is required for the optimal induction of ethylene synthesis in response to ROS stress, while the JA pathway is antagonistic to ethylene synthesis and programmed cell death can be halted by the application of methyl jasmonate (Wang *et al.*, 2002). Ethylene is involved in the early response to stress, and its synthesis is transient, while JA and salicylic acid have longer lasting and systemic effects to limit cellular damage or pathogen infection (Wang *et al.*, 2002). The production of ROS is adverse to fruit quality. Therefore the retention of anti-oxidants and reduction in stress are critical to maintaining fruit quality. While manipulating JA and salicylic acid may be useful, it would be best to not elicit the stress response and limit ethylene synthesis.

2.7. Anti-Oxidants

An anti-oxidant is “any substance that, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or prevents oxidation of that

substrate" (Halliwell, 1995). ROS are highly reactive because of unpaired valence shell electrons. Anti-oxidants limit cellular damage caused by ROS, particularly during stress conditions. ROS are by-products of normal cellular functioning. ROS are effectively reduced during normal cellular functioning, but when homeostasis is disturbed during stress conditions ROS may accumulate to damaging concentrations. Membranes, proteins and DNA can be damaged if ROS are not reduced to less reactive species (Borg and Schaich, 1988). Anti-oxidants are therefore essential for continued cellular functioning. Non-enzymatic (e.g. ascorbic acid, tocopherol and phenols) and enzymatic (superoxide dismutase, catalase, peroxidase and glutathione peroxidase) anti-oxidants exist. A more detailed review of anti-oxidants was recently made by Tesfay (2009).

2.7.1. Non-Enzymatic Anti-Oxidants

The majority of work on anti-oxidants in avocados has focussed on the phenols in relation to mesocarp browning, but this is caused by dysfunctional cellular physiology when membrane integrity is compromised. In normal functioning, phenols are important anti-oxidants. The mesocarp contains very low anti-oxidant capacity, compared with the seed and exocarp; the major anti-oxidants are ascorbic acid and mannoheptulose (Tesfay *et al*., 2010). Besides being a broad spectrum anti-oxidant, ascorbic acid is also used in the regeneration of tocopherol - an anti-oxidant that limits membrane damage (Thomas *et al*., 1992). The concentration of ascorbic acid in the mesocarp is low, compared with the seed and exocarp (Tesfay *et al*., 2010) and declines during ripening (Wills and Tirmazi, 1982). Mannoheptulose was recently found to be a major anti-oxidant in the mesocarp, but only has the 1% of the anti-oxidant power of ascorbic acid (Tesfay *et al*., 2010) and also declines during ripening (Liu *et al.*, 1999a). The decline in anti-oxidant capacity and cell wall break down would account for increased browning potential at ripeness.

2.7.2. Enzymatic Anti-Oxidants

Enzymatic anti-oxidants function to reduce the superoxide radical ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2) and then oxygen and water. The dismutation (simultaneous oxidation and reduction) of superoxide to H_2O_2 and O_2 is catalysed by superoxide dismutase (EC 1.15.1.1); the reaction depends on the metal (copper, manganese, zinc or nickel) co-factor. The decomposition of H_2O_2 is catalysed by peroxidase (EC 1.11.1.x) and catalase (EC 1.11.1.6). Catalase has a very high turnover rate, indicating its importance in cellular functioning (Dat *et*

al., 2000).

2.1. Ripening Temperature

Ripening temperature is a highly important factor affecting fruit quality (Eaks, 1978; Hopkirk *et al.*, 1994). The optimal ripening temperature is dependent on the growing temperature and storage temperature: fruit grown at a lower temperature should be ripened at 15-18°C while those grown at a higher temperature can be ripened at 20-25°C without a loss of quality (Hopkirk *et al.*, 1994). During ripening, there is also compromise between proper colour development in black-skinned cultivars and the risk of developing postharvest diseases (Hofman *et al.*, 2002a). At lower temperatures (15°C) there are fewer occurrences of fungal rots, but also less ripe fruit with acceptable colour, and at higher ripening temperatures (18-20°C) the percentage of ripe fruit with black colour increased but the percentage of the area of the flesh with body rots also increased. It was noted that 'Hass' fruit ripened at 17°C took longer to reach a satisfactory exocarp colour compared with those ripened at 24°C, and the fruit ripened at 17°C were usually over-ripe by the time it had developed the desired black colour (Hofman *et al.*, 2002a). A very high ripening temperature (30-40°C) will increase the risk of fungal decay, vascular browning and mixed ripening (Eaks, 1978; Hopkirk *et al.*, 1994). Fruit which ripen faster, at lower temperatures, are less prone to pathological rots (Hopkirk *et al.*, 1994). This may be because the fruit spend less time in a vulnerable state of having highly permeable cell membranes and weak cell walls, providing easy access and suitable growth conditions for a pathogen. The ripening temperature affects the metabolic rate of the fruit, especially the activity of the ripening enzymes. Ripening was slowed by seven days and PG activity delayed and decreased by reducing the ripening temperature from 20 to 14°C (Zauberman and Fuchs, 1981). A temperature of approximately 18-20°C is most suitable for ripening fruit (Hopkirk *et al.*, 1994).

3. Cold Storage

Temperature management is the most effective tool for maintaining fruit quality because of its profound effects on fruit physiology and pathology (Kader, 2002; Kader and Rolle, 2004). The objective of cold storage is to maintain fruit firmness and quality until the fruit are removed from cold storage and for normal ripening physiology to commence after the fruit

are returned to room temperature, so that fruit are able to ripen normally (Bower, 2005a). The optimum shelf-life of fruit is reduced two to three times for every 10°C increase in storage temperature (Kader and Rolle, 2004). The maintenance of fruit quality is best achieved by suspending the ripening processes using cold storage, but in a manner that will not result in chilling injury. In conjunction with cold storage, treatments such as controlled and modified atmosphere storage, ethylene inhibitors, scrubbers and removal and waxing are used to reduce chilling injury, delay ripening and maintain fruit quality. Some treatments have proven more effective while others have limited commercial applicability (Bower and Cutting, 1988). Mitigating treatments are required for the cold storage of tropical and subtropical fruit because these crops have a lower chilling tolerance than temperate fruits and need to be stored at higher temperatures, for a shorter storage period, or suffer quality loss from chilling injury.

3.1. Objectives of Cold Storage

The aim of cold storage is to put the fruit into a state of reduced physiological activity and to be able to resume normal physiological functioning once the fruit are removed from cold storage. The storage temperature and duration is ultimately determined by the interaction between fruit deterioration and decay and its susceptibility to chilling injury (Wills *et al.*, 1998). Produce has to be cooled to slow senescence and prolong the shelf life. In order to maximise shelf life, the lowest temperature possible needs to be used. The limit to which products can be subjected is usually defined by the induction of chilling injury, which then becomes a limiting factor in postharvest management. Cold storage and chilling injury are hence closely linked and are discussed together.

Avocados are subtropical to tropical and have a high respiration rate and are hence susceptible to chilling injury, making postharvest management challenging, especially if long transit times are required to reach export markets and if a cold disinfestation treatment is required for phytosanitary purposes. A single mitigating treatment will not be effective in maintaining fruit quality. An integrated approach, using a number of methods, has to be utilised. South African avocados are stored for up 30 days at 4-6°C, depending on fruit maturity (PPECB, 2008) and fruit are generally of a good condition when they arrive in Europe, although quality is dependent on season, grower and fruit maturity. However, increasing competition, costs and the desire to enter new markets make it imperative to improve technology to deliver quality fruit to consumers while maintaining profitability.

3.2. Chilling Injury

Chilling injury is defined as "the permanent or irreversible physiological damage to plant tissues, cells or organs, which results from the exposure of plants to temperatures below some critical threshold for the species or tissue" and chilling temperature as "any temperature below the critical threshold temperature, but above freezing, that causes injury" (Lyons and Breidenbach, 1987). The nature and severity of chilling injury is dependent on species, cultivar, organ, maturity, severity and duration of exposure to chilling temperatures, the ambient environment before and after chilling and other stresses which may be experienced by the plant tissue (Kader, 2002; Kader and Rolle, 2004). Commodities generally exhibit increased chilling injury once they are removed from cold storage and the metabolic activity of the fruit increases (Crisosto *et al.*, 2004; Paull, 1990). The symptoms of external chilling injury (ECI) in avocados are skin pitting and shrivelling and is described as 'discrete patches' in the International Avocado Quality Manual (White *et al.*, 2004). External chilling injury is the result of desiccation and necrosis of the exocarp during cold storage and is increased with reduced storage temperature but is limited by packaging fruit in a manner that limits water loss (Bower and Magwaza, 2004). Internal chilling injury (ICI) symptoms are caused by damage to membrane and organelle structures (Platt-Aloia, 1980) and symptoms are various types of tissue discolouration. These symptoms are thought to be physiological disorders that are exacerbated during cold storage (van Rooyen, 2006).

It was previously thought that the postharvest storage life of tropical and subtropical crops exhibits a bell shape curve with the optimum range between 7 and 10°C. At higher temperatures there is continued senescence and ripening; at temperatures less than 7°C storage life decreases because of the development of chilling injury and ripening is severely reduced (Kader and Rolle, 2004; Paull, 1990). This is apparent in tropical crops, but being subtropical, avocados can be stored at lower temperatures without exhibiting chilling injury (Bower and Papli, 2006; Van Rooyen and Bower, 2006). It is thought that temperatures between 2 and 7°C are in the 'killing temperature zone' and when fruit are stored at near 0°C chilling injury is reduced (Crisosto *et al.*, 2004; Lurie and Crisosto, 2005).

3.2.1. Chilling Injury Model

Raison and Orr (1990) proposed that the process of chilling injury be divided into two stages (Figure 6). The primary phase is the temperature-induced stage which initiates the metabolic dysfunctions that lead to tissue damage in the secondary phase. The secondary

phase includes the many metabolic processes that cause visible chilling injury symptoms. This division allows the more instantaneous primary event to be separated from the time-dependant secondary events. The primary event is initiated if the temperature of the tissue falls below the critical temperature, which is specific for each tissue. This causes a disproportionate inhibition or acceleration in the reactions of various metabolic processes. If chilling is prolonged there is an imbalance in metabolism and loss of cellular integrity. Cellular damage is reversible if the chilling stress is removed before the loss of cellular integrity; if not, a return to a higher temperature accelerates the development of visible chilling injury symptoms. In the short term the primary and secondary phases are reversible. If the temperature is raised during the reversible stage there is a sudden rise in metabolic activity, linked to the metabolism of intermediates accumulated during chilling, but the metabolic balance is restored. If the chilling stress continues beyond the period of reversibility and cellular damage occurs, an increase in temperature accelerates the appearance of chilling symptoms because the anabolic repairs cannot match the catabolic processes and the cell death and decay accelerates.

Avocado fruit are most sensitive to chilling at the climacteric peak because of the very high metabolic rate during this period; fruit that are pre- or post-climacteric are much less sensitive (Couey, 1982; Paull, 1990). At the climacteric peak, the rates of respiration and metabolism are greatest and pools of intermediates are maximal. Chilling injury is partially caused by damage to some regulatory enzymes at low temperatures allowing intermediates to accumulate to toxic levels (Kosiyachinda and Young, 1976). This accumulation of intermediates could be reduced by further lowering the temperature to thermally inhibit the enzymes with a lower temperature threshold. There is obviously a lower limit, but results indicate that it is much lower than 5°C for avocados (Van Rooyen and Bower, 2006; Van Rooyen, 2009). There is increased risk of ECI at the lower temperatures, although this is reduced with preconditioning treatments (Van Rooyen, 2009). In avocados, it is suggested that the imbalance is between ethylene and the activities of cellulase and PG with increased membrane degradation during cold storage (Hershkovitz *et al.*, 2005).

3.2.2. Physiology of Chilling Injury

Chilling temperatures cause a number of physiological and biochemical responses in plant cells. These include changes in membrane structure and function, termination of cytoplasmic streaming and alterations in the respiration and ethylene production rates and

eventually cell death (Morris, 1982).

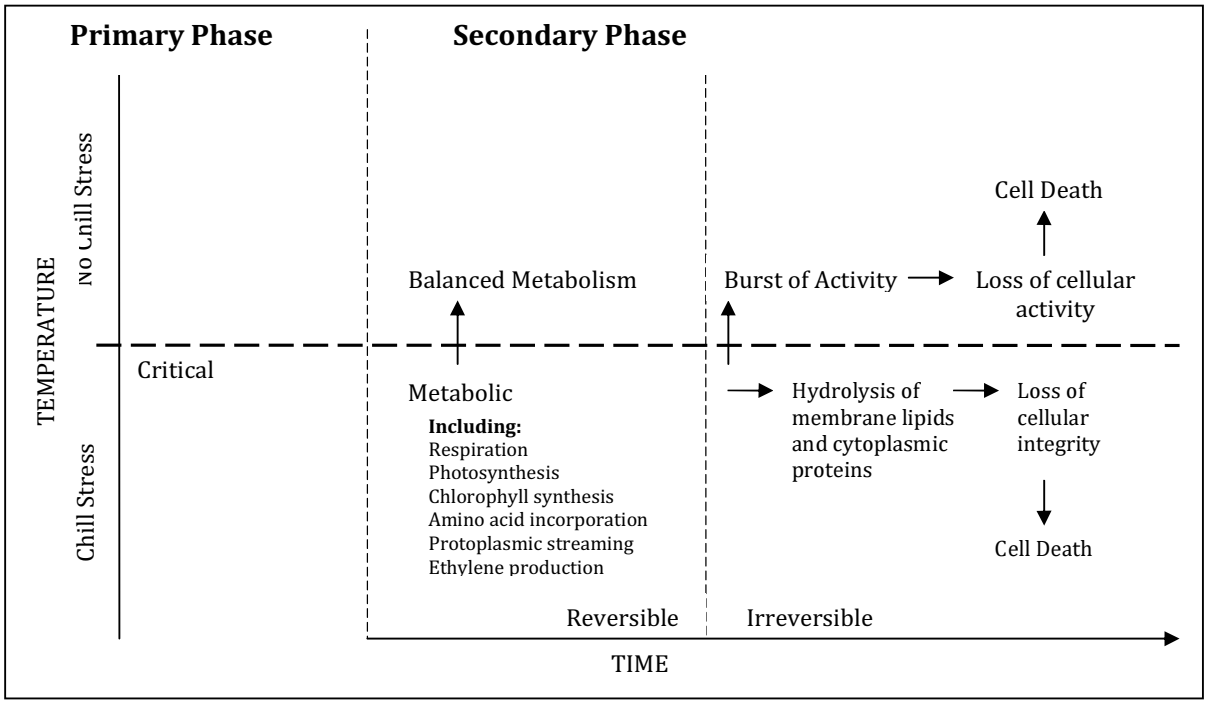

Figure 6: Schematic representation of the relationship between the primary and secondary phases of chilling injury, taken from Raison and Orr (1990).

3.2.2.1. Membrane Changes

Chilling stress rapidly affects the ultrastructure of cells, inducing the malfunction of organelles and membranes well before the appearance of visible chilling injury. Membrane permeability is an expression of the freedom with which water and solutes can pass through the membrane, and naturally increases during ripening and senescence (Murata, 1990). Membrane permeability is also increased in chill-injured tissue because of phase changes in the lipid membrane (Platt-Aloia, 1980). The combination of natural and induced membrane permeability greatly increases the total permeability and make the fruit more susceptible to phenolic browning such as mesocarp discolouration (Murata, 1990). Mitochondria and membrane functioning is impaired by the oxidation of chlorogenic acid (a phenolic compound) by PPO; this oxidation causes the swelling of mitochondria, degradation of the tonoplast, increased potassium ion leakage and decreased phospholipids in the tonoplast and cell membrane (Abe, 1990).

The ultrastructure of cell membranes of unripe, ripe and chilling-injured fruit is compared in Figure 7 (Platt-Aloia, 1980). The cell membranes of healthy unripe (Figure 7A) and ripe (Figure 7B) fruit have an even distribution of intra-membrane protein particles in the

lipid bi-layer, but cell membranes of fruit that had chilling injury underwent changes in organisation, whereby membrane components re-distributed to form areas in the lipid bi-layer that are depleted of protein particles (Figure 7C, indicated with arrow). This re-distribution is indicative of a phase separation of membrane lipids into gel (semi-solid) and fluid regions with the proteins being excluded from the gel phase. This phase separation occurs only in chilling-injured avocados and results in increased membrane leakiness and a loss of selective permeability (Murata and Nishida, 1990; Platt-Aloia, 1980). The loss of selective permeability results in cell death and tissue collapse, causing visible chilling injury. It was later found that mesocarp discolouration was intensified at temperatures which increased electrolyte leakage during cold storage (Van Rooyen and Bower, 2006).

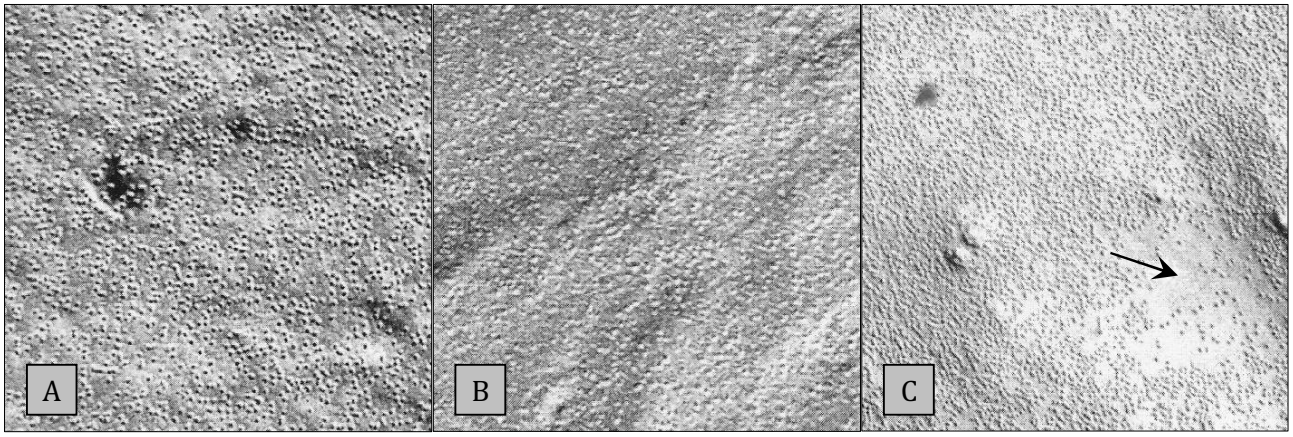

Figure 7: Electron micrograph of a freeze fracture replica of the cell membrane from an (A) unripe, (B) ripe and (C) chilling injured fruit, taken from Platt-Aloia (1980).

3.2.2.2. Mesocarp Discolouration

Phenolic browning, most commonly presenting as mesocarp discolouration, is caused by the enzymatic oxidation of phenols to melanin. The reaction is a two step process. In the first step, the oxidation of o-diphenols to the corresponding o-quinone is catalysed by PPO. In the presence of oxygen, the o-quinones are then irreversibly oxidised to brown melanin pigments (Bower and Cutting, 1988). In healthy tissue, PPO is primarily located in the thylakoid membranes of chloroplasts and other plastids while the phenolics are located in the vacuole, the enzyme and substrate are therefore spatially separated. If the cell is damaged and compartmentalisation is compromised, PPO and the phenolics are released into the cytoplasm and oxidation results in the presence of oxygen (Hershkovitz *et al.*, 2005).

An increase in PPO activity near ripeness resulted in an increase in mesocarp discolouration (Cutting *et al.*, 1989). Electrolyte leakage, an indicator of membrane

permeability, also increased in fruit more prone to mesocarp discolouration (van Rooyen, 2006). Good correlations were found between mesocarp discolouration, PPO and peroxidase (EC 1.11.1.7) activity, indicating that the disorder is positively related to reduced cellular compartmentalisation (Hershkovitz *et al.*, 2005). Nutrient imbalances predispose fruit mesocarp discolouration because of weak cell walls and more permeable membranes (van Rooyen, 2006). Mesocarp discolouration was largely attributable to excess tree and fruit nitrogen and was not necessarily chilling injury, but is exacerbated by cold storage (van Rooyen, 2006).

3.2.2.3. Ethylene in Chilling Injury

The exposure of fruit to ethylene before or during cold storage increases the risk of internal chilling injury. The combination of ethylene and low temperature accelerates skin injury and pulp browning in 'Ettinger', 'Fuerte' and 'Hass' fruit after just two weeks of cold storage (Pesis *et al.*, 1999). 'Fuerte' fruit stored in an atmosphere containing 100 $\mu L.L^{-1}$ ethylene had increased mesocarp discolouration at 12°C, compared to fruit stored in an ethylene-free atmosphere (Lee and Young, 1984). The fruit did not produce a climacteric when exposed to ethylene during cold storage (6 and 9°C) but the ethylene production rate was elevated (Lee and Young, 1984). ACC synthase is sensitive to chilling injury because the enzyme is labile and membrane-bound, so membrane integrity must be maintained for the continued functioning of ACC and synthesis of ethylene. Perhaps contradictorily, ACC oxidase activity is increased in a fruit after chilling injury (Fuchs *et al.*, 1995). It was observed that fruit that were removed from six weeks cold storage at 2°C and had chilling injury, had a much smaller ethylene peak than fruit stored at the same temperature for only three weeks and without chilling injury (Fuchs *et al.*, 1995).

When cellular ethylene reception was blocked by 1-methylcyclopropene (1-MCP), mesocarp discolouration was reduced (Hershkovitz *et al.*, 2005; White *et al.*, 2003; Woolf *et al.*, 2005). This evidence supports the role of ethylene in the development of internal chilling injury and the disorder is a symptom of an imbalance in metabolism rather than cold damage (Pesis *et al.*, 2002), especially when reduced storage temperatures are known to reduce chilling injury (Van Rooyen and Bower, 2006). The lower temperature prevents the accumulation of metabolic intermediates during cold storage (Section 3.2.1), *i.e.* a reduction in storage temperature will reduce respiration and ethylene production, reduce the accumulation of metabolic intermediates and reduce internal chilling injury. In summary,

ethylene is involved in the occurrence of mesocarp discolouration and treatments that reduce the metabolic activity and stress during storage reduce the development of the disorder (Hershkovitz *et al.*, 2005; Pesis *et al.*, 2002; Van Rooyen and Bower, 2006).

3.2.2.4. Sugars in Chilling Injury

Generally, sugars can influence the chilling sensitivity in three ways: sugars decrease the osmotic potential of the cytoplasm, thereby decreasing water loss and lowering the freezing temperature; by acting as osmoprotectants to stabilise cell membranes and enzymes by binding to constitutive molecules; and by serving as an energy source for anabolic processes (Purvis, 1990). The role of sugars in chilling resistance is complicated by the holistic nature of plant systems, but generally an increased concentration of sugars (for a particular cultivar) will indicate better fruit quality because environmental conditions which favour photosynthesis, and therefore the accumulation of sugars, also reduce the chilling sensitivity of plant tissues (Purvis, 1990). Also, glucose is the precursor of ascorbic acid – a major anti-oxidant in avocado mesocarp (Tesfay *et al.*, 2010) – so an increased concentration of glucose will permit a higher anti-oxidant capacity (Section 2.7.1). The sugar concentration in avocado mesocarp is very low and mannoheptulose is a major anti-oxidant in the mesocarp (Tesfay *et al.*, 2010) and thought to be used in respiration as an energy source (Liu *et al.*, 1999a). This dual function would place a strain on the limited pool of mannoheptulose and make the fruit vulnerable to physiological disorders. With the limited sugar concentration, the solution effect is limited in avocados. The best way of ensuring fruit storage ability, with respect to sugars, is to maximise them at harvest and minimise losses during cold storage. It is therefore crucial to optimise preharvest orchard practices and cold storage, to maximise carbohydrate accumulation and minimise carbohydrate losses.

3.3. Cold Chain Maintenance

Transient temperature increases during cold storage are known to be damaging to fruit quality. Fruit are moved through a number of handling points during exportation. There is a risk of transient temperature increases, or cold chain breaks, at these handling points, making the maintenance of the cold chain difficult (Dodd *et al.*, 2007). The Perishable Products Export Control Board of South Africa (PPECB) has set out protocols for shipping avocados (PPECB, 2008, 2009). The time and temperature between harvest and consumption are critical in

determining eating quality. Refrigerated trucks are not able to cool fruit and the pulp temperature can rise by 2°C during road transport. The PPECB stipulates that the maximum time a container of avocados can be without cooling is three hours (PPECB, 2008) and adherence to the time-temperature tolerance (TTT) is only 2°C.h. The repercussions of a cold chain break are serious and deserve further research because fruit quality is highly dependent on temperature management and fruit quality affects product price (Dodd *et al.*, 2007). A temperature break for two or three days at 25°C increased mass loss, colour change and softening if fruit were stored for 30 or 40 days (Undurraga *et al.*, 2007)

3.4. Phytosanitary Cold Sterilisation

The expansion of South African avocado fruit sales into new markets is hampered by phytosanitary requirements imposed by many countries because of the insect pests present in the country. The three preferred options for phytosanitary disinfestation are ionising radiation, heat treatment and cold sterilisation. Fumigation and pesticide applications are not preferred because of residual toxicity (Heather and Hallman, 2008). Radiation and heat treatment are not useful for avocados due to physiological damage caused by the required protocols (Woolf and Lay-Yee, 1997), so the only remaining option is cold sterilisation. In the last decade, the South African Avocado Growers' Association have invested considerable funding for research on ultra-low temperature storage and the integrated postharvest methods to better maintain fruit quality at these temperatures because if a suitable integrated method can be developed and implemented, many more markets will be opened, easing pressure on the European market.

4. Mitigating Treatments for Cold Storage

Fruit quality is maximal at harvest and can only decline postharvest, so the aim is to have excellent quality fruit at harvest and maintain this quality as much as possible until consumption (Hewett, 2006). Significant losses in fruit quality can occur pre-harvest, but most quality loss is at harvest or during postharvest handling, so it these areas that receive the most attention. To minimise chilling injury, pre- and postharvest stress needs to be reduced as much as possible (Bower, 1988, 2005a, b). Cold temperature acclimation, water loss reduction and respiration and ethylene management are focal areas for maintaining fruit quality during

cold storage (Sevillano *et al.*, 2009).

4.1. Pre-harvest Factors

Pre-harvest management, especially water stress and nutrition, is well documented to affect postharvest quality (Arpaia and Eaks, 1990; Arpaia *et al.*, 2004; Bower, 1985, 1988; Bower and Cutting, 1988; van Rooyen and Bower, 2003; van Rooyen, 2006). Fruit with a low calcium accumulation due to water stress are prone to have poor quality, partly because of a higher PPO concentration at ripeness (Bower and van Lelyveld, 1986) (Section 3.2.2.2). The inability to commercially increase the calcium concentration postharvest (Eaks, 1985) is indicative that pre-harvest management is critical to postharvest fruit quality. Hewett (2006) provides an overview, and Arpaia *et al*. (2004) deals specifically with production factors affecting avocado postharvest quality. Genetics, site and microclimate, light availability, growing temperature, rainfall and irrigation, soil mineral content and fertilisation, growth regulators and harvest maturity all affect fruit quality at various points in the supply chain (Arpaia *et al.*, 2004; Hewett, 2006).

4.1.1. Genetics

Certain cultivars are more tolerant of cold storage than others. Lowland-type cultivars are particularly prone to chilling injury (Lahav and Lavi, 2002). 'Hass' can be stored at slightly lower temperatures because the skin colour development during ripening masks slight blemishes and external chilling injury.

4.1.2. Orchard Management

The fundamental principle of crop production is to harvest light and convert it dry matter. This can be improved with correct canopy management, irrigation, fertilisation and soil management (Arpaia *et al.*, 2004; Lahav and Whiley, 2002; Whiley, 2002; Wolstenholme, 2002). Water has numerous effects on crop physiology and fruit quality by affecting vegetative growth, yield, fruit size and storability (Bower, 1985; Meyer *et al.*, 1990). Water movement is required for calcium accumulation (Witney *et al*., 1990a) and calcium is required for cell wall strength, membrane stability (Bangerth, 1979) and cell messenging (Zielinski, 1998). Water stress will also reduce carbohydrate accumulation by limiting photosynthesis. Also, sun exposed fruit are more tolerant to cold storage because of an accumulation of heat shock

proteins, and may account for postharvest variation in quality (Woolf *et al*., 1999).

Harvest can be delayed to improve cold tolerance so fruit can be shipped at a lower temperature (PPECB, 2008, 2009), but this may be increase the risk of mesocarp discolouration (Cutting and Bower, 1987).

4.1.3. Growth Regulators

Growth regulators are used to control vegetative growth and improve the sink strength of fruit relative to leaves. Gibberellic acid may be used to manipulate flowering, but is not used widely (Whiley, 2002). Triazoles, which inhibit gibberellin biosynthesis, are used commercially to reduce vegetative growth to increase fruit retention, fruit size and yield by increasing the dry matter accumulation in fruit (Wolstenholme *et al*., 1990). Paclobutrazol (Cultar®) and uniconazole (Sunny® or Magic®) are used commercially in the avocado industry for this purpose (Whiley, 2002).

4.1.4. Nutrition

Balanced nutrition is critical for normal plant functioning. Plants require a range of macro- and micro-nutrients and all these interact. Avocados are a relatively new horticultural crop and fertilisation was still being refined during the initial research into cold temperature storage (1970s and 80s in South Africa) so perhaps chilling injury results were confounded by nutrient imbalances in fruit and not the chilling injury as such, as was found in 'Pinkerton' (van Rooyen, 2006).

Calcium is important in the development of the cell wall and provides an example of the effect of preharvest management on postharvest quality. A more in-depth discussion on the roles of the various nutrients is provided by van Rooyen (2006). The effect of calcium on avocado physiology was extensively studied in the 1980s and is known to affect cell membrane and wall structure and function (Bower and Cutting, 1988; Ferguson, 1984; Wills and Tirmazi, 1982; Witney *et al.*, 1990b). Little research on the effect of calcium on avocado physiology has been forthcoming since then, so the review by Bower and Cutting (1988) still provides a review of the current understanding. Calcium moves in the xylem with the transpiration flow so calcium accumulation is dependent on the transpiration rate. Both water stress and a low demand for water from the environment limit the transpiration rate. There is also a reciprocal movement of auxin and calcium in plant tissues (Section 2.6.1). The spring vegetative flush and early fruit development coincide in avocados, causing competition

between leaves and fruit. The terminal bud exports more auxin than the fruit so more calcium accumulates in the leaves than the fruit (Cutting and Bower, 1989). Therefore, any cultural practice that limits the spring growth, such as the application of a triazole (Section 4.1.3), can improve fruit quality (Bower and Cutting, 1988). Fruit growth also has to be controlled because if fruit grow faster than calcium is deposited, calcium deficiency will result in the fruit. Nitrogen was found to contribute most to the incidence of mesocarp discolouration (accounting for 38% of variance) while calcium was not significant, only contributing 3% (van Rooyen, 2006) – it must be noted that calcium may play a greater direct role with correct nitrogen fertilisation. However, the impact of nitrogen is far-reaching because of nutrient and carbohydrate interactions. Excess nitrogen causes an increase in vegetative growth, reducing the calcium and carbohydrate accumulation in the fruit. This would reduce membrane stability, energy reserves and cell wall strength, affecting ripening and fruit quality.

Ripening rate is linked to the fruit calcium concentration (Tingwa and Young, 1974; Wills and Tirmazi, 1982; Witney *et al.*, 1990b). The Ca^{2+} ion is thought to form cross linkages between pectin fibrils, strengthening the cell wall strength and providing resistance to chilling injury and enzymatic action (Chaplin and Scott, 1980; Jarvis, 1984; Matteo *et al.*, 2005; Willats *et al.*, 2001). By infusing calcium into fruit, respiration and ethylene production were severely reduced (Tingwa and Young, 1974; Wills and Tirmazi, 1982; Witney *et al.*, 1990b), leading Tingwa and Young (1974) to suggest that the variability in ripening is related to the calcium concentration. Unfortunately, orchard sprays and dipping fruit in a calcium solution do not provide adequate calcium uptake, and although vacuum infiltration is a much more effective method, it is not useful commercially (Davenport, 1984; Eaks, 1985; Veldman, 1983; Wills and Tirmazi, 1982). Proper soil fertilisation and irrigation are therefore the most effective means of ensuring calcium uptake in fruit and the interactions with vegetative growth and nitrogen in particular, needs to be kept in mind (Bower, 1988; van Rooyen, 2006).

4.2. Postharvest Methods

Once harvested, fruit are very susceptible to quality loss and deterioration. This can be minimised with proper postharvest management. Cold storage is the primary means of maintaining fruit quality. Postharvest treatments focus on limiting water loss and the effect of ethylene because a reduction in water loss during storage reduces the potential for physiological disorders and desiccation (Bower and Jackson, 2003) and ethylene in the storage atmosphere can intensify mesocarp discolouration (Pesis *et al.*, 1999). The effect on

internal and external quality must both be considered to determine the success of a postharvest treatment (Bower, 2005a).

4.2.1. Postharvest Handling

Although avocados have a waxy cuticle that reduces water loss, the lenticels (nodules) on the skin have no active mechanism to limit water loss. Mechanical damage to the cuticle and lenticels greatly increases water loss, desiccation and external chilling injury (Everett *et al.*, 2008). This highlights the importance of adhering to good picking practices of not harvesting immediately after rain and handling fruit gently (Everett *et al.*, 2008; PPECB, 2008). Cold chain maintenance, as already mentioned, is crucial in maintaining fruit quality (Section 3.3.).

4.2.2. Storage Temperature

As already discussed, cold storage is the principal means of maintaining fruit quality, but there are limits to storage temperature and the duration of storage. It is generally accepted that non-West Indian cultivars, e.g. 'Fuerte' and 'Hass', can be stored for 28 days at 5-6°C before physiological disorders start to increase drastically. Lowering the storage temperature can prolong the maximum storage period by reducing mesocarp discolouration (Dixon *et al.*, 2004a) but may increase external chilling injury. Reduced temperature storage (2°C for green skin cultivars and 1°C for black skin cultivars) has repeatedly proven to be an effective method of reducing flesh browning disorders such as mesocarp discolouration (Van Rooyen and Bower, 2006) and semi-commercial trials yielded very positive results for 'Hass' fruit stored at 1°C (Van Rooyen, 2009). Early research showed that after 2 weeks 'Hass' stored at 0°C were not significantly different from fruit stored at 5°C and after 4 weeks at 0°C chilling injury was 'moderate' but production location was significant in determining the efficacy of this treatment (Eaks, 1983).

Increased internal chilling injury occurs when fruit are stored in the 'killing zone' and is much reduced if fruit are stored at a lower temperature – but above the freezing point of the fruit (Crisosto *et al.*, 1999; Crisosto *et al.*, 2004; Lurie and Crisosto, 2005). The concept of the 'killing zone' was proposed when it was found that the postharvest life of a number of peach cultivars (also climacteric) was reduced by shipping at 5°C instead of 0°C (Crisosto *et al.*, 1999). While not a perfect comparison because peaches are temperate fruit, it does illustrate that the optimal storage temperature for subtropical avocados may, counter-intuitively, be

below the current commercial threshold.

It is imperative that field heat is removed as soon after harvest as possible to maintain quality, but this needs to be done in a controlled manner. During cooling, air speed must be moderated to minimise desiccation and the delivery air temperature must not be excessively low or chilling injury will result. The incidence and severity of mesocarp discolouration and occurrence of decay increased with increased fruit temperature and holding time before cold storage (Arpaia *et al*., 1992). With an increase in cooling from 6h to 12h after harvest, the percentage of moderate or severe mesocarp discolouration increased four-fold and decay by 4.4%; a pulp temperature of 40°C at harvest resulted in significantly higher mesocarp discolouration. This may be because of membrane instability, a longer time to move through the killing zone during cooling, water stress at harvest or carbohydrate depletion. What is clear is that fruit must be chilled and put into a state of reduced physiological activity as soon after harvest as possible, but in a controlled manner to reduce stress during cooling.

4.2.3. Heat Treatments

Heat shock is known to elicit the production of heat shock proteins which confer thermo-tolerance to high and low temperatures (Woolf and Lay-Yee, 1997). A hot water or vapour heat treatment can be used but hot water treatments are commercially favourable to vapour treatments because of the lower capital investment (Hofman *et al.*, 2002b). Mixed results have been obtained regarding the efficacy of a hot water treatment for avocados. Researchers in Australia and New Zealand found a hot water treatment (40-42°C for about 30 min) reduced chilling injury during cold storage (Hofman *et al.*, 2002b; Woolf, 1997). A slightly more severe treatment (43°C for 43 min) significantly reduced pathological decay (Hofman *et al.*, 2002b). Researchers in South Africa found vapour heat treatment to be detrimental to fruit quality (Donkin and Wolstenholme, 1995; Weller *et al.*, 1998) and results were highly variable for hot water treatment, depending on cultivar and fruit maturity, but a similar optimal treatment to the antipodean researchers was found (Kritzinger *et al.*, 1998). A later study found that a hot water treatment of 38°C for 15-30 min reduced the spread in ripening and improved colour development, especially of early season fruit and fruit stored at 1°C and slightly reduced pathological decay (Blakey and Bower, 2006). In light of these variable results and the high costs of heating, hot water treatments are not used commercially in the avocado industry.

4.2.4. Low Temperature Conditioning and Acclimation

Low temperature conditioning is the exposure of chilling sensitive fruit to a temperature slightly above the chilling temperature threshold to induce cold tolerance before reducing the storage temperature further. The exposure to low, non-chilling temperatures can help in reducing chilling injury by reducing the utilisation of sugars as respiration is reduced (Purvis, 1990). In South Africa, avocado fruit are acclimated using a step down treatment to acclimate the fruit to low temperature storage (PPECB, 2009). This is based on earlier findings that fruit are most sensitive to chilling injury during the first week of cold storage (Toerien, 1986). Low temperature conditioning at 6°C for 3 days before storage at 1°C for 16 days also reduced external chilling injury and allowed effective disinfestation of Queensland fruit fly at the lower temperature (*Bactrocera tryoni* Froggatt) (Hofman *et al.*, 2003). Similarly, a treatment of 6-8°C for 3-5 days reduced external chilling injury in 'Hass' fruit compared to the non-conditioned control fruit (Woolf *et al*., 2003). Conditioning 'Pinkerton' fruit at 10°C for two days prior to cold storage at 2°C significantly reduced external chilling injury compared to non-conditioned control fruit (van Rooyen, 2006).

4.2.5. Ethylene Control

The presence of ethylene in the storage atmosphere is highly detrimental to fruit quality, resulting in a marked increase in mesocarp discolouration intensity (Pesis *et al.*, 2002). Much postharvest research is focussed on limiting and controlling the effect of ethylene on fruit physiology (Terry *et al.*, 2007b). Ethylene can either be removed from the storage atmosphere or its perception blocked.

4.2.5.1. Scavengers

Ethylene scrubbers are used to oxidise ethylene, removing it from the storage atmosphere. Any oxidising agent can be used to do this, but potassium permanganate ($KMnO_4$) is most commonly used. This is supplied either in a sachet or incorporated into a film (de Kruijf and van Beest, 2003). The advantage of using an ethylene scrubber is that there is not a prolonged effect on the ripening physiology after storage (Porat *et al.*, 1999). However $KMnO_4$ has limited efficacy in an atmosphere with high relative humidity and avocados are stored at 90-95% relative humidity. More recently, a palladium-promoted material (e+®) has been shown to be far more effective in scavenging ethylene than $KMnO_4$ (Ilkenhans *et al.*,

2007; Meyer and Terry, 2010; Terry *et al.*, 2007a; Terry *et al.*, 2007b). This material can scavenge up to 10 mL of ethylene per gram at 100% relative humidity and does not have a residual effect once removed (Terry *et al.*, 2007a). If this material proves to be commercially viable, it will offer an alternative treatment to $KMnO_4$ and 1-methylcyclopropene (1-MCP).

4.2.5.2. Inhibitors and 1-MCP

The ethylene receptor can be blocked by a range of inhibitors. Silver thiosulphate and 2,5-norbornadiene are effective ethylene inhibitors while CO_2 is an ethylene antagonist (Sisler *et al.*, 1996; Sisler and Serek, 1997), but 1-MCP has become the eminent ethylene inhibitor because it is odourless, non-toxic and highly effective at very dilute concentrations (Porat *et al.*, 1999; Sisler *et al.*, 1996; Sisler, 2006). The inhibitory effect of 1-MCP on ethylene reception was discovered by Edward Sisler (2006). Initially it was named Sis-X to protect patent rights, but the real name was released in 1994 (Serek *et al.*, 1994). 1-MCP is the subject of much recent postharvest research on various crops because of its effect of blocking ethylene receptors at extremely low doses. Typical dose rates are: 5 $nL.L^{-1}$ for flowers (Serek *et al.*, 1995), 20 $nL.L^{-1}$ for potted plants (Serek *et al.*, 1994) and 100 $nL.L^{-1}$ for oranges (Porat *et al.*, 1999).

There are conflicting results regarding the usefulness of 1-MCP for avocados because the effect of 1-MCP continues post-storage. Treatment with 1-MCP reduced the occurrence of ethylene-related disorders and softening during cold storage (Hershkovitz *et al.*, 2005; Pesis *et al.*, 2002), but the inhibition of ripening can be too severe and result in fruit developing postharvest rots because ripening is delayed (Jeong and Huber, 2004). A similar problem with oranges has been encountered (Porat *et al.*, 1999). 1-MCP reduced the rate of mass loss, ripening rate and respiration of avocados during ripening at 20°C (Jeong *et al.*, 2002; Jeong *et al.*, 2003). This delay in ripening increased the susceptibility of fruit to fungal rots. Fruit treated with 1-MCP (900 $nL.L^{-1}$) lost an extra 2.0% of the initial mass compared to untreated fruit and 6.6% compared to waxed fruit when ripened at 20°C; for fruit stored at 13°C, the difference between waxed fruit and waxed and 1-MCP treated fruit was 3.0% because the fruit took four days longer to ripen, losing more dry mass and water (Jeong *et al.*, 2003). A lower dose of 100 $nL.L^{-1}$ has been recommended (Woolf *et al.*, 2005), but the results are not consistent because the effect is highly dependent on the source of fruit and can aggravate mixed ripening (Adkins *et al.*, 2005; Ochoa-Ascencio *et al.*, 2009). Only orchards with a low inoculum loads are recommended for 1-MCP treatment (Adkins *et al.*, 2005). But inoculum

loads in orchards vary within and between seasons and farms (Dixon *et al.*, 2004a). The effect of 1-MCP cannot be overcome by treatment with exogenous ethylene (100 $\mu L.L^{-1}$ for 12h); fruit are only ethylene sensitive when new ethylene receptors have been synthesised (Jeong and Huber, 2004). Despite the mixed results, avocados have been treated commercially with 1-MCP since 2003 in South Africa at a dose rate of 300-600 $nL.L^{-1}$ to prevent fruit softening in cold storage and physiological disorders, based on the findings of the Agricultural Research Council – Institute for Tropical and Subtropical Crops (ARC-ITSC) (Kruger and Lemmer, 2007). The researchers at the ARC-ITSC found that 1-MCP treatment was comparable to controlled atmosphere storage when applied at the optimum dosage (Lemmer *et al.*, 2003), but did delay ripening after cold storage (Lemmer *et al.*, 2005). Later recommendations were to have a minimum maturity of 70% moisture content (30% dry matter) before treating with 1-MCP (Kruger and Lemmer, 2007). The cold storage period for South African fruit exported to Europe is sufficient time for the re-synthesis of new ethylene receptors and the delay in ripening is much reduced and the reduced risk of internal browning disorders out-weighs the delay in ripening.

1-MCP has proven to be a very effective tool for postharvest quality management, but does not address the increased water loss that causes the increased ABA and ethylene synthesis. The control of water loss still needs attention in the avocado industry.

4.2.6. Water Loss Reduction

Postharvest conditions, especially during storage, need to be optimised to minimise water and respiratory losses. It was found that the degree of external chilling injury is influenced by mass loss during storage (Bower *et al.*, 2003; Bower and Jackson, 2003; Bower and Papli, 2006). During cold storage, water loss is likely to be the major contributor to mass loss, because of the reduced respiration rate (Bower and Jackson, 2003). The threshold mass loss value is about 6%; if fruit lose more than 6% during storage, chilling injury is likely to be severe (Bower *et al.*, 2003; Bower and Jackson, 2003; Bower and Papli, 2006).

While air movement in the storage container is needed to minimise temperature gradients during cold storage, high air speeds desiccate the fruit (Mitchell *et al.*, 1992; Wills *et al.*, 1998). The constant removal of the thin boundary layer of nearly saturated air around the fruit increases the vapour pressure deficit and this deficit is met by moisture from the fruit (Wills *et al.*, 1998). Water loss results in desiccation, stress, shrivelling and even necrosis in severe cases. Carton packaging reduces water loss to some extent, by slowing the air speed

over the fruit, but additional methods are required to prevent desiccation and external chilling injury (Wills *et al.*, 1998).

4.2.6.1. Humidification

Increasing the relative humidity, also thought of as reducing the vapour pressure deficit, is a simple means of limiting the water loss from fruit. The humidification of the storage atmosphere to reduce moisture loss from the fruit significantly reduced the incidence of pathological and internal physiological disorders in avocado fruit (Bower *et al.*, 1989). This is probably because of water stress and ethylene stimulating the growth of anthracnose and cell death resulting in increased fungal growth by reducing cell defences and providing nutrients for fungal growth. However, relative humidity greater than 95% increases the risk of pathological decay.

4.2.6.2. Waxing

Waxing is used extensively in the South African industry because of its effective control of postharvest water loss. Wax is favoured because of its ease of application even though it has also been found that certain waxes increase postharvest disorders by not permitting sufficient gaseous exchange (Bower *et al.*, 2003; van Rooyen and Bower, 2007). Unpublished data (Blakey and Bower) confirmed that some commercial wax treatments can restrict the respiration rate by about 50% by clogging the lenticels. The effect of a wax treatment depends on the concentration, formulation, method of application and application duration which vary between packhouses. Fruit size may also be important, depending on the method of application, because in a volume-fed system large fruit receive more wax than smaller fruit because larger fruit move more slowly through the wax applicator compared to smaller fruit. Also the stem- and blossom ends of fruit receive less wax compared to the equatorial region. Wax needs to crack or develop small holes during the pre-cooling stage to allow sufficient gaseous exchange during ripening, but the degree of cracking depends on the wax formulation (Bower *et al.*, 2003).

4.2.7. Atmosphere Modification

Modification of the concentrations of oxygen and CO_2 is used to limit respiration and ripening during storage. This can be done using a pre-storage CO_2 shock or by altering the

storage atmosphere. Ethylene synthesis is oxygen dependant and CO_2 is a competitive inhibitor of ethylene (Burg and Burg, 1967; Chaves and Tomas, 1984), so increasing the CO_2:O_2 ratio has direct inhibitory effects on ethylene reception as well as respiration (Kanellis *et al.*, 1989). The reduction in the concentration of oxygen limits the conversion of AdoMet to ACC thereby reducing the ethylene production rate (Burg and Burg, 1967). A reduction in oxygen can also reduce ethylene sensitivity without affecting respiration (Burg and Burg, 1967).

4.2.7.1. Controlled Atmosphere Storage

Controlled atmosphere storage (CAS) is cold storage where the concentrations of CO_2 and O_2, and the ratio between the two gases, are set at a particular value at the start of storage (static CAS) or controlled during storage (dynamic CAS) (Burdon *et al.*, 2008; Heather and Hallman, 2008). CAS is used to reduce the respiration rate without reducing the storage temperature, and is very effective storage technique for chilling sensitive crops. However, it is expensive and cheaper technologies exist to prolong storage. CAS (2% O_2, 10% CO_2) decreased postharvest physiological changes, specifically of cellulase activity, and limited mesocarp discolouration (Bower *et al.*, 1989; Kanellis *et al.*, 1989). Static CAS (5% O_2, 5% CO_2) is better than storage in air and dynamic CAS (<3% O_2, 0.5% CO_2) is better than static CAS, in terms of limiting fungal rots and physiological disorders, but there are increased costs with these benefits (Burdon *et al.*, 2008). The use of CAS is limited in South Africa because of the increased use of 1-MCP[2].

4.2.7.2. Modified Atmosphere Storage

A modified atmosphere can be generated when fresh produce is stored in a confined atmosphere and the CO_2:O_2 ratio increases due to the produce respiring. This increase reduces respiration and ethylene production during cold storage. The major concern with the use of modified atmosphere packaging (MAP) is that the internal CO_2 will accumulate to toxic concentrations. This is averted by the micro-perforations which allow gaseous exchange during storage, while still allowing a high relative humidity to develop (van Rooyen, 2006). This is termed modified humidity packaging. The use of modified atmosphere/modified humidity packaging (MA/MHP) has been shown to be a better alternative to waxing: microperforated polypropylene bags (polybags) and microperforated polyethylene bags with an ethylene absorbing coating limited moisture loss to a greater extent than any wax coating

[2] Pers. comm. J.J. Bezuidenhout, Westfalia Technological Services, Tzaneen, South Africa

(Bower, 2005b). Polybags were preferred because the anti-mist coating and micro-perforations prevented free water accumulation, which promotes postharvest decay, and allowed greater gaseous exchange, preventing an accumulation of CO_2 which can cause anaerobiosis (Bower, 2005b). Comparing fruit stored at 5.5°C, wax, MA/MHP and carton wrapping reduced mass loss by 6.6, 12.2 and 10.1% compared to the control; differences at 2°C are not so noticeable because the control fruit lost much less water at the lower temperature (Bower *et al.*, 2003). The inclusion of an ethylene scavenger in the MAP reduced mesocarp discolouration and decay (Pesis *et al.*, 2002). MA/MHP, possibly with the inclusion of an ethylene scrubber, may offer to viable alternative to CAS, but the delay in cooling needs to be improved, especially when the ambient temperature is high (Bower and Blakey, 2008).

4.2.7.3. Carbon Dioxide Shock

Although not used commercially, a CO_2 shock treatment (20% CO_2) for three days severely reduced ethylene production, internal ethylene concentration and ACC concentration (van Eeden *et al.*, 1990). These authors found that while there was a rapid increase in ACC concentration after removal from the high CO_2 atmosphere, the amount of ACC produced was reduced, indicating that the CO_2 inhibited ACC formation due to reduced ACC synthase production and/or activity. Higher concentrations of CO_2 (40-50% for two days) were also investigated but found that fruit quality was significantly reduced compared to control, controlled atmosphere storage and 1-MCP treated fruit (Maré *et al*., 2002).

5. Near-Infrared Spectroscopy

The use of near-infrared (NIR) spectroscopy is increasing in the horticultural industry as a means to determine various internal attributes of fruit and vegetables. Avocados are a highly variable crop, due to factors mentioned previously and NIR spectroscopy may well prove to be useful in the avocado industry as a means to sort fruit with similar internal characteristics, particularly fruit maturity.

5.1. Background

NIR spectroscopy is a non-destructive measurement technique using electromagnetic radiation in the region of approximately 780-2500 nm (Nicolaï *et al.*, 2007). Light energy is

directly related to frequency or wavelength. NIR spectroscopy uses the principle that only certain frequencies are absorbed by a particular molecule, due to the stretching and bending of bonds, particularly C-H, O-H and N-H bonds, in the molecule (Workman and Shenk, 2004). It is this very selective nature of the interaction between the light and molecule that makes analytical spectroscopy possible and provides information about its molecular composition.

In this branch of spectroscopy, NIR radiation is projected onto a sample and some of the reflected or transmitted radiation is captured and related to variables of interest using multivariate analysis. As this backscattered radiation spectrum is affected by both the scattering and absorption properties of the tissue, it provides information about the physical structure and chemical composition of the sample (Nicolaï *et al.*, 2006; Nicolaï *et al.*, 2007). NIR spectra are complicated, but result from fundamental molecular vibration mechanisms that are well understood and reproducible. The prediction of chemical compounds is achieved by calibrating and validating NIR spectral information against reference data and is termed a model (Workman and Shenk, 2004). This model is then used to measure the parameter in subsequent samples.

There are three basic data collection methods (DCM) in NIR: reflectance, transmission and interactance. In reflectance mode, the light source and detector are located on the same side of the sample. In transmission mode, the two components are located on opposite sides of the sample, while interactance measures the internally reflected light from a sample, on the same side as the light emitter (Nicolaï *et al.*, 2007).

This branch of spectroscopy has greatly expanded the options available to agricultural researchers because of its speed in testing, sample and parameter versatility, sensitivity (Burns and Ciurczak, 2001; Williams and Norris, 2001).

5.2. NIR in Horticulture

Research on postharvest quality has advanced from simply evaluating external parameters such as colour, size and absence of blemish to measuring internal parameters such as taste, texture, nutrition, health attributes and safety (Nicolaï *et al.*, 2006). With advances in NIR technology it is now possible to use NIR spectroscopy to measure these parameters. NIR spectroscopy has been used for decades for analysing grains and seeds but had limited use in horticultural crops because fruit and vegetables contain large amounts of water (water dominates the spectrum, complicating the prediction of more dilute compounds), have an irregular shape (which results in pathlength differences), are highly heterogeneous and have a

thick rind or skin (Nicolaï *et al.*, 2007; Slaughter and Abbott, 2004). The larger sample size of fruit – compared to grains – decreased the accuracy of prediction because reduced light penetration in earlier instruments (Workman and Shenk, 2004), but the development of the interaction probe solved the problems with sample shape and presentation (Kawano, 2002). Advances in processing power now mean that NIR spectrophotometers have an integration time of much less than 0.1s, allowing integration into high speed grading lines (Walsh *et al.*, 2004) and improved light penetration even with a reflectance DCM.

Apples are the most commonly studied fruit in NIR spectroscopy. A number of compounds and attributes have been successfully determined: fructose, glucose, sucrose, total soluble solids, sorbitol, malic acid, titratable acidity, pH, dry matter, water content, nitrogen, maturity, external colour, firmness, bruised tissue and water core (Slaughter and Abbott, 2004).

For each fruit species and cultivar a new calibration model may be required and the calibration models should be based on large datasets, incorporating factors such as orchards, season and temperature (Peirs *et al.*, 2002; Peirs *et al.*, 2003). However a calibration can be done rapidly and remotely by the manufacturer, so this may not be so disadvantageous.

Recent reviews on the use of NIR technology in agriculture and the food industry are available (Roberts *et al.*, 2004; Williams and Norris, 2001). These reviews provide detailed chemical, physical and statistical theory behind NIR spectroscopy and its use in the analysis of agricultural commodities. Nicolaï *et al.* (2007) provide an excellent extensive review of the use of NIR spectroscopy in horticulture.

5.3. NIR in the Avocado Industry

The use of NIR spectroscopy in the avocado industry is limited despite promising results (Clark *et al.*, 2003; Schmilovitch *et al.*, 2001), and the need for a means to measure maturity non-destructively (Hofman *et al.*, 2002a). At present the maturity of large batches of fruit is determined by measuring the dry matter or moisture content on a very small sample of fruit. This method is destructive, laborious, time-consuming and non-representative of the population mean. Fruit within the same orchard differ significantly in maturity, so a sample mean is determined which provides a very general indication of a batch of fruit and is likely to be non-representative of the population mean. Also, the best indicator of maturity is oil concentration, but this method requires specialised equipment and training (Section 2.4.4). The measurement of either dry matter or oil concentration is possible with NIR, and it may

also be able to quantify oil, sugar and protein concentrations using NIR spectroscopy. The ability to measure these parameters can be used to improve production practices and greater profitability (Kawano, 2002; Walsh and Subedi, 2010).

Early season fruit fetch a premium price, tempting the grower to harvest immature fruit for increased profit. Immature fruit, by definition, will not ripen and marginally mature fruit will be of poor quality because of the very low concentration of oil (Schmilovitch *et al.*, 2001). If these immature and marginally mature fruit are harvested and ripened, there will high amounts of wastage because few of the fruit will be mature, and the fruit that reach the consumer will be of poor quality and cause consumer dissatisfaction. This practice would be discouraged if a packhouse could scan fruit to determine maturity and reject any immature fruit. There is therefore potential and a need to use NIR spectroscopy as an analysis tool in the avocado industry.

Ripening has recently been modelled to determine the potential postharvest life of avocados using firmness and simulated enzyme activity; fruit maturity was estimated using firmness and enzyme activity (Ochoa-Ascencio *et al.*, 2009). This model was able to predict the ripening rate at harvest and after storage with an R^2 of 0.92. These authors acknowledged that the terms used in the model represent net effects of complex systems. NIR spectroscopy can measure many of these contributory effects, particularly dry matter, oil and protein. Clark *et al.* (2003) were able to measure dry matter of 'Hass' avocados and found that the correct choice in the DCM of the instrument is critical: a DCM with enhanced penetration is necessary for optimal results for avocados. The use of an instrument using a reflectance DCM resulted in development of a model with an R^2 of 0.75 and standard error of 2.6% DM for avocados, while the use of an interactance DCM increased the predictive ability, increased the R^2 to 0.88 and decreased the standard error to 1.8% DM (Clark *et al.*, 2003).

Avocados are highly variable, because of a number of parameters already mentioned, so having technology to quantify some of these parameters and sort fruit to have greater consistency within cartons and pallets as well as insight into quality and shelf life is highly desirable to the avocado industry.

6. Conclusion

Considerable advances in the understanding of avocado physiology have been made in the last fifty years but there is much about avocado physiology that is still poorly understood. The storage of 'Hass' at 1°C while maintaining external fruit quality is important for the South African avocado industry as it is a means of phytosanitary disinfestation required for potential export markets. There is scope for further research in ripening physiology, especially the effects of cold storage and cold storage mitigating treatments on avocado physiology.

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Chapter 2
The relationships between sugars, protein and oil of ‘Hass’ avocado fruit during ripening

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A better understanding of avocado (*Persea americana* Mill. 'Hass') postharvest physiology is required for improved management of this crop. The heptoses mannoheptulose and perseitol are thought to be important compounds in avocado physiology, but there is uncertainty about their functions. The aim of this study was to better understand the relationships between these and other minor sugars, protein and oil during ripening. Fruit were harvested from a warm subtropical area and a cool subtropical area in South Africa, thrice each during a season. Mannoheptulose and perseitol consistently declined to very low concentrations within 10 days of harvest. Glucose and fructose were present in very low concentrations throughout, but increased during ripening. The concentration of glucose in fast ripening fruit increased significantly three days before slow ripening fruit. The concentration of sucrose generally decreased slightly during ripening. The concentration of protein increased sigmoidally during ripening. The concentration of oil did not change significantly during ripening. It is suggested that mannoheptulose and perseitol are a major carbon source in avocado fruit and may also be used as an energy source.

1. Introduction

Avocado (*Persea americana* Mill.) is an oleaginous, climacteric fruit that contains high concentrations of the heptoses D-mannoheptulose (D-manno-2-ketoheptose) and perseitol (D-glycero-D-galacto-heptitol) (Liu *et al.*, 1999a). The functions of these sugars are not fully known, although their presence in avocado has long been known (Muntz and Marcano, 1884). The respiratory climacteric requires substantial energy reserves and the respiratory quotient (CO_2:O_2) during ripening is approximately one, indicating that the substrate used during ripening is a carbohydrate (Blanke, 1991). However the mesocarp contains little glucose and starch, while mannoheptulose and perseitol are the most abundant sugars in the mesocarp and are depleted at ripeness (Landahl *et al.*, 2009; Liu *et al.*, 1999a), suggesting that they are utilised during ripening. Besides this, mannoheptulose is the predominant anti-oxidant in avocado mesocarp (Tesfay *et al.*, 2010), and perseitol is a storage compound (Tesfay, 2009). Furthermore, it has been suggested that mannoheptulose may be a ripening inhibitor (Liu *et al.*, 2002).

Extensive cell wall alteration and break down is required to achieve palatability. A number of enzymes are required, but cellulase (β-1,4-endoglucanase; EC 3.2.1.4) and endo-polygalacturonase (EC 3.2.1.15) are regarded as being primarily responsible for softening and are synthesized *de novo* (Awad and Young, 1979), suggesting an increase in protein concentration - depending on the rate of protein catabolism. *De novo* synthesis requires both an energy and carbon source, which has not been categorically identified in avocado.

The health benefits of avocado due to its high percentage of monounsaturated fatty acids (50-60% of total fatty acids) are well documented (Meyer and Terry, 2010). Fruit maturity is determined by the concentration of oil, usually estimated by percent dry matter (Hofman *et al.*, 2002), and greatly affects eating quality, but the information on the changes in the concentration of oil during ripening is scarce.

It is proposed that the heptose sugars are important sources of carbon and energy during avocado ripening. The main aim of this study was to determine the changes in the concentrations and relations between individual sugars, protein and oil during ripening from two distinct production locations in South Africa and secondarily to identify potential differences between fast and slow ripening fruit.

2. Materials and Methods

2.1. Fruit Material

Export grade 'Hass' fruit (236-265 g) were harvested from a commercial orchard near Tzaneen, South Africa (23°43'S, warm subtropical climate) on 18 May, 1 June and 15 June (27.0, 28.6 and 30.3% dry matter, respectively) and from a commercial orchard near Howick (29°27'S, cool subtropical climate) on 12 July, 7 August and 31 August 2007 (28.5, 29.9 and 36.7% DM). Fruit were harvested from the same orchard on each farm to reduce variability between harvest dates and seasons. Twenty fruit were harvested from the same orchard on each sampling date. Fruit from Tzaneen reached the laboratory within two days of harvest, using refrigerated transport (~15°C). Fruit from Howick were delivered to the laboratory on the day of harvest. Fruit were ripened at 21 ± 2°C. Dry matter was determined on a subset of 100 fruit.

2.2. Sampling

Avocado fruit are highly heterogeneous, so to reduce sampling variation, two cores 15 mm in diameter were taken from each fruit on each sampling day, according to Kanellis *et al.* (1989). The sampled area of the fruit was immediately sealed with warm petroleum jelly to prevent oxidation. Fruit from Tzaneen were sampled two, five, eight, 11, 13 and 15 days after harvest. Fruit from Howick were sampled on the day of harvest and three, seven, 10 and 13 after harvest. The sampled mesocarp (~6.5 g fresh mass) was flash frozen in liquid nitrogen, lyophilised, hand-milled and stored at -20°C until further use.

2.3. Firmness

Firmness was measured according to Köhne *et al* (1998) using a hand-held densimeter (5 mm tip) (Bareiss, Oberdischingen, Germany). For comparison, a firmometer (300 g weight) has a scale of 0 (hard) to 120 (soft), a 5 mm densimeter has equivalent readings of 85-90 (hard) to 55-60 (soft). Readings were taken on the area to be sampled immediately before sampling. Fruit were deemed ripe when the reading on the densimeter was less than 60 and removed from the experiment if the reading was below 50 or if there were symptoms of fungal infection.

Fast and slow ripening fruit were defined as ripening in 6-7 days and 12-13 days

postharvest, respectively.

2.4. Respiration rate

The concentration of CO_2 was measured using an infrared gas analyser (EGM-1, PP Systems, Hitchin, UK). Individual fruit were incubated in a 1 L container for 15 min. The headspace CO_2 concentration was converted to respiration rate taking into account fruit mass, fruit volume, free space in the jar and the ambient CO_2 concentration.

2.5. Ethylene production rate

Individual fruit were incubated in a 1 L container for 30 min, with a 20 mL autosampler vial enclosed. Ethylene was measured using GC-FID (DANI 1000, DANI Instruments, Monzese, Italy). The GC was fitted with a stainless steel column packed with alumina-F1 stationary phase. The injector, column and detector were set at 160°C, 100°C and 180°C, respectively. The mobile phase was instrument grade nitrogen gas at 35 kPa. The autosampler injected 1 mL from the 20 mL vial. The ethylene production rate (EPR) was calculated taking into account fruit mass, fruit volume and free space in the jar.

2.6. Sugar Analysis

Sugars were analysed according to Liu *et al.* (1999a), with slight modifications. A 10 mL aliquot of 80% ($v.v^{-1}$) ethanol was added to 100 mg of mesocarp, heated in an 80°C water bath for 60 min and then incubated at 4°C for 24 h. Samples were then filtered through glass wool and dried in a vacuum concentrator (Savant, Farmingdale, NY). Dried samples were re-suspended in 2.0 mL ultrapure water, placed in a sonic bath for 10 min, and filtered through a 0.45 µm nylon syringe filter. The analysis was performed using an HPLC (Shimadzu, Kyoto, Japan) equipped with a differential refractometric detector (RID–10A) and a Phenomenex column (Rezex RCM-Monosaccharide, 200 mm × 780 mm × 8 µm). The elution was isocratic, using ultrapure water as the mobile phase. Individual sugars were identified by co-elution with standards of glucose, fructose, sucrose (Sigma-Aldrich, St Louis, MO), mannoheptulose and perseitol (Glycoteam, Hamburg, Germany). Sugars were quantified by applying a standard curve for each sugar. Samples were analysed twice and the mean taken.

2.7. Protein Analysis

Protein extraction was according to Kanellis and Kalaitzis (1992), with slight modifications. Milled tissue (100 mg) was thawed in 5 mL extraction buffer containing 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 20 mM $MgSO_4$,, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.5 mM PMSF, 10 µM leupeptin and 10% ($v.v^{-1}$) glycerol. The mixture stood on ice for 15 min with gentle shaking, was filtered through Miracloth and stored at -75°C. Protein quantification was done using the micro-assay of Bradford (1976), in duplicate, and a calibration curve using bovine serum albumin applied.

2.8. Oil analysis

Oil was quantified according to Meyer and Terry (2008), with slight modifications. Hexane (9.0 mL) was added to 300 mg mesocarp and the test tubes placed into a sonic bath for 10 min. The supernatant was filtered under vacuum and another 6 mL hexane added to the sample test tube. This was left for 5 min and the tube emptied into the Büchner funnel. The test tube was then rinsed with 3 mL hexane. The 18 mL of hexane was combined and dried using a vacuum concentrator (Savant, Farmingdale, NY). The oil was weighed and converted to percent dry mass (% DM).

2.9. Statistical analysis

Single fruit replications were used, with 20 fruit per treatment. The eight fruit closest to the mean respiration, ethylene and firmness values were used for further analysis. Statistical analysis was conducted using Genstat 12.1 (VSN International, Hemel Hempsted, UK) using a repeated measures REML analysis, using harvest and time after harvest as factors, at a 95% confidence level. Fruit from the two production areas were analysed separately because of the additional effect of time between harvest and sampling. Means were separated using individual LSDs. Data are presented as the mean ± standard error of the mean (SEM). Tests for correlation were done using Spearman's rank correlation (r_s).

3. Results

Although there were significant differences between production locations and harvest dates, the trends in concentrations of mannoheptulose, perseitol, protein and oil during ripening were consistent throughout. The heptoses declined during ripening, protein increased considerably and oil remained fairly constant. The hexoses and sucrose were present in low concentrations and did not exhibit a consistent trend. All the parameters from both production locations showed significant changes during ripening ($P<0.001$), except for glucose from Tzaneen fruit ($P=0.072$), and oil (0.036) and sucrose (0.002) from Howick. P-values for time, harvest and the interaction between the two factors for each production location are given in Table 2.

3.1. Firmness, respiration and ethylene production

At arrival, the fruit from each harvest had similar firmness and, as would be expected, softened significantly during ripening ($P<0.001$).There was no significant differences between the harvest dates ($P=0.350$ and $P=0.130$) but the fruit from Howick showed a significant interaction effect ($P<0.001$) for firmness loss during ripening (Figure 8A-B).

The climacteric response was observed in all the fruit. There were significant differences between the fruit from the three harvests for Tzaneen ($P<0.001$), but not Howick ($P=0.563$) although the interaction between time and harvest was significant for both locations ($P<0.001$ and $P=0.038$, respectively) (Figure 1E-F). There were highly significant differences between the harvest dates ($P<0.001$) and the interaction between time and harvest date ($P<0.001$) for the respiration rate for fruit from both production locations (Figure 8C-D).

3.2. Sugars

Avocado fruit can a number of sugars, sugar alcohols and oligosaccharides but fructose, glucose, sucrose, mannoheptulose and perseitol were consistently present during ripening. Ribose, volemitol, and the oligosaccharides stachyose and raffinose were infrequently detected.

Harvest date had a highly significant effect on the five sugars in the Tzaneen fruit ($P=0.007$ for mannoheptulose and $P<0.001$ for the other four sugars). In the Howick fruit, harvest date did not have a significant effect on the concentrations of fructose and glucose

(P=0.408 and 0.227, respectively), but sucrose (P=0.016), mannoheptulose (P<0.001) and perseitol (P=0.012) were. The interaction between time and harvest had a significant effect on all the sugars in fruit from Tzaneen, but only had a significant effect on mannoheptulose (P=0.001) and perseitol (P=0.041) in the Howick fruit. Glucose (Figure 9A-B) and fructose (Figure 9C-D) were present in very low concentrations, compared to the other three sugars, and the concentration of both sugars increased in an irregular manner during ripening. The concentration of sucrose was intermediate between the hexoses and the heptoses (Figure 9E-F). Sucrose was present at a higher concentration in the first harvest, from both locations, than the subsequent harvests, but there were no consistent trends. Mannoheptulose (Figure 9G-H) and perseitol (Figure 9I-J) were confirmed as the predominant non-structural carbohydrates in mature, unripe avocado mesocarp. There were significant losses of mannoheptulose and perseitol in the first week postharvest in fruit from both production locations but none in the second week postharvest.

3.3. Protein

Harvest date did not have a significant effect on protein concentration in Tzaneen fruit (P=0.121), but did on the fruit from Howick (P=0.003). The interaction between time and harvest date was not significant in fruit from either location (P=0.158 and 0.311, respectively). The concentration of protein increased sigmoidally during ripening. The concentration of protein in fruit from the first harvest from Tzaneen increased significantly every sampling day from day 2 until day 11 (Figure 9K). In the second harvest there was no increase between day 2 and day 5, but the concentration increased significantly between day 5 and day 8, and day 8 and day 11, with no significant difference thereafter. In the last harvest there was a significant increase from day 2 until day 8 and then a significant decline in the concentration of protein on day 15. In the fruit from the first harvest from Howick, there was no significant increase in the initial concentration of protein by day 3; thereafter it increased on day 7 and day 10 (Figure 9L). In the second harvest, there was a significant increase in the concentration of protein on every sampling day, similarly for the last harvest, except between day 7 and day 10.

3.4. Oil

Harvest date had a significant effect on the concentration of oil in Tzaneen and Howick fruit (P<0.001), but the interaction between time and harvest was not significant (P=0.234 and P=0.834, respectively). The initial concentration of oil increased with each harvest from

Tzaneen (Figure 10A), but there was no significant increase between the latter harvests. The first harvest from Howick had the highest oil concentration, but there was no significant difference between the first and third harvests, although there was an 8% difference in dry matter percentage; the fruit from the second harvest had a significantly lower concentration of oil (Figure 10B).

3.5. Correlations between parameters

The correlations between protein and mannoheptulose, perseitol and total heptose were significant ($P<0.001$) in both production locations, but the correlations were fair for the fruit from Tzaneen (r_s = -0.68, -0.68, and -0.69, respectively) and weak for the fruit from Howick (r_s = -0.48, -0.56, -0.52, respectively; Table 3). In fruit from both production locations there was a significant ($P<0.001$) and strong positive correlation between the total heptose concentration and firmness ($P<0.001$, r_s = 0.80, 0.79, respectively, Table 3).

3.6. Fast vs. slow ripening fruit

Only fruit from Howick were used in this section of the study because there was no delay between harvest and sampling with these fruit. There were few significant differences between the fast (6-7 days to ripen) and slow (12-13 days to ripen) ripening fruit (Table 4). Slow ripening fruit had a significantly higher ethylene climacteric peak, and a higher concentration of mannoheptulose and lower respiration rate on the day of harvest. The concentration of protein was also slightly higher in the fast ripening fruit. The concentration of glucose showed different trends in the fast and slow ripening fruit where the fast ripening fruit had a significant increase, compared to the day of harvest, on day 7, while in the slow ripening fruit there was only a significant difference on day 10.

4. Discussion

4.1. Roles of individual sugars

Sugars have multiple roles in metabolism regulation in general (Rolland *et al.*, 2002), and avocado in particular (Richings *et al.*, 2000; Tesfay, 2009). Although the importance, and loss, of the heptoses during ripening is well-known, there is conjecture about their specific roles (Meyer and Terry, 2010), having been suggested as a ripening inhibitor (Liu *et al.*, 2002), energy source (Liu *et al.*, 1999a) and anti-oxidant (Cowan, 2004; Tesfay *et al.*, 2010).

As evidence that these sugars are a major energy source during ripening: the respiratory substrate during ripening is a carbohydrate (Blanke, 1991) and since starch is present in very low concentrations (less than 1% DM) in the mesocarp (Liu *et al.*, 1999a), compared to other fruit, and the increase in glucose during ripening, considering the very low concentration of starch, does not suggest the use of glucose as a major source of energy while the decline in the heptose sugars during ripening suggests that these sugars are used as a major source of energy and/or carbon during ripening. However, this would need to be confirmed with isotopic labelling of the heptoses. The multi-functionality of the heptose sugars would cause a reduction in the correlations between the sugars and protein and respiration, which was also noted by Meyer and Terry (2010). As a source of energy, carbon and anti-oxidant capacity, these sugars are critical in avocado postharvest physiology and the management of these sugars postharvest will have affect on ripening and internal disorders, particularly if the fruit are placed into cold storage before ripening as this will deplete the limited reserves of these sugars (Meyer and Terry, 2010). The suggestion of a minimum heptose concentration is fraught with assumptions on the ripening temperature, cell wall composition, ripening enzyme activity and the method of extraction, and is not attempted since it is highly unlikely to influence commercial practices.

The slight changes and dissimilar trends in the concentration of sucrose observed in the six harvests concur with Meyer and Terry (2010), who found sucrose to be important, but called into question the suggested role of sucrose in the carbon energy store (Liu *et al.*, 1999a). Sucrose may be of more importance pre-harvest as a transport sugar, as glucose is required for cellulose synthesis and fructose for lipid synthesis (Basciano *et al.*, 2005; Zavaroni *et al.*, 1982).

The results of the individual hexoses are often not reported because they are present in very low concentrations in avocado mesocarp (Landahl *et al.*, 2009; Liu *et al.*, 1999b; Liu *et al.*, 2002; Meyer and Terry, 2010), but there is a close relationship between these sugars and ABA

and hence ripening (Richings *et al.*, 2000). The cell wall is predominantly composed of cellulose, which is an unbranched D-glucose polymer (Heldt and Heldt, 2005). Avocado fruit produce prolific amounts of cellulase during ripening (O'Donoghue *et al.*, 1994), suggesting an increase in the concentration of glucose during ripening, as was noted in this experiment. Some glucose may come from the hydrolysis of starch, but the concentration of starch is low in the mesocarp (Liu *et al.*, 1999a). The differences in the rate of increase of glucose between fast and slow ripening fruit (Table 4) may provide an indication of ABA activity (Richings *et al.*, 2000) and cellulase activity, and suggests a highly integrated response mechanism to modulate fruit growth and development (Richings *et al.*, 2000). With the numerous sugars in avocado mesocarp (Bean, 1958), and the importance of sugars in various physiological processes, for instance as sources of carbon, energy and signalling molecules, further research into avocado sugar physiology is warranted to better understand the roles of individual sugars and how these sugars can be manipulated to provide a higher quality fruit in terms of improved storage- and shelf life.

4.2. Oil

During ripening, the concentration of oil remained fairly constant, only showing a significant increase late in the ripening process in fruit from Tzaneen, which may be because of increased cell wall degradation resulting in an improved extraction (Meyer and Terry, 2008; Mostert *et al.*, 2007). It is suggested that oil plays a limited physiological role in avocado postharvest physiology.

4.3. Commercial Implications

Postharvest losses of heptoses should be minimised because of their importance in avocado ripening (Liu *et al.*, 1999a; Meyer and Terry, 2010) and internal fruit quality (Tesfay *et al.*, 2010). This can be achieved by removing field heat soon after harvest, lowering storage temperatures, reducing the duration of storage and eliminating cold chain breaks, all of which are already recommended handling procedures (PPECB, 2008). Van't Hoff's quotient (Q_{10}) for the effect of temperature on respiration for 5 to 25°C is 2.5 to 4.0 (Robertson, 2006). The elevated metabolic rate caused by a supra-optimal storage temperature will result in a rapid depletion of the finite carbohydrate reserves and ethylene production during storage, increasing the risk of partial ripening and physiological disorders such as mesocarp discolouration (Pesis *et al.*, 2002).

5. Conclusion

Mannoheptulose and perseitol appear to be a major carbon source in avocado fruit and an important energy source, especially for protein synthesis. The management of the respiration rate during postharvest handling, cold storage and ripening to limit the loss of these sugars is important to reduce the risk of fruit not achieving eating ripeness and developing internal disorders.

Table 2: P-values for REML analysis for time, harvest and the interaction between time and harvest for fruit harvested thrice from a commercial orchard near Tzaneen and (warm subtropical climate) another near Howick (cool subtropical climate) South Africa in the 2007 season.

		P-value										
Location	Factor	EPR	Firmness	Fructose	Glucose	Heptose	Manno	Oil	Perseitol	Protein	Resp	Sucrose
Tzaneen	Time	<0.001	<0.001	<0.001	0.072	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Harvest	<0.001	0.350	<0.001	<0.001	<0.001	0.007	<0.001	<0.001	0.121	<0.001	<0.001
	Time.Harvest	<0.001	0.068	<0.001	<0.001	<0.001	0.010	0.234	<0.001	0.158	<0.001	<0.001
Howick	Time	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.036	<0.001	<0.001	<0.001	0.002
	Harvest	0.563	0.130	0.408	0.227	<0.001	<0.001	<0.001	0.012	0.003	<0.001	0.016
	Time.Harvest	0.038	<0.001	0.584	0.385	0.007	0.001	0.834	0.041	0.311	<0.001	0.094

EPR, Ethylene production rate; Manno, mannoheptulose; Resp, respiration rate.

Table 3: Spearman's rank correlation co-efficients between, ethylene production rate, firmness, fructose, glucose, heptose, mannoheptulose, oil, perseitol, protein, respiration and sucrose during the ripening of 'Hass' avocado fruit. Values are from fruit that were harvested thrice from a commercial orchard near Tzaneen (warm subtropical climate) and another in Howick (cool subtropical climate), South Africa in the 2007 season. Fruit were ripened at 21 ± 2°C.

Location	Parameter	EPR	Firmness	Fructose	Glucose	Heptose	Manno	Oil	Perseitol	Protein	Resp
Tzaneen	Firmness	-0.28									
	Fructose	0.37	-0.46								
	Glucose	0.18	-0.08	0.41							
	Heptose	-0.38	0.80	-0.44	-0.01						
	Manno	-0.38	0.74	-0.38	-0.01	0.96					
	Oil	0.10	-0.07	0.08	0.04	-0.14	-0.10				
	Perseitol	-0.34	0.85	-0.48	-0.01	0.85	0.75	-0.19			
	Protein	0.34	-0.74	0.33	0.07	-0.69	-0.68	0.19	-0.68		
	Resp	0.21	-0.10	0.18	0.29	-0.11	-0.13	-0.05	-0.07	-0.05	
	Sucrose	0.05	0.05	0.06	-0.14	-0.03	-0.04	0.01	0.07	0.09	-0.36
Howick	Firmness	-0.61									
	Fructose	0.40	-0.47								
	Glucose	0.14	-0.28	0.42							
	Heptose	-0.67	0.79	-0.54	-0.22						
	Manno	-0.64	0.75	-0.53	-0.21	0.98					
	Oil	0.10	-0.17	0.03	0.19	-0.05	-0.03				
	Perseitol	-0.64	0.75	-0.50	-0.21	0.91	0.84	-0.10			
	Protein	0.43	-0.59	0.39	0.24	-0.52	-0.48	0.34	-0.56		
	Resp	-0.07	0.12	-0.18	0.07	0.18	0.14	-0.01	0.24	-0.21	
	Sucrose	0.07	0.23	0.08	-0.18	0.32	0.33	-0.03	0.27	0.05	-0.26

EPR, Ethylene production rate; Manno, mannoheptulose; Resp, respiration rate.

Table 4: Comparison between fast and slow ripening 'Hass' avocado fruit from a commercial orchard near Howick (cool subtropical climate) South Africa, averaged for three harvests in the 2007 season. Fruit were ripened at 21 ± 2°C.

		Day Postharvest				
	Ripening Rate	0	3	7	10	13
Firmness	Fast[1]	85.4 ± 1.6 A[2,3]	76.4 ± 2.7 B	60.2 ± 2.2 C	50.6 ± 3.0 D	-
(densimeter units)	Slow	86.7 ± 1.6 A	78.0 ± 2.4 B	76.2 ± 1.4 B*	62.7 ± 0.8 C*	58.0 ± 1.2 C
Respiration	Fast	2421 ± 62 A	3299 ± 298 B	2999 ± 122 B	1912 ± 128 C	-
($\mu mol.kg^{-1}.h^{-1}$	Slow	1577 ± 224 A*	2772 ± 405 B	2555 ± 260 B	1756 ± 161 A	1594 ± 142 A
Ethylene	Fast	1.8 ± 1.7 A	137.2 ± 49.3 A	880.1 ± 244.1 B	403.3 ± 104.8 AB	-
($\mu mol.kg^{-1}.h^{-1}$)	Slow	0.0 ± 0.0 A	215.2 ± 182.0 B	2021.6 ± 376.2 C*	660.3 ± 127.7 D	516.7 ± 151.7 BD
Glucose	Fast	0.28 ± 0.17 A	0.82 ± 0.36 AB	1.43 ± 0.26 B	0.91 ± 0.36 AB	-
($mg.g^{-1}$ DM)	Slow	0.07 ± 0.07 A	0.61 ± 0.51 AB	0.52 ± 0.43 AB	1.76 ± 0.56 B	1.09 ± 0.54 AB
Fructose	Fast	0.0 ± 0.0 A	0.0 ± 0.0 A	0.61 ± 0.22 A	0.43 ± 0.11 A	-
($mg.g^{-1}$ DM)	Slow	0.0 ± 0.0 AB	0.0 ± 0.0 A	0.62 ± 0.32 ABC	0.62± 0.25 BC	1.09 ± 0.29 C
Sucrose	Fast	3.17 ± 0.62 A	2.20 ± 0.47 A	2.56 ± 0.58 A	2.11 ± 0.42 A	-
($mg.g^{-1}$ DM)	Slow	4.29 ± 0.55 A	3.02 ± 0.37 A	3.86 ± 0.48 A	3.91 ± 0.53 A*	2.70 ± 0.79 A
Mannoheptulose	Fast	7.93 ± 1.76 A	4.05 ± 1.00 AB	1.01 ± 0.10 B	0.83 ± 0.14 B	-
($mg.g^{-1}$ DM)	Slow	13.97 ± 1.90 A*	8.36 ± 1.52 B	2.49 ± 0.48 BC	1.22 ± 0.18 C	1.31 ± 0.41 C
Perseitol	Fast	6.62 ± 1.00 A	2.88 ± 0.63 B	0.59 ± 0.04 C	0.53 ± 0.10 C	-
($mg.g^{-1}$ DM)	Slow	7.76 ± 0.26 A	5.05 ± 0.77 B	1.40 ± 0.40 C	0.49 ± 0.07 C	0.48 ± 0.07 C
Protein	Fast	17.74 ± 4.80 A	24.10 ± 5.95 AB	32.94 ± 4.80 BC	35.52 ± 4.81 C	-
($mg.g^{-1}$ DM)	Slow	15.28 ± 2.57 A	21.00 ± 2.02 AB	27.02 ± 2.40 BC	33.68 ± 3.15 C	39.27 ± 5.73 C

[1] Fast ripening fruit ripened in 6-7 days after harvest, slow ripening fruit ripened in 12-13 days.

[2] Values are means ± SEM; $n_{fast} = 5$, $n_{slow} = 6$.

[3] Different letters indicate significant differences ($P < 0.05$) between sampling days.

* Significant difference ($P < 0.05$) between fast and slow ripening fruit on that sampling day.

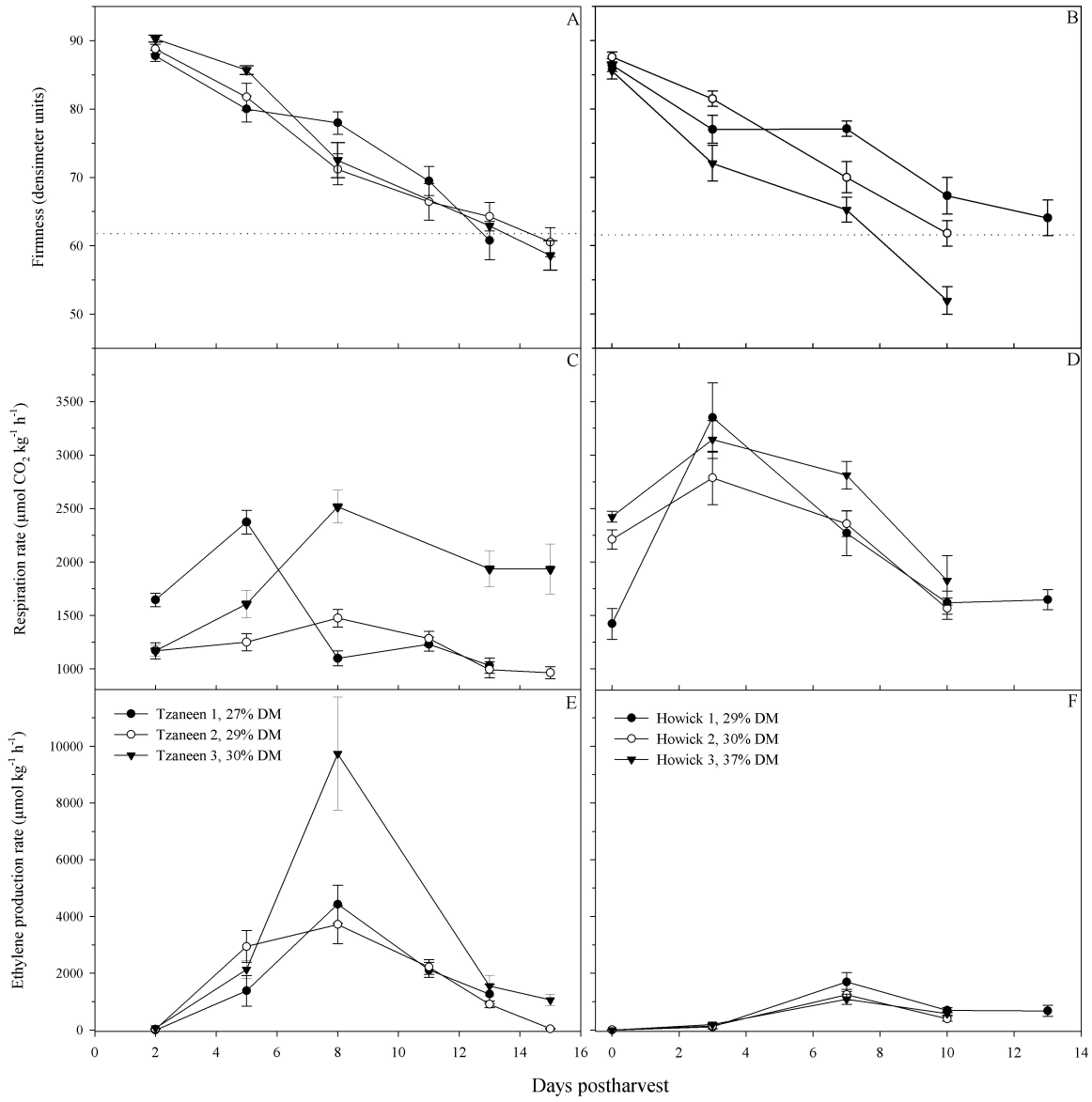

Figure 8: Changes in firmness (A-B), respiration rate (C-D) and ethylene production rate (E-F) of South African 'Hass' avocados during ripening at 21±2°C in the 2007 season. Three harvests were made from a commercial orchard near Tzaneen (warm subtropical climate; left column) and another near Howick (cool subtropical climate; right column). Vertical bars = SEM; n = 8. Horizontal lines in A and B indicate ripeness.

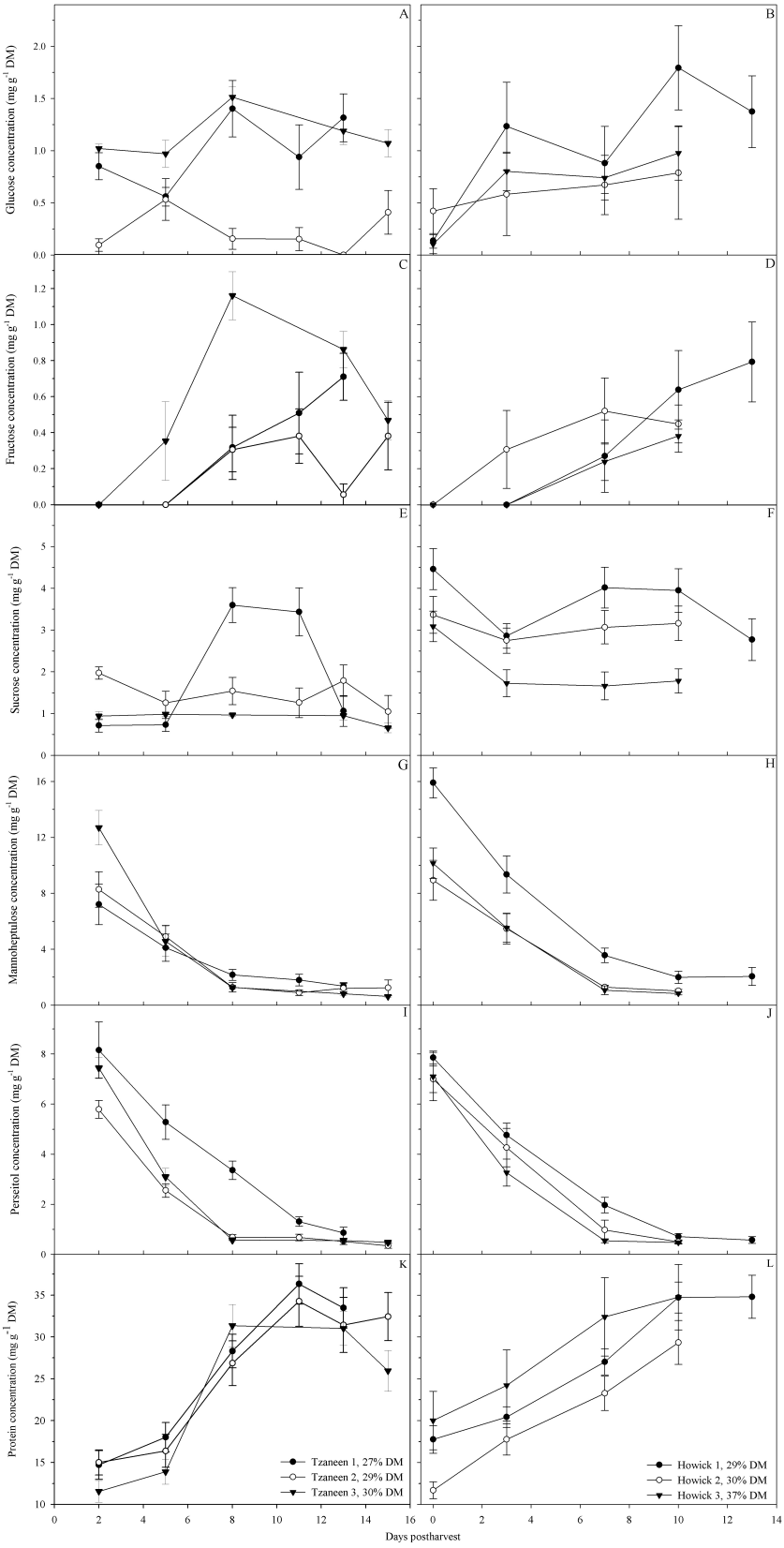

Figure 9: Changes in the concentrations of glucose (A-B), fructose (C-D), sucrose (E-F), mannoheptulose (G-H), perseitol (I-J) and protein (K-L) in the mesocarp of South African 'Hass' avocados during the 2007 season. Three harvests were made from a commercial orchard near Tzaneen (warm subtropical climate; left column) and another near Howick (cool subtropical climate; right column). Vertical bars = SEM; n = 8.

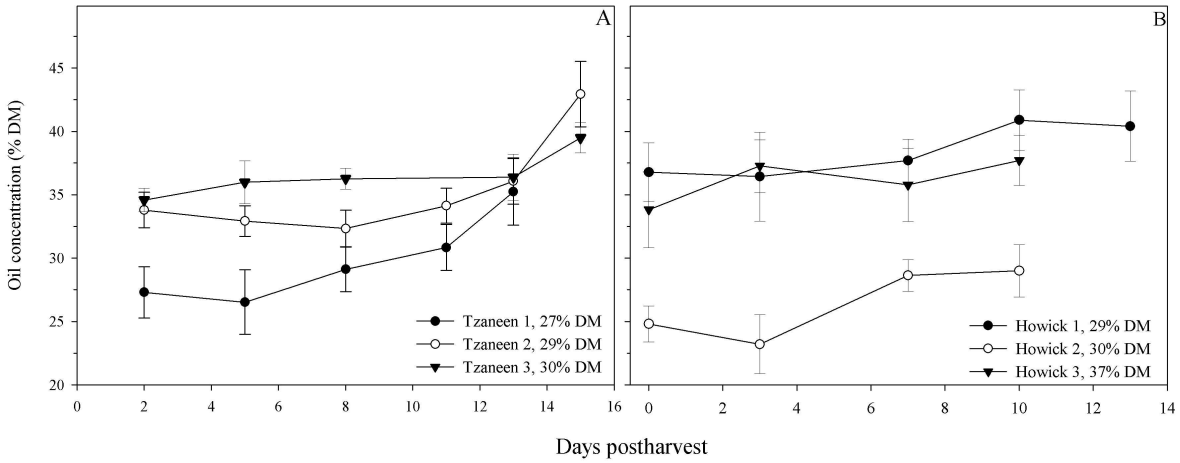

Figure 10: Changes in the concentration of oil in the mesocarp of South African 'Hass' avocados during the 2007 season. Three harvests were made from a commercial orchard near Tzaneen (warm subtropical climate; A) and another near Howick (cool subtropical climate; B). Vertical bars = SEM; n = 8.

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Chapter 3
Effects of reduced storage temperature and modified humidity packaging on ‘Hass’ avocado physiology and quality

Robert Blakey, Isa Bertling and John Bower

The exportation of avocado fruit into markets with phytosanitary requirements has become a high priority due to increased competition in the European market. For South African fruit phytosanitary disinfestation can be achieved by storing fruit at 1°C for greater than 22 days. Storage at 1°C and with modified humidity packaging (MHP) have shown promise, but research into the physiological effects of these treatments was required. Compared to conventional storage (5.5°C), fruit stored at 1°C had significantly reduced respiration, ethylene production, softening, water loss, mass loss and mannoheptulose consumption and the use of MHP further reduced these parameters. Cold storage resulted in increased cellulase and polygalacturonase activity during ripening; but cellulase, polygalacturonase and pectin methylesterase activity during ripening were not significantly affected by storage treatments. Days to ripen and lenticel damage were slightly increased after storage at 1°C, but the percent sound fruit was not significantly reduced. The significantly reduced mass and water loss from fruit stored in MHP resulted in significantly reduced lenticel damage. These results show that the storage of 'Hass' avocado fruit at 1°C for phytosanitary disinfestation is possible.

1. Introduction

In order to ship fruit from producer to market, as well as create flexibility in sales time for the marketer, it is necessary to store the product for as long as possible, but at the same time maintain a quality acceptable to the consumer. Postharvest management of fruit must reduce metabolic processes that result in undesirable changes; reduce water loss that will cause loss in marketable mass, shrivelling and premature softening; and minimise physical damage, physiological disorders and pathological diseases (Thompson, 1996).

Avocado is a high value subtropical crop that is highly variable and perishable with limited storage life (White *et al.*, 2003). Improvements in postharvest handling of avocado fruit are required because current storage conditions may exacerbate ripening disorders (Burdon *et al.*, 2008), and do not meet phytosanitary disinfestation requirements set by certain countries (Bower, 2005). Research into the cold disinfestation of South African avocados, especially the 'Hass' cultivar, to meet these requirements has been on-going. A treatment of 1°C for 22 continuous days is thought to be adequate, but there is concern that a cold disinfestation treatment for the fruit fly species (*Ceratitis cosyra, C. rosa* and *C. capitata*) present in South Africa will reduce fruit quality. However, recent studies have shown that 'Hass' can be stored at 1°C for 30 days and have good internal quality, without compromising external quality (Van Rooyen, 2009). Storing fruit in modified humidity packaging (MHP) has been found to be an effective method to reduce water loss and maintain avocado fruit quality during storage (Bower *et al.*, 2003; Bower, 2005).

Cellulase, endo-polygalacturonase (PG) and pectin methyl esterase (PME) have been extensively studied in relation to avocado ripening, but there is a paucity of information on the effects of reduced temperature storage and MHP on ripening physiology, which is needed to optimise storage and shipping protocols.

The objectives of this study were to determine the effects of storage at 1°C and MHP on ripening physiology, compared to non-stored fruit and commercial storage conditions to establish the possibility of cold disinfestation of South African 'Hass' avocados.

2. Materials and Methods

2.1. Plant material

Export grade 'Hass' avocado fruit were obtained from the same orchards of a commercial farm in Tzaneen, South Africa (23°43'S, warm subtropical) on 18 May, 1 June and 15 June (27.0, 28.6 and 30.3% dry matter) and 12 July, 7 August and 31 August 2007 (28.5, 29.9 and 36.7% DM) from a commercial farm in Howick (29°27'S, cool subtropical). The following season fruit were harvested from the same orchards on 22 April, 13 May, 3 June (22.4, 24.3 and 28.0% DM) and 2 July, 23 July and 12 August (28.7, 29.6 and 32.2% DM). Fruit were harvested from the same orchard on each farm to reduce variability between harvest dates and seasons.

Fruit from Tzaneen reached the laboratory within two days of harvest, using refrigerated transport (~15°C) while the fruit from Howick was delivered to the laboratory on the day of harvest. The experiment was deemed to have started at arrival in the laboratory.

2.2. Fruit treatment

Fruit were randomly assigned into one of five treatments: either non-stored, stored for 28 days at 1.0 or 5.5°C (air delivery temperature) and fruit at each temperature were either stored in regular atmosphere (RA) or in MHP. The MHP was 30 µm thick polypropylene bags with 9 µm micro-perforations (2 × 2cm spacing) and an anti-mist coating (Polylam Packaging, Johannesburg, South Africa). Four fruit were sealed in each MHP bag, and removed from the bag after cold storage. Fruit were stored in standard 4 kg cartons in a reefer container. The relative humidity in the reefer containers was 70-90% and 100% in the MHP bags. Twenty fruit per treatment combination per harvest were used. Fruit were ripened at 21±2°C.

2.3. Fruit sampling

To reduce sampling variability during ripening, the same fruit were repeatedly sampled using a cork-borer, and the sampled area immediately sealed with warm petroleum jelly (Kanellis *et al*., 1989). Two samples were taken at the fruit equator using a 15 mm diameter cork borer. The mesocarp (~5 g) was flash-frozen in liquid nitrogen, lyophilised, milled in liquid nitrogen with pestle and mortar and stored at -20°C.

2.4. Firmness

Firmness was measured according to Köhne *et al.*(1998) using a hand-held densimeter (5 mm tip) (Bareiss, Oberdischingen, Germany). For comparison, a firmometer (300 g weight) has a scale of 0 (hard) to 120 (soft), a 5 mm densimeter has equivalent readings of 85-90 (hard) to 55-60 (soft). Readings were taken on the area to be sampled immediately before sampling. Fruit were deemed ripe when the reading on the densimeter was less than 60 and removed from the experiment if the reading was below 50 or if there were symptoms of fungal infection.

2.5. Respiration rate

The concentration of CO_2 was measured using an infrared gas analyser (EGM-1, PP Systems, Hitchin, UK). Individual fruit were incubated in a 1 L container for 15 min. The headspace CO_2 concentration was converted to respiration rate taking into account fruit mass, fruit volume, free space in the jar and the ambient CO_2 concentration.

2.6. Ethylene production rate

Individual fruit were incubated in a 1 L container for 30 min, with a 20 mL autosampler vial enclosed. Ethylene was measured using GC-FID (DANI 1000, DANI Instruments, Monzese, Italy). The GC was fitted with a stainless steel column packed with alumina-F1 stationary phase. The injector, column and detector were set at 160°C, 100°C and 180°C, respectively. The mobile phase was instrument grade nitrogen gas at 35 kPa. The autosampler injected 1 mL from the 20 mL vial. The ethylene production rate (EPR) was calculated taking into account fruit mass, fruit volume and free space in the jar.

2.7. Determination of water loss

Fruit water concentration was estimated according to the model developed in Chapter 4. To calculate water loss during storage, the water content of individual fruit was measured pre- and post-storage and the values subtracted. The R^2 of the model was 0.92 and the standard error of prediction = 1.8% MC.

2.8. Determination of sugar concentration

Sugars were analysed according to Liu *et al.* (1999), with slight modifications. A 10 mL aliquot of 80% ($v.v^{-1}$) ethanol was added to 100 mg of mesocarp, heated in an 80°C water bath for 60 min and then incubated at 4°C for 24 h. Samples were then filtered through glass wool and dried in a vacuum concentrator (Savant, Farmingdale, NY). Dried samples were re-suspended in 2.0 mL ultrapure water, placed in a sonic bath for 10 min, and filtered through a 0.45 µm nylon syringe filter. The analysis was performed using an HPLC (Shimadzu, Kyoto, Japan) equipped with a differential refractometric detector (RID–10A) and a Phenomenex column (Rezex RCM-Monosaccharide, 200 mm × 780 mm × 8 µm). The elution was isocratic, using ultrapure water as the mobile phase. Individual sugars were identified by co-elution with standards of glucose, fructose, sucrose (Sigma-Aldrich, St Louis, MO), mannoheptulose and perseitol (Glycoteam, Hamburg, Germany). Sugars were quantified by applying a standard curve for each sugar. Samples were analysed twice and the mean taken.

2.9. Determination of total protein concentration

Protein extraction was according to Kanellis and Kalaitzis (1992), with slight modifications. Milled tissue (100 mg) was thawed in 5 mL extraction buffer containing 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 20 mM $MgSO_4$,, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.5 mM PMSF, 10 µM leupeptin and 10% ($v.v^{-1}$) glycerol. The mixture stood on ice for 15 min with gentle shaking, was filtered through Miracloth and stored at -75°C. Protein quantification was done using the micro-assay of Bradford (1976), in duplicate, and a calibration curve using bovine serum albumin applied.

2.10. Determination of enzyme activity

Assay: To measure the activity of cellulase (β-1,4-endoglucanase; EC 3.2.1.4), PG (EC 3.2.1.15) and PME (EC 3.2.1.11), the protein extract was thawed on ice and vortexed for 1 s before 250 µL of the extract was added to 750 µL of each enzyme substrate and the blank. The mixture was incubated at 40°C for 15 min, 1,500 µL dinitrosalicylic acid (DNS) reagent added, vortexed briefly and boiled for 5 min. Thereafter the test tubes were cooled in water and centrifuged at 3,500 × g for 20 min. Absorbance of the supernatant was measured at 575 nm using a UV/Vis spectrophotometer (LKB4050, Biochrom, Cambridge, UK). Each sample was blanked for any reducing sugar artefacts in the protein extract. Standard curves of glucose

(cellulase) and D-galacturonic acid (PG and PME) were prepared daily.

DNS Reagent: A 1.0% (w.v^{-1}) solution of DNS with 1.6% (w.v^{-1}) NaOH, 30.0% (w.v^{-1}) Rochelle salt and 0.05% (w.v^{-1}) sodium sulphite in distilled water was prepared (Miller, 1959). The solution was slowly heated to 45°C and filtered through Whatman® #1 filter paper. The reagent was stored in an amber-coloured bottle at 4°C for the maximum of two weeks.

Enzyme Substrates: A 1.0% (w.v^{-1}) hydroxyethylcellulose solution in sodium citrate buffer (0.05 M, pH 4.8) was used as a substrate for cellulase and a 0.3% (w.v^{-1}) pectin solution in the same buffer was used as a substrate for PG (Awad and Young, 1979). An unbuffered 0.5% (w.v^{-1}) 95% esterified pectin in 0.1 M NaCl solution was made and was brought to pH 7.5 using 0.1 N NaOH immediately prior to use for PME (Hagerman and Austin, 1986). Enzyme substrates were prepared daily.

2.11. Determination of fruit quality

Lenticel damage (skin spotting) and external chilling injury (ECI; discrete patches) of stored fruit was rated according to the International Avocado Quality Manual (White *et al.*, 2004) using a scale 0 (no discrete patches) to 3 (50% of fruit surface area covered). Fruit were rated before being placed into cold storage and at removal from cold storage to determine net lenticel damage. Sound fruit is defined as being eating ripe and free from any diseases and disorders.

2.12. Statistical Analyses

Twenty fruit per treatment combination per harvest were evaluated and the eight fruit closest to the mean respiration, ethylene and firmness values were used for sugar and enzyme analysis. These 48 fruit were used as the source of replication in physiological measurements while the 120 fruit per season were used for fruit quality analyses. REML analysis (Genstat 12.1) was conducted for the measurements immediately after removal from cold storage, with storage treatment as a factor and blocking by harvest date. For the enzyme and firmness analyses, repeated measure REML, using the same treatment structure, was conducted. Means were separated using individual LSDs at a 95% confidence level. All data are presented as the mean ± standard error of the mean (SEM).

3. Results

3.1. Respiration and ethylene production

The respiration rate and EPR had similar trends and were highly affected by storage treatment ($P<0.001$). Fruit stored in MHP, at both temperatures, had significantly lower rates of respiration and ethylene production, compared to fruit stored in RA, and did not increase significantly after 28 days of cold storage, compared to the non-stored fruit immediately after harvest (Table 5). The fruit stored at 1°C in RA had significantly higher rates of respiration and ethylene production, compared to the non-stored and MHP-stored fruit. The fruit stored at 5.5°C in RA (*i.e.* conventional storage) was significantly higher still.

3.2. Firmness

The fruit stored at 5.5°C in RA softened significantly during storage ($P<0.001$; Figure 11) and four days after removal from storage were significantly softer than the other four treatments. After four days only the fruit stored at 1°C in MHP were not significantly softer than the non-stored fruit.

3.3. Water loss

Water loss during storage was significantly ($P<0.001$) decreased by the use of MHP, especially for fruit stored at 5.5°C (Table 5). The fruit stored at 5.5°C in MHP showed a slight gain (0.45% ± 0.43%) in water concentration during storage which is likely due to the saturation of the atmosphere in the MHP limiting water loss from the fruit, combined with experimental error.

3.4. Mass loss

Fruit stored in MHP lost significantly less mass than fruit stored in RA ($P<0.001$; Table 5). Mass loss is related to both respiration rate and water loss and similar trends were observed in these parameters, where the conventionally stored fruit had the highest values and fruit stored in MHP significantly lower values.

3.5. Sugars

The postharvest treatment of fruit had significant effects on the concentration of mannoheptulose ($P<0.001$) and perseitol ($P=0.01$). Storage for 28 days resulted in a significant loss of mannoheptulose, relative to the non-stored fruit, but the MHP-stored fruit at 1°C lost significantly less mannoheptulose than the other storage treatments (Table 5). Storage at 5.5°C resulted in a significant loss of perseitol, but storage at 1°C did not. Fructose, glucose and sucrose are present in low concentrations in avocado mesocarp, but give an indication of metabolic activity during cold storage. Sucrose decreased significantly ($P<0.001$) during storage, although there were no significant differences between storage treatments. The monosaccharides increased significantly during cold storage ($P<0.001$), compared to the non-stored fruit. The concentrations of fructose and glucose was significantly higher in the fruit stored in RA at 5.5°C, although there was no significant difference in the concentration of glucose between the fruit stored in RA and MHP at 5.5°C.

3.6. Protein and enzyme activity

The differences in the concentration of protein between the treatments were not significant ($P=0.120$; Table 5), but the fruit stored at 5.5°C had a higher concentration of protein compared to the non-stored fruit.

Cellulase activity increased until approximately day 8 and then declined slightly in the post-climacteric stage (Figure 12A). Although the trend was similar between stored and non-stored fruit, cellulase activity was significantly higher in the stored fruit ($P<0.001$). At removal from cold storage, the only treatment that had a significantly higher activity than the non-stored fruit was the fruit stored at 5.5°C in RA. On day 4 the fruit stored in RA, at both temperatures, had a higher activity compared to the fruit stored in MHP. Differences were no longer significant 8 days after removal from storage.

At removal from storage, no differences in PG activity were noted (Figure 12B). Thereafter, all storage treatments showed a significant increase ($P<0.001$) and significantly higher activity compared to non-stored fruit, but there were no significant differences between the storage treatments.

No significant differences ($P=0.354$) were found in PME activity between treatments, although the RA stored fruit had a slightly higher PME activity compared to the fruit stored in MHP (Figure 12C).

3.7. Fruit quality

In first season, higher levels of lenticel damage occurred in fruit stored at 1°C than at 5.5°C, and fruit stored in RA had a higher degree of injury than fruit stored in MHP, although differences were slight ($P<0.001$; Table 6). However, lenticel damage was commercially acceptable because colour development during ripening of 'Hass' fruit masks this slight damage – which is about 5% of the fruit surface. No significant differences ($P=0.299$) were found in the severity of ECI, which was also slight and infrequent in all storage treatments. The incidence of body rots was low and no storage treatment was significantly different to the non-stored fruit ($P=0.140$; Table 6), although the fruit stored at 5.5°C in RA had a significantly higher incidence of body rots than the fruit that were stored at 1°C in RA and those stored at 5.5°C in MHP. The incidence of stem end rot was negligible throughout the season. Vascular browning was the only notable physiological disorder and was low and non-significant ($P=0.190$) with only the fruit stored at 5.5°C in RA significantly higher than non-stored fruit and the fruit stored at 1°C in MHP. There were no significant differences in the percentage of sound fruit between the four storage treatments, but the non-stored fruit had a significantly lower percentage (Table 6). This is due to the slow ripening rate of fruit from the first harvests from both locations.

When the experiment was repeated the following season similar results were obtained with few significant differences in fruit quality between the two seasons. The higher incidence of body rots in the non-stored and RA 5.5°C treatments in 2007 were not observed in 2008. The incidence of vascular browning was significantly higher in the fruit stored in RA at 5.5°C in 2008 and was significantly higher than the other treatments ($P<0.001$). The low percent of sound fruit in the non-stored fruit was not repeated in 2008, but the high incidence of vascular browning in the fruit stored in RA at 5.5°C resulted in a significantly reduced percentage of sound fruit, compared to all treatments except the MHP 1°C treatment.

4. Discussion

4.1. Fruit physiology

The aim of cold storage is to minimise physiological changes and maintain fruit quality. The results confirmed that storage at 1°C and the use of MHP resulted in limited physiological changes during cold storage, and specifically reduced the respiration rate, EPR, heptose depletion and protein accumulation. The reduction in the EPR is critical because many postharvest disorders are directly or indirectly attributable to ethylene (Pesis *et al.*, 2002). Also, water loss is known to stimulate ethylene synthesis (Lallu *et al*., 2004) via ABA signalling (Bower and Cutting, 1988) and can increase ethylene-related disorders (Pesis *et al.*, 2002) and susceptibility to fungal rots (Dixon *et al*., 2005). Therefore the reduction in the loss of mass and water by lowering the storage temperature, and particularly the use of MHP, should reduce the risk of internal disorders, fungal rots and lenticel damage (Bower *et al.*, 2003).

Ethylene is also linked to softening during and post-storage as it is the gene transcriptor for cellulase (Buse and Laties, 1993), which is involved in early fruit softening (Awad and Young, 1979). This is supported by the finding that the fruit stored at 5.5°C in RA had a significantly higher EPR and cellulase activity with a significant loss in firmness. Reduced temperature storage and/or MHP could therefore be used to reduce softening during storage, without negatively affecting fruit quality and ripening. The post-storage effects on enzyme activity seem to be minimal because there were only transient differences in cellulase activity between the storage treatments and no significant differences in the activity of PG and PME. As noted previously (Fuchs and Zauberman, 1987), PG activity did not increase during cold storage. The increased days to ripen of non-stored fruit may be explained by the delayed and reduced cellulase and PG activity compared to stored fruit. PME is crucial in modulating ripening, but there is disagreement in the literature concerning PME activity postharvest. Initially, PME activity was found to decline during ripening in 'Fuerte' (Awad and Young, 1979), however, later results showed that PME activity fluctuated considerably during ripening in 'Fuerte' and 'Hass' (Fuchs and Zauberman, 1987; Kaiser *et al.*, 1996; Zauberman and Jobin-Décor, 1995) and in this trial the PME activity of individual fruit was also highly variable, which may explain why no trend was observed.

Mannoheptulose is believed to be important in avocado physiology as a possible source of energy (Liu *et al.*, 1999) and the primary anti-oxidant in the mesocarp (Tesfay *et al*., 2010) and possibly a major source of carbon (Chapter 3). Perseitol has been identified as a storage

carbohydrate that is easily converted to mannoheptulose (Tesfay, 2009). This pool of heptose sugars is very limited and susceptible to depletion during postharvest handling. The reduction in respiration rate achieved by the reduction in storage temperature, and use of MHP, will better maintain the heptose pool during cold storage, compared to fruit stored at 5.5°C in RA, reducing the risk of physiological disorders (Tesfay *et al*., 2010). It is proposed that the increase in glucose is primarily due to cellulase activity during cold storage.

4.2. Fruit quality and ripening

The storage of fruit at 1°C reduced important physiological changes during storage without negatively affecting the external and internal quality in two consecutive seasons. Although fruit stored at 1°C had a reduced rate of softening compared to fruit stored at 5.5°C, which is to be expected because of the reduced metabolic activity during cold storage, this should not be of concern commercially, especially if phytosanitary disinfestation is achieved.

It was previously found that a pre-conditioning treatment was required for the successful storage of fruit at 0-1°C (Hofman *et al.*, 2003; Van Rooyen, 2009; Woolf *et al.*, 2003), but results from two seasons showed that 'Hass' fruit can be successfully stored at 1°C without a significant loss in fruit quality. It must be noted that the fruit from Tzaneen were pre-conditioned during transportation, but the fruit from Howick were not – although this fruit was grown in a much cooler climate and was perhaps acclimated to the lower storage temperature. However, the use of a pre-conditioning treatment commercially may be warranted to reduce the risk of poor quality consignments, especially in early season fruit.

5. Conclusion

It can be concluded that the cold disinfestation of South African 'Hass' avocados is possible; additionally this treatment reduced cellular metabolism and reduced fruit quality loss compared to the fruit stored at 5.5°C in RA. MHP can be used to limit cellular metabolism and external chilling. The reduction in cellular metabolism will reduce the risk of internal chilling injury symptoms which are a result of ethylene in the storage atmosphere and imbalances in enzymatic activity.

Table 5: Initial respiration rate, ethylene production rate, water loss, fresh mass loss, and concentrations of mannoheptulose, perseitol, fructose, glucose, sucrose and total protein of non-stored 'Hass' avocado fruit and at the removal from cold storage. Fruit were stored in modified humidity packaging or regular atmosphere at 1°C or 5.5°C. Results from six harvest dates in the 2007 season are combined. Fruit were harvested thrice from a commercial orchard near Tzaneen (warm subtropical climate) and another near Howick (cool, subtropical climate), South Africa.*

Treatment	Respiration ($mmol.kg^{-1}.h^{-1}$)	Ethylene ($\mu mol.kg^{-1}.h^{-1}$)	Water Loss (%)	Mass Loss (%)	Mannoheptulose ($mg.g^{-1}$ DM)	Perseitol ($mg.g^{-1}$ DM)	Heptose ($mg.g^{-1}$ DM)	Fructose ($mg.g^{-1}$ DM)	Glucose ($mg.g^{-1}$ DM)	Sucrose ($mg.g^{-1}$ DM)	Protein ($mg.g^{-1}$ DM)
Non-stored	1.63 ± 0.075 A	16.23 ± 4.22 A	-	-	10.44 ± 0.64 C	7.18 ± 0.28 B	17.70 ± 0.74 C	0.00 ± 0.00 A	0.46 ± 0.07 A	2.35 ± 0.21 B	14.92 ± 0.84 A
MHP 1.0°C	1.85 ± 0.120 A	69.81 ± 9.67 A	0.14 ± 0.34 A	0.50 ± 0.03 A	7.78 ± 0.73 B	6.31 ± 0.36 AB	14.09 ± 1.02 B	0.70 ± 0.19 B	0.81 ± 0.08 B	1.15 ± 0.11 A	15.60 ± 0.85 AB
MHP 5.5°C	1.99 ± 0.144 A	72.47 ± 22.41 A	-0.45 ± 0.43 A	0.51 ± 0.03 A	5.60 ± 0.62 A	5.63 ± 0.39 A	11.23 ± 0.89 A	0.64 ± 0.11 B	0.94 ± 0.09 BC	1.05 ± 0.12 A	17.45 ± 0.85 B
RA 1.0°C	2.46 ± 0.133 B	332.50 ± 58.87 B	0.65 ± 0.30 AB	3.91 ± 0.15 B	6.25 ± 0.51 A	6.36 ± 0.45 AB	12.61 ± 0.85 AB	0.47 ± 0.10 B	0.71 ± 0.06 B	1.12 ± 0.14 A	17.12 ± 0.89 AB
RA 5.5°C	3.13 ± 0.172 C	551.54 ± 106.38 C	1.56 ± 0.54 B	7.59 ± 0.24 C	5.25 ± 0.51 A	5.69 ± 0.25 A	10.88 ± 0.66 A	1.12 ± 0.10 C	1.14 ± 0.11 C	1.32 ± 0.12 A	17.34 ± 1.03 B
P-value	<0.001	<0.001	0.001	<0.001	<0.001	0.01	<0.001	<0.001	<0.001	<0.001	0.120

MHP, modified humidity packaging; RA, regular atmosphere.

*Each point represents the mean of 48 fruit ± SEM. Different letters indicate significant differences within each column.

Table 6: Severity of external chilling injury and net lenticel damage at removal from cold storage, and incidence of sound fruit*, body rots and vascular browning at ripeness of non-stored 'Hass' avocado fruit and fruit stored in modified humidity packaging (MHP) or regular atmosphere (RA) at 1°C or 5.5°C¶. Fruit were harvested thrice from a commercial orchard near Tzaneen (warm subtropical climate) and another near Howick (cool, subtropical climate), South Africa in 2007 and 2008 and the fruit from each season combined.

	2007					2008				
Treatment	ECI (0-3)[1]	Lenticel (0-3)[1]	Body Rots (%)	VB (%)	Sound fruit (%)	ECI (0-3)[1]	Lenticel (0-3)[1]	Body Rots (%)	VB (%)	Sound fruit (%)
Non-stored	-	-	6.0 ± 2.2 AB§	0.0 ± 0.0 A	70.0 ± 4.2 A§	-	-	0.8 ± 0.8 AB	0.0 ± 0.0 A	94.2 ± 2.2 B
MHP 1.0°C	0.05 ± 0.02 A	0.52 ± 0.03 AB	3.3 ± 1.9 AB	0.0 ± 0.0 A	90.8 ± 2.7 B	0.03 ± 0.01 AB	0.53 ± 0.05 AB	0.8 ± 0.8 AB	0.8 ± 0.8 A	86.7 ± 3.1 AB
MHP 5.5°C	0.01 ± 0.01 A	0.41 ± 0.03 A	2.5 ± 1.3 A	1.7 ± 1.3 AB	93.3 ± 2.3 B	0.01 ± 0.01 A	± 0.04 A	0.0 ± 0.0 A	0.8 ± 0.8 A	94.2 ± 2.2 B
RA 1.0°C	0.05 ± 0.03 A	0.64 ± 0.03 C	2.5 ± 1.3 A	1.7 ± 1.1 AB	83.3 ± 3.4 B	0.09 ± 0.03 BC	0.63 ± 0.05 B	1.7 ± 1.1 AB	1.7 ± 1.7 A	95.8 ± 1.8 B
RA 5.5°C	0.02 ± 0.01 A	0.60 ± 0.03 BC	8.3 ± 2.7 B§	3.3 ± 1.9 B§	89.2 ± 2.9 B	0.15 ± 0.06 C	0.76 ±0.06 C	3.3 ± 3.3 B	16.7 ± 5.4 B	80.8 ± 3.6 A
P-value	0.299	<0.001	0.140	0.190	<0.001	0.057	<0.001	0.200	<0.001	<0.001

[1]Sound fruit are ripe and free from diseases and disorders after 12 days postharvest or removal from cold storage.

*Each value is the mean of 120 fruit ± SEM. Different letters indicate significant differences within each parameter.

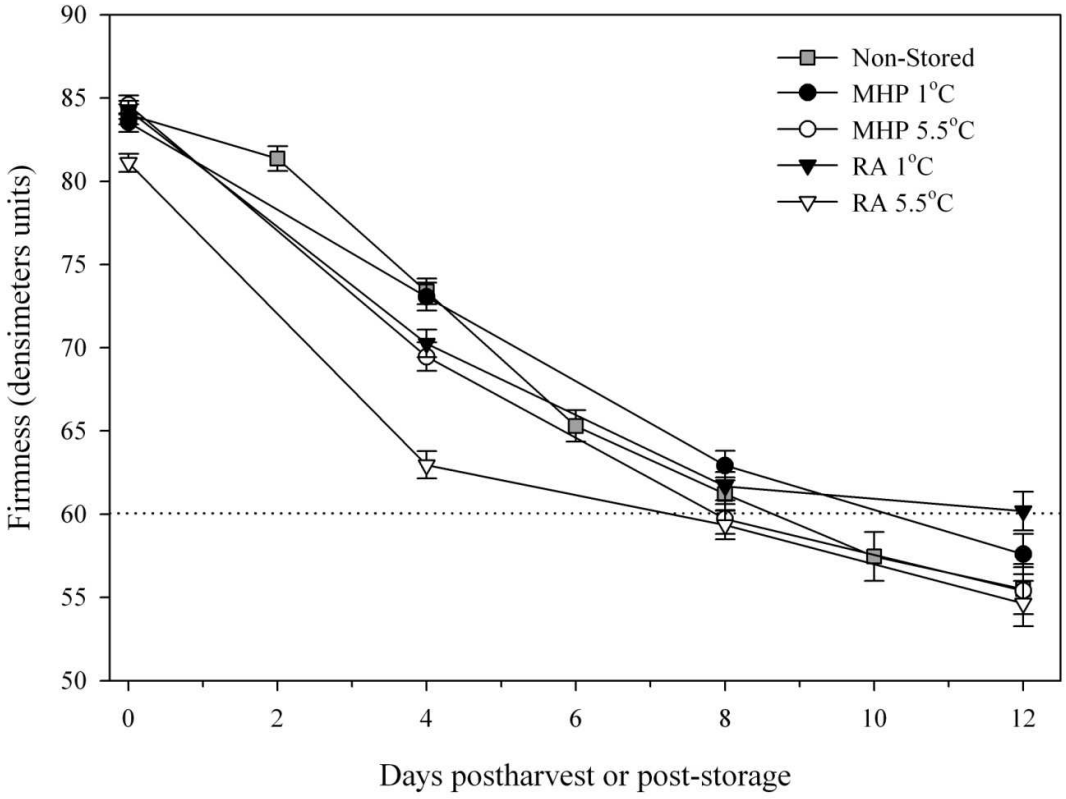

Figure 11: Loss of firmness of non-stored fruit and fruit stored in modified humidity packaging (MHP) or regular atmosphere (RA) at 1°C or 5.5°C during ripening. The dotted line at 60 units indicates eating softness. Fruit were harvested from commercial orchards near Tzaneen (warm subtropical climate) and Howick (cool subtropical climate), South Africa, thrice in a season. Vertical bars = SEM; n = 120

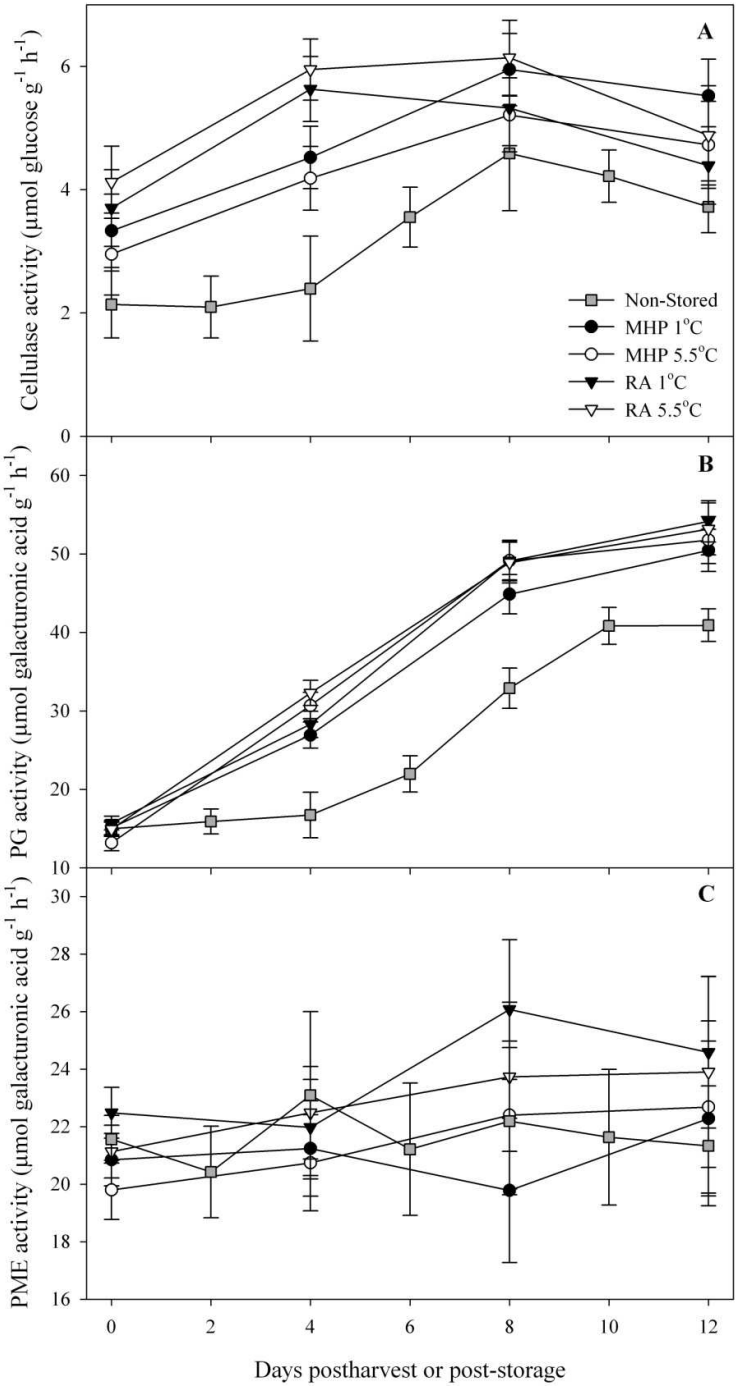

Figure 12: Cellulase (A), polygalacturonase (B) and pectin methylesterase (C) activity of non-stored fruit and fruit stored in modified humidity packaging (MHP) or regular atmosphere (RA) at 1°C or 5.5°C during ripening. Fruit were harvested from commercial orchards near Tzaneen (warm subtropical climate) and Howick (cool subtropical climate), South Africa, thrice in a season. Vertical bars = SEM; n = 48

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Chapter 4
Influence of water and ABA supply on the ripening pattern of 'Hass' avocado fruit and the prediction of water content using near-infrared spectroscopy

Robert Blakey, Isa Bertling and John Bower

Avocado fruit are highly variable, and even those graded for similar size and appearance do not behave in the same manner after harvest. This is particularly problematical for those involved in sales to the "ready-ripe" market. These operations are faced with a high variation in the rate of ripening within a consignment, causing logistical difficulties. Fruit water content (or its complement dry matter) has been suggested as a major role player in ripening and has hence been used as the maturity marker in the South African avocado industry. Presently, fruit water content is destructively measured using a representative sample as an indicator of when to harvest. In order to investigate if fruit water content and/or abscisic acid trigger fruit ripening, water or ABA was infused into commercially mature, but non-ripe avocado fruit. The fruit ripening, mass, respiration and ethylene production patterns were determined over the ripening period. The infusion of ABA into fruit significantly reduced the time to the climacteric peak and reduced the variation while the infusion of water only slightly reduced in variation in the time to the climacteric peak. It is suggested that the fruit water stress, simulated by ABA infusion, may be the trigger for ripening. Furthermore, an equation was developed using near-infrared (NIR) spectroscopy to measure mesocarp water content (R2 = 0,92, SE = 1,8% MC). It is postulated that on-line sorting of fruit using NIR spectroscopy, based on time to ripen, would result in consignments of fruit with less ripening variation, thereby solving the industry's logistical problem of fruit which have a wide spread of ripening being packed into one carton.

1. Introduction

The avocado is an unusual fruit in that ripening, which is associated with, but not dependent on, softening and colour change, does normally not occur on the tree but only after harvest (Bogin and Wallance, 1965). The decision to harvest must therefore be made independent of visible measurements and is currently either based on the oil content of the fruit (Young and Lee, 1978), dry matter content or water content - as in South Africa (Clark *et al.*, 2003; Hofman *et al.*, 2002).

Variable ripening in consignments of avocados is creating a substantial logistical problem, especially when fruit is ripened in pre-packaging facilities (Bower *et al.*, 2007). While some of the causes of the variation in the rate of ripening amongst fruit of similar size and origin are known, these factors do not fully explain this variation. The protracted flowering period, which occurs in most avocado production areas (Salazar-García *et al.*, 1998), can result in a high variation in the physiological age of fruit from a single tree. This is exacerbated by the position of the fruit on the tree. An even greater variation can hence be expected within one orchard, and when fruit from a variety of orchards are shipped on one consignment to overseas markets.

The rate of postharvest ripening is deemed to be related to fruit maturity, as fruit picked at the beginning of the picking season take longer to ripen than fruit picked at the end of the season (Adato and Gazit, 1974; Zauberman and Schiffman-Nadel, 1972). This may be related to water stress and the interaction between water stress, ABA and ethylene synthesis (Bower and Cutting, 1988; Cutting *et al.*, 1986; Lieberman *et al.*, 1977).

Acceptable norms for the water content or dry matter have been calculated from large numbers of fruit, individual fruit variability calls into question the accuracy and usefulness of these norms, particularly as the diagnostic methods are destructive, and can thus only be applied to small samples of consignments. In order to solve the problem of variability in ripening within the avocado trade, it is necessary to ascertain whether measuring fruit water content at the time of harvest is indeed a useful parameter of the ripeness of a fruit, ascertain if there is any physiological basis to using such a measurement, and if so, devise a means of accurately determining the parameter non-destructively. Near-infrared (NIR) spectroscopy is increasingly used for the non-destructive determination of internal and external parameters of horticultural crops (Nicolaï *et al.*, 2007). The use of NIR spectroscopy to evaluate avocado quality parameters has been very limited. Only dry matter has been predicted in whole fruit

(Clark *et al.*, 2003; Schmilovitch *et al.*, 2001; Walsh *et al.*, 2004). The purpose of this investigation was therefore to establish first whether fruit water content at the time of harvest is indeed physiologically related to ripening, and if so, whether an acceptable prediction of fruit maturity using NIR spectroscopy can be achieved.

2. Materials and Methods

2.1. Fruit

Export grade 'Hass' fruit (236-265 g) were sourced from Tzaneen (23°43'S, 30°07'E, Limpopo Province) and Howick (29°27'S, 30°16'E, KwaZulu-Natal). Fruit from Howick were picked and immediately transported to the laboratory in Pietermaritzburg 40 km away. Fruit from Tzaneen reached the laboratory within two days of picking, after refrigerated transport (~15°C). Fruit were harvested on 18 May, 1 June, 15 June 2007, 22 April, 13 May and 3 June 2008 from Tzaneen, and 14 March, 18 April, 12 July, 7 August, 31 August 2007, 2 July, 23 July and 12 August 2008 from Howick to provide a spread in maturity and water content. Each batch in 2007 contained 100 fruit and 60 fruit in 2008. Additional fruit were harvested with 2 cm long pedicles from the Howick batches of 12 July, 7 August and 31 August 2007 to be used in the infusion experiment.

2.2. Treatment with water and ABA

Silicon tubing was attached to the pedicle (retained after harvest and re-cut prior to treatment to remove any dead tissue) of treated fruit, and sealed with petroleum jelly at the base according to Cutting and Bower (1987). A single application of 1.5 mL water or aqueous ABA solution (48 $\mu g.fruit^{-1}$, as 1.5 mL of a 32 $\mu g.mL^{-1}$ solution) was applied to 10 fruit per treatment. Control fruit were untreated.

2.3. Ethylene Measurement

Individual fruit were incubated in a 1 L container for 30 min, with a 20 mL autosampler vial enclosed. Ethylene was measured using GC-FID (DANI 1000, DANI Instruments, Monzese, Italy). The GC was fitted with a stainless steel column packed with alumina-F1 stationary

phase. The injector, column and detector were set at 160°C, 100°C and 180°C, respectively. The mobile phase was instrument grade nitrogen gas at 35 kPa. The autosampler injected 1 mL from the 20 mL vial. The ethylene production rate (EPR) was calculated taking into account fruit mass, fruit volume and free space in the jar.

Days to the climacteric was calculated as the number of days from harvest to the peak in ethylene production.

2.4. Ripeness

Ripening time was calculated as the number of days from harvest to eating soft stage. Fruit were deemed when ripe the average reading on a densimeter was less than 60. A firmometer (300 g weight) has a scale of 0 (hard) to 120 (soft), a 5 mm densimeter has equivalent readings of 85-90 (hard) to 55-60 (soft) (Köhne *et al.*, 1998).

2.5. Near-infrared Spectroscopy

Fruit (1,200 in total) from all harvests were used for this analysis. On arrival at the laboratory, each fruit was scanned on a NIRS6500 spectrophotometer (Foss NIRSystems, Silver Spring, MD) in reflectance mode, and Visible/NIR (400-2500 nm) spectra of intact fruit were acquired. Vision© v2.22 (Foss NIRSystems, Silver Spring, MD) was used to analyse the data with a Teflon block as reference measurement. Fruit were automatically scanned 16 times by the NIRS6500, and the average taken as the sample spectrum. Approximately 3 cm^2 of the fruit surface was scanned. Immediately after scanning, a tissue sample was taken from each fruit using a 5 mm diameter cork borer and the sampled area immediately sealed with petroleum jelly to prevent oxidation (Kanellis *et al.*, 1989). The exo- and endocarp were removed from the core sample, the mesocarp portion weighed (~0.8 g fresh mass), frozen in liquid nitrogen, transferred to -20°C and subsequently lyophilised to calculate water content. Fruit were allowed to ripen at 21±2°C. An external validation of the model was performed on fruit from the 2008 season.

2.6. Statistical analysis

Statistical analysis was performed using Genstat 12.1. A factorial analysis of variance using harvest and infusion treatment was conducted at a 95% confidence level. Ten replicates of single fruit were used per treatment. Means were separated using Tukey's test at a 95% confidence level.

3. Results

3.1. Effect of ABA and water infusion on fruit ripening

Harvest date and infusion treatment were both highly significant ($P<0.001$ and $P=0.004$, respectively), however the interaction between these treatments was not significantly different ($P=0.172$), therefore only these main treatment effects will be discussed. Fruit from the last harvest reached the climacteric significantly earlier than the fruit from the former two harvests and had lower variation (Table 7). The variation in the number of days to reach the climacteric declined through the season (Table 7).

The infusion of 48 μg ABA into each fruit resulted in faster ripening, with fruit reaching the climacteric significantly faster and with less variation. Fruit infused with 1.5 mL of water were intermediate in the number of days to reach the climacteric and was no significant difference between this and the other two treatments; but there was a slight decrease in the variation to the number of days to the climacteric (Table 8).

3.2. Prediction of water content using NIR spectroscopy

A Partial Least Squares (PLS) equation with 14 factors, with a number of mathematical pre-treatments available in the software, was used to construct the equation to predict water content. The equation was developed using long wave NIR region (1100-2000 nm. Multiplicative Scatter Correction (MSC), Savitzky-Golay (7-point convolution window, quadratic convolution polynomial, second derivative smoothing), Detrend (second degree smoothing) and Standard Normal Variate were used as pre-treatments. Table 9 provides details of the equation. The equation developed for prediction of water content, was used to measure the water content of the fruit infused with water and ABA. This allowed the non-destructive

determination of the water content of fruit at the commencement of the experiment, and account for some of the variation in the fruit. The predicted and actual values for the water content of fruit from the first two harvest dates (12 July and 7 August and 31 August) had very small error: 1.0, 1.4 and 0.7% water respectively (Table 10). Compared to the destructively measured results, only the second harvest showed a slight significant difference.

4. Discussion

Water has for some time been considered an important factor in avocado ripening (Adato and Gazit, 1974; Bower, 1985; Bower and Cutting, 1987, 1988). Based on this, water content is used in South Africa for maturity indexing, and determining the start and termination of the normal packing period (Hofman *et al.*, 2002). The start of picking is linked to the ability of fruit to ripen normally without shrivelling. Bower *et al.* (2007) found that water was the single most important factor in the variation of ripening of avocado fruit. The non-destructive measurement of moisture content using NIR spectroscopy was attempted to determine the feasibility of this technique in the avocado industry. The development of the model outlined here suggests that NIR spectroscopy could be used commercially, with a suitable spectrometer which may be able to reduce the standard error of prediction (SEP) (Clark *et al.*, 2003). The use of a data collection method with greater penetration into the fruit should reduce the SEP, as was shown by Clark *et al.* (2003) who improved their model for the prediction of dry matter by changing from reflectance to interactance mode to $R^2 = 0.88$ and SEP=1.8% DM from $R^2 = 0.75$ and SE=2.6% DM. NIR spectroscopy could be used to measure the maturity of a large number of samples in a packhouse to provide a better estimate of fruit maturity for quality control purposes or be used to sort fruit according to maturity online. Predicting the time to ripen of avocado fruit, based on moisture content at harvest has proven challenging, but this may be possible if ripening conditions can be better controlled and an NIR spectrometer with improved penetration is used.

Postharvest water loss, and the related increase in ABA, may be an important factor in avocado ripening. This is supported by the evidence that packing fruit in polypropylene bags – so that water loss is decreased – before cold-storage delays ripening after removal from cold storage (Bower, 2005; Bower and Papli, 2006; van Rooyen and Bower, 2007) and from observations that retaining the packing after removal from cold storage prevents ripening

completely. Water loss, because of its relation with ABA, is suggested to initiate ripening processes (Bower, 1985; Bower *et al.*, 1986; Bower and Cutting, 1988). ABA has previously been shown to increase in avocados during pre-harvest water stress (Bower *et al.*, 1986) and as the fruit matured (Bower, 1985). In this experiment water stress was simulated by the infusion of ABA in the fruit, which was found to hasten ripening and reduce the variation in ripening particularly in comparison to control fruit. More mature fruit (*i.e.* fruit with lower water content) ripen sooner, possibly because the fruit are more stressed and have a higher endogenous ABA concentration.

5. Conclusion

It has been shown that the infusion of ABA can hasten and reduce the variation in avocado ripening, possibly by simulating water stress. NIR spectroscopy can be used to non-destructively measure the water content of whole avocados, however the main goal is to predict the ripening rate of avocados at harvest and further experimentation, minimising environmental variation and using an NIR spectrometer with a greater field of view and penetration depth, is required to be able to estimate ripening time of avocado fruit.

Table 7: Mean days to reach the climacteric and standard deviation for three harvest dates of 'Hass' avocado fruit from a commercial orchard near Howick South Africa in the 2007 season. Means are separated using Tukey's test at 95% confidence level.

Harvest	Days to Climacteric	SD
12/07/2007	10.9 b	2.36
07/08/2007	10.8 b	2.05
31/08/2007	9.4 a	1.51

SD, Standard deviation.

Table 8: Mean days to reach the climacteric and standard deviation for control, water- and ABA-infused fruit combined for three harvest dates of fruit from a commercial orchard near Howick South Africa in the 2007 season. Means are separated using Tukey's test at 95% confidence level

Treatment	Days to Climacteric	SD
Control	10.3 b	2.47
Water	9.2 ab	2.33
ABA	8.6 b	1.65

SD, Standard deviation.

Table 9: Correlation co-efficient (R^2), range in moisture content, mean, standard error of calibration, number of data points in the calibration set (n_c), standard error of prediction and number of samples in the validation set (n_v) for the NIR spectroscopy equation for measuring avocado fruit water content.

R^2	Range (%)	Mean (%)	SEC	n_c	SEP	n_v
0.92	55.4 - 87.4	75.6	1.8	604	2	513

SEC, standard error of calibration; SEP, standard error or prediction

Table 10: Predicted water content using NIR spectroscopy equation and actual water content (% fresh mass) of three batches of avocado fruit from Howick for three harvest dates. Data are means ± standard error of the mean.

	Moisture Content	
Harvest Date	Predicted	Actual
12/7/2007	70.5 ± 0.4	71.5 ± 0.4
7/8/2007	68.7 ± 0.5	70.1 ± 0.4
31/8/2007	63.9 ± 0.4	63.3 ± 0.5

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Chapter 5
The importance of cold chain maintenance and storage temperature to 'Hass' avocado ripening and quality

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The South African avocado industry is export-based, with the majority of fruit exported to Europe. Fruit are cold-stored for a maximum of 28 days with a number of handling points in the cold chain. Breaks in the cold chain at these handling points are thought to increase postharvest losses but have not been previously quantified. Fruit (cv. Hass) were harvested from Howick and subjected to a delay of 24h before cold storage or a 9 h break in cold storage at 5, 10 or 20 days during a 28 days cold storage period at 1°C or 5.5°C. After the storage period respiration, ethylene production, firmness and fruit mass were measured and fruit quality assessed. A break in the cold chain five, 10 or 20 days after cooling resulted in a reduction in the respiration rate immediately after removal from cold storage for fruit stored at 1°C but there was a significant reduction in the respiration rate of fruit stored at 5.5°C immediately after removal from cold storage if there was a delay in cooling and if the cold chain was broken 20 days after cooling. There were no significant differences in the ethylene production rate between the treatments until seven days after removal from cold storage. A break 10 days after cooling reduced the mass loss during ripening for both storage temperatures. If the cold chain was broken during 1°C storage, softening was accelerated, but the effect of cold chain breaks during 5.5°C on fruit softening was less severe. No significant differences were found in the percentage sound fruit between the control fruit and any cold chain break treatment if fruit were stored at 5.5°C, because the percentage sound fruit in the control was low, but if fruit were stored at 1°C and the cold chain broken there was a significant decrease in the percentage sound fruit. In a related study, the concentration of individual sugars was measured in fruit sequentially over a 24 h period. Fruit lost 45% of mannoheptulose and 17% of perseitol, the predominant sugars in avocado flesh, after 24 h at room temperature. If a cold chain break occurred, storage at 1°C resulted in there being more sound fruit than storage at 5.5°C, but a break in the cold chain, anywhere in the shipping process, severely reduced fruit quality.

1. Introduction

Transportation of fruit from the northern provinces of South Africa (Limpopo and Mpumalanga) to the port in Cape Town and eventually supermarkets in Europe requires considerable logistical management to deliver quality fruit to the consumer. The transportation of fruit from these areas has improved over the last 20 to 30 years (Toerien, 1986), but continued improvements have to be made to remain globally competitive. While it is known that breaking the cold chain should be prevented (Dodd *et al.*, 2007; Eksteen, 1995, 1999; Vorster *et al.*, 1991), the effects of a cold chain break on fruit quality and physiology have not been studied in South Africa. Successful storage of avocados (and all horticultural crops) requires the earliest possible removal of field heat and the maintenance of the optimal temperature throughout storage (Ginsberg, 1985). The Perishable Produce Export Control Board of South Africa (PPECB) has recommendations and guidelines available for the handling of avocados due for export that outline the correct handling of fruit with respect to refrigerated storage to minimise fluctuations in pulp temperature (Eksteen, 1995, 1999). By concentrating efforts on compliance with these protocols and procedures, fruit quality has been notably improved, but could be further improved because it was shown that inadequate temperature management was common in the South African avocado industry and increased costs and poor fruit quality; it was suggested that controlled atmosphere and 1-MCP treatments may be superfluous with correct cold chain management (Dodd *et al.*, 2007). It was recently shown that breaking the cold chain reduced avocado quality by increasing transpiration and mass loss (Undurraga *et al.*, 2007). These authors also found that a break in the cold chain near the end of storage resulted in the fruit entering the ripening process prematurely resulting fruit softening and colour change in 'Hass'.

It has been shown that 'Hass' can be successfully stored at 1°C, rather than the conventional 5.5°C (Van Rooyen, 2009). Reduced temperature storage may mitigate some of the effects of a cold chain break by maintaining the pulp temperature below critical levels for a longer period than fruit stored 5.5°C.

The aim of this study was to quantify the effect of cold chain breaks at various points in a simulated shipping trial for fruit stored at 1 and 5.5°C and determine the changes in concentrations of individual sugars during a 24 h period.

2. Materials and Methods

2.1. Fruit Treatment

Organic, local grade fruit (cv. Hass) were sourced from a commercial farm in Howick, South Africa (29°27'S, cool subtropical climate) on 2 July, 22 July and 11 August 2008 and randomly assigned to the ten treatment combinations with 10 fruit per treatment combinations. The average dry matter for each harvest date was 26.6, 29.6 and 32.2%.

A delay of 24 h and breaks in the cold chain at 5, 10 and 20 days into cold storage for approximately 9 h were applied once during a 28 days storage period. Fruit were stored at 1 or 5.5°C. In the control treatment, the cold chain was maintained for the entire storage period. After cold storage fruit were ripened at 21±2°C and measurements taken 0, 2, 5 and 7 days after the fruit were removed from cold storage.

Five fruit from the latter two harvests were used to ascertain the changes in the sugar profile over a 24 h period. These fruit were directly ripened at 21±2°C and sampled 24 h after harvest and then 2, 4, 6 and 24h subsequently.

2.2. Respiration Rate

The respiration rate was measured using an infrared environmental gas monitor (EGM-1, PP Systems, Hitchin, UK). Individual fruit were incubated in a 1 L container for 15 min. The headspace CO_2 concentration (ppm) was measured and converted to respiration rate (mL $CO_2.kg^{-1}.h^{-1}$), taking into account the fruit mass and volume and the ambient CO_2 concentration.

2.3. Ethylene Production Rate

To measure ethylene production rate, individual fruit were incubated in a 1 L container for 15 min. Analysis was done using a DANI 1000 gas chromatograph (DANI Instruments, Monzese, Italy) fitted with an HT250D autosampler (HTA, Brescia, Italy), stainless steel column packed with alumina F1 stationary phase and a flame ionization detector. The temperatures for the injector, column and detector were 160°C, 100°C and 180°C respectively. The mobile phase was instrument grade nitrogen gas at 35 kPa. The autosampler injected 1 mL from the 20 mL autosampler vial.

2.4. Ripeness

Ripeness was assessed using a hand-held firmness tester (Bareiss, Oberdischingen, Germany) according to Köhne *et al.* (1998). Fruit were considered ripe when the average reading from opposite ends at the equator was less than 60 units.

2.5. Fruit Quality Evaluation

The incidence and severity of shrivel, external chilling injury and pathological diseases were assessed at each evaluation day. Internal quality was assessed at ripeness. Sound fruit are defined as being ripe, free from diseases and disorders and with good colouring, *i.e.* marketable fruit. Good colouring was defined as the achievement of skin colour change to predominantly black colouration. External chilling injury (discrete patches) was rated according to the International Avocado Quality Manual using a scale of 0 (no discrete patches) to 3 (50% of fruit surface area covered) (White *et al.*, 2004).

2.6. Sugar Analysis

For fruit that were sampled sequentially, samples were taken using a cork borer and the area immediately filled with warm petroleum jelly to prevent oxidation (Kanellis *et al.*, 1989). The mesocarp (~2 g fresh mass) was flash-frozen in liquid nitrogen, lyophilised, milled with pestle and mortar and stored at -20°C.

Sugar analysis was done according to Liu *et al.* (1999), with slight modifications. Sugars were extracted from 100 mg of lyophilised mesocarp in 10 mL 80% ($v.v^{-1}$) ethanol by heating to 80°C in a water bath for 60 min followed by incubation at 4°C for 24 h. Samples were filtered through glass wool and dried in a vacuum concentrator (Savant, Farmingdale, NY). Dried samples were re-suspended in 2 mL ultrapure water, placed in a sonic bath for 10 min and filtered through a 0.45 µm nylon syringe filter. The analysis was performed using HPLC (LC-20AT, Shimadzu, Kyoto, Japan) equipped with a refractive index detector (RID-10A, Shimadzu) and a Rezex RCM-Monosaccharide column (200 mm × 780 mm × 8 µm; Phenomenex, Torrance, CA). The elution was isocratic, using ultrapure water as the mobile phase. Individual sugars were identified by co-elution with standards of glucose, fructose, sucrose (Sigma-Aldrich, St Louis, MO), mannoheptulose and perseitol (Glycoteam GmbH, Hamburg, Germany). Quantification of sugars was done using five standard solutions of each sugar. Samples were

analysed twice and the mean taken.

2.7. Statistical Analysis

Analysis was performed using Genstat 12.1. A repeated measures analysis of variance was done using cold chain break treatment and storage temperature as factors and harvest date in the blocking structure, providing 30 replications per treatment combination. Significant differences were calculated at the 95% confidence level.

3. Results

Storage temperature ($P<0.001$) and cold chain break ($P<0.001$) both had a highly significant effect on respiration after removal from cold storage, but their interaction did not ($P=0.059$). Only the fruit stored at 1°C consistently had a significantly lower respiration rate after removal from cold storage (Table 11). For fruit stored at 5.5°C, only fruit that had cooling delayed and the cold chain broken on day 20 showed a significant difference at removal from cold storage, compared with the control. Fruit that had cooling delayed showed a significant decline in respiration on day seven. Although the fruit stored at 1°C had an initially lower respiration rate, by the second day after removal, the differences between the temperatures (comparing within treatments) were insignificant, indicating that the lower storage temperature does not have a post-storage effect on respiration.

Storage temperature ($P<0.001$) and cold chain break ($P<0.001$) both had a highly significant effect on ethylene production, but their interaction did not ($P=0.631$). The only significant differences in the EPR were on day seven (Table 12). The large rise in ethylene production from day five to day seven suggests that some of the fruit started to decay. With there being no significant differences between treatments until after day five ($P=0.631$), data were combined to compare the effect of storage temperature ($P<0.001$) on ethylene production during ripening (Table 13). There were significant differences between the two storage temperatures on every sampling day. The fruit stored at 1°C initially had a lower EPR than those stored at 5.5°C, but by day two had a higher rate of ethylene production which once again declined below the level of the higher storage temperature on day five and day seven. There was no difference in the EPR of fruit stored at 5.5°C at removal from cold storage and day two.

This may be because fruit stored at 5.5°C had already entered the climacteric stage during cold storage, suggesting that the storage temperature was too high to adequately lower the metabolic processes of ripening.

The cold chain break did not have a significant effect (P=0.052) on mass loss, although storage temperature (P<0.001), and the interaction were significant (P=0.009). The fruit stored at 1°C lost significantly less mass during storage and this difference was maintained until ripeness (Table 14). This reduced mass and water loss during storage is thought to have resulted in improved fruit quality, with fruit stored at 1°C having up to 80% sound fruit compared to only 30% for fruit stored at 5.5°C (Figure 13).

Storage temperature, cold chain break and their interaction all had significant effects on fruit softening (P0.015, P<0.001 and P=0.019, respectively). Softening during storage can be a major problem (Dodd *et al.*, 2007; Ginsberg, 1985; Undurraga *et al.*, 2007), but this was greatly reduced by reducing the storage temperature to 1°C. Storage at 1°C resulted in non-significant softening during storage - except for fruit with a cold chain break on day 10 - while fruit stored at 5.5°C showed a significant loss of firmness during storage. Fruit stored at 5.5°C ripened slightly before those stored at 1°C because of the loss of firmness during storage. At 1°C, the control fruit took slightly longer to ripen than the treated fruit, which shows that the break in the cold chain did have a prolonged effect on ripening. It is suggested that the cold chain break resulted in increased synthesis of ripening enzymes that modulate softening in avocados (Awad and Young, 1979; O'Donoghue *et al.*, 1994; Pesis *et al.*, 1978).

The predominant disorder throughout the season was shrivelling, while fruit with poor colour and anthracnose occurred infrequently. There was no significant difference in external chilling injury between the treatments, with the mean damage being 0.6 on a scale of 0-3. Significant differences may have been found if a finer rating scale was used. Storage temperature (P=0.024), cold chain break (P=0.40) and their interaction (P=0.01) all had significant effects on the percent sound fruit at ripeness. At 5.5°C, there was no significant difference between the treatments and the control because all the results were below 30% (Figure 13). But at 1°C, the fruit that had a constant cold storage period had significantly better quality. The control had 80% sound fruit while the delay, break 5, break 10 and break 20 treatments had 60, 57, 31 and 40% sound fruit respectively. The break on day 10 had the worst quality fruit of the treatments for fruit stored at 1°C. This may be related to the reduced respiration rate and decreased mass loss during ripening which may be indicative of a malfunction in the ripening metabolism of these fruit. Comparing fruit within the cold chain

treatments, fruit stored at 1°C were all significantly better, except for the fruit that had the cold chain break on day 10, where there was no significant difference.

Storage at 5.5°C hastened fruit colour development compared to fruit stored at 1°C, which exacerbated the appearance of variable ripening, but not necessarily the number of days to ripen (Figure 14). The fruit stored at 1°C were still green after removal from storage, but showed similar colour development to the fruit stored at 5.5°C five days after removal from cold storage (Figure 15). Fruit stored at 5.5°C had extensive, severe shrivelling while the fruits stored at 1°C still had a good appearance (Figure 16). While fruit stored at lower temperatures did have slower colour development, the fruit quality was superior and with reduced mass loss.

4. Discussion

It is known that temperature greatly affects the respiration rate of fruit (Biale, 1941; Eaks, 1978). Once fruits are harvested, finite energy reserves are utilised for respiration. The storage temperature should be set to minimise respiration during storage without causing chilling injury. Avocados are climacteric fruit with a very high respiration rate and, therefore, require a large energy reserve to reach eating ripeness; if energy reserves are depleted below a certain level, ripening may not be achieved or physiological disorders may occur. A break in the cold chain will cause a spike in respiration that will reduce the energy reserves of the fruit. This was shown to occur in a few hours where mannoheptulose and perseitol declined 45% and 17% respectively during a 24 h period (Figure 17). If the total heptose concentration is considered, the loss after was 18% only 3 h and 33% after 24 h. Avocado fruit also have a limited energy reserve compared to other climacteric fruit - only about 0.1 °brix. Although avocados have a very high lipid reserves, there is evidence that the lipids are not used as a major energy source during ripening because the concentration of oil on a dry mass basis increased during ripening (Meyer and Terry, 2008). This highlights the need to maintain the cold chain and ensure the fruit are stored at the lowest possible temperature, without causing chilling injury, to minimise respiration and maintain fruit quality as much as possible.

5. Conclusion

In conclusion, breaking the cold chain is known to be detrimental to avocado fruit quality. While fruit largely recovered from a cold chain break physiologically, fruit quality was severely reduced. Avocado fruit are also susceptible to the rapid depletion of energy reserves. These results show the importance of maintaining the cold chain and storing avocado fruit at the lowest possible temperature, without causing chilling injury, for maintaining postharvest fruit quality.

Table 11: Respiration rate (mL.kg^{-1}.h^{-1}) of 'Hass' avocados from Howick, South Africa (cool subtropical climate) during ripening at 21±2°C averaged for three harvest dates in the 2008 season ± standard error of the mean, for five storage treatments[1], after cold storage at 1 or 5.5°C for 28 days.

Storage temp	Days after	Cold Chain Treatment				
(°C)	cold storage	Control	Delay	Break 5	Break 10	Break 20
1	0	67.6 ± 6.2 A	62.8 ± 5.2 A	49.0 ± 4.1 B	44.4 ± 4.3 B	51.8 ± 6.4 B
	2	80.3 ± 4.4 A	82.7 ± 6.2 A	80.8 ± 5.5 A	76.4 ± 4.0 A	85.6 ± 6.4 A
	5	71.4 ± 7.2 A	76.4 ± 5.5 A	75.4 ± 6.5 A	69.8 ± 6.3 A	72.5 ± 6.7 A
	7	62.8 ± 4.0 A	64.5 ± 4.1 A	67.0 ± 4.3 A	63.4 ± 2.1 A	66.5 ± 2.1 A
5.5	0	86.9 ± 4.7 A	79.2 ± 5.4 B	86.4 ± 8.6 A	80.9 ± 9.7 A	78.0 ± 5.1 B
	2	81.1 ± 6.7 A	80.4 ± 6.5 A	81.7 ± 5.4 A	80.2 ± 4.3 A	81.7 ± 4.8 A
	5	76.3 ± 6.3 A	74.6 ± 4.2 A	74.3 ± 6.3 A	72.2 ± 6.1 A	79.8 ± 6.2 A
	7	70.6 ± 5.5 A	61.4 ± 3.0 B	77.2 ± 5.5 A	69.7 ± 3.4 A	67.5 ± 2.8 A

LSD = 6.7 mL.kg^{-1}.h^{-1}. Different letters indicate a significant difference to that treatment's control, on the same day.

[1] Control, no cold chain break during 28 day cold storage;
Delay, cooling was delayed for 24 h after harvest;
Break 5, cold chain broken for 9 h five days after harvest;
Break 10, cold chain broken for 9 h five days after harvest;
Break 20, cold chain broken for 9 h five days after harvest.

Table 12: Ethylene production rate ($\mu L.kg^{-1}.h^{-1}$) of 'Hass' avocados from Howick, South Africa (cool subtropical climate) during ripening at 21±2°C averaged for three harvest dates in the 2008 season ± standard error of the mean, for five storage treatments[1], after cold storage at 1 or 5.5°C for 28 days.

Storage temp	Days after	Cold Chain Treatment				
(°C)	cold storage	Control	Delay	Break 5	Break 10	Break 20
1	0	72.5 ± 6.8 A	78.7 ± 4.5 A	61.2 ± 7.3 A	60.4 ± 7.9 A	66.9 ± 9.4 A
	2	93.2 ± 9.0 A	104.8 ± 12.4 A	95.6 ± 11.2 A	93.2 ± 9.6 A	102.2 ± 17.0 A
	5	85.3 ± 11.7 A	97.3 ± 10.0 A	91.3 ± 7.9 A	87.1 ± 12.4 A	91.4 ± 11.5 A
	7	97.0 ± 7.5A	114.7 ± 10.8 A	126.5 ± 13.0 B	95.6 ± 7.4 A	134.4 ± 14.9 B
5.5	0	84.8 ± 7.5 A	97.7 ± 9.8 A	83.9 ± 6.5 A	89.0 ± 6.5 A	91.0 ± 9.0 A
	2	90.6 ± 4.7 A	93.7 ± 6.4 A	90.1 ± 6.9 A	85.4 ± 7.5 A	87.0 ± 6.3 A
	5	113.4 ± 14.6 A	129.7 ± 30.6 A	103.3 ± 15.9 A	125.6 ± 24.4 A	126.0 ± 19.5 A
	7	172.1 ± 20.3 A	166.2 ± 33.8 A	207.8 ± 30.8 B	171.1 ± 13.3 A	225.1 ± 46.6 B

LSD = 21.5 $\mu L.kg^{-1}.h^{-1}$. Different letters indicate a significant difference to that treatment's control on the same day.

[1] Control, no cold chain break during 28 day cold storage;

Delay, cooling was delayed for 24 h after harvest;

Break 5, cold chain broken for 9 h five days after harvest;

Break 10, cold chain broken for 9 h five days after harvest;

Break 20, cold chain broken for 9 h five days after harvest.

Table 13: Ethylene production rate ($\mu L.kg^{-1}.h^{-1}$) ± standard error of the mean of 'Hass' avocados from Howick, South Africa (cool subtropical climate) during ripening at 21±2°C averaged for three harvest dates in the 2008 season after cold storage at 1 or 5.5°C for 28 days.

Days after cold storage	Storage temp (°C)	
	1	5.5
0	67.9 ± 7.6 ax	89.3 ± 8.0 Ay
2	97.8 ± 12.1 bx	89.4 ± 6.4 Ay
5	90.5 ± 10.8 cx	119.6 ± 21.7 By
7	113.6 ± 12.6 dx	188.4 ± 30.9 Cy

LSD between temperatures = 4.8 $\mu L.kg^{-1}.h^{-1}$. Significant differences are indicated with xy.

LSD between days at the same temperature = 6.5 $\mu L.kg^{-1}.h^{-1}$. Significant differences are indicated with ABC at 5.5°C and abcd at 1C.

Table 14: Percent mass loss of 'Hass' avocados from Howick, South Africa (cool subtropical climate) during ripening at 21±2°C averaged for three harvest dates in the 2008 season, for five storage treatments[1] ± standard error of the mean, after cold storage at 1 or 5.5°C for 28 days.

Storage temp	Days after	Cold Chain Treatment				
(°C)	cold storage	Control	Delay	Break 5	Break 10	Break 20
1	0	4.6 ± 1.2 A	5.1 ± 1.0 A	4.6 ± 1.3 A	4.4 ± 1.6 A	4.5 ± 0.7 A
	2	8.2 ± 2.1 A	8.3 ± 1.7 A	7.9 ± 2.1 A	7.7 ± 2.6 A	7.8 ± 1.3 A
	5	11.9 ± 1.8 A	11.5 ± 2.4 A	11.2 ± 2.1 A	11.0 ± 2.7 A	11.3 ± 1.1 A
	7	13.9 ± 0.5 A	13.6 ± 1.0 A	13.2 ± 2.6 A	12.1 ± 0.9 B	13.2 ± 0.7 A
5.5	0	10.3 ± 1.9 A	10.0 ± 2.4 A	10.7 ± 2.5 A	9.8 ± 2.5 A	9.7 ± 1.5 A
	2	13.8 ± 2.7 A	13.2 ± 3.2 A	14.1 ± 3.4 A	13.1 ± 3.4 A	12.9 ± 2.1 A
	5	17.2 ± 2.5 A	16.3 ± 3.0 A	17.3 ± 3.2 A	16.2 ± 3.3 B	16.0 ± 1.9 A
	7	18.7 ± 1.5 A	17.9 ± 1.9 A	18.8 ± 0.7 A	17.4 ± 1.3 B	17.6 ± 1.0 A

LSD = 0.9% mass. Different letters indicate a significant difference to that treatment's control, on the same day.

[1] Control, no cold chain break during 28 day cold storage;
Delay, cooling was delayed for 24 h after harvest;
Break 5, cold chain broken for 9 h five days after harvest;
Break 10, cold chain broken for 9 h five days after harvest;
Break 20, cold chain broken for 9 h five days after harvest.

Table 15: Fruit firmness (densimeter units) of 'Hass' avocados from Howick, South Africa (cool subtropical climate) during ripening at 21±2°C averaged for three harvest dates in the 2008 season, for five storage treatments[1] ± standard error of the mean, after cold storage at 1 or 5.5°C for 28 days.[2]

Storage Temp (°C)	Days after cold storage	Treatment				
		Control	Delay	Break 5	Break 10	Break 20
1	Harvest	86 ± 0.9 Aa	86 ± 0.9 Aa	86 ± 0.8 Aa	87 ± 0.8 Aa	87 ± 1.0 Aa
	0	85 ± 0.8 Aa	86 ± 0.7 Aa	85 ± 0.8 Aa	84 ± 1.1 Ab	85 ± 0.8 Aa
	2	74 ± 2.5 Ab	73 ± 2.4 Ab	73 ± 2.5 Ab	72 ± 2.7 Ac	73 ± 2.9 Ab
	5	62 ± 2.4 Ac	59 ± 2.1 Bc	59 ± 2.0 Bc	59 ± 2.0 Bd	58 ± 1.6 Bc
	7	61 ± 1.3 Ac	58 ± 1.3 Bc	58 ± 1.5 Bc	58 ± 1.2 Bd	55 ± 1.1 Bd
5.5	Harvest	87 ± 0.9 Aa	88 ± 0.8 Aa	85 ± 0.9 Aa	88 ± 0.9 Aa	87 ± 0.9 Aa
	0	79 ± 1.5 Ab	81 ± 1.4 Ab	78 ± 1.6 Ab	80 ± 1.4 Ab	80 ± 1.3 Ab
	2	64 ± 2.3 Ac	66 ± 2.3 Ac	64 ± 2.3 Ac	64 ± 2.2 Ac	63 ± 1.8 Ac
	5	60 ± 2.1 Ad	61 ± 1.8 Ad	59 ± 1.7 Ad	59 ± 1.8 Ad	57 ± 1.6 Bd
	7	58 ± 1.9 Ad	59 ± 1.4 Ad	59 ± 0.9 Ad	55 ± 1.6 Be	58 ± 2.1 Ad

LSD = 2.3 units when comparing means of two treatments; Significant difference between a treatment and that treatment's control, on the same day.

LSD = 2.1 units when comparing firmness at two days, with the same storage temperature and treatment.

[1] Control, no cold chain break during 28 day cold storage;
Delay, cooling was delayed for 24 h after harvest;
Break 5, cold chain broken for 9 h five days after harvest;
Break 10, cold chain broken for 9 h five days after harvest;
Break 20, cold chain broken for 9 h five days after harvest

[2] Fruit were ripe when the average densimeter reading was below 60.

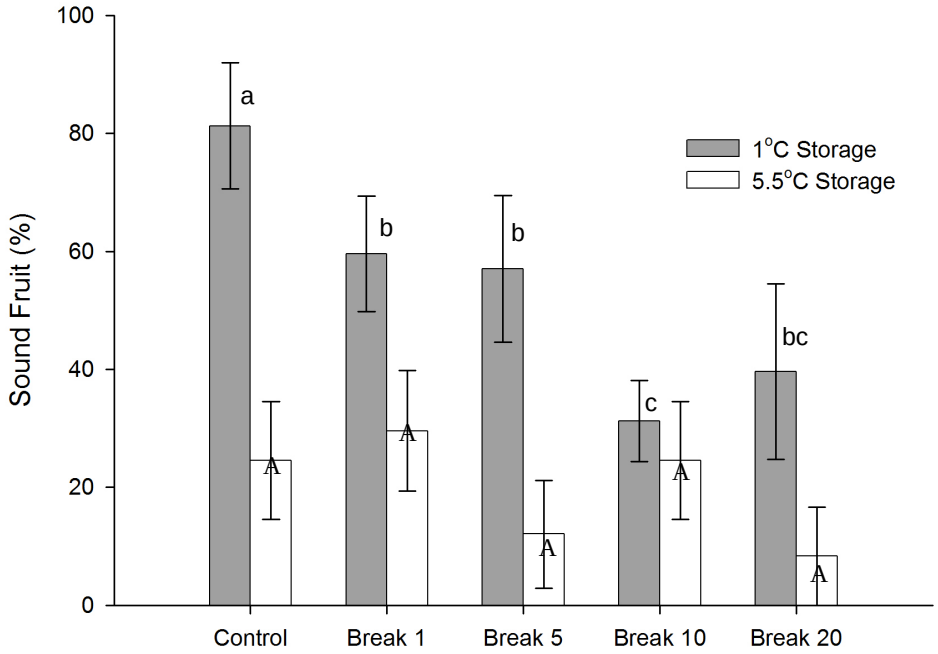

Figure 13: Percentage of sound fruit of 'Hass' avocados from Howick, South Africa (cool subtropical climate) during ripening at 21±2°C averaged for three harvest dates in the 2008 season, for five storage treatments[1], after cold storage at 1 or 5.5°C for 28 days and ripening at 21±2°C. Sound fruit are defined as being ripe and free from diseases and disorders. Error bars indicate standard error of the mean.

Lowercase letters indicate significant differences at 1°C storage temperature.

Uppercase letters indicate significant differences at 5.5°C.

Control, no cold chain break during 28 day cold storage;

Delay, cooling was delayed for 24 h after harvest;

Break 5, cold chain broken for 9 h five days after harvest;

Break 10, cold chain broken for 9 h five days after harvest;

Break 20, cold chain broken for 9 h five days after harvest

Figure 14: Fruit two days after removal from cold storage. Fruit in the top row were stored at 1°C, and fruit in the bottom row at 5.5°C. The columns, from left to right are: delay, break 5, break 10, break 20 and the control.

Control, no cold chain break during 28 day cold storage;

Delay, cooling was delayed for 24 h after harvest;

Break 5, cold chain broken for 9 h five days after harvest;

Break 10, cold chain broken for 9 h five days after harvest;

Break 20, cold chain broken for 9 h five days after harvest

Figure 15: Fruit five days after removal from cold storage. Fruit in the top row were stored at 1°C, and fruit in the bottom row at 5.5°C. The columns, from left to right are: delay, break 5, break 10, break 20 and the control.

Control, no cold chain break during 28 day cold storage;

Delay, cooling was delayed for 24 h after harvest;

Break 5, cold chain broken for 9 h five days after harvest;

Break 10, cold chain broken for 9 h five days after harvest;

Break 20, cold chain broken for 9 h five days after harvest

Figure 16: Fruit at ripeness showing the most damaging treatment of breaking the chain at 10 days. The fruit on the left were stored at 1°C, those on the right at 5.5°C.

Control, no cold chain break during 28 day cold storage;

Delay, cooling was delayed for 24 h after harvest;

Break 5, cold chain broken for 9 h five days after harvest;

Break 10, cold chain broken for 9 h five days after harvest;

Break 20, cold chain broken for 9 h five days after harvest

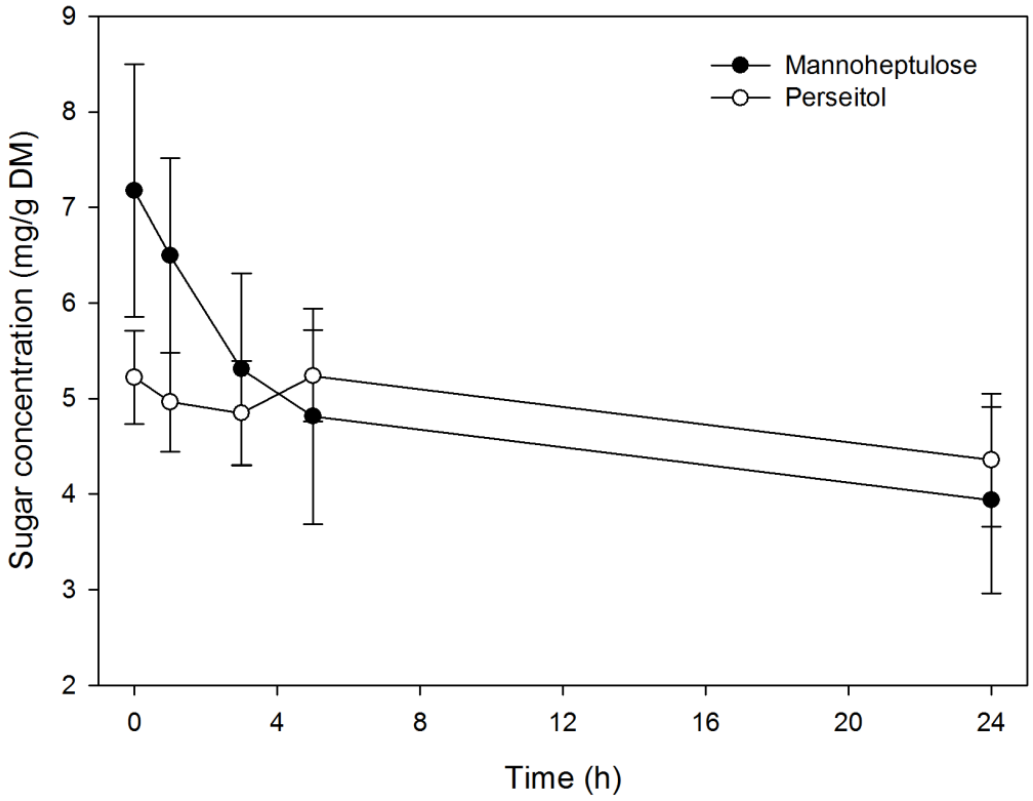

Figure 17: The concentration of mannoheptulose and perseitol 24-48 h postharvest of fruit from two harvests from Howick, South Africa (cool, subtropical climate). Error bars indicate standard error of the mean.

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Chapter 6
Changes in ‘Hass’ avocado stress physiology following a postharvest application of ethephon

Robert Blakey, Samson Tesfay, Nhlanhla Mathaba, Isa Bertling and John Bower

Ethylene is used commercially to hasten ripening of avocados, but the presence of ethylene in the storage atmosphere can increase the risk of physiological disorders. To investigate the short term effect of ethylene on avocado physiology, fruit were dipped in a low concentration ethephon solution. The ethephon treatment significantly increased the ethylene production rate of avocados after 6 h, compared to untreated fruit. After 6 h, the respiration rate of treated fruit increased significantly during the investigation while that of the untreated fruit did not show a significant increase from the initial measurement. Lipid peroxidation increased after dipping, reaching a peak after 4 h, and then declined to a level similar to untreated fruit. Mannoheptulose declined significantly during the 6 h investigation in both the untreated and treated fruit. It is concluded that avocado fruit can undergo significant physiological changes, and quality loss, in a short amount of time, which is exacerbated by exposure to ethylene.

1. Introduction

Avocado is a climacteric fruit with a high ethylene production rate during ripening (Sitrit *et al.*, 1986). Endogenous ethylene stimulates ripening and the gas is supplied exogenously to hasten ripening commercially (Hofman *et al.*, 2002), but the presence of ethylene in the storage atmosphere is detrimental to fruit quality (Pesis *et al.*, 1999; Pesis *et al.*, 2002; Zauberman and Fuchs, 1981). Softening and mesocarp discolouration increased significantly in stored fruit treated with ethephon (2-chloroethylphosphonic acid), especially when stored at 4 or 6°C (Pesis *et al.*, 2002). Fruit treated with ethylene before storage showed significantly higher external and internal chilling injury and increased stem end rot (Pesis *et al.*, 1999).

Interruptions in the cold chain can occur at handling points and during transit (Dodd *et al.*, 2007). These breaks or delays in cooling are typically only a few hours, but nonetheless can have a severe detrimental effect on fruit quality, and are mostly avoidable with correct handling of fruit. Breaks or delays in the cold chain can result in the initiation of the climacteric (Biale, 1941), which can increase the risk of partial softening, quality loss and limited shelf life (Blakey *et al.*, 2009a; Dodd *et al.*, 2007; Undurraga *et al.*, 2007).

Ethephon releases ethylene as a breakdown product and is used as a ripening promoter in a number of horticultural crops. The application of ethephon to mangoes was found to rapidly decrease superoxide dismutase activity and catalase activity, but increase ascorbate peroxidase activity, lipid peroxidation and the concentration of H_2O_2 (Singh and Dwivedi, 2008). This indicates that exposure to ethylene can cause rapid physiological changes which increase the risk of quality loss because of changes to the anti-oxidant system.

In this experiment fruit were dipped in ethephon to investigate some of the initial physiological changes that occur during exposure to low concentrations of ethylene, which may occur during improper postharvest handling, such as a cold chain break or a delay in cooling. The changes in respiration rate, ethylene production rate, total anti-oxidant capacity, lipid peroxidation and sugars of avocado fruit were measured sequentially during a 6 h period after ethephon dipping to ascertain the effect on fruit stress physiology and potential causes of quality loss in this short period of time.

2. Materials and Methods

2.1. Plant Material

Export grade 'Hass' avocados (236-265 g) were obtained from a commercial farm in Howick ((29°27'S, cool subtropical climate, South Africa) on 23 July 2008 (29.6% dry matter) and 12 August 2008 (32.2% dry matter) and immediately transported to the laboratory 40 km away in Pietermaritzburg. Fruit maturity was determined on a subset of 60 fruit from a related experiment.

2.2. Ethephon Treatment

For the main experiment, fruit were held at 21±2°C for three days for the fruit to become ethylene-sensitive. Treated fruit were then dipped in a 50 $mL.L^{-1}$ ethephon solution for 5 min and allowed to air dry. This low concentration of ethephon was used to simulate exposure to low ambient ethylene. Untreated fruit were kept in a separate laboratory to the treated fruit. For comparison, 10 fruit per harvest from the same orchard were allowed to ripen at 21±2°C; ethephon-treated fruit were treated at the same concentration 8 h after harvest.

2.3. Sampling

Avocado fruit have large endogenous variation between fruit (Schroeder, 1985) so to reduce the variation between fruit, samples were taken sequentially from the equator of the same fruit and the fruit sealed with petroleum jelly to prevent oxidation according to Kanellis *et al.* (1989). Fruit were sampled immediately after dipping and then two, four and six hours after treatment. The sample core - taken using a 15 mm diameter cork borer - of mesocarp tissue (~2.5 g fresh mass) was flash-frozen in liquid nitrogen, lyophilised and stored at -20°C. Samples were later hand-milled in liquid nitrogen.

2.4. Respiration rate

The concentration of CO_2 was measured using an infrared gas analyser (EGM-1, PP Systems, Hitchin, UK). Individual fruit were incubated in a 1 L container for 15 min. The headspace CO_2 concentration was converted to respiration rate taking into account fruit mass, fruit volume, free space in the jar and the ambient CO_2 concentration.

2.5. Ethylene production rate

Individual fruit were incubated in a 1 L container for 30 min, with a 20 mL autosampler vial enclosed. Ethylene was measured using GC-FID (DANI 1000, DANI Instruments, Monzese, Italy). The GC was fitted with a stainless steel column packed with alumina-F1 stationary phase. The injector, column and detector were set at 160°C, 100°C and 180°C, respectively. The mobile phase was instrument grade nitrogen gas at 35 kPa. The autosampler injected 1 mL from the 20 mL vial. The ethylene production rate (EPR) was calculated taking into account fruit mass, fruit volume and free space in the jar.

2.6. Sugars

Sugars were analysed according to Liu *et al.* (1999), with slight modifications. A 10 mL aliquot of 80% ($v.v^{-1}$) ethanol was added to 100 mg of mesocarp, heated in an 80°C water bath for 60 min and then incubated at 4°C for 24 h. Samples were then filtered through glass wool and dried in a vacuum concentrator (Savant, Farmingdale, NY). Dried samples were re-suspended in 2.0 mL ultrapure water, placed in a sonic bath for 10 min, and filtered through a 0.45 µm nylon syringe filter. The analysis was performed using an HPLC (Shimadzu, Kyoto, Japan) equipped with a differential refractometric detector (RID–10A) and a Phenomenex column (Rezex RCM-Monosaccharide, 200 mm × 780 mm × 8 µm). The elution was isocratic, using ultrapure water as the mobile phase. Individual sugars were identified by co-elution with standards of glucose, fructose, sucrose (Sigma-Aldrich, St Louis, MO), mannoheptulose and perseitol (Glycoteam, Hamburg, Germany). Sugars were quantified by applying a standard curve for each sugar. Samples were analysed twice and the mean taken.

2.7. Total anti-oxidant capacity

Total anti-oxidant capacity (TAOC) was measured according to Benzie and Strain (1996), with slight modifications. Lyophilised mesocarp (100 mg) was extracted with 1 N perchloric acid, vortexed and centrifuged at 12,400 × g for 10 min at 4°C. The ferric reducing anti-oxidant power (FRAP) reagent solution (300 mM sodium acetate buffer pH 3.6, 10 mM (II)-TPTZ (***2***,4,6-Tris (***2***-pyridyl)-1,3,5-triazine) in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ (10:1:1 – $v.v^{-1}.v^{-1}$)) was freshly prepared prior to measurement. Subsequently, a 30 µL aliquot of the sample was mixed with 900 µL FRAP reagent and the absorbance at 593 nm was measured after 10 min. The TAOC was expressed as µmol $FeSO_4.7H_2O$ eq.g DM^{-1}.

For confirmation, anti-oxidant activity was also determined according to Re *et al.* (1999), with slight modifications. 2,2'-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was prepared as a 7 mM solution in water and the ABTS radical cation ($ABTS^{\cdot+}$) produced by reacting the 7 mM ABTS solution with 2.45 mM ammonium persulfate and allowing the mixture to stand in the dark at room temperature for 6 h. Thereafter 10 µL sample solution - from extracts of freeze-dried mesocarp (200 mg) in acetate buffer (0.1 M, pH 4.0) - was added to 1.0 mL activated ABTS solution. The absorbance at 734 nm was recorded before the addition of the sample and 6 min after and the difference calculated to determine the percent radical scavenging capacity.

2.8. Lipid peroxidation

Lipid peroxidation was quantified according to Dipierro and De Leonardis (1997). Lyophilised mesocarp (200 mg) was extracted with 10 ml of ice-cold 0.1% ($v.v^{-1}$) trichloroacetic acid and homogenised using an ultrasonic cell disrupter (VirTis VirSonic 100, Gardiner, NY, USA) for 30 s. The homogenate was centrifuged at 20,000 × g for 10 min at 4°C. A 1.0 mL aliquot of the supernatant was thoroughly mixed with 4.0 mL of 20% ($v.v^{-1}$) trichloroacetic acid containing 0.5% ($v.v^{-1}$) thiobarbituric acid. The mixture was incubated in a 95°C water bath and then quickly cooled in an ice bath. After centrifugation at 20,000 × g for 10 min at 4°C, the absorbance of the supernatant at 532 nm was recorded and corrected for non-specific absorbance at 600 nm using a spectrophotometer (Beckman Coulter DU-800, Fullerton, CA, USA). The malondialdehyde (MDA) concentration was calculated using an extinction coefficient of 155 $mM^{-1}.cm^{-1}$. Results are expressed in nmol MDA.g DM^{-1}.

2.9. Statistical analysis

Repeated measures ANOVA using ethephon treatment as a factor and with 10 replications was performed using Genstat v12.1 at a 95% confidence level.

3. Results

Replications were pooled because no significant differences were found between the two harvest dates and the maturity of the fruit from both harvest dates was similar – 29.6 and 32.2% dry matter, respectively. Overall there were very few significant differences between the treated and untreated fruit, which was expected because of the short duration of the experiment, so significant differences results are telling.

3.1. Ethylene

The ethephon treatment significantly increased (P=0.002) the EPR of the treated fruit, although the interaction between time and treatment was not significant (P=0.363). Although the ethephon treated fruit had a higher EPR than the untreated fruit, there was no significant difference until 6 h after treatment, as the EPR of the ethephon treated fruit increased whilst the EPR of the untreated fruit declined (Figure 18A). The EPR of the untreated fruit after 6 h was similar to the EPR of fruit that were ripened, but the ethephon treated fruit had a much higher EPR compared to the ethephon treated fruit that were ripened (Figure 18B). The EPR of the fruit used in this experiment was lower and the ripening rate higher because the fruit were more mature compared to most commercial 'Hass' which is mature at 23.0% dry matter (Milne, 1994). The production area where the fruit was obtained is used to extend the South African avocado season by harvesting fruit with higher dry matter.

3.2. Respiration

The ethephon treatment had no significant effect on the respiration rate during the 6 h investigation (P=0.170), there was also no significant effect caused by the interaction between time and treatment (P=0.323), although the effect of time was significant (P<0.001). The untreated fruit showed a significant increase after 4 h compared to the initial rate, but then declined to a non-significant level after 6 h. The respiration rate of treated fruit increased significantly after 6 h compared to the untreated fruit at the same time (Figure 19A). The respiration rate of the untreated and ethephon-treated fruit was similar to the respiration rate of fruit that were ripened after 6 h (Figure 19B).

3.3. Lipid Peroxidation

There were significant treatment (P=0.035) and time (P<0.001) effects on lipid peroxidation, although the interaction between the two factors was not significant (P=0.125). There were significant differences between the treatments at 2 and 4 h, but results were similar after 6 h (Figure 20). In the treated fruit, lipid peroxidation increased significantly 4 h after treatment and then declined significantly 6 h after treatment. Within the untreated fruit, lipid peroxidation declined significantly from the initial value 6 h after treatment. No significant differences were found in the lipid peroxidation of flesh in fruit treated with ethylene previously (Meir *et al.*, 1991), but lipid peroxidation was measured daily and not on the same fruit, so the transient changes noticed here would not have been recorded.

3.4. Total Anti-oxidant Capacity

The trends for the two anti-oxidant assays were very similar hence only the results from the FRAP assay are reported. The TAOC of treated fruit remained fairly stable while the TAOC of untreated fruit declined slightly during the period of investigation, but treatment (P=0.360), time (P=0.651) and interaction effect (P=0.588) were not significant (Figure 21). A longer evaluation time may have found significant differences.

3.5. Sugars

Only mannoheptulose, perseitol and sucrose are presented because glucose and fructose were present in trace amounts. There was no significant treatment (P=0.408, 0.382 and 0.611, respectively), time – except for mannoheptulose – (P<0.001, P=0.099 and 0.074, respectively) or the treatment-time interaction (P=0.326, 0.739 and 0.487) effect on any of the sugars. There was a significant loss of mannoheptulose between 2 and 4h in the untreated and ethephon-treated fruit (Figure 22A). There were no significant changes in perseitol during the investigation (Figure 22B). The sucrose concentration of the untreated fruit increased linearly and was significantly higher than the initial concentration after 6 h while there was no significant change in the concentration of sucrose in the ethephon-treated fruit during the investigation (Figure 22C).

4. Discussion

The majority of postharvest disorders are directly or indirectly attributable to ethylene, so limiting the effect of ethylene - either its production or reception – pre-ripening is critical in maintaining postharvest fruit quality (Pesis *et al.*, 1999; Pesis *et al.*, 2002). These results suggest that unripe fruit have enough energy and cell integrity to recover from exposure to a low concentration of ethephon, but there was a significant decline in the concentration of mannoheptulose and an elevated EPR. This is important because mannoheptulose has been shown to be a potential quality indicator in avocado because it is both a major energy, carbon and anti-oxidant in the mesocarp (Blakey *et al.*, 2009b; Tesfay, 2009; Tesfay *et al.*, 2010). Indeed, in this experiment there was a strong negative correlation between mannoheptulose and respiration rate, R^2 = 0.84 in the untreated and 0.96 in the treated fruit, and a strong positive correlation between mannoheptulose and TAOC, 0.90 and 0.75, respectively. The concentration of soluble sugars in avocados is very low compared to other climacteric fruit, making the loss of mannoheptulose in the 6 h period of investigation particularly concerning. A loss in TAOC, along with the elevated EPR, will increase the risk of physiological disorders developing. If the autocatalytic generation of ethylene is triggered by exposure to a low concentration of exogenous ethylene, elevated storage temperature or a cold chain break for instance and the fruit are returned to cold storage, a metabolic imbalance may result (Raison and Orr, 1990) and physiological disorders associated with ethylene exposure during cold storage, such as mesocarp discolouration and vascular browning, may develop (Pesis *et al.*, 1999; Pesis *et al.*, 2002; White *et al.*, 2003; Woolf *et al.*, 2005) – especially if 1-MCP was not applied soon after harvest. The commercial implications of this are large financial losses because of quality loss and variable ripening which makes management of the fruit at ripening facilities difficult.

5. Conclusion

Avocado fruit are susceptible to potential quality loss after only a few hours of improper handling, especially if exposed to ethylene, because of an elevated ethylene production and loss of mannoheptulose, which can increase the risk of physiological disorders and therefore fruit quality loss.

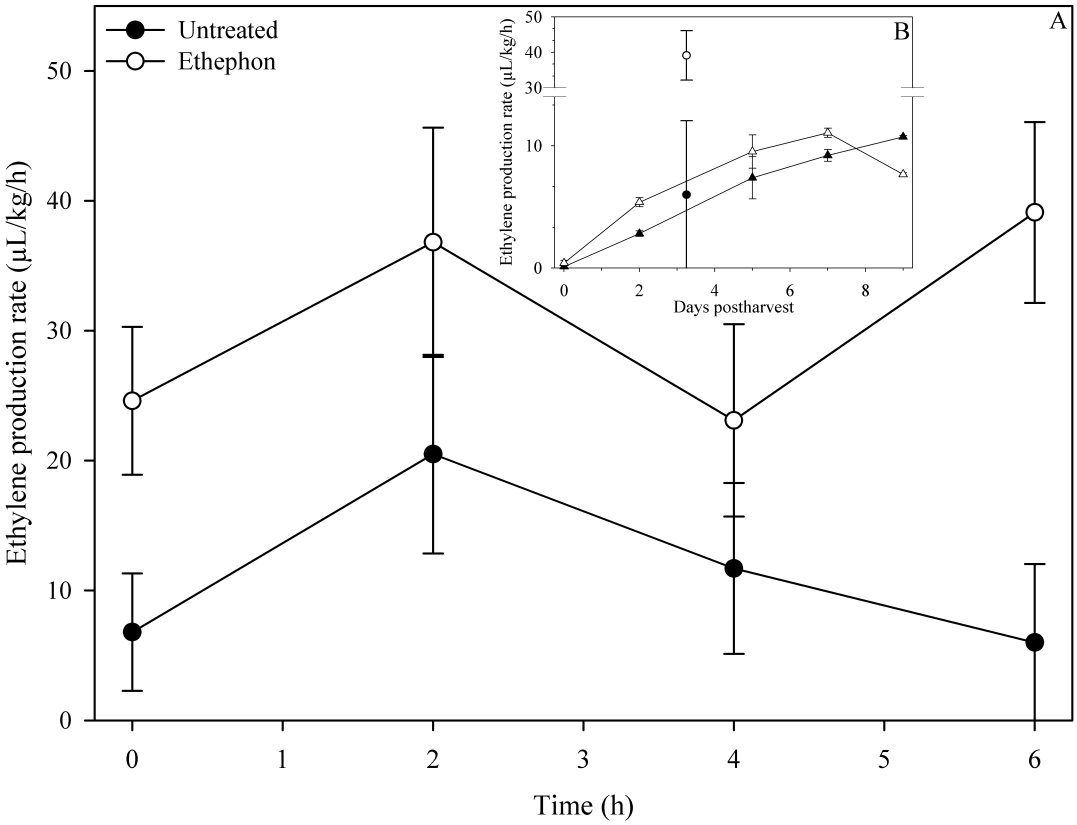

Figure 18:

A. Ethylene production rate over a 6 h period for untreated and ethephon treated fruit. Means are calculated from 10 late season fruit from two harvests from a commercial farm in Howick, South Africa. LSD within treatment = 18.9 $\mu L.kg^{-1}.h^{-1}$, LSD between treatment = 19.8 $\mu L.kg^{-1}.h^{-1}$.

B. Ethylene production rate for fruit from the same orchard and harvest date as Fig. 1A that were ripened at 21±2°C with 6 h point from Fig. 1A included.

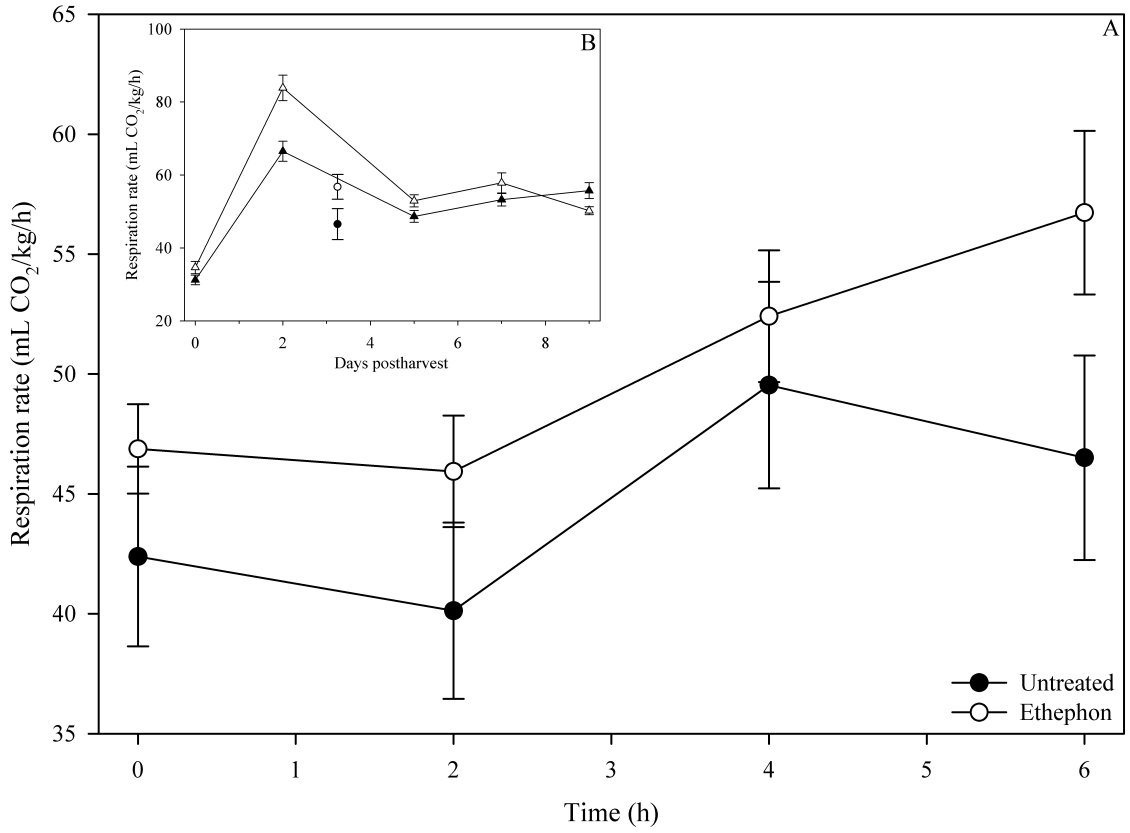

Figure 19:

A. Respiration rate over a 6 h period for untreated and ethephon treated fruit. Means are calculated from 10 late season fruit from two harvests from a commercial farm in Howick, South Africa. LSD within treatment = 6.2 mL CO_2.kg^{-1}.h^{-1}. LSD between treatment = 10.3 mL CO_2.kg^{-1}.h^{-1}.

B. Respiration rate for fruit from the same orchard and harvest date as Fig. 1A that were ripened at 21±2°C with 6 h point from Fig. 2A included.

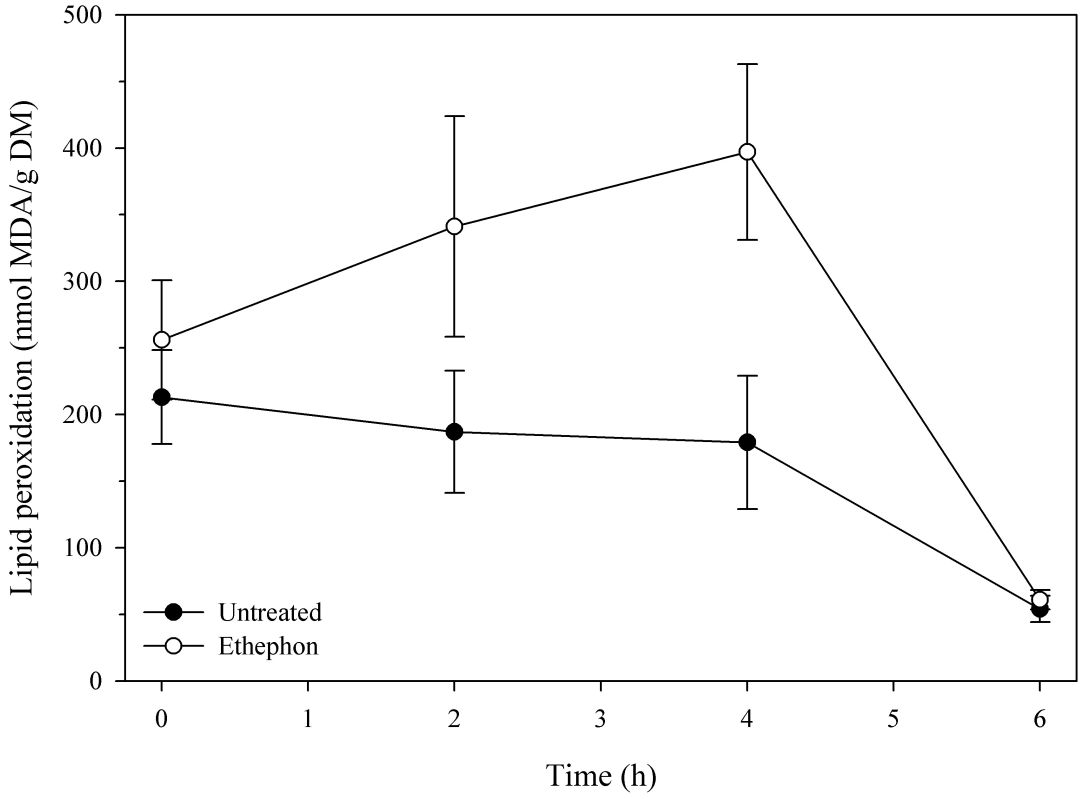

Figure 20: Lipid peroxidation over a 6 h period for untreated and ethephon treated fruit. Means are calculated from 10 late season fruit from two harvests from a commercial farm in Howick, South Africa. LSD within treatment = 140 nmol MDA.g DM^{-1}. LSD between treatment = 142 nmol.g DM^{-1}.

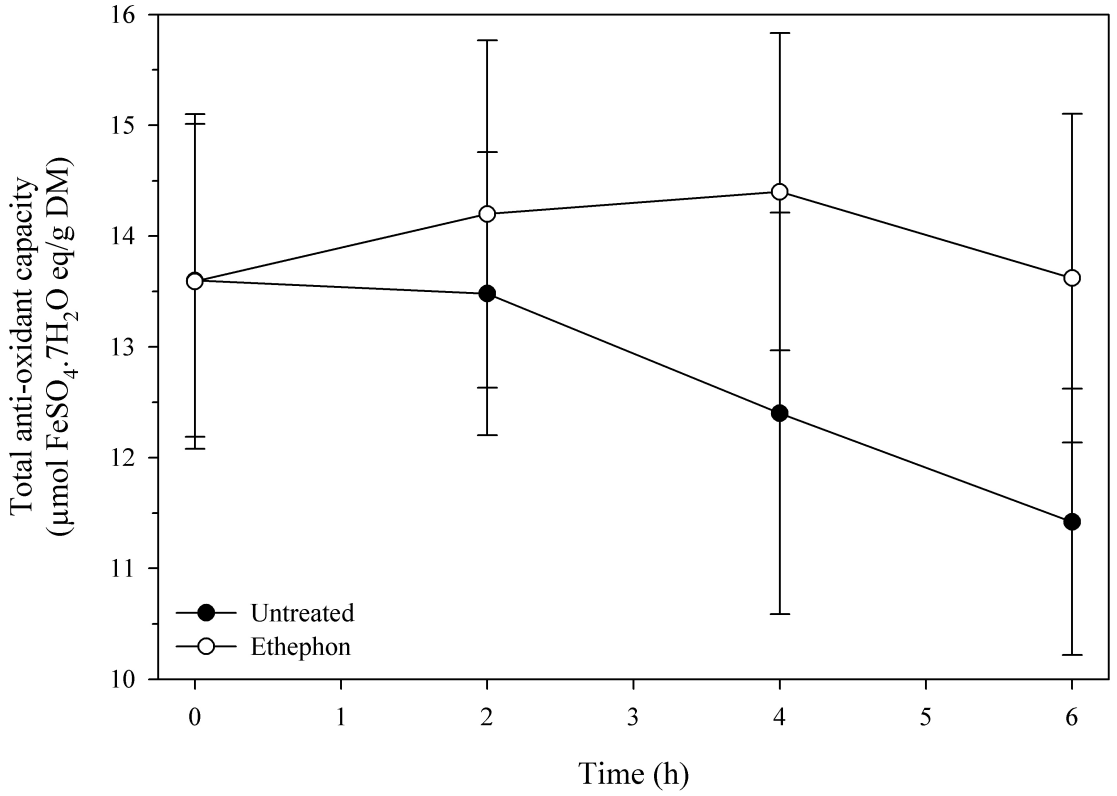

Figure 21: Total anti-oxidant capacity, measured using the FRAP assay over a 6 h period, for untreated and ethephon treated fruit. Means are calculated from 10 late season fruit from two harvests from a commercial farm in Howick, South Africa. LSD within treatment = 3.1 µmol FeSO4.7H2O eq.g DM^{-1}. LSD between treatment = 4.0 µmol FeSO4.7H2O eq.g DM^{-1}.

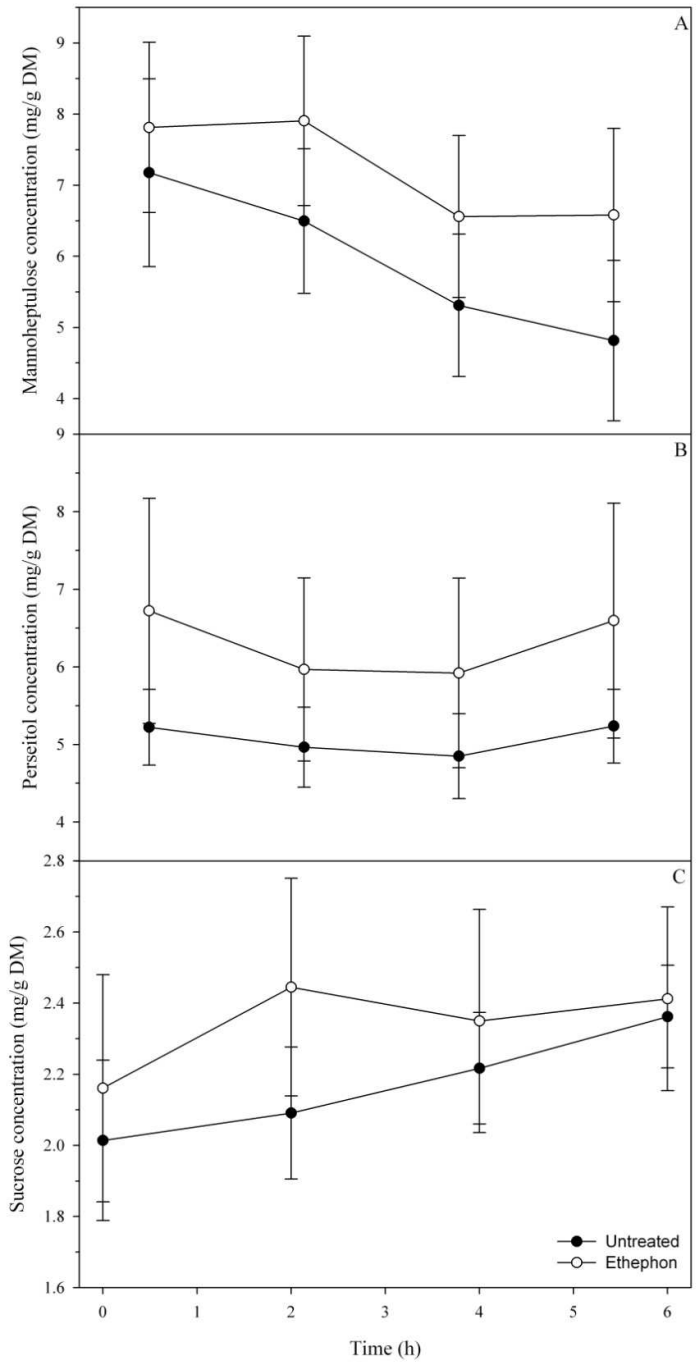

Figure 22: Mannoheptulose (A), perseitol (B) and sucrose (C) concentration over a 6 h period, for untreated and ethephon treated fruit. Means are calculated from 10 late season fruit from two harvests from a commercial farm in Howick, South Africa. LSD_{manno} within treatment = 0.9 mg.g DM^{-1}. LSD_{manno} between treatment = 3.4 mg.g DM^{-1}. $LSD_{perseitol}$ within treatment = 0.8 mg.g DM^{-1}. $LSD_{perseitol}$ between treatment = 3.1 mg.g DM^{-1}. $LSD_{sucrose}$ within treatment = 0.3 mg.g DM^{-1}. $LSD_{sucrose}$ between treatment = 0.8 mg.g DM^{-1}.

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Chapter 7
General Discussion

1. Introduction

Ripening is a complex, highly ordered process that requires interaction between sugars, ripening enzymes, cell walls, lipids, phytohormones, water and temperature. It is not sufficient to consider these factors in isolation, and a better understanding of these interactions is needed for a greater understanding of avocado postharvest physiology. The trends in these parameters are of more interest than the numerical values, because considerable variation has been shown between fruit within the same orchard, between production locations within South Africa (Chapter 3) and even more so between different countries (Donetti and Terry, 2010). The above factors are discussed and a ripening model that integrates these factors is proposed. The commercial implications of this research are then discussed.

2. Sugars

There is still no definitive answer to what the primary carbon and energy sources in avocado is although the heptoses are the most likely candidates, because they are the only non-structural carbohydrates present in sufficient concentration to sustain respiration during ripening, but there was no conclusive evidence from previous reports or this study. This is probably because the heptoses are multi-functional, being utilised as carbon and energy sources, as an anti-oxidant (Tesfay *et al.*, 2010) and possibly as a cryoprotectant (Häfliger *et al.*, 1999). The heptose concentration generally declines during fruit maturation, but depends on source-sink relations, so rather than recommending a certain preharvest threshold value for the mannoheptulose and perseitol, it is recommended that tree health is maintained, using appropriate horticultural practices, as discussed in Whiley *et al.* (2002), to maximise fruit quality and fruit are only harvested when horticulturally mature. Similarly good postharvest management will reduce the loss of water and sugars and maintain fruit quality for longer. This is the critical stage for fruit quality because fruit can deteriorate after a few hours of mismanagement because of increased water loss and carbohydrate consumption (Chapters 6 and 7). These good postharvest management principles include: removing the field heat as soon as possible after harvest, limiting handling, reducing water loss, limiting storage period

and maintaining recommended temperature protocols during cold storage and ripening.

Although glucose and fructose are present in much lower concentrations, these sugars are still critical in postharvest quality and should not be dismissed. These sugars are involved in ABA metabolism (Richings *et al.*, 2000), and therefore will affect ripening. Also, glucose is the monomer of cellulose and therefore a major component of the cell wall. The earlier increase in glucose concentration in fast- compared with slow-ripening fruit (Table 4, Chapter 3) is thought to be because of increased cellulase activity soon after harvest - which was observed. The effect of this increased glucose is thought to stimulate ABA turnover (Richings *et al.*, 2000) and stimulate the climacteric response (Adato *et al.*, 1976). As the cell wall is increasingly solubilised and depolymerised, the activity of cellulase declines post-climacteric (Figure 12A, Chapter 4), resulting in a decrease in glucose production. Glycolysis - for utilisation in the Krebs Cycle - results in the decline in glucose concentration.

Fructose is present in very low concentrations and the slight increase may be due to the activity of glucose isomerase (D-xylose aldose-ketose-isomerase, EC 5.3.1.5) converting glucose to fructose and the hydrolysis of sucrose. Fructose is the precursor of triglyceride synthesis (Basciano *et al.*, 2005; Zavaroni *et al.*, 1982); it is suggested that the low concentration of fructose in avocado mesocarp is due to the high concentration of triglycerides in the tissue. This is noticeable when one compares the fructose:triglyceride ratio in the West Indian (Lowland) race and Guatemalan and Mexican races: The West Indian race has a higher concentration of fructose and lower concentration of triglycerides while the other two races have a lower fructose:triglyceride ratio. Since lipid synthesis is so highly favoured in avocado fruit, and since triglycerides are not actively involved in postharvest physiology (Chapter 3), the concentrations of monosaccharides are low and this limits the carbon source for other compounds such as proteins.

It is suggested that sucrose is predominantly a transport sugar to deliver glucose and fructose to the fruit, although it is still important in cellular functioning (Cripps, 2001; Meyer and Terry, 2010). Sucrose is therefore indirectly critical for fruit development as a provider of the substrate for the cell wall and lipids – the major contributors to dry matter in avocado fruit.

3. Enzymes and Cell Wall

Cellulase, PG and PME are critical in avocado ripening. It was shown that there were post-storage effects of the cold storage treatments on cellulase activity, but less so on PG activity while PME activity was highly variable and no conclusion could be drawn on the effect of cold storage treatments on PME activity (Figure 12, Chapter 4). Cold storage, in general, increased the activity of both cellulase and PG, and the fruit stored in regular atmosphere exhibited a faster rise in cellulase activity post-storage probably because of increased water loss compared to the bagged fruit (Table 5, Chapter 4). PME activity has been shown to be highly variable (Fuchs and Zauberman, 1987; Jeong and Huber, 2004; Kaiser *et al.*, 1996; Zauberman and Jobin-Décor, 1995) and it is becoming increasingly evident that PME activity does not simply decline postharvest as previously thought (Awad and Young, 1979), although some fruit do exhibit this trend.

The considerable variation in enzyme activity between fruit is thought to contribute to variable ripening, but even fruit with comparable enzyme activity showed differences in the days to ripen, suggesting that differences in the cell wall structure contribute to variable ripening. The cell wall structure has only been studied in relation to ripening, not in relation to ripening rate. This may prove to be an important aspect of avocado ripening physiology.

4. Lipids

Being an oleaginous fruit, the oil concentration and quality is important to the eating quality and health benefits of the fruit. The oil concentration was about 15% lower than reported elsewhere for unripe 'Hass', but did increase according to previous findings, using a similar method of extraction (Meyer and Terry, 2008; Meyer and Terry, 2010; Mostert *et al.*, 2007). In individual fruit, oil concentration fluctuated 5-15% around the equator during ripening, which may be due to differences in both cell wall structure and oil deposition during fruit development.

It is a concern that the moisture content or dry matter (MC/DM) did not correlate well with oil concentration in this small dataset (Chapter 3). This has been noted before, but the use of MC/DM as a maturity marker will persist because it provides an acceptable measure of maturity, if done correctly, that is cheap and easy to measure. Hand-held NIR could be used to measure oil concentration, but the cost of instrumentation is currently prohibitive to most growers.

5. Phytohormones

Ethylene and ABA have been extensively studied in avocado fruit because these phytohormones, with their interactions with other cellular compounds, are critical in fruit ripening and quality. There appears to be differences in ethylene sensitivity or ACC synthase functioning in fruit from the different production locations, with Howick fruit having a significantly lower ethylene production rate but still capable of completing ripening (Fig 1E-F, Chapter 3). The causes of this difference in ethylene production are uncertain, but it seems to be enzymatic and environmental, rather than substrate-limited. The methionine substrate is recycled during ethylene biosynthesis, so an increasing pool of methionine is not required and the ethylene biosynthesis is regulated by the isoforms of ACC synthase (Wang *et al.*, 2002; Wills *et al.*, 1998). ACC synthase is in turn up-regulated by abiotic stress, which fits the proposed ripening model (Figure 23). Perhaps cultivar and production location differences are related to ACC synthase synthesis, and therefore the development of ethylene-related physiological disorders during cold storage. This is worth investigation in avocados. The ethylene production rate (measured at removal from cold storage) was significantly reduced by the use of modified humidity packaging and 1°C storage, which is thought to be because these treatments reduced water stress and prevented the stimulation of the climacteric and therefore ripening during cold storage (Chapter 4).

ABA is more difficult to quantify than ethylene and research on ABA in avocados has been scarce in the last twenty years, although there have been many genetic, molecular and biochemical studies on ABA biosynthesis recently (Seo and Koshiba, 2002) which should be related to avocado physiology. The involvement of ABA in ripening and fruit quality was confirmed in previous studies and ABA synthesis should be limited by reducing fruit stress (Cutting and Bower, 1987; Cutting *et al.*, 1989).

6. Water

Water can be considered the most important compound for plant growth regulation, and its importance is often under-acknowledged. Fruit quality is reduced by pre- and postharvest water stress (Bower *et al.*, 1989). Water is 50-78% of mature avocado fruit at harvest. Product shrinkage, due to small losses in water, should be minimised to improve returns. Besides product shrinkage, water loss and temperature are major factors that affect postharvest fruit quality. Water loss during cold storage must be minimised by reducing the vapour pressure deficit of the storage atmosphere. This can be done by reducing the storage temperature,

increasing the relative humidity or altering the atmosphere using MH bags; wax can be used to limit transpiration. It is proposed that water is the ripening inhibitor and as fruit lose water ABA synthesis is stimulated, triggering ripening (Adato, 1974; Adato *et al.*, 1976; Bower and Cutting, 1988). The present literature supports this theory but another study investigating the role of ABA specifically in ripening, in relation to water status, ethylene and quality would yield more direct answers.

7. Ripening Model

An aim of this research was to construct an integrated ripening model for avocados. A model, taking into account water loss, ABA, ethylene, respiration, cellulase, polygalacturonase and sugars, is proposed in Figure 23. Ripening is exceedingly complex and this is an attempt to produce a simple model, drawing together decades of avocado research and results from this project. It must be stressed that this model is not complete, but it does lay a foundation for future research on avocado ripening. The following explanation is offered: the supply of water and nutrients from the mother plant to the fruit is terminated at harvest and the resultant water stress stimulates ABA synthesis (Walton, 1980). This in turn stimulates ethylene synthesis (Adato *et al.*, 1976; Cutting *et al.*, 1986). Ethylene causes the autocatalytic response by stimulating ACC synthase and ACC oxidase activity (Blumenfeld *et al.*, 1986), but cellulase and PG synthesis is stimulated and controlled by ethylene and in turn stimulates ethylene synthesis. At low concentrations ethylene is a gene transcriptor for cellulase and PG, but these enzymes also up-regulate the ethylene biosynthesis pathway, which would increase ethylene synthesis (Buse and Laties, 1993; Fuchs *et al.*, 1986; Fuchs and Zauberman, 1987). At higher concentrations, ethylene is also involved in the translational control of these enzymes (Buse and Laties, 1993). The increase in cell wall solubilisation and depolymerisation, from the activity of cellulase in particular, causes an increase in the concentration of glucose and other sugars. This increase in glucose results in an increase in ABA synthesis and breakdown (Richings *et al.*, 2000; Seo and Koshiba, 2002), which would up-regulate the cycle.

The increase in metabolism from these responses causes the dephosphorylation of ATP to ADP. Since the availability of ADP limits the respiration rate, the transient increase in ADP would unlimit phosphorylation and increase the respiration rate, generating increased ATP which would allow an increase in metabolism, particularly protein synthesis and cellulase (Brady, 1976; Tucker and Laties, 1984). This elevated respiration, would be sustained until the energy reserves are depleted. From the results from this experiment, it is suggested that the

major energy reserve is mannoheptulose early in the ripening process and once mannoheptulose and perseitol are depleted, glucose is utilised as an energy source (Figure 10, Chapter 3). Perseitol is a storage form of mannoheptulose and is converted to mannoheptulose as the concentration of mannoheptulose declined. Mannoheptulose is oxidised to perseitol as an anti-oxidant (Tesfay *et al.*, 2010). It is suggested that mannoheptulose is also used as a source of carbon for protein synthesis. The increase in the concentration of glucose that occurs during cell wall solubilisation and depolymerisation would decline as the fruit approaches ripeness and the glucose is used to fuel respiration, reducing ABA turnover, concomitantly the fruit enters the senescent phase where catabolic processes predominate, and the ripening process is down-regulated. It is acknowledged that respiration is highly complex, and highly sensitive to the ambient temperature, so there are other means and mechanisms to increase the respiration rate and this would feedback to increase the rate of ripening.

The proposed model explains why exogenous ethylene has a greater effect on fruit softening in more mature fruit (Pesis *et al.*, 1978). The concentrations of ABA and cellulase are higher in more mature fruit, so exogenous ethylene would have a greater effect on the up-regulation of cellulase synthesis and ethylene production. In immature and less mature fruit, exogenous ethylene has no effect - or even an adverse effect - on ripening, because the concentrations of ABA and cellulase are too low, so the response is much less and fruit senesce rather than ripen (Bower and Cutting, 1988; Pesis *et al.*, 1978).

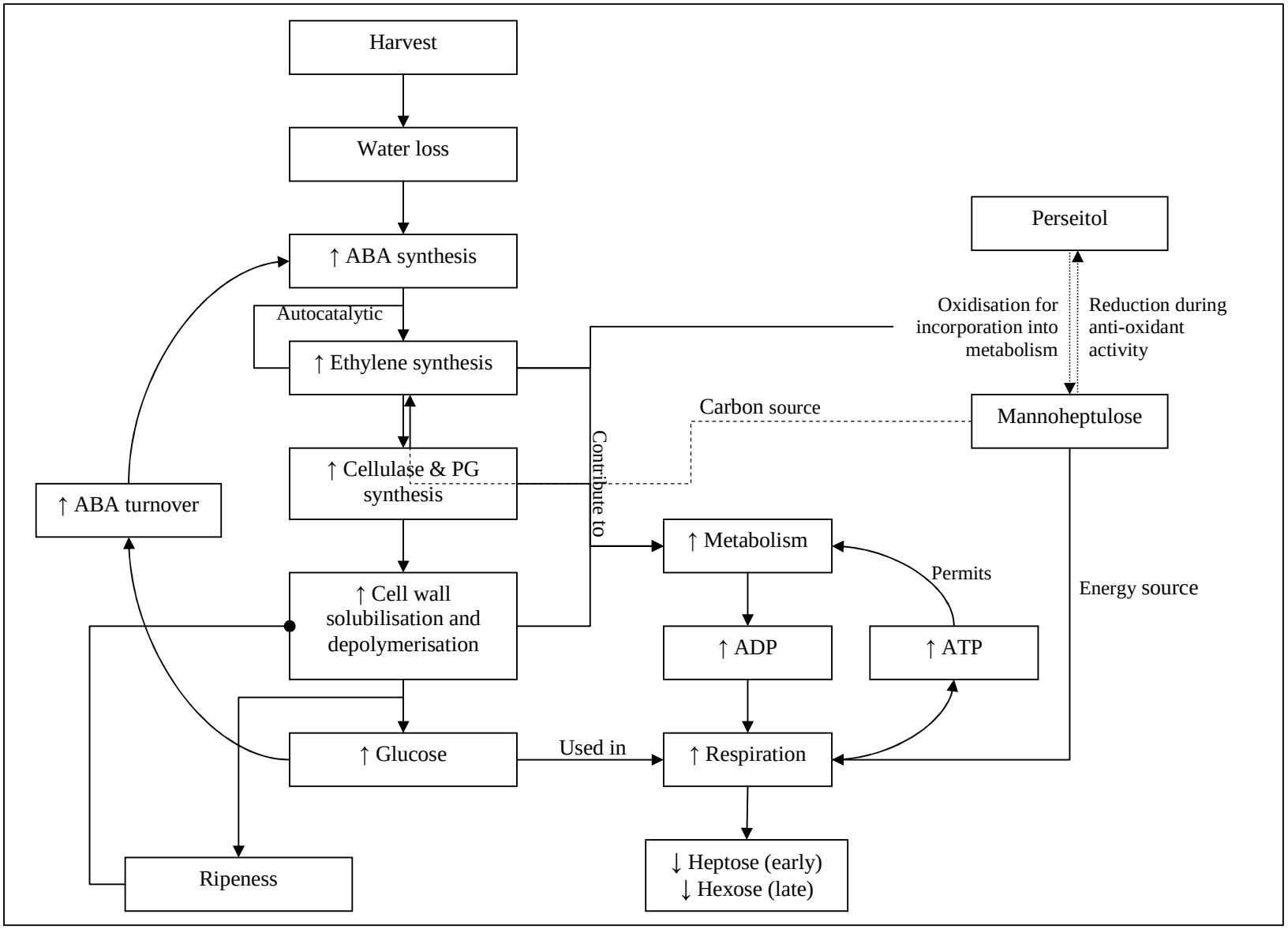

Figure 23: Proposed ripening model for avocado fruit

↑ = increase, ↓ = decrease, ▼ = initiates/causes, ● = terminates

8. Commercial Considerations

Temperature and water management are critical in maintaining fruit quality (Bezuidenhout, 1992; Bower and Cutting, 1987; Dodd *et al.*, 2007; Hofman *et al.*, 2002). There is still opportunity to apply this knowledge in the avocado industry and the PPECB and industry in South Africa is committed to improving this. There is potential for quality losses at all stages of handling in the value chain (Figure 24). Delays in delivering fruit to the packhouse, for the removal of field heat, still exist, although there are guidelines that recommend fruit are taken to the packhouse every four hours and not exposed to direct sunlight and fruit only left outside overnight in there is a substantial chance of cooling (PPECB, 2008). The problems from packhouse to wholesaler have largely been solved with logistics management, monitoring and dynamic temperature management[3]. Incorrect ripening protocols, poor temperature management at the supermarket and consumer ignorance are responsible for further quality losses. If fruit quality is to be improved, there has to be buy-in from and co-operation between all role-players in the industry. Growers can contribute to improved fruit quality by adhering to guidelines and transporting fruit to the packhouse with minimum delays. The real challenge is to get buy-in from role-players at the end of the value chain. It needs to be demonstrated that it is in the best interest of the ripeners, supermarkets and consumers to handle fruit correctly. Perhaps ripening protocols need to be developed by the industry for ripeners abroad to follow, as there seems to be a lack of understanding in avocado physiology and management at some facilities. Educational material for fresh produce managers in the supermarkets and consumers may help improve fruit quality at consumption – but this is much easier proposed than implemented.

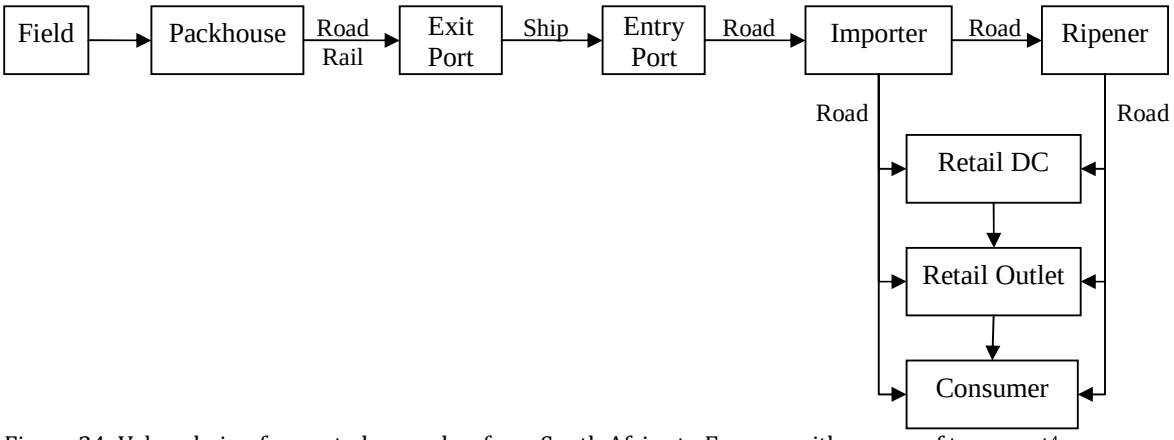

Figure 24: Value chain of exported avocados, from South Africa to Europe, with means of transport[4].
DC = Distribution Centre

[3] Pers. comm. J.J. Bezuidenhout, Westfalia Technological Services, Tzaneen, South Africa
[4] Pers. comm. H. Boyum, Westfalia Marketing, Tzaneen, South Africa

9. Commercial Implications of Study

The commercial aim of this research was to study the effects of 1°C cold storage and MHP on fruit physiology. These treatments result in improved fruit quality compared to the commercial control, in terms of reduced mass loss, water loss, ethylene production, respiration, softening, heptose consumption, after removal from cold storage, without commercially affecting fungal rots, physiological disorders or external chilling injury (Chapter 4). In response to the repeated success of reduced temperature shipping trials, commercial shipping temperatures for late season South African fruit to Europe were reduced to 3.0°C, at the time of writing, and will be reduced further, once suitable containers are available and fruit quality is acceptable[5]. The application of MHP needs to be adapted for commercial operations (Hofman *et al.*, 2002) because there is a reduction in cooling especially on warm days (Bower and Blakey, 2008). A carton insert, similar to table grapes may be a feasible option. This will increase the packaging and cost of shipment, but will be balanced by the reduction in mass loss, no need for waxing, reduce d variable ripening and improved fruit quality.

Ancillary to this was the investigation of the potential of NIR in the avocado industry. This aspect of the project has progressed to commercial testing of handheld and online NIR for a range of parameters at Westfalia Technological Services in 2010 and 2011. This technology has progressed incredibly and it is now possible to accurately sort fruit at a high throughput (up to 12 fruit.s^{-1}.$lane^{-1}$) for up to four parameters[6]. It is a given that avocados are highly variable, due to various uncontrollable genetic and environmental reasons, NIR offers the opportunity to sort fruit into less variable subsets (cartons or pallets) while also separating fruit with poor internal quality from those with good internal quality.

10. Future Research

Avocado physiology is exceedingly complex. To truly understand a species' physiology, a combination of genomics, transcriptomics, metabolomics and proteomics, with focus on the latter, preferably with non-destructive sampling, is needed. As very little is known at the genetic and molecular level of the avocado, understanding of the physiology together with potential for disorders and the best manner in which to store and transport fruit to ensure a desired quality is still incomplete. Identification of the critical enzymes, metabolites and structural carbohydrates for in-depth studies is needed. The use of chemometrics is

[5] Pers. comm. J.J. Bezuidenhout, Westfalia Technological Services, Tzaneen, South Africa
[6] Taste Technologies Ltd, Auckland, New Zealand

increasing, and would be a very useful tool in these studies to understand these complex interactions and relationships.

11. Conclusion

This project has improved the understanding of avocado ripening physiology and the interactive effects of production location, maturity, low temperature storage and modified humidity packaging. This is important for future design of optimal storage and shipping protocols. Previously, various aspects of avocado fruit physiology have been studied, but not in a comprehensive manner which takes into account important interactions of pre- and postharvest conditions which impact on final fruit quality at consumer level. Using some of the important parameters measured, it has also been possible to demonstrate the potential for NIR spectroscopy as a valuable tool in the avocado industry for non-destructive and possibly orchard or packline prediction of quality outcomes, and thereby allowing the packer or marketer to better target logistic requirements and sales opportunities.

Beyond the obvious recommendation for improved postharvest management to better maintain fruit quality, a model explaining fruit ripening is postulated. There are many factors which contribute to ripening and fruit quality which still need to be investigated, so this model is not complete, but lays the foundation for further research into avocado ripening physiology. It is envisioned that as NIR technology improves and is used more extensively in the avocado industry, non-destructive sampling will provide a window into fruit to allow a greater understanding of how these factors influence ripening. Once calibration models are developed for each factor of interest, many more samples can be analysed repeatedly, at a much faster rate and lower cost, to provide more conclusive answers. It is hoped that some of the results from this research, particularly NIR, will be used commercially in the near future.

The paradigm that avocados cannot be stored below 5°C without developing chilling injury has again been shown to be incorrect. South African ‘Hass’ avocados can be successfully stored at 1°C. The use of modified humidity packaging provides a means of limiting metabolism during cold storage, particularly for fruit stored at 5.5°C and may offer a viable commercial postharvest treatment. Semi-commercial testing including fruit from different production areas, maturities and a number of seasons is required before these treatments are used commercially.

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