# SPECIAL CARBOHYDRATES OF AVOCADO – THEIR FUNCTION AS ‘SOURCES OF ENERGY’ AND ‘ANTI-OXIDANTS’

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# DECLARATION

I, **SAMSON ZERAY TESFAY**, 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.

______________________________

**Samson Zeray Tesfay**
December 2009

I certify that the above statement is correct.

______________________________

**Dr. Isa Bertling**
Supervisor
December 2009

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To my creator for giving me life, energy and ability to conduct the experiment.

# ABSTRACT

There is increasing interest in special heptose carbohydrates, their multifunctional roles from a plant physiological view point in fruit growth and development as well as in the whole plant in general due to their potential in mitigating photo-oxidative injury to the whole plant system and the image of avocado as 'health fruit'. Studies have been carried out to investigate the role of avocado heptoses, rare carbohydrates predominantly produced in avocado. Several authors have documented various research findings and speculated on multifunctional roles of avocado special sugars. However, few reports have made an attempt to elucidate the multifunctional roles of avocado heptose carbohydrates as: 'sources of energy', storage and phloem-mobile transport sugars, and precursors for formation of anti-oxidants.

Assessing the avocado carbohydrates over the plant growth and development during ontogeny may, therefore, offer clues to better understand whole plant behaviour. Plant sampling was carried out over different developmental stages. Using plants grown in the light versus etiolated seedlings; sugar determinations were also done to determine what sugar is produced from which storage organs. The sugars were extracted and analysed by isocratic HPLC/RID. The embryo had 47.11 % hexose and 52.96 % heptose sugars. The seed, however, also released significant amounts of *D*-mannoheptulose (7.09 ± 1.44 mg $g^{-1}$ d. wt) and perseitol (5.36 ± 0.61 mg $g^{-1}$ d. wt). Similarly fruit and leaf tissues had significant amounts of heptoses relative to hexoses at specific phenological stages. In postharvest 'ready-to-eat' fruit the following carbohydrate concentrations were as follows:exocarp heptoses 13 ± 0.8; hexoses 4.37 ± 1.6 mg $g^{-1}$ d. wt, mesocarp heptoses 8 ± 0.2; hexoses 3.55 ± 0.12 mg $g^{-1}$ d. wt), seed heptoses (only perseitol) 13 ± 1.1; hexoses 5.79 ± 0.53 mg $g^{-1}$ d. wt. The results of

this experiment was the first to demonstrate that the heptoses *D*-mannoheptulose, and its polyol form, perseitol, are found in all tissues/organs at various phenological stages of avocado growth and development. Secondly, heptoses, as well as starch are carbohydrate reserves that are found in avocado. The heptoses, beyond being abundantly produced in the avocado plant, are also found in phloem and xylem saps as mobile sugars.

The study also presents data on the interconversion of the C7 sugars *D*-mannoheptulose and perseitol. It is deduced that *D*-mannoheptulose can be reduced to perseitol, and perseitol can also be oxidized to *D*-mannoheptulose by enzymes present in a protein extract of the mesocarp. The potential catalyzing enzyme is proposed to be an aldolase, as electrophoretic determinations prove the presence of such an enzyme during various stages of development in various plant organs.

Avocado heptoses play an important role in plant growth and development and in fruit in particular. Moreover, they are reported as sources of anti-oxidants, and contribute significantly to fruit physiology if they function in coordination with other anti-oxidants in fruit tissues. To evaluate the presence of anti-oxidant systems throughout avocado fruit development, various tissues were analysed for their total and specific anti-oxidant compositions. Total anti-oxidant levels were found to be higher in the exocarp and in seed tissue than in the mesocarp. While seed tissues contained predominantly ascorbic acid (AsA) and total phenolics (TP), the anti-oxidant composition of the mesocarp was characterised by the C7 sugar, *D*-mannoheptulose. Among the anti-oxidant enzymes assayed, peroxidase (POX) and catalase (CAT) were present in higher concentrations than superoxide dismutase (SOD) in mesocarp tissue. Different anti-oxidant systems seem to be dominant within the various fruit tissues.

Carbohydrates are the universal source of carbon for cell metabolism and provide the precursors for the biosynthesis of secondary metabolites, for example via the shikimic acid pathway for phenols. The preharvest free and membrane-bound phenols, catechin and epicatechin, are distributed differently in the various fruit tissues. Membrane-bound and free phenols also play a role as anti-oxidants, with free ones being more important. KSil (potassium silicate) application to fruit as postharvest treatment was used to facilitate the release of conjugates to free phenols via lysis. This treatment improved fruit shelf life. Western blotting also revealed that postharvest Si treatment affects the expression of enzymatic anti-oxidant-catalase (CAT).

Overall the thesis results revealed that C7 sugars have anti-oxidant properties and that *D*-mannoheptulose is the important anti-oxidant in the edible portion of the avocado fruit. *D*-mannoheptulose is furthermore of paramount importance as a transport sugar. Perseitol on the other hand acts as the storage product of *D*-mannoheptulose, which can be easily converted into *D*-mannoheptulose.

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# DEDICATION

This thesis is dedicated to my beloved wife Nonkululeko A. Zwane, without you this would have never happen. I love you.

# CHAPTER 1
# GENERAL INTRODUCTION

Carbohydrates are molecules containing carbon, hydrogen and oxygen in the molecular formula of $(CH_2O)_n$ where n is greater than 3. They also comprise polyhydroxy aldehydes (aldoses), ketones (ketoses), alcohols or acids and their simple derivatives as well as any compound that may be catagorized to these, which have characterstics of carbohydrates and furthermore as defense mechanisms (Cowan, 2004). Carbohydrates are the major source of energy and are used as building blocks in plant biosynthetic reactions, participating in the formation of proteins and lipids (Duffus and Duffus, 1984). Owing to this multi-functional image in plant growth and development, various studies have been undertaken on carbohydrate synthesis, productions and distribution.

All carbohydrates in plants are synthesized via the process of photosynthesis in leaves. The Calvin cycle describes the net fixation of carbon dioxide (Figure 1). The principal product of photosynthetic carbon assimilation in the chloroplast is triose-P, which is exported either to the cytosol to make soluble sugars, or retained within the chloroplast to make starch or to regenerate RUBP via Calvin cycle intermediates (Leegood, 1996). Various plant parts are supplied with carbohydrates over the course of growth and development in response to plant source-sink strength. This carbohydrate partitioning to plant organs and the transport of such compounds to their cells, as sink destiny, depends on active cell metabolism (González-Real et al., 2009). The major transport form in plants is the disaccharide sucrose, but in some plants also tri- and tetrasaccharides or sugar alcohols. During the night carbohydrates are stored in leaves, supplying the rest of the plant with carbohydrates. Plants store carbohydrates most commonly in the form of starch, but other low molecular weight oligosaccharides

(Heldt, 2005) or sugar alcohols to take over this function (Liu et al., 1999a; Bertling et al., 2007). However, polyols have also been postulated to fulfil a function as storage compounds and/or energy sources depending on their specific site in particular plant organs. Due to the lack of enzymes which catabolize polyols, such compounds are not rapidly utilized, and thus, are particularly useful as storage and transport forms. Polyols may also play a role as compatible solutes in source leaves, allowing the continuation of photosynthetic activity and carbon metabolism under adverse environmental conditions such as water stress (Loescher et al., 1985).

Carbohydrates are a major contributor to plant respiratory activity (Duffus and Duffus, 1984). Respiration is a process of the degradation of metabolites via glycolysis and/or the oxidative pentose phosphate pathway (OPPP), with its products being oxidized by the tricarboxylic acid cycle (TCA) and the resultant pyridine nucleotides used in the synthesis of ATP via oxidative phosphorylation in mitochondria. The primary substrates of carbohydrate respiration are hexose phophates, which originate from the degradation of sucrose and reserve polysaccharides such as starch (Duffus and Duffus, 1984), as well as other sugar alcohols (Liu et al., 1999a). *D*-mannoheptulose and its polyol form, perseitol, are found in all parts of the avocado (*Persea americana* Mill.) tree (Liu et al., 1999a, b). In avocado, C7 sugars have been postulated to be associated with metabolic processes of fruit development as well as being involved in respiratory processes associated with fruit maturation, postharvest quality and fruit ripening (Liu et al., 1999b).

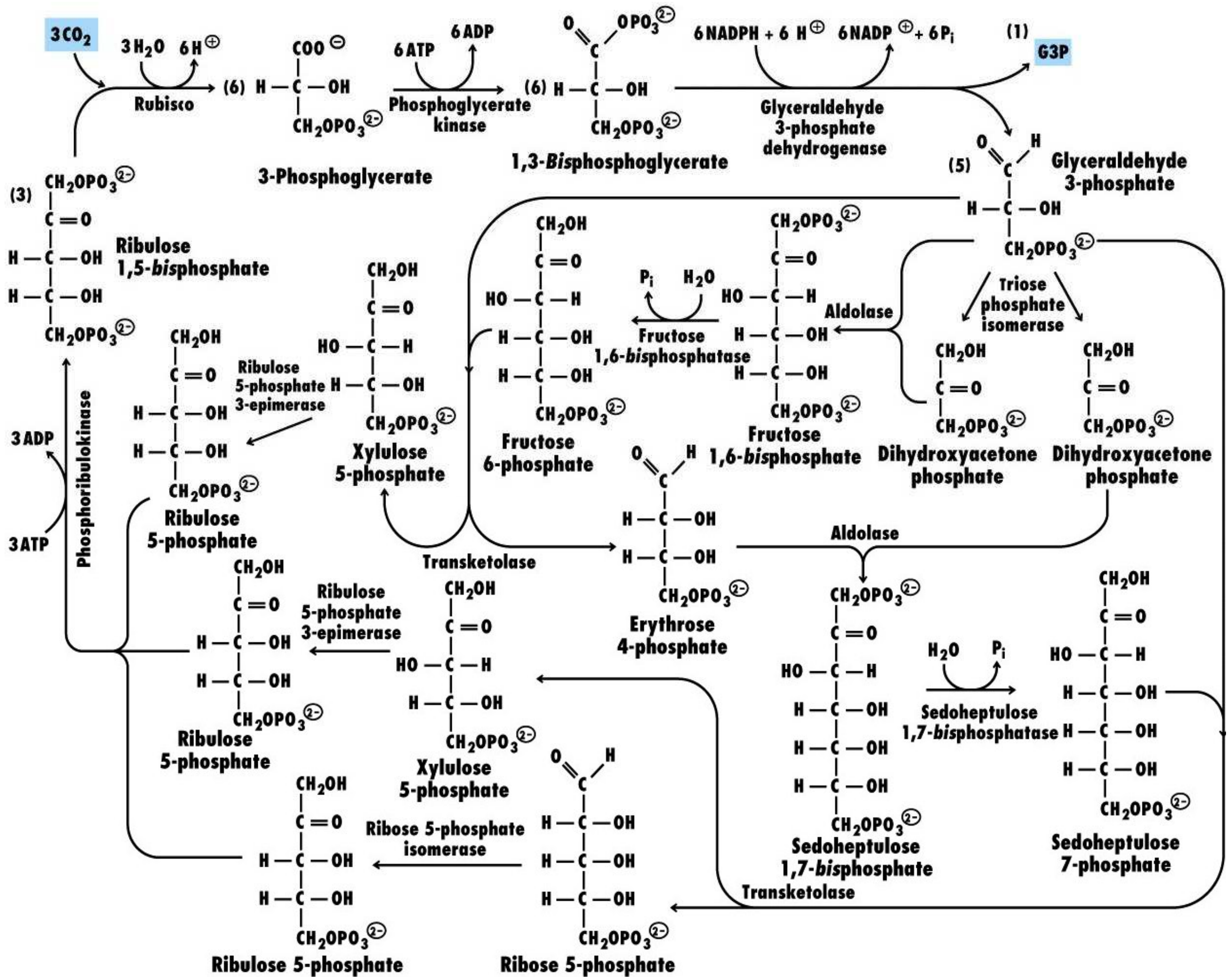


Figure 1. Calvin cycle depicting formation of sugar compounds, initially formed from $CO_2$ (Horton, 2006).

Carbohydrates also play an essential part in the synthesis of numerous anti-oxidant compounds that are involved in the protection against different harmful compounds (free radicals or ROSs), which can cause damages to proteins, lipids and DNA, leading ultimately to cell death. Glucose feeding into primary metabolism can result in enhanced reducing power in the form of NADH or NADPH through glycolysis and the OPP pathway, respectively

(Averill-Bates and Przybytkowski, 1994; Zhang et al., 2000; Ryu et al., 2004). Glucose is also a sugar precursor for certain anti-oxidants, for carotenoids, which comeoff the OPP pathway (Pallett and Young, 1993) and for ascorbate synthesis (Foyer, 1993; Smirnoff et al., 2001), and yields the carbon skeletons of amino acids, including cysteine (Cys), glutamate (Glu), and glycine (Gly), which are the building blocks of the anti-oxidant glutathione (Noctor and Foyer, 1998). These amino acids are involved in defence systems to counteract oxidative stress through the ascorbate-glutathione cycle, in redox homeostasis, peroxide detoxification, or in the production of anti-oxidants.

Generally, carbohydrates are the universal source of carbon for cell metabolism and provide precursors in the biosynthesis of metabolites (Mann, 1987). The non-oxidative glycolysis of glucose, which yields phosphoenolpyruvate (PEP), and erythrose-4-phosphate, underlines all metabolic functionality (Haslam, 1993). Glucose is also the reported precursor for synthesis of ascorbic acid (Smirnoff, 2000).

## SUGARS AS CARBOHYDRATE RESERVES

The avocado fruit is characterized by its nutritious flesh, which contains an exceptionally high percentage of unsaturated oils (20 to 30%, of fresh weight) (Davenport and Ellis, 1959; Biale and Young, 1971; Whiley and Schaffer, 1994). Avocado requires a high energy input during fruit growth and development, as a large amount of energy is not only needed for oil production but also for the development of the relatively large avocado seed. This energy is provided by carbohydrates derived from photosynthesis (Wolstenholme, 1986). Carbohydrate storage and metabolism in fruit tissues are therefore important aspects of fruit

growth and development, as oil accumulation and beneficial anti-oxidant compounds are affected (Oliveira and Priestley, 1988).

Carbon allocation in the avocado plant differs over the growing season. As avocados are growing during the winter season, the plant supplements its stored reserves with recently fixed carbon via photosynthesis. Liu et al. (1999a) reported an increase in stem total soluble solutes (TSS) and starch as a result of winter photosynthesis, confirming storage of more carbon forms. This suggests that sugar accumulation might be acting to enhance shoot cold hardiness (Kramer and Kozlowski, 1979). The decrement in stem TSS from winter peak levels coincides with an increase in stem starch in spring, indicating that soluble sugars are converted to starch. Starch accumulation may also occur due to leaf photosynthesis during the spring season. Stem carbohydrate reserves might therefore be an important energy source for flower development (Jackson and Sweet, 1972). Liu et al. (1999a) also reported that avocado uses its stored stem reserves more than its trunk and root reserves during active tree growth (spring). Active tree growth reflects the stem reserves used to support developmental phases which are: vegetative growth, flowering, fruit set and fruit development until maturity. Similarly, the decline in stem TSS and the transport of canopy photosynthetic assimilates during early active vegetative tree growth are aligned with the spring vegetative shoot flush and the periods of fruit set and rapid fruit expansion. It has been postulated that avocado productivity is dependent upon the accumulation and utilization of carbohydrates within the tree (Wolstenholme, 1986; Whiley and Wolstenholme, 1990). It is thought that carbohydrate allocation within a tree might determine adequate vegetative growth, annual fruit set, fruitlet abscission, and fruit production. Similarly, fruit size depends on fruit expansion due to cell division and cell expansion (Cowan et al., 1997), both processes require carbohydrate

allocation. The tree carbohydrate status is affected by factors such as weather condition, water availability, and nutrient status (Robinson et al., 2002).

Avocado fruit are known for their high respiration rate throughout growth and during postharvest ripening (Bower and Cutting, 1988; Whiley et al., 1992; Blanke and Whiley, 1995). Liu et al. (1999b) reported that the C7 sugars, *D*-mannoheptulose and perseitol, were the major nonstructural carbohydrates present in avocado fruit tissues. TSS is also found as a major biomass component of young fruit during early developmental stages. Hence, it was postulated that early fruit growth depends specifically on the accumulation of fructose, glucose, *D*-mannoheptulose, and perseitol reserves (Liu et al., 1999b).

In general, the attainment of greater fruit size is linked to: carbon accumulation and storage. During increased fruit growth, soluble sugars are the major carbon source resulting in an increase in biomass. The fruit is, hence, a strong sink and stores photoassimilates (Cannell and Dewar, 1994) during the early developmental stage. Once fruit growth slows, the plant experiences a gradual decrement in the amount of accumulated sugar. This is also correlated with the accumulation of oil. The significant decrease in the common sugars (sucrose, fructose, and glucose) and *D*-mannoheptulose in the peel and flesh during postharvest cold storages (1 or 5 ºC), is due to sugars being used as the energy source for the respiration (Liu et al., 1999b).

## SUGAR ALCOHOLS IN HIGHER PLANTS

Sugar alcohols comprise aldose and ketose sugars (and their derivatives) that have been reduced to form straight or branched chain polyols. The sugar alcohols are compounds consisting of three or more carbons bearing one or more hydroxyl group. Since these sugars

are derived from simple sugars, they can usually be regarded as “special kinds of sugar or carbohydrates” (Lewis and Smith, 1967). This summary on polyols will mainly focus on three main pentitols and hexitols: dulcitol (or galactitol), sorbitol (or glucitol) and mannitol, as well as the heptitols perseitol, D-mannoheptulose and volemitol.

### *Distribution of polyols*

In higher plants, seventeen alditols have been described and thirteen different alditols have been identified in angiosperms (Lewis, 1984). The three most frequent hexitols in angiosperms are galactitol, sorbitol and mannitol. Galactitol is predominantly present in the Celastraceae (*Euonymus*) and the Scrophulariaceae (*Antirrhinum*, *Digitalis*). Sorbitol is common in the Rosaceae such as *Malus*, *Pyrus*, *Prunus*, and is also found in the Plantaginaceae. Mannitol, the most widely distributed hexitol, is present in over 100 higher plant species distributed among several families including the Rubiaceae (*Coffea*), Oleaceae (*Lingustrum*, *Sorbus*, *Olea*), and Apiaceae (*Apium*, *Daucus*, *Carum*) (Lewis, 1984). Inositol is the substrate for the production and accumulation of methylated derivatives like *D*-ononitol (1-*D*-4-*O*-methyl-*myo*-Inositol) and *D*-pinitol (1-*D*-3-*O*-methyl*chiro*-inositol). These cyclitols are found in common ice plant (*Mesembryanthemum crystallinum*) and in other plants such as tropical legumes (*Cajanus cajan*), mangrove fern (*Acrostichum aureum* L.), maritime pine (*Pinus pinaster*) and mistletoes (*Viscum flavescens*) (Richter and Popp, 1992; Wanek and Richter, 1997). Some species of *Primula* contain the heptitol volemitol. *D*-mannoheptulose, has been reported in a wide variety of plants, but predominantly produced in avocado (LaForge, 1916). The polyols are most commonly derived from hexose sugars. Thus,

mannitol, sorbitol (or glucitol) and dulcitol (or galactitol) are derived from glucose, fructose, and galactose, respectively (Noiraud et al., 2001).

Table 1. Commonly-distributed sugar alcohols found in plants

| No. | Sugar alcohols | Common name of selected sugar alcohol | Reported occurrence in plant family | |
|---|---|---|---|---|
| 1 | Tetritols and pentitols | Erythritol | Fruits | Shindou et al.1989 |
| | | Xylitol | Rosaceae | Washüttl et al. 1973 |
| 2 | Hexitols | Sorbitol | Rosaceae, Plantaginaceae | Zimmermann and Ziegler, 1975; Bieleski, 1982 |
| | | D-Mannitol | Apiaceae, Oleaceae, Rubiaceae | Zimmermann and Ziegler, 1975; Bieleski, 1982 |
| | | Galactitol | Celestraceae, Scrophulariaceae | Lewis, 1984 |
| | | Polygalitol | Polygalaceae | Sakuma and Shoji, 1981 |
| | | Styracitol | Styracaceae | Pauletti et al., 2006 |
| 3 | Heptitols | Volemitol | Primulaceae | Häfliger, 1999 |
| | | Perseitol | Lauraceae | LaForge, 1916 |
| | | D-mannoheptulose | Lauraceae | LaForge, 1916 |

*Synthesis of polyols*

The most prevalent plant polyols are the sorbitol, mannitol, and galactitol. Although some polyols are photosynthetically produced by plants, in most living organisms, the first stage of the metabolism of polyols is their direct oxidation to sugars but preliminary phosphorylation of the polyol before oxidation to a sugar phosphate may also occur. Polyols are also produced by direct reduction of sugars or sugar phosphates (Lewis and Smith, 1967).

The biosynthetic pathways of the hexitols sorbitol (glucitol), mannitol, galactitol (dulcitol), and the pentitol ribitol have been established in higher plants. They use NADPH as a hydrogen donor and aldose phosphate as a hydrogen acceptor, together with the corresponding phosphatases. Galactitol, however, is formed directly from unphosphorylated Gal (and NADPH) (Negm, 1986). It has been demonstrated the most common alditols sorbitol, mannitol, and galactitol are phloem-mobile sugars (Lewis, 1984; Davis and Loescher, 1990; Moing et al., 1992; Flora and Madore, 1993).

Mannitol is syntheisized by NADPH-dependent mannose-6-phosphate reductase that catalyzes the conversion of mannose-6-phosphate to mannitol-1-phosphate, followed by dephosphorylation by a phosphatase (Rumpho et al., 1983). In most cases in various different plant species, mannitol synthesis occurs simultaneously with either sucrose synthesis, as in celery (Rumpho et al., 1983), or with raffinose oligosaccharide synthesis, as in Oleaceae (Zimmermann and Ziegler, 1975).

Sorbitol is also synthesized in source organs by the activity of aldose-6-P-reductase (Negm and Loescher, 1981) catalysing conversion of glucose-6-phosphate to sorbitol-6-phosphate. Sorbitol-6-phosphate is further converted to sorbitol by a specific phosphatase.

Sink organs have little or no capacity to synthesize sugar alcohols like mannitol or sorbitol (Loescher and Everard, 1996).

The cyclitol myo-inositol is also produced from glucose-6-phosphate (Loewus and Loewus, 1983). *myo*-Inositol is important in the synthesis of phytin, galactinol, raffinose, and stachyose in soybean seeds and is synthesized by *myo*-inositol phosphate synthase (MIPS; EC 5.5.1.4; Hegeman et al., 2001; Hitz et al., 2002) and *myo*-inositol monophosphatase (IMP1; EC 3.1.3.25; Ishitani et al., 1996; Styer et al., 2004).

Volemitol is synthesized by the action of a NADPH-dependent ketose reductase. Reductases involved in vascular plant alditol biosynthesis use aldoses (not ketoses) and phosphate esters (not free sugars) as their substrates (for review, see Loescher and Everard, 1996, Häfliger, 1999).

In avocado, three known enzymatic reactions generate a C7 intermediate: a) an aldolase reaction: erythrose-4-P + dihydroxyacetone-P↔sedoheptulose-1,7-bis-P; b) a transketolase reaction: xylulose-5-P + ribose-5-P↔sedoheptulose-7-P + glyceraldehyde-3-P; or c) a transaldolase reaction: fructose-6-P + erythrose-4-P↔sedoheptulose- 7-P + glyceraldehyde-3-P. However, the main route actively involved in the Calvin cycle for synthesis of C7 sugar alcohols has not been documented to date (Liu et al., 2002).

The physiological roles of polyols are similar to disaccharides and oligosaccharides, in that they are multifunctional in plants and are produced in response to different factors (e.g. stress factors).

*General function of sugar alcohols*

Numerous roles have been attributed to polyols. Polyols are osmotically active solutes, notably in response to abiotic stress. Their concentration increases in response to the stress in order to compensate for reduced cell water potential inside the cell (Popp and Smirnoff, 1995). Their hydroxyl groups can effectively replace water by establishing hydrogen bonds under water-deficit stress conditions and, therefore, protect enzyme activities and membranes, by functioning as osmoprotectant. It has been shown that in several plant species, sugar alcohols like mannitol, sorbitol or dulcitol are transport sugars (Zimmermann and Ziegler, 1975). The advantages of transporting sugar alcohols are that these compounds are highly soluble and also chemically inert. In addition to functioning as a transport sugars, sugar alcohols have also been shown to serve as storage for reduced carbon.

Inositol, cyclohexane hexitol, is an ubiquitous cellular component in a wide variety of organisms. Inositol phosphates function in signal transduction in cells (Hegeman et al., 2001). In contrast to inositol, mannitol and sorbitol are primary photosynthetic products in mature leaves (Stoop et al., 1996). High productivity and high photosynthetic rates in celery are linked to the synthesis of mannitol, which represents an additional sink for photosynthetically fixed $CO_2$ (Pharr et al., 1995). As a storage compound, mannitol is more reduced than hexoses and thus stores more reducing power (Cochrane, 1958). Furthermore, mannitol, in addition to being a versatile metabolite and osmoprotectant, is also an anti-oxidant (Smirnoff and Cumbes, 1989). Sorbitol may function as a compatible solute for providing osmotic adjustment, and as a protective molecule for proteins and membrane in plant cells under stress conditions such as high salt concentrations, drought and low temperature (Deguchi et al., 2002).

### *Seven-carbon sugars*

Avocado (*Persea americana* Mill., Lauraceae) contains the seven carbon (C7) sugar *D*-mannoheptulose and related C7 sugar perseitol (Figure 2) as major forms of nonstructural carbohydrates (Liu et al., 1999b, 2002). The plant is able to synthesize and translocate significant amount of seven-carbon (C7) sugar alcohols. The seven-carbon (C7) sugars, *D*-mannoheptulose and its polyol form, perseitol, are found in all parts of the avocado (*Persea americana* Mill.) at various phenological stages (Liu et al., 1999a). The concentration of C7 sugars and starch has been reported to be similar in various organs. It has therefore been postulated that both carbohydrates are major forms of carbohydrate reserves in avocado. The synthesis and abundance of these sugars over the entire life cycle of avocado furthermore indicates their importance in growth and development.

Liu et al. (2002) postulated that C7 sugars fulfil an important function not only in the storage of carbon but also in carbon assimilation and translocation. Furthermore, these compounds are predicted to provide reducing power and protect against different types of stresses. However, evidence for such a function has not been provided yet. However, Volemitol (D-glycero-D-manno-heptitol, a-sedoheptitol), an unusual seven-carbon sugar alcohol, has been reported to occur in avocado (Cowan, 2004) and in certain species of *Primula*, In the latter genus volemitol has a key role in photosynthetic assimilation, translocation and storage of carbon, provision of reducing power, as well as protection against different types of stresses (Häfliger et al., 1999).

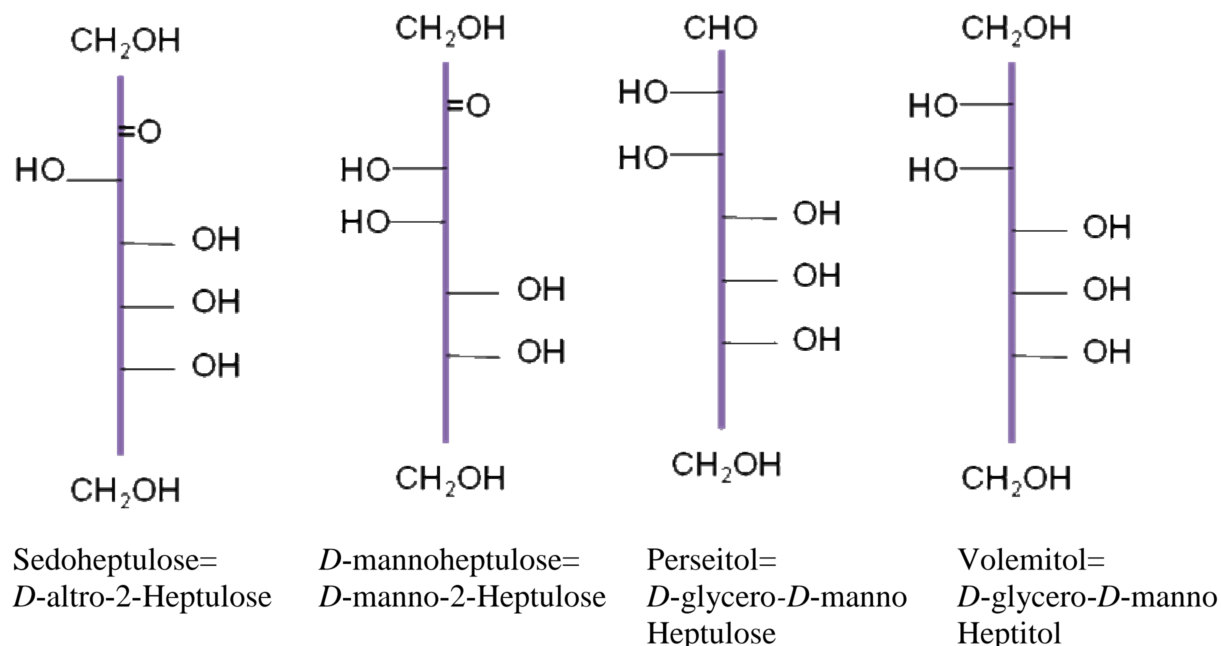


Figure 2. Fischer projections of four structurally related C7 sugars (Collins, 1987).

Polyols may perform two specific functions in metabolism: (a) storage of reducing power, and (b) co-enzyme regulation. This is substantiated by the fact that polyols are more reduced than ketoses and that the reversible synthesis of polyols from ketoses is coupled to co-enzyme oxido-reduction. Performance of these functions might also help to explain why polyols often coexist in abundance with other soluble carbohydrates (Lewis and Smith, 1967).

## ANTI-OXIDANTS

An anti-oxidant is “any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate” (Halliwell, 1995). To control the level of reactive oxygen species (ROS) and to protect cells under stress conditions, plant tissues contain several non-enzymatic anti-oxidants

(e.g. L-ascorbic acid, phenols) and enzymes scavenging ROS (e.g. SOD, CAT, peroxidases and glutathione peroxidase).

The avocado fruit (*Persea americana* Mill.) is well known for its high nutritive value (Bergh, 1992) and its favourable unsaturated to saturated fatty acid ratio of 14.3 to 1 (Slater et al., 1975). Avocados also contain relatively high amounts of iron and potassium (Wolstenholme, 1990). Besides the occurrence of rare C7 sugars, avocado is also unusual as it accumulates high amounts of fat-soluble substances, such as $\beta$-carotene (Human, 1987) and $\alpha$-tocopherol (Terasawa et al., 2006). Avocado furthermore contains high concentrations of phenol anti-oxidants, responsible for mesocarp browning of avocado fruit (Lidster et al., 1986).

From a plant physiological viewpoint the purpose of anti-oxidants is to scavenge reactive oxygen species (ROS), most commonly hydrogen peroxide, the superoxide radical and the hydroxide radical (Vranová et al., 2002) produced within the plant cell. Such ROS are formed during oxidative stress and have to be eliminated before oxidising vital cell components. Therefore, the occurrence of damaging concentrations of ROS needs to be counteracted to avoid permanent tissue damage (Seifried et al., 2007). Such counteraction occurs through a system of low molecular mass non-enzymatic anti-oxidants as well as through certain enzymes, such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (POX), these enzymes catalyse the destruction of ROS resulting in the production of non-damaging oxygen-containing compounds (Blokhina et al., 2003).

ROS are produced during normal cell metabolism as by-products of the energy-generating processes, photosynthesis and respiration (Vranová et al., 2002; Apel and Hirt, 2004). When the production of ROS is relatively low, the anti-oxidant capacity is sufficient to

maintain a balance between ROS production and anti-oxidant, thus re-establishing a redox homeostasis and allowing normal cell metabolism to continue. Under growth limiting environmental conditions, this redox balance is easily disturbed, potentially leading to a damaging ROS accumulation (Foyer and Noctor, 2005). Under unfavorable environmental conditions, such as temperature extremes, drought, or salt stress, the rate of carbon fixation is limited, causing an increase in photoinhibition potentially steering the photosystem toward overproduction of superoxide radicals and $H_2O_2$ (Foyer and Noctor, 2005) (Figure 3). Overproduction of ROS can cause membrane rigidification, peroxidation of membrane lipids, protein denaturation and DNA mutation (Borg and Schaich, 1988). Moreover, ROS are implicated in leaf senescence, fruits ripening and postharvest fruit spoilage (Leshem et al., 1986). To counteract the effects of ROS, anti-oxidant strength is essential to maintain normal plant growth and development.

As the avocado fruit requires a long developmental period of six to more than twelve months from flowering to maturity (Scora et al., 2002), it is likely that the fruit is exposed to environmental stresses during this extended period. Fruit must therefore be fitted with "stress-relieving" mechanisms in order to allow counteraction of stress and ROS accumulation during their long developmental period.

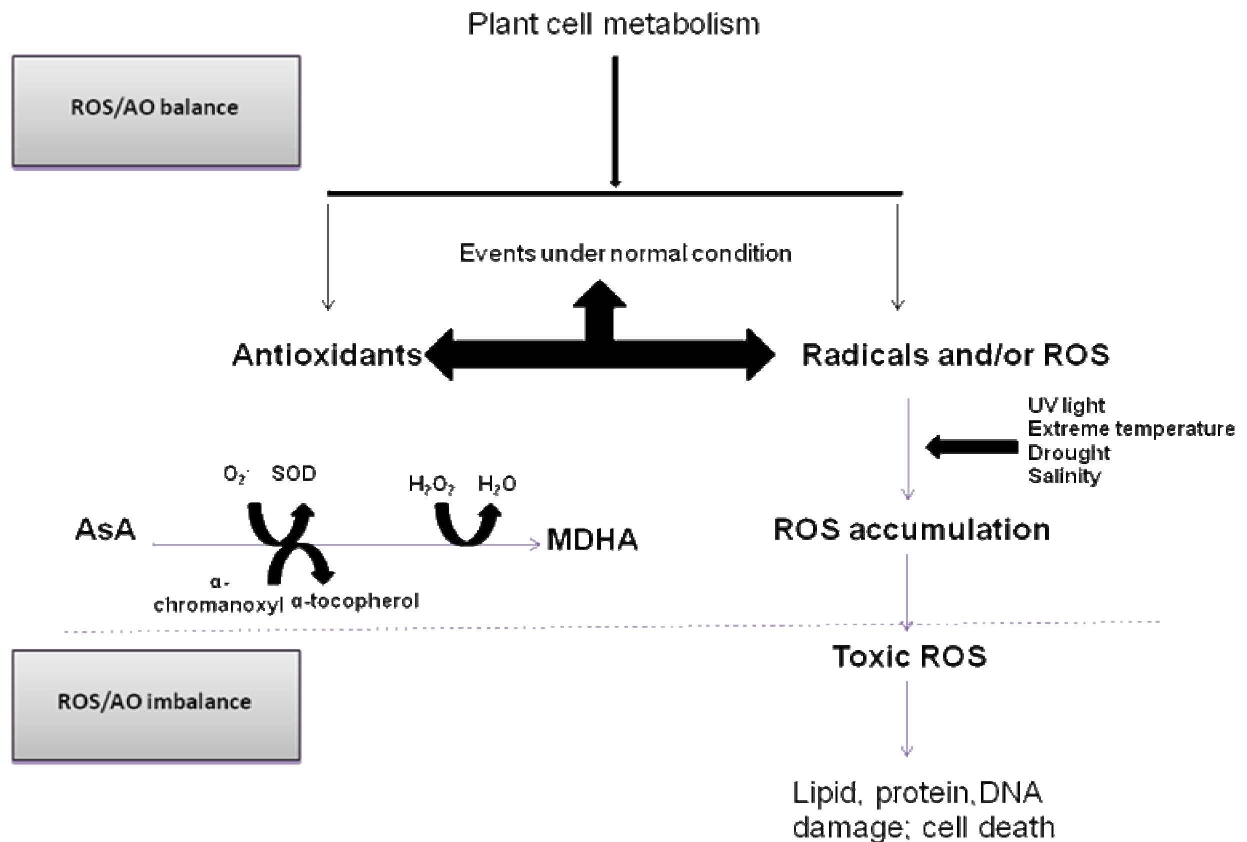


Figure 3. Schematic diagram of cellular production of anti-oxidants (AO) and reactive oxygen species (ROSs), and their state of metabolic equilibrium affected by different stress factors which may result in cell death. Ascorbic acid (AsA), monodehydroascorbate (MDHA), α-tocopherol and superoxide dismutase represent cell anti-oxidants; hydrogen peroxide ($H_2O_2$), singlet oxygen radical ($O_2^-$) represent ROS

*Ascorbic acid*

Ascorbic acid (AsA) has an important role in environmental stress resistance, including photooxidation (Conklin et al., 1996; Foyer, 1993; Noctor and Foyer, 1998; Smirnoff, 2000), where it acts as a free radical reductant in plant tissues, thereby reducing oxidative damage (Torres del Campo, 2005). The ability to donate electrons in a wide range of enzymatic and non-enzymatic reactions makes ascorbic acid the main ROS-detoxifying

compound in the aqueous phase (Figure 4). Ascorbic acid is a very important anti-oxidant compound for generating another important anti-oxidant compound, tocopherol. AsA regenerates tocopherol from tocopheroxyl radical providing membrane protection (Thomas et al., 1992).

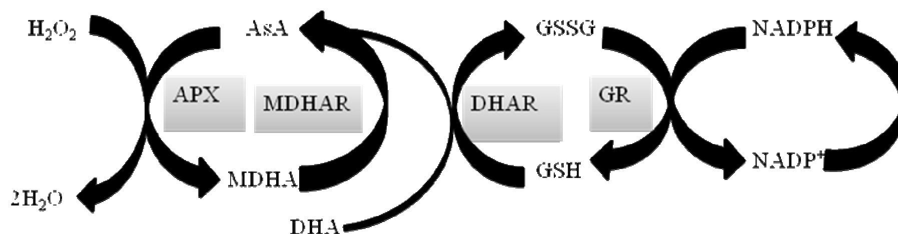


Figure 4. Ascorbate-glutathione cycle (Halliwell-Asada pathway). ascorbate-peroxidase (APX),; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase (May et al., 1998)

### *Phenolic compounds*

Phenolic phytochemicals are secondary metabolites, which constitute one of the most abundant groups of natural metabolites and form an important part of both human and animal diets (Bravo, 1998). Phenolic compounds posses an aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular weight polymer (Strube et al., 1993; Harborne, 1994). However, several plant phenolics and other plant products exist conjugated either to sugars (primarily glucose), as glycosides, or to other moieties. An increase in accumulation of free phenols improves the anti-oxidant pool of a tissue, thus enhancing post-harvest fruit quality and possibly improving the health benefit of fruit (Vattem and Shetty, 2002; Zheng and Wang, 2003).

*Superoxide dismutase (SOD)*

SODs are thought to be the primary cell enzymatic defense against its potential cytotoxicity. Under normal cell metabolism, the superoxide radical is a normal product of the univalent reduction of molecular oxygen. The scavenging of $O_2^{\cdot -}$ is achieved through an upstream enzyme, SOD, which catalyses the dismutation of superoxide to $H_2O_2$, which has a protective role against oxygen toxicity in all aerobic organisms (Bowler et al., 1992). The ability of plants to overcome oxidative stress only partly relies on the induction of SOD activity and other factors can regulate the availability of the substrate for SOD.

*Catalase (CAT) and peroxidases (POX)*

Catalase (CAT) and peroxidase (POX) catalyze the reduction of $H_2O_2$ to molecular oxygen ($O_2$) and water ($H_2O$). These enzymes have high reaction rates to remove the bulk amounts of $H_2O_2$ (Dat et al., 2000). The intracellular level of $H_2O_2$ is regulated by a wide range of enzymes, most importantly catalases (Willekens et al., 1995) and peroxidases. Catalase metabolizes $H_2O_2$ to produce water and dioxygen.

Under normal conditions plants produce ROS and anti-oxidants to maintain cellular metabolism. ROS accumulation is regulated by different stress conditions, such as salinity, drought and UV light (Foyer and Noctor, 2005). Plants therefore need to counterbalance ROS accumulation by producing different types of anti-oxidants, such as catalase (CAT), and then continue their normal cellular metabolism as well as plant growth and development. Basically, plant anti-oxidant production capacity is determined by the plant's energy sources, such as carbohydrates. Plant cultural and management practices could be applied to manipulate the plant's anti-oxidant levels and improve fruit quality (Figure 3).

## SILICON TREATMENT AND ANTI-OXIDANTS

Silicon (Si) plays numerous roles in plant growth and development, including enhancement of resistance to salinity, metal toxicities as well as drought stress, and increasing the accumulation of plant phenols (Savant et al., 1999; Bekker et al., 2007). Silicon can partially offset the negative impacts of NaCl stress by raising SOD and CAT activities, leaf chlorophyll content, and the photochemical efficiency of photosystem II (Al-Aghabary et al., 2004). Addition of Si to salt-treated barley significantly increased SOD activity and decreased malondialdehyde (MDA) concentration, a measure of membrane lipid peroxidation, in plant leaves (Liang, 1999). The effect of Si addition on the alleviation of Mn toxicity of rice plants, especially on peroxidase activity, has also been reported (Horiguchi, 1988). The effects of Si on anti-oxidant activity have been previously reported. Silicon treatment of plants increases stress tolerance by accumulation of anti-oxidant compounds, anti-oxidants can therefore counteract the negative effects of accumulated oxidants.

## AIM OF THE THESIS

Sugars acting as 'sources of energy', are manufactured in leaves, and are transported via the vascular tissue to various parts of plant. Avocado is one of the rare fruit trees, which predominantly contains C7 sugars. There are numerous speculations on the role of these sugars. Various researchers have reported on their production, storage and transport, roles as a fruit-ripening factor or as precursors of anti-oxidant.

Therefore this thesis was initiated to elucidate the role of hexoses versus heptoses in avocado plants. The following approaches were taken:

1. Ontogenic avocado plants produce hexoses and heptoses over different plant developmental stages. The sugar concentrations also vary according to developmental stages as well as fruit phenological phases. Plant ontogenic studies were undertaken to test the hypothesis that avocado produces different hexoses versus heptoses depending on plant growth and developmental stages, and that the two sugars are, hence, produced as result of a switch from C6 sugars to C7 sugars as fruits approach maturity.
2. The C7 sugars, perseitol and *D*-mannoheptulose, are produced predominantly by avocado plants throughout their growth cycles. Since these sugars also play multifunctional roles, as an 'energy source', and in carbohydrate storage and transport, there is a huge demand for improving their production by the plant. However, knowledge of plant sugar biosynthesis, metabolism and physiology is very limited. Similarly, volemitol, an isomer to C7 sugars is reported to be formed from sedoheptulose using reducing co-factors NADP(H). Therefore it is hypothesized that there is an enzymatic interconversion between *D*-mannoheptulose and perseitol, using reducing co-factors NADP(H).
3. The avocado fruit is rich in its content, it contains different types of beneficial compounds, such as low molecular non enzymatic hydrophilic (or lipophilic) and enzymatic anti-oxidants, and C7 sugars alcohols. It is reported that these anti-oxidants are distributed within fruit tissues; seed and exocarp accumulate more low molecular weight anti-oxidants than mesocarp tissue. It was also reported that *D*-mannoheptulose inhibits fruit ripening. Therefore it is hypothesised that *D*-mannheptulose is an anti-oxidant, and it plays a major part in fruit mesocarp tissue to maintain normal cellular metabolism.
4. The avocado phenolics are produced in both free and conjugated forms. The free phenolics are reported to be accumulated in fruit peel and seed. Whereas fruit mesocarp accumulates

more conjugated phenolics. It has also been indicated that exogenous Si application increases plant resistance to diseases by increasing level of anti-oxidants, such as free phenolics. Therefore it is hypothesized that potassium silicate improves fruit quality by increasing the plant's anti-oxidant pool.

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# CHAPTER 2

## THE QUEST FOR THE FUNCTION OF ‘HASS’ AVOCADO CARBOHYDRATES: CLUES FROM FRUIT DEVELOPMENT, SEED DEVELOPMENT AND SEED GERMINATION

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## ABSTRACT

**Background and Aims**: *D*-mannoheptulose (*D*-manno-2-Heptulose) and perseitol (*D*-glycero-*D*-manno-Heptose) are unusual seven-carbon sugars that could fulfill several important physiological functions in avocado. Although these sugars have been postulated to act as storage and transport sugars, there is no satisfactory evidence reported so far with regard to such functions. An ontogenic approach, through the determination of carbohydrate (CHO) concentrations in various tissues at different stages of the life cycle of avocado, could potentially elucidate partitioning of carbohydrates, transport and storage in avocado during its development and provide clues of the function of these sugars in avocado.

**Methods**: Plant material was collected at different plant developmental phases over an extended time. An ontogenic approach, i.e. investigating sugar profiles of tissues at different developmental stages such as seed germination, early seedling development, leaves, flower buds and fruit ripening to post-harvest fruit development, was used.

**Key Results**: Fruit and leaf tissues recorded significantly higher amounts of heptoses than hexoses at specific phenological stages of fruit development. Furthermore, postharvest 'ready-to-eat' fruit recorded the following concentrations in the exocarp: hexoses 4.37 ± 1.6 mg $g^{-1}$ d. wt,; heptoses 13 ± 0.8 mg $g^{-1}$ d. wt); in the mesocarp:(hexoses 3.55 ± 0.12 mg $g^{-1}$ d. wt ; heptoses 8 ± 0.2 mg $g^{-1}$ d. wt); in the seed:  hexoses 5.79 ± 0.53 mg $g^{-1}$ d. wt ; heptoses (only perseitol) 13 ± 1.1 mg $g^{-1}$ d. wt. At the pre-organ differentiation stage, when the seed was cracking, embryo sugars were 47 % hexoses and 53 % heptoses sugars. The germinating seed, however, also produced significant amounts of *D*-mannoheptulose (7.09 ± 1.44 mg $g^{-1}$ d. wt)

and perseitol (5.36 ± 0.61 mg $g^{-1}$ d. wt). Overall, this study reports patterns of sugar dominance; hexoses are predomintly produced at early fruit development while heptoses form the major component of the sugar pool as fruit approach maturity as fruit are 'ready-to-eat'. Finally heptoses dominate at embryo and germinating seed.

**Conclusions**: It is postulated that perseitol functions as a storage reserve sugar which is converted to *D*-mannoheptulose during early seedling development. These sugars are also transported together to sink tissues for utilization. Similarly, starch is hydrolized from the cotyledons to support the embryo/juvenile CHO metabolism to produce sucrose that function as a transport sugar. Avocado seed perseitol only accumulates in the late postharvest stages of fruit ripening.

*Keywords*: avocado C6 and C7 sugars, seed germination to fruit maturity

## INTRODUCTION

Plants are capable of producing all organic materials required for growth, metabolism, and reproduction from very simple inorganic precursor molecules obtained from the atmosphere and the soil (Pattanagul *et al*., 2002). An ontogenic approach might provide clues to understand plant CHO partitioning, transport and storage in specific tissues at a specific developmental stage. Synthesis and transport of CHOs may change during plant growth and development and become important determinants of plant productivity and, possibly survival. Sucrose and polyols have been reported to act as transport CHOs (Zimmermann and Ziegler, 1975). Similar to starch, sugar alcohols have also been shown to serve as storage compounds,

and, additionally, are synthesized to function as osmoprotectants (Nadwodnik and Lohaus, 2008).

Avocado plants are able to synthesize and translocate significant amounts of seven-carbon (C7) sugars (Liu *et al*., 1999). The C7 sugar heptose, *D*-mannoheptulose and its polyol form, perseitol, are found in all parts of the avocado plant, however, at different concentrations depending on tissue type and phenological stage of the plant (Liu *et al*., 1999). Similar amounts of C7 sugar alcohols and starch have been reported in avocado (Scholefield *et al*., 1985) and have been postulated to act as major forms of carbohydrate reserves (Oliveira and Priestley, 1988). Similarly, high volemitol concentrations in *Primula* leaves also clearly indicate that volemitol functions as storage compound (Häfliger *et al*., 1999). The synthesis and abundance of these sugars over the entire life cycle of the avocado plant clearly indicates their importance in growth and development. Special CHOs are often synthesized at specific development stages of a species. Additionally, Bruneau *et al.* (1991) indicated that in apple sorbitol production changes with photoperiod, affecting NADPH production, thereby possibly influencing sorbitol synthesis and the ratios of sorbitol to other carbohydrates.

Therefore, tissues from different ontogenic stages of avocado were analysed to elucidate carbohydrate partitioning, transport and storage. There have been various reports on C7 sugars as well as sugar alcohols functioning as transport (Noiraud *et al*., 2001; Liu *et al*., 2002) and/or reserve molecules and osmoprotectants in plants (Morgan, 1984).

Therefore, the following questions were addressed: Do leaf and different fruit tissues differ in their composition at specific phenological stages? What happens to the carbohydrate profile of fruit tissues postharvest? How does the carbohydrate profile of various tissues, from

mature seed to embryo to seedling and during fruit development vary? The answer to such questions could elucidate the role of C7 sugars in avocado and help increase the knowledge of the function of these rare special carbohydrates.

## MATERIALS AND METHODS

*Chemicals*

All chemicals were obtained either from Sigma-Aldrich®, Saarchem®, Fluka®, or Glycoteam GmbH.

*Plant Material*

Tissue from mature 'Hass' avocado (*Persea americana* Mill,) seed, dark- and light-grown seedlings, flower buds (closed pistillate stage) (Ish-Am and Eisikowitch, 1991), fruit (mesocarp, exocarp and seed) and leaves were used. The latter two were sampled at different phenological stages, from January to June from orchards in the KZN Midlands (30°16´E, 29°28´S). Additionally the CHO profile of commercially mature fruit was followed over a 28 day cold storage period plus the following softening period (approximately 7days).

*Preharvest sampling from leaf and fruit tissue*

Leaf and fruit material was sampled from 112 days after full bloom (DAFB) (starting January) on a monthly basis, to June, when fruit had reached commercial maturity (62-67 % moisture content) (McOnie and Wolstenholme, 1982). Each month, the youngest fully mature leaves from ten trees (eight leaves per tree) and twenty fruit (two fruit per tree) at specific stages of tree phenology (from early fruit set to 32 % oil in the mesocarp) were sampled. Fruit

tissues (exocarp, mesocarp and seed) as well as leaf tissue were shock-frozen, freeze-dried, ground and subsequently stored at -20 ºC until further analysis.

*Postharvest sampling from fruit tissue*

Mature 'Hass' fruit were stored at 5.5 ºC for 28 days (commercial shipping period and temperature for South African exports) and ripened at room temperature until they achieved the 'ready to eat' softness stage (fruit firmness < 600 N). Fruit tissues (exocarp, mesocarp and seed) were separated, freeze-dried, ground and subsequently stored at -20 °C until further analysis.

*Seed germination and seedling developmental stages*

Avocado seeds were submerged in tap water, which was exchanged every five days for six to eight weeks until seeds showed cracking, the sign of germination. Seeds were then placed in hot beds (25-28 ºC) filled with Perlite® for further development (after ± 8 weeks of radicle development, shoots protruded, showing green, active cell differentiation indicating shoot development). Seedlings were transplanted at the one-leaf stage into 200 mm diameter pots filled with a mixture of composted pine bark, peat moss, and sand (1:1:1 ratio). Seedlings were then taken to growth rooms, to simulate ambient growth conditions at a 24 ºC (light) and 16 ºC (dark) 12 h daylength cycle with a, light intensity of 314 µmol $m^{-2}$ $s^{-1}$ PAR (photosynthetically active radiation). Etiolated seedlings were produced by transferring seeds with a visible radicle to a growth room set to 24 ºC for 12h followed by 16 ºC for 12h. Seedlings were kept under these conditions for 60 days until sufficient leaf and shoot biomass

had developed. The two growth conditions were applied mainly to investigate the partitioning and production of avocado seedling sugars in response to lack of light to inhibit photosynthesis. Plant sampling strategies and stages were (Fig. 1A to 1E): (A) mature seed, prior to imbibition; (B) embryonic stage, 15 days of imbibition ; (C) radicle emergence, -30 days of imbibition) with daily sampling of seed portions) ; (D) seedling grown under light and (E) under dark condition. Samples were freeze-dried, ground and kept at -75 ºC for further analysis.

Non-structural soluble and non-soluble carbohydrates were analysed for different tissues and organs. The plant materials were collected during both, preharvest and postharvest stages.

*Phloem Exudation*

Phloem exudates were obtained by the EDTA (ethylenediaminetetraacetic acid) method described previously by Bachmann *et al.* (1994). Briefly, the buffer was prepared using 2 % starch agarose in 5 mM phosphate buffer and 5 mM EDTA, pH 7.5. Agarose was used because it is free of $O_2$ and improves survival of cut plant sections. The solution was prepared by boiling the mixture for 10 min and then 2 mL was poured into a small plastic container and left until solidified. The fresh cut leaf-petioles and shoots were placed into the plastic container with agarose buffer (Fig. 2A; 2B) for exudate collection. Plant material was left in the buffer for 12 h under controlled environmental conditions (8.42 µmol $m^{-2}$ $s^{-1}$ light intensity, 25 ºC temperature, 70-75 % relative humidity). After plant material was removed

from the agarose buffer, sugars were extracted from the agarose and analysed according to Liu *et al*. (1999) using an HPLC (LC – 20AT, Shimadzu Corporation, Kyoto, Japan) equipped with a refractive index detector (RID-10A, Shimadzu Corporation, Kyoto, Japan) and a Rezex RCM–Monosaccharide column (300 mm x 7.8 mm) (8 micron pore size; Phenomenex®, Torrance, CA, USA).

*Determination of soluble sugar concentration*

Freeze-dried, ground material (0.05 to 0.10 g d. wt) was mixed with 10 mL 80 % (v/v) ethanol and homogenized for 60 s. Thereafter, the mixture was incubated in an 80 ºC water bath for 60 min and kept at 4 ºC overnight. After centrifugation at 12,000 x *g* for 15 min at 4 ºC, the supernatant was filtered through glass wool and taken to dryness in a Savant Vacuum Concentrator (SpeedVac, Savant, NY, USA). Dried samples were resuspended in 2 mL ultra-pure water, filtered through 0.45 µm nylon filters and analysed using the above mentioned HPLC system. The concentration of individual sugars was determined by comparison with authentic sugar standards.

*Starch determinations*

Starch was determined according to Sluiter and Sluiter (2005). The dried pellet (0.1 g d. wt) obtained from the soluble sugar extracts was mixed with 2 mL of dimethyl sulfoxide (DMSO) and vortexed for 20 s, the test tube tightly capped and placed in a boiling water bath for 5 min. After adding 2.9 mL 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (pH 7.0) and 0.1 mL thermostable *α*-amylase (equivalent to 300 Units), tubes were vortexed

vigorously and incubated in a boiling water bath for 6 min with mixing in 2 min intervals. After addition of 4 mL sodium acetate buffer (1M, pH 4.5) and 0.1 mL (equivalent to 20 Units) amyloglucosidase, samples were incubated at 50 ℃ for 30 min. The samples were filtered through a 0.45 µm nylon filter and analyzed using the isocratic HPLC/RID system described above.

*Statistical analysis*

Analyses of variance were performed using GenStat (version 9.1; VSN International, Hemel Hempstead, UK). Standard deviation values were calculated and differences among treatments were separated by the least significant difference (LSD) at $P < 0.05$ level.

## RESULTS

*Sugar profile of flower, fruit and leaf tissue*

Whereas the C6 sugars glucose and sucrose, as well as the C6 storage carbohydrate starch, were major components of the sugar profile of juvenile avocado tissue, at maturity a different sugar composition was present. In flower buds sucrose was found to be the dominant carbohydrate while volemitol, *D*-mannoheptulose and perseitol were present at a lower concentration (Fig. 6).

Analysis of fruit tissue revealed that carbohydrates fluctuate substantially during the fruit growing period (Fig. 7). At the earliest sampling date in January (early fruit development, fruit size of 87 mm x 61 mm) the C7 sugars, particularly *D*-mannoheptulose and perseitol, were the dominant carbohydrates, while the C6 sugars sucrose, fructose and glucose were

present in lesser amounts. The C6 polysaccharide starch was only dominant in seed tissue and showed an increase during seed maturation. *D*-mannoheptulose concentrations found in the mesocarp and exocarp were the highest sugar concentrations detected in all analysed tissues. Perseitol concentrations were consistently higher in mesocarp and seed tissue than in exocarp and leaf tissues. Leaves predominantly contained sucrose followed by *D*-mannoheptulose, while only small amounts of fructose and, in particular glucose, were detected in this organ. Furthermore, C7 sugars were found in lower amounts in leaf tissue than in any of the other fruit tissues (Fig. 7; Table 2).

*Sugar profile of mature, soft fruit*

All three fruit tissues (mesocarp, exocarp and seed) contained sucrose and perseitol when soft, ‘eat-ripe’, with the highest perseitol concentration found in the seed (Fig. 8). At this stage of fruit development the highest soluble sugar concentration was that of perseitol in the seed, consisting predominantly of the cotyledons. *D*-mannoheptulose was the predominant sugar in the exocarp. The seed contained no *D*-mannoheptulose, and this sugar was present at the lowest concentration in the mesocarp, perseitol, however, was the dominant sugar in mesocarp tissue.

*Sugar profile of seed, embryo and juvenile avocado tissue*

The embryo contained three heptoses (*D*-mannoheptulose, volemitol and perseitol) and three hexoses (glucose, fructose sucrose). The dominating embryo sugar was *D*-mannoheptulose, followed by sucrose (Fig. 3A). The soluble sugar profile of the cotyledons

(Fig. 3B) revealed sucrose and *D*-mannoheptulose to be present in the highest concentrations, followed by glucose and perseitol, with fructose at the lowest level. Volemitol was not detected in the cotyledons.

*D*-mannoheptulose concentrations in the cotyledon peaked on day 3 and 4, while perseitol levels declined and then remained at a similar level over the following 3 days, increasing again towards day 5 of radicle emergence. During the first six days following radicle emergence cotyledon starch levels declined (Fig. 4). Although glucose levels in the cotyledons were low compared to other soluble sugars, these increased on day 4 of germination, with sucrose following a similar trend. Starch and glucose concentrations were negatively correlated (r = -0.61).

*Effect of light on carbohydrate profile of juvenile plant tissue*

Shoots of etiolated seedlings made up the bulk of the biomass of such seedlings and contained significantly higher concentrations of C7 sugars and glucose (Fig. 5B) than shoots of seedlings grown under light (Fig. 5A; Table 1). Cotyledons of light-grown seedlings contained a significantly higher percentage of starch than those of dark-grown plants. On the other hand, cotyledons of dark-grown seedlings contained higher amounts of C7 sugars, whereas leaves of both treatments contained similar amounts of C7 sugars. Sucrose and starch were not detectable in leaves of etiolated seedlings. Most seedling organs contained the three major hexoses and the two major C7 sugars (Fig. 5 A; Fig. 5 B; Table 1).

*Transported sugars*

Carbohydrate exudates collected from the primary as well as the mature leaf contained similar amounts of sucrose. However, mature leaves exuded predominantly the C7 sugar *D*-mannoheptulose, while its sugar alcohol, perseitol, was present in the exudates in similar amounts as sucrose (Fig. 9). In mature leaf petiole exudates glucose and fructose were not detectable, while the C7 sugar *D*-mannoheptulose was dominating, followed by perseitol and sucrose. Together with its components glucose and fructose, sucrose exuded from seedling stems. The C7 sugars *D*-mannoheptulose and perseitol were, however, the dominating sugars exuded from the seedling stem.

# DISCUSSION

The presence of the two major C7 sugars, *D*-mannoheptulose and perseitol in different tissues and at different ontogenic stages seems to indicate that these are synthesized concurrently, supporting earlier reports on their simultaneous presence of *D*-mannoheptulose and perseitol in various avocado tissues (Liu *et al*., 1999). The plant also produces another rare C7 sugar, volemitol, but only at the embryonic and flowering stage. This confirms results by Cowan (2004) who found the production of this sugar to be related to certain stages of the ontogenic development.

Mesocarp and seed *D*-mannoheptulose declined as fruit approached maturity; however, levels of *D*-mannoheptulose remained higher in the mesocarp than in other fruit tissues. Mesocarp perseitol concentrations dropped sharply at the first fruit drop stage (January, Fig. 7) and reached lower levels than the seed at fruit maturity (June), confirming the suggestion by Bertling and Bower (2006) on the importance of this C7 sugar as markers for post-harvest

quality. The further decline in *D*-mannoheptulose from harvest maturity to the “eat-ripe” stage (Fig. 8) confirms suggestions by Liu *et al*. (2002) that this carbohydrate is the respiratory substrate of the ripening fruit. Similarly, in banana, fruit storage at lower temperature is aligned with a slowed down respiration rate and a reduction in enzyme activities and increased postharvest life through a reduction in the utilization rate of storage compounds (Kader, 1986; Bhande *et al*., 2008). The dominance of hexoses during early ontogenic stages compared to the dominance of heptoses during the later stages seems to be a typical feature of the ontogenic development of ‘Hass’ avocado.

During the early growth period the young seedling derives its energy requirements from storage carbohydrates in the seed’s cotyledons and/or endosperm (Chong *et al*., 2002). The only soluble carbohydrates present in significant amounts (more than 5 mg $g^{-1}$ d. wt) in mature seeds of ‘eat-ripe’ avocado were found to be perseitol and, at lower concentration, sucrose (Fig. 8, July), while the universal storage carbohydrate, starch, was only detectable in seeds increasing as fruit developed to physiological maturity in June (Fig. 7), findings coherent with reports by Liu *et al*. (1999). The seed starch increased pre-harvest, while seed perseitol was the major carbohydrate in fruit postharvest (Fig. 8). This might suggest that both, starch and perseitol, act as storage carbohydrates as they accumulate in typical ‘sink’ tissues. This hypothesis is further strengthened by the decline in starch and perseitol in the cotyledons during early germination (Fig. 4).

At the onset of germination the embryo of the avocado seed contained relatively equal amounts of glucose, fructose, sucrose, *D*-mannoheptulose and perseitol (Fig. 3). This suggests that hexoses are, ontogenically, the first carbohydrates produced via the breakdown of starch.

Hence, the emerging avocado seedling uses glucose and fructose as sources of energy to maintain plant cell metabolism, similar to other seedlings (Duffus and Duffus, 1984). The non-reducing sucrose was found in the young seedling, primarily in the cotyledon (Fig. 3B), but also in the stem of the seedling, a distribution pattern in line with the common role of sucrose as transport sugar supplying energy to plant organs (Duffus and Duffus, 1984). Furthermore, leaf and stem sap contained sucrose, perseitol and *D*-mannoheptulose, confirming results by Liu *et al*. (2002) that these carbohydrates are transportable sugars in avocado (Fig. 9).

The variation in *D*-mannoheptulose levels in avocado in comparison with much less variation in its reduced counterpart, perseitol, might reflect stages in the metabolism of the heptoses. As storage carbohydrates typically accumulate in seeds, perseitol should be the likely reserve carbohydrate. This sugar could be converted by an aldose into *D*-mannoheptulose (Cowan, 2004). Both sugars were found in, and exuded from, the stem of seedlings (Fig. 5; Fig. 9), indicating that both sugars are transportable. However, while perseitol is the major C7 sugar in the seed (Fig. 8; carbohydrate profile of mature seed), *D*-mannoheptulose must have been converted from its reduced form, the likely storage product, perseitol. Therefore, the enzymes synthesizing C7 sugars must be able to fulfil diverse functions, a finding in support of earlier suggestions of such a function for C7 sugars by Liu *et al*. (2002). The higher sugar concentrations in stem tissue of etiolated seedlings compared with light-grown avocado seedlings confirm the results by Häfliger *et al*. (1999) that higher concentrations of sugars are transported in darkness than light. An under-storey rainforest tree, like avocado (Schaffer and Whiley, 2002), benefits from a rapid period of seedling growth, as

it is critical for survival of the species to grow out of the forest floor area ,where available light is a limiting factor, into greater sunlight for efficient photosynthesis. Quick carbohydrate availability will result in quick development and allow the avocado to reach its light compensation point. This point (approx. 660 µmoles $m^{-2}$ $s^{-1}$ PPF (Photosynthetic Photon Flux) (Bower *et al.,* 1978) is relatively low compared with other crops, hence, an initial boost of carbohydrate (hexose) availability will support the necessary initial growth spurt to grow out into an area of the forest where the light compensation point is reached. If sucrose is the initial transport sugar, it must become available via the hydrolysis of starch and/or the addition of glucose to fructose to form sucrose, the transport sugar of the young seedling. However, no fructose could be found in the seed (cotyledon) tissue in the early germination period (Fig. 4), which suggests that the activity of glucose-P-isomerase (in favour of fructose-6-phosphate) (Schnarrenber and Oeser, 1974) is lower than that of the sucrose forming enzymes (Leloir and Cardini, 1955) as well as sucrose synthase and sucrose phosphate synthase at this stage of seedling development, thus rendering fructose levels below the detection limit during this developmental period.

The prevalence of certain sugar alcohols, such as *D*-pinitol in pigeonpeas (*Cajanus cajan*) (Keller and Ludlow, 1993), *myo*-Inositol in Brassicaceae/ Fabaceae (Frias *et al*., 1996) and sorbitol in apple (Webb and Burley, 1962), concomitant with glucose and fructose has been widely reported. Various functions have been ascribed to polyols, such as osmolytes and stress protectants (Morgan, 1984; Keller and Ludlow 1993). Hence, the presence of perseitol, similar to *myo*-Inositol, in the seed might be due to the osmolyte function of sugar alcohol (Loewus and Murthy, 2000). *myo*-Inositol has been further postulated to play an important

role in signal transduction, hormonal homeostasis, and cell wall biosynthesis (Moore *et al*., 1990). Because sugar alcohols are produced in considerable amounts, exceeding those of signalling molecules, a further physiological role of heptoses in avocado might be as cryoprotectant (Häfliger *et al*., 1999). It might, hence, be an ecological advantage to store a sugar which is easily convertible to a transport sugar and could also, as sugar alcohol, be used as an osmoprotectant. The carbohydrate composition of the seed, followed through germination and early seedling growth, confirmed that stored starch is released from the cotyledons, seemingly to form glucose units (Fig. 4). Although fructose, glucose and sucrose were found to be present in the embryo, upon germination no more fructose was detectable (Fig. 4), possibly indicating a quick turnover by sucrose (phosphate) synthase, quicker than the fructose is isomerized from glucose - a hypothesis that can be substantiated by the $K_m$ of fructose phosphate to glucose phosphate (Schnarrenber and Oeser, 1974) compared with the production of sucrose from glucose and fructose. Therefore, fructose could have been instantly used for sucrose formation and, in the form of sucrose, been transported to sinks. Shoots of the etiolated seedlings showed a sugar profile different to light-grown seedlings. First, shoot monosaccharide concentrations of both, C6 and C7, sugars (Fig. 5) were significantly higher in etiolated seedlings. Second, in etiolated seedlings starch reserves were almost depleted from the cotyledons, while the cotyledons of light-exposed seedlings still contained significantly higher starch reserves. These findings agree with results by Kazama (1978) who found the sugar concentration of cucumber to be higher in dark- than light-grown plants, whereas starch was the opposite. These patterns confirm that the hexoses glucose and fructose are generated by the breakdown of starch (Duffus and Duffus, 1984), while the

heptoses perseitol and to *D*-mannoheptulose can also be generated from starch via the Calvin cycle (Cowan, 2004).

While leaf tissue of avocado seedlings (juvenile plants) does not seem to differ significantly in carbohydrate composition from that of leaves on flower or fruit bearing branches (adult plants) (Fig. 5A; Figs. 7-8), the sugar profile of reproductive tissue seems to be dominated by different sugars. Such a dominance of C7 over C6 sugars is already visible in flower buds (Fig. 6), and even more pronounced in fruit tissue (Fig. 7). Hence, it seems that the sugar profile of avocado is linked to the ontogenic age of the tissue. Initially, during germination, starch supplies hexoses (Duffus and Duffus, 1984), and perseitol supplies *D*-mannoheptulose (Fig. 4). Wang *et al*. (1997) reported similar results in apples where the special sugar alcohol of that species, sorbitol, is produced in higher amounts in mature than in young leaves early stages of ontogeny. However, sucrose decreased in mature leaves with increased daylength. In avocado, young, juvenile and vegetative tissue is characterized by the presence of hexoses, while adult reproductive tissue is characterised by the presence of heptoses. Although significant amounts of hexoses are produced during the juvenile stage, at tree maturity avocado predominantly produces C7 sugars, especially in mesocarp and exocarp tissues.

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TABLE 1. Soluble sugar concentrations of light grown and etiolated avocado seedlings. The data show the carbohydrate concentrations of seedlings in light and dark growing conditions, which potentially emphasizes the storage carbohydrates at seedling stage. Similarly it also reveals the role of hexoses (glucose, fructose and sucrose) and heptoses (*D*-mannoheptulose and perseitol) as sources of energy as well as transport sugar

| Rooms | Tissue | mg $g^{-1}$ d. wt | | | | | | Starch (% ) |
|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | *D*-Mannoheptulose | Perseitol | Mean | |
| Light | Leaf | nd | [1]$1.91 \pm 0.96^{jklm}$ | $1.51 \pm 0.38^{klmn}$ | $5.80 \pm 2.1^{f}$ | $6.83 \pm 0.91^{ef}$ | [2]$3.20^{d}$ | $2.4 \pm 0.13$ |
| | Root | nd | $2.80 \pm 2.28^{hijk}$ | $0.811 \pm 0.37^{mn}$ | $1.74 \pm 1.33^{klm}$ | $2.52 \pm 1.01^{ijk}$ | $1.58^{f}$ | $3.2 \pm 0.32$ |
| | Seed | nd | $0.81 \pm 0.31^{lmn}$ | $2.23 \pm 0.16^{ijkl}$ | $2.68 \pm 1.15^{ijk}$ | $2.33 \pm 0.12^{ijk}$ | $1.61^{f}$ | $13.8 \pm 1.76$ |
| | Shoot | nd | $3.30 \pm 2.66^{ghij}$ | $1.94 \pm 1.33^{jklm}$ | $2.39 \pm 1.35^{ijk}$ | $4.34 \pm 2.42^{g}$ | $2.40^{e}$ | $2.7 \pm 0.24$ |
| | Mean | - | [3]$2.21^{e}$ | $1.62^{ef}$ | $3.15^{d}$ | $4.01^{c}$ | | |
| Dark | Leaf | [1]$2.26 \pm 0.11^{ijk}$ | $4.17 \pm 0.48^{gh}$ | nd | $6.44 \pm 0.32^{ef}$ | $7.59 \pm 0.27^{e}$ | [2]$4.09^{c}$ | nd |
| | Root | $0.24 \pm 0.03^{n}$ | $0.63 \pm 0.22^{mn}$ | nd | $1.49 \pm 0.24^{klmn}$ | $3.55 \pm 0.36^{ghi}$ | $1.18^{f}$ | $0.2 \pm 0.02$ |
| | Seed | $2.17 \pm 0.32^{ijkl}$ | $5.80 \pm 0.17^{f}$ | $2.10 \pm 0.22^{jkl}$ | $9.17 \pm 0.51^{d}$ | $10.55 \pm 0.41^{d}$ | $5.96^{b}$ | $4.4 \pm 0.76$ |
| | Shoot | $30.20 \pm 0.1^{a}$ | $28.6 \pm 0.05^{b}$ | $1.95 \pm 0.03^{jklm}$ | $21.67 \pm 0.02^{c}$ | $30.94 \pm 0.07^{a}$ | $22.67^{a}$ | $1.4 \pm 0.24$ |
| | | [3]$9.89^{b}$ | $9.8^{b}$ | $1.01^{f}$ | $9.69^{b}$ | $13.15^{a}$ | | |
| [1]Room x Tissue x Sugar | | $LSD_{(0.05)} = 1.439$ | | | | | | |
| [2]Room x Tissue | | $LSD_{(0.05)} = 0.644$ | | | | | | |
| [3]Room x Sugar | | $LSD_{(0.05)} = 0.720$ | | | | | | |
| Tissue x Sugar | | $LSD_{(0.05)} = 1.018$ | | | | | | |

Values are means ± SD. (n = 5)

[1, 2, 3] Values followed by a different lower-case letter are significantly different at P = 0.05.

nd – not detected.

TABLE 2. Sugar concentrations of fruit tissues (mesocarp, exocarp and seed) and leaves of avocado over six months of developmental

| Tissue | mg $g^{-1}$ d. wt | | | | | |
|---|---|---|---|---|---|---|
| | Fructose | Glucose | Sucrose | *D*-Mannoheptulose | Perseitol | Mean |
| Mesocarp | [1]6.5 ± 6.27 [fgh] | 3.03 ± 2.47 [i] | 4.88 ± 3.4 [ghi] | 22.63 ± 11.19 [b] | 13.63 ± 7.6 [c] | [3]10.13 [a] |
| Exocarp | 3.77 ± 5.16 [hi] | 3.52 ± 2.01 [hi] | 4.73 ± 2.14 [ghi] | 26.65 ± 4.66 [a] | 8.05 ± 3.9 [efg] | 9.34 [a] |
| Seed | 12.93 ± 18.2 [cd] | 5.62 ± 4.44 [ghi] | 7.86 ± 4.7 [efg] | 10.51 ± 7.4 [cde] | 12.54 ± 4.6 [cd] | 9.89 [a] |
| Leaf | 5.71 ± 6.3 [ghi] | 2.75 ± 1.91 [i] | 9.76 ± 5.2 [def] | 6.63 ± 2.91 [fgh] | 6.13 ± 2.7 [ghi] | 6.2 [b] |
| | [2]7.23 [c] | 3.73 [d] | 6.81 [c] | 16.61 [a] | 10.09 [b] | |

[1]Tissue x Sugar LSD $_{(0.05)}$ = 3.462

[2]Sugar LSD $_{(0.05)}$ = 1.731

[3]Tissue LSD $_{(0.05)}$ = 1.548

Values are means ± SD. (n = 5)

[1, 2, 3] Values followed by a different lower-case letter are significantly different at P = 0.05.

FIG. 1. Avocado seedlings' germinated and grown under two growth conditions (light vs. dark): embryo (Panel A; embryo in the center is indicated); cotyledon (Panel B; with newly emerged shoot); seedling was grown for 60 days under light (Panel C; one block measures 1 cm.); seedling was grown for 60 days under light exclusion (etiolated) (Panel D; one block measures 3 cm.).

FIG. 2. Sap collection procedures from leaf petioles (A) and cut stem of seedlings (B).

FIG. 3. Carbohydrates (% of total soluble sugars) in germinating avocado embryo after 7 days of imbibition (Panel A) and cotyledon (Panel B) (mg $g^{-1}$). $LSD_{0.05} = 1.805$ (Panel A); $LSD_{0.05} = 1.324$ (Panel B)

FIG. 4. Soluble and non-soluble carbohydrates of cotyledon tissue during the early germination period. $LSD_{0.05} = 2.454$ (soluble sugars); $LSD_{0.05} = 4.039$ (starch)

FIG. 5. Partitioning of carbohydrates in different organs of seedlings grown under light (Panel A) and darkness (etiolated) (Panel B); ↓ symbol denotes ('not detected'). Bars represent SE of mean values.

FIG. 6. Sugar profiles of avocado flower buds. $LSD_{0.05} = 1.5$ (n=5)

FIG. 7. Seasonal changes in non-structural soluble and insoluble carbohydrate concentration in fruit mesocarp, exocarp, seed tissue and leaf of 'Hass' avocado during experimental period. $LSD_{0.05} = 3.79$ (soluble sugars), $LSD_{0.05} = 5.40$ (starch) Vertical bars represent ± SE. (n=5)

FIG. 8. Carbohydrates of avocado fruit tissues at the ‘eat-ripe’ stage. Physiologically mature fruit were picked and stored for 28 days in cold storage (5.5 ºC) followed by ripening at room temperature (fruit firmness was < 60 %); nd (not detectable), $LSD_{0.05}$ = 1.564. Vertical bars represent ± SE. (n=5)

FIG. 9. Sugars exuded from primary leaf petioles (13 L x 6 W (cm)), seedling stem (28-30 cm height) and fully expanded mature leaves (18 L X 7.5 W (cm)) of avocado seedlings. Vertical bars represent ± SE. (n=3)

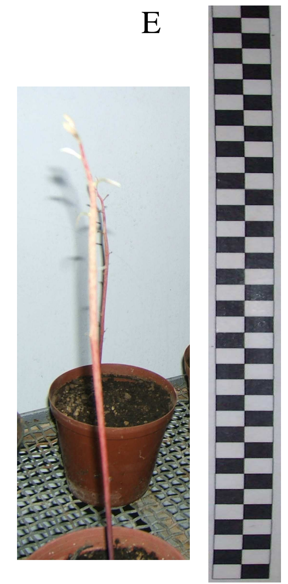
A

B

C

D

E



A

Leaf petioles

B

Seedling stem as seed attached to as carbohydrate source

FIG. 2.

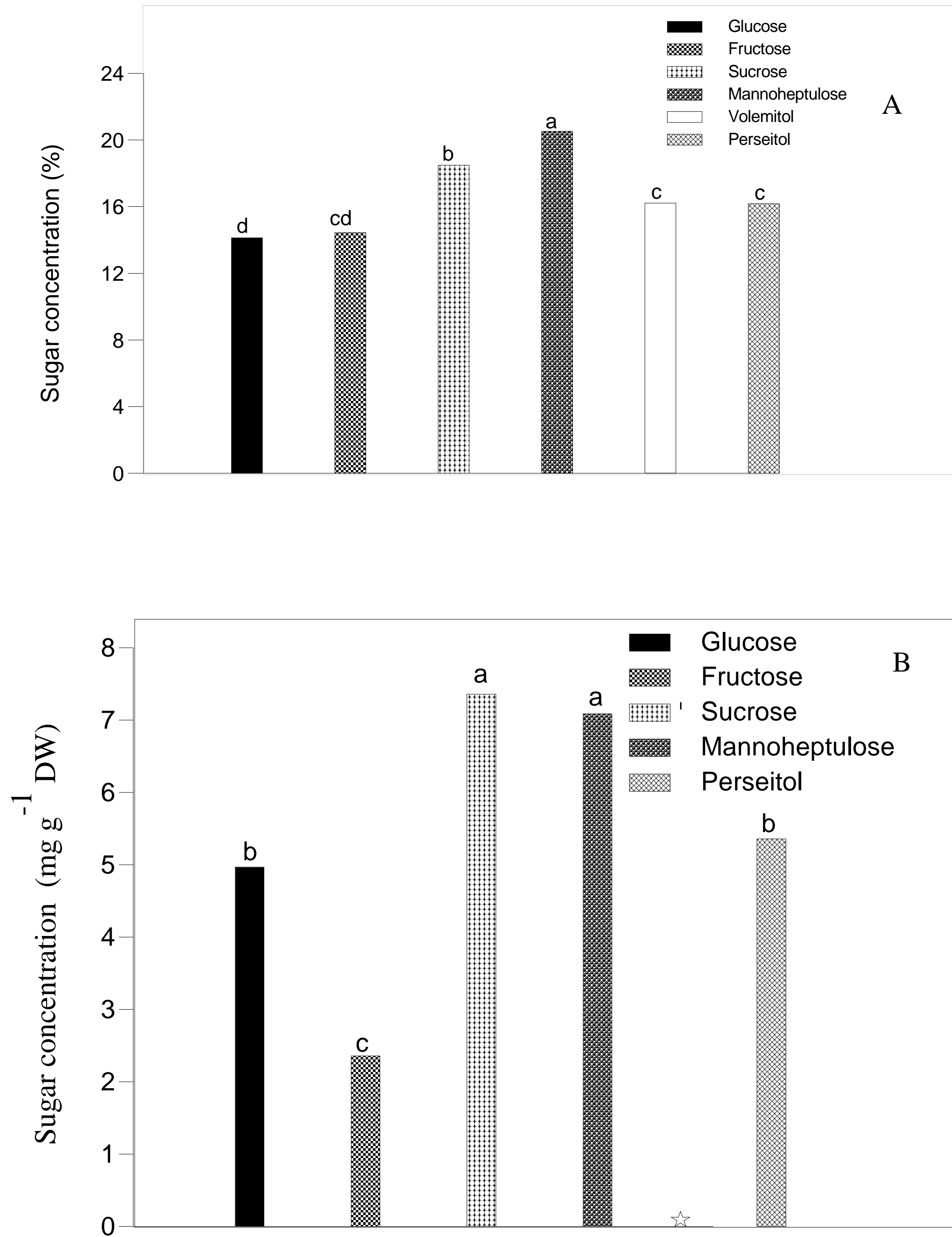



FIG. 3.

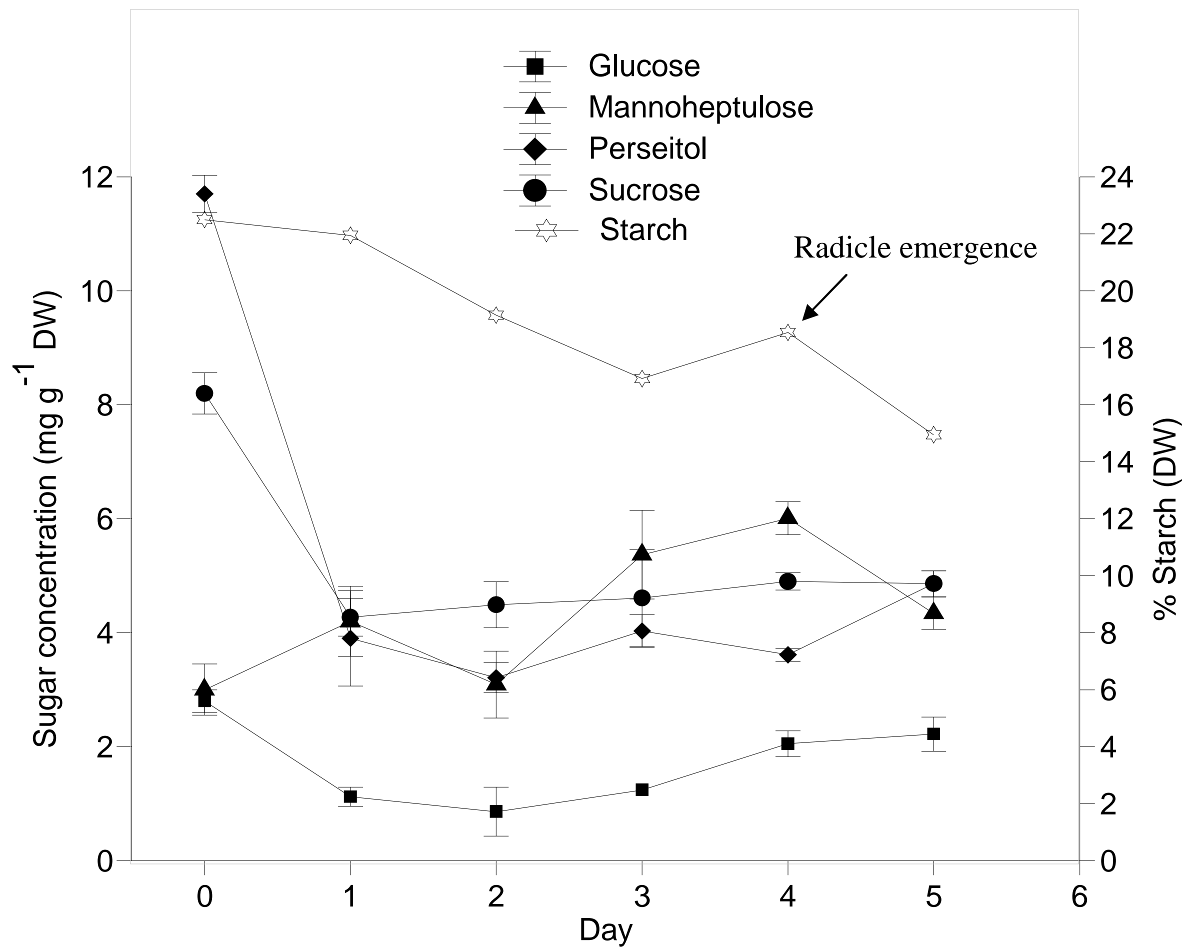



FIG. 4.

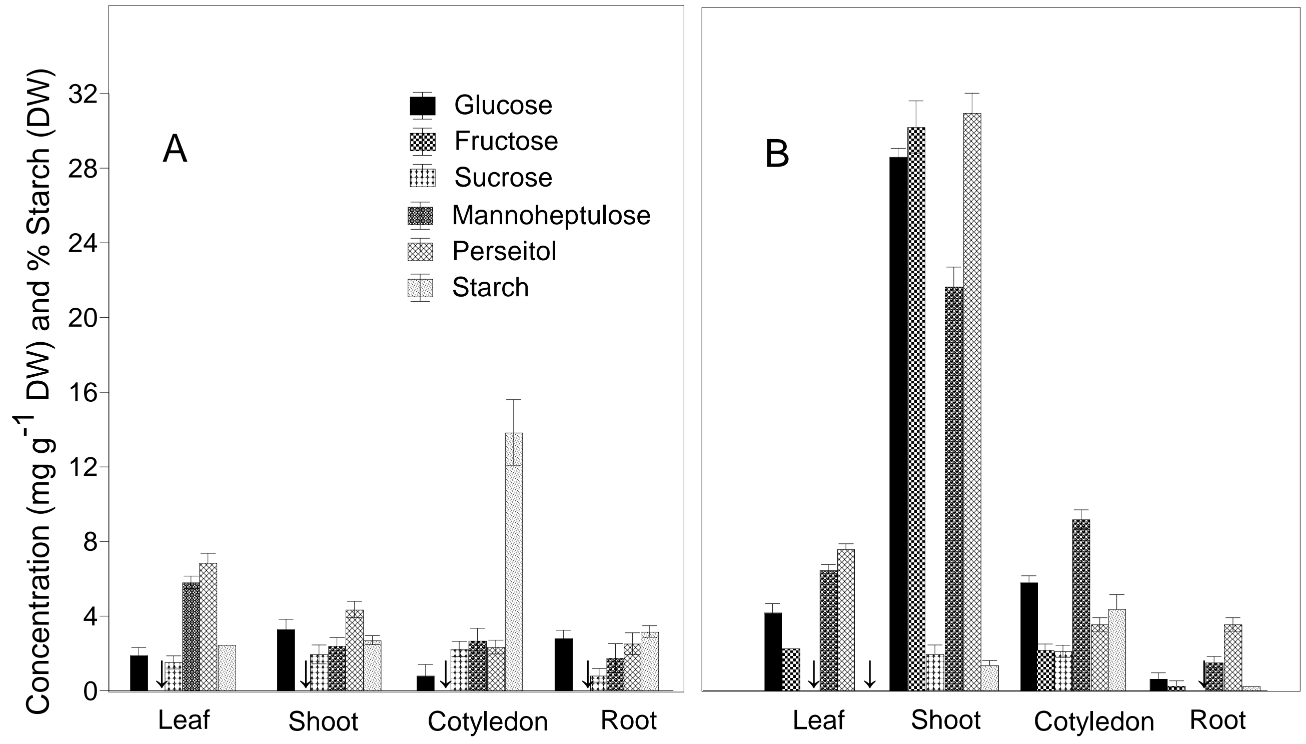



FIG. 5.

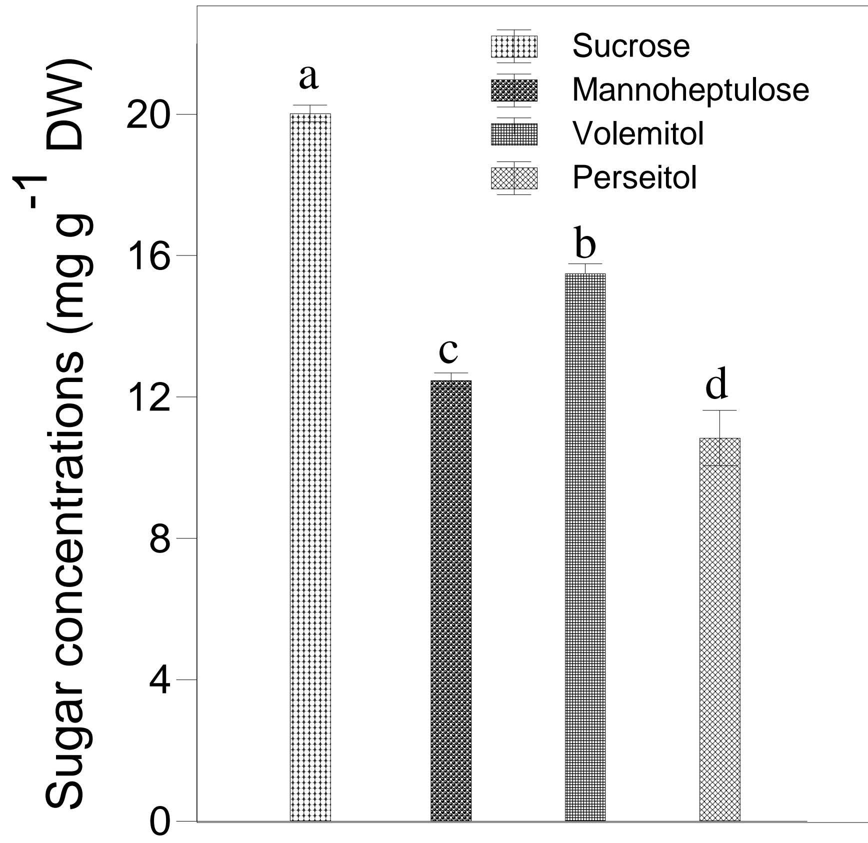



FIG. 6.

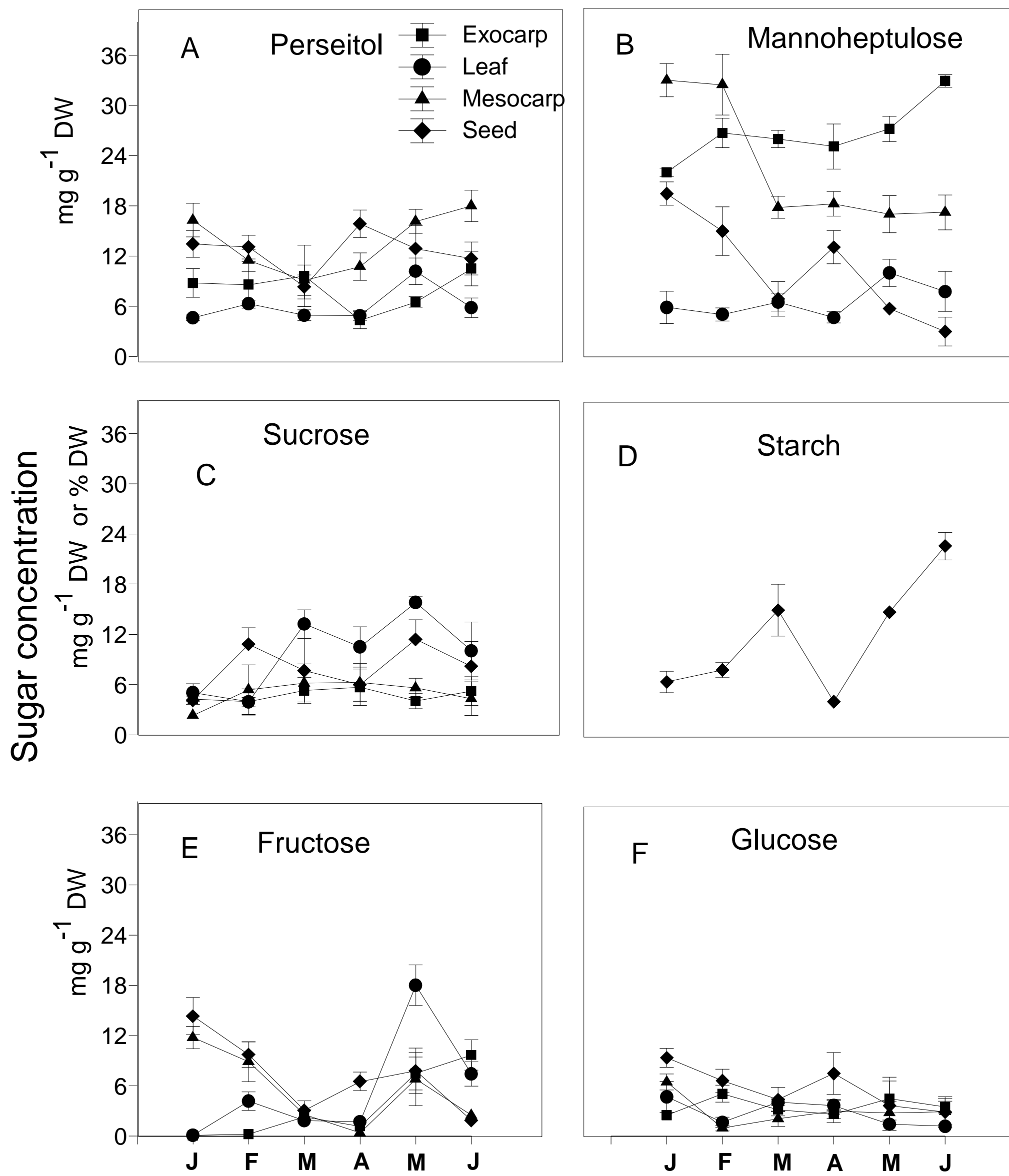



FIG. 7.

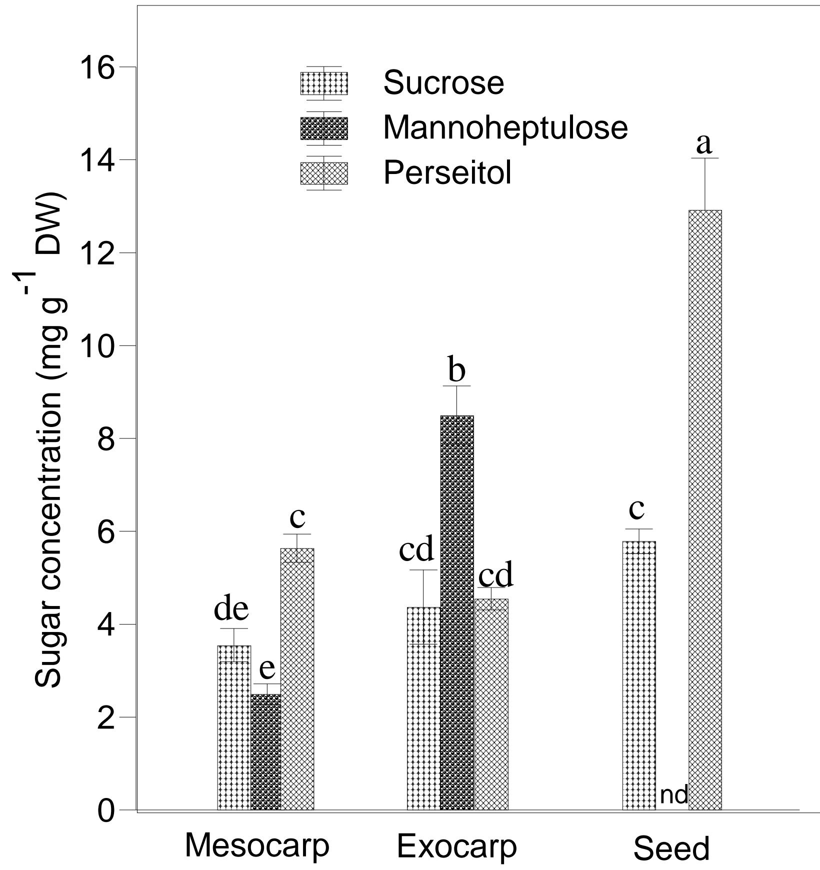



FIG. 8.

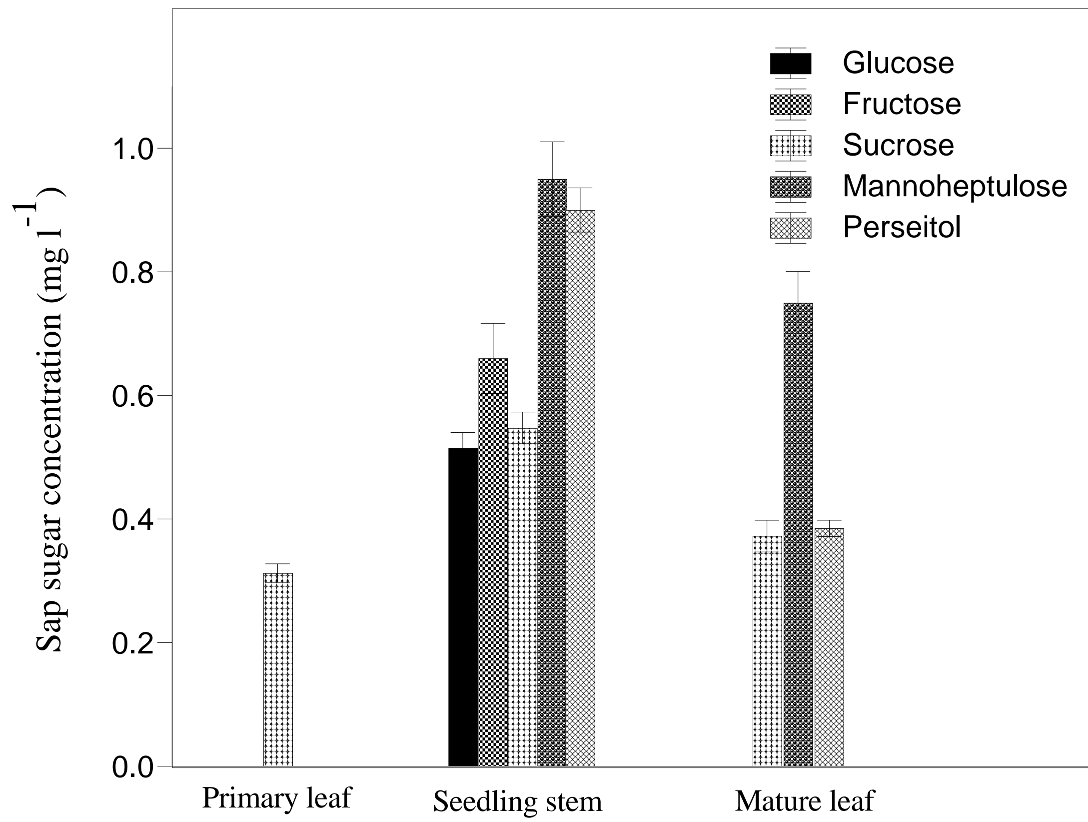



FIG. 9.

# CHAPTER 3

## D-MANNOHEPTULOSE AND PERSEITOL IN 'HASS' AVOCADO: METABOLISM IN SEED AND MESOCARP TISSUE

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Abstract

Mature avocado plants produce a higher amount of the heptose *D*-mannoheptulose and its polyol form, perseitol, than hexoses. These heptoses have various functions as anti-oxidants and energy sources. Although C7 sugars are abundantly produced in avocado, knowledge of their metabolism and synthesis is limited. Therefore the synthesis of these sugars in seed and mesocarp tissue was investigated, in particular the interconversions of perseitol to *D*-mannoheptulose and of *D*-mannoheptulose to perseitol were followed in different tissues. The ability of cotyledonsof etiolated seedlings to convert perseitol to *D*-mannoheptulose was significantly higher than that of the cotyledons of germinating seedlings grown in the light as well as that of dormant seeds. The cotyledons of light-grown, germinating plants had a greater ability to convert *D*-mannoheptulose to perseitol than the cotyledons of etiolated plants and the dormant seed. The cotyledons of etiolated plants showed higher activity in converting perseitol to *D*-mannoheptulose than the cotyledons of light- grown plants; whereas the cotyledons of light-grown plants had a greater ability to convert *D*-mannoheptulose to perseitol than cotyledons of etiolated seedlings. In mesocarp tissue, a higher percentage of perseitol was converted to *D*-mannoheptulose than *vice versa*. Furthermore, aldolases could be identified in mesocarp as well as in dry seed tissue. Therefore, the production of these heptoses and their enzymatic inter-conversion is dependent on the plant's ontogenic stage. This paper describes the following basic findings a) conversion of *D*-mannoheptulose to perseitol as well as conversion of perseitol to *D*-mannoheptulose in dry seed, cotyledons and mesocarp tissue b) detection of aldolase enzymes and c) interconversion of perseitol and *D*-mannoheptulose in bark exudes.

*Keywords:* Perseitol, *D*-mannoheptulose, ontogeny, *Persea americana* Mill.

## 1. Introduction

The major non-structural carbohydrates in avocado (*Persea americana* Mill.) are the heptoses *D*-mannoheptulose and perseitol (Liu et al., 1999, 2002). Although there have been reports on photosynthetic assimilation, translocation and storage of carbon, reducing power, and protection against different types of stresses (Bieleski, 1982; Lewis, 1984; Loescher and Everard, 1996; Stoop et al., 1996; Cowan, 2004; Bertling et al., 2007), little is known about their physiology and metabolism in avocado. However, the biosynthetic pathways of most pentols and hexitols in higher plants have been elucidated (Moing et al., 1992; Flora and Madore, 1993). Häfliger et al. (1999) investigated the metabolism of the C7 sugar alcohol volemitol and characterized a novel ketose reductase enzyme in the horticultural hybrid polyanthus (*Primula* x *polyantha*).

Isomers of volemitol, perseitol, as well as its hydrogenated form *D*-mannoheptulose, have been reported to be produced in substantial amounts in avocado (Liu et al., 1999). However, avocado seeds also contain the common carbohydrate storage forms sucrose and starch (Liu et al., 2002), which is commonly the case in plants producing rare carbohydrates (Häfliger et al., 1999). In avocado the sugar profile changes when seeds and seedlings are grown in the light or dark (Kazama et al., 1978). It has been postulated that avocado plants use *D*-mannoheptulose for a variety of purposes, ranging from energy sources to anti-oxidants and transport sugars (Liu et al., 2002; Bertling and Bower, 2005, 2006; Bertling et al., 2007), and that perseitol, the reduced form of *D*-mannoheptulose, may also function in storage and/or as a transport carbohydrate.

It has been proposed by Häfliger et al. (1999) that the heptose formation in avocado is catalysed by three known enzymatic reactions that will form C7 intermediates. a) an aldolase

reaction- erythrose-4-P + dihydroxyacetone-P ↔ sedoheptulose- 1,7-bis-P; b) a transketolase reaction- xylulose-5-P + ribose-5-P ↔ sedoheptulose-7-P + glyceraldehyde-3-P; or c) a transaldolase reaction- fructose-6-P + erythrose-4-P ↔ sedoheptulose- 7-P + glyceraldehyde-3-P (Liu et al., 2002). This formation of heptoses requires NADPH/NADP as redox power (Häfliger et al., 1999).

Negm et al. (2001) speculated that heptoses occur by aldolase condensation of a dihydroxyacetone with erythrose-4-phosphate to form sed-1-7-bis-phosphate and this reaction, in avocado, is followed by isomerization to a phosphorylated *D*-mannoheptulose derivative, probably perseitol. Avocado seeds, as storage organs, contain starch as a storage carbohydrate (Liu et al., 2002) but also contain the C7 sugar perseitol, a likely storage form of D-mannoheptulose (Tesfay et al., 2010).

The current experiment was designed to investigate formations of heptoses, especially D-mannoheptulose-perseitol interconversions, in light-and dark-grown seedlings.

## 2. Materials and methods

### *2.1. Chemicals*

All chemicals were obtained either from Sigma-Aldrich®, Saarchem®, Fluka®, or Glycoteam GmbH.

### *2.2. Plant materials*

Dry seed

Mature 'Hass' avocado fruit were collected from trees in the KwaZulu-Natal Midlands (30°16´E, 29°28´S), the exo- and mesocarp removed, and the seed freeze-dried and stored at -75ºC for further analysis.

Seed germination

Seeds from the same batch of fruit were imbibed by submerging them in tap water. The water was replaced every five days for 6-8 weeks until seeds cracked, a sign of germination. Seeds were then transferred to heated propagation beds (26 ºC) for seedling development, for about eight weeks until the seedling reached a height of 30 cm. Seedlings which had not yet developed leaves, but only roots and stems, were then transferred to a dark growth room for eight weeks to etiolate seedlings. . Seedlings were etiolated until they reached a height of 100 cm producing pale leaves of reduced size, an indication of restricted photosynthesis. Thereby it was ensured that the energy for seedling development was only obtained from CHOs derived from the cotyledons. Sampling of cotyledon portions was carried out from (a) plants grown under light and (60 days exposure to light) (b) etiolated plants (60 days exposure to darkness) while they were germinating. Cotyledon samples were freeze-dried, ground and kept at -75 ºC for further analysis.

Fruit

Physiologically mature fruit were sampled from the same orchard, the mesocarp immediately separated from other fruit tissues, freeze-dried and ground. Samples were stored at -75 ºC until further analysis.

*2.3. Enzyme extraction and activity determination of seed and mesocarp tissue*

The extraction of proteins was carried out according to Helmerhorst and Stokes (1980). Milled, frozen fruit tissue (1.0 g) was thawed and extracted on ice in a glass homogenizer containing 5 volumes of extraction buffer (20 mM Hepes/KOH, pH 7.5, 5 mM DTT, 5 mM $MgCl_2$, 2% (w/v) PEG-20,000, and 2% (w/v) PVP K30). The mixture was allowed to stand on ice for 15 min and then centrifuged at 20,000 x *g* for 20 min. The supernatant was passed through Miracloth® and immediately centrifuge-desalted through Sephadex G-25 pre-equilibrated with the appropriate assay buffer. The desalted supernatant was used for the enzyme assay. The Bradford microassay (Bradford, 1976) was used to determine the protein concentration of the samples.

Enzyme activity was measured spectrophotometrically by monitoring the continuous reduction of NADPH and oxidation of NADP at $A_{340\ nm}$ ($\Delta$ $A_{340\ nm}$ $min^{-1}$ $mg^{-1}$ protein) over a period of 10 min (Häfliger et al., 1999; Zhang et al., 2000), with slight modification. The spectrophotometric assay mixture (1.0 ml) contained 25 mM *D*-mannoheptulose or perseitol, 1 mM NADPH/NADP, extraction buffer, and 250 µl desalted crude enzyme extract, (approximately contained 0-2 mg $\mu l^{-1}$ protein). The chromatographic assay mixture was prepared as above and was incubated for 0, 10, 20 min with 250 µl extract used for HPLC analysis. Each reaction was stopped by placing the sample in boiling water for 5 min. Thereafter 5.0 ml 90% (v/v) ethanol was added to the sample for the extraction of carbohydrates. The mixture was then taken to dryness using a Savant Vacuum Concentrator (SpeedVac, Savant, NY, USA). Subsequently, samples were suspended in ultra-pure water and aliquots analysed by HPLC according to Liu et al. (1999). The product of reversible conversions of *D*-mannoheptulose and perseitol was quantified chromatographically based on the final concentration of the end products.

### *2.4. Electrophoretic detection of mesocarp and seed aldolase proteins*

Crude mesocarp and seed protein extracts were loaded onto a 12% acrylamide running gel and a 5% acrylamide stacking gel, and were separated by SDS-PAGE using a Mini-PROTEAN® Tetra Cell (Bio-Rad, USA) at 200 V. Amount of 100 µg of seed protein and 50 µg of mesocarp protein were loaded. Spinach aldolase was used as the standard for identification of aldolases in the samples.

### *2.5. Carbohydrate composition of exudates from girdled branches*

Several branches (10-15 cm diameter) of different trees were girdled using Optima Girdling Pliers (Optima, RSA). Exudates from the cut branches were collected on Whatman® no. 1 filter paper, which was placed onto the girdled area immediately after the girdling process. The filter paper was removed after 30 min and rinsed with 5 ml 80% (v/v) ethanol. The liquid collected in a 20 ml glass vial. Sample collections were carried out during the early hours of the morning. The sugar quantification method described by Liu et al. (1999) was followed. Furthermore, the dry residue which had formed on the girdled branches was collected seven days after the girdling process. The residue was suspended in ultra-pure water and filtered through 0.45 µm filters before analysis by HPLC.

### *2.6. Statistical analysis*

Analyses of variance were performed using GenStat version 9.1 (VSN International, Hemel Hempstead, UK). Standard error values were calculated and differences among treatments were separated by the least significant difference at $P < 0.05$ level.

## 3. Results

The dry dormant seed could only convert perseitol to *D*-mannoheptulose at 20% of the rate of the cotyledons of light-grown seedlings. The cotyledons of etiolated seedlings, however, displayed a four to five times greater ability to convert perseitol to *D*-mannoheptulose than the cotyledons of germinating seedlings (Figure 2A). On the other hand, cotyledons of seedlings germinating under light had a three times greater ability than mature, dormant seeds to convert *D*-mannoheptulose to perseitol (Figure 2B). An attempt was also made to elucidate whether there was a correlation between the enzyme activity and actual tissue carbohydrate concentrations. The dormant seed had a higher perseitol than *D*-mannoheptulose concentration, whereas the cotyledons of seedlings germinating in the light had a different pattern, similar to those germinating in the dark: *D*-mannoheptulose concentrations were higher than perseitol concentrations (Figure 3).

In mesocarp tissue, a higher percentage of *D*-mannoheptulose was converted to perseitol than perseitol to *D*-mannoheptulose (Figures 4A and B). Therefore, the enzyme catalyzing the reduction of *D*-mannoheptulose to perseitol had a relatively higher activity than the enzyme catalysing the oxidation of perseitol to *D*-mannoheptulose.

Fresh branch exudates collected immediately after girdling contained *D*-mannoheptulose, perseitol and sucrose, while only perseitol was detected in the dry exudates collected seven days after girdling (Table 1; Figure 5).

Electrophoresis of mesocarp and seed protein extracts (Figures 6 and 7) showed that various aldolases were present. Spiking the protein extracts with spinach aldolase resulted as very intense protein bands, with similar aldolases being detected in sample extracts (Figure 6). In mature, dormant seed aldolase was significantly more expressed than in the cotyledons of etiolated seedlings as well as light-grown seedlings.

## 4. Discussion

The C7 sugars are a special group of carbohydrates, which are abundantly produced in avocado plants and have variety of functions, such as carbon and energy storage, as well as anti-oxidants (Tesfay et al., 2010). At the onset of germination the embryo of the avocado seed contains relatively equal proportions of glucose, fructose and perseitol, with higher concentrations sucrose, and even more so *D*-mannoheptulose. (Chapter 2, Figure 3). As most seeds accumulate carbohydrates as storage compounds (Duffus and Duffus, 1984), perseitol has been postulated to be the likely reserve carbohydrate of avocado (Cowan, 2004), which would be converted into *D*-mannoheptulose by an aldolase. Both sugars were found in and exuded from the stem of seedlings (Figure 5), indicating that both sugars are transportable, as described by Liu et al. (2002). However, while perseitol is the major C7 sugar in the seed, and could have been exported from the seed, *D*-mannoheptulose was not present in substantial amount in the seed. It is likely that this C7 sugar was released from its reduced form, perseitol, which therefore acted as the storage form of *D*-mannoheptulose.

Liu et al. (2002) reported *D*-mannoheptulose to be a primary photosynthetic product, which is translocated from leaves to different sink cells of the avocado plant. The plant also produces perseitol, the reduced form of *D*-mannoheptulose, in the leaf and both sugars can be detected in phloem sap (Liu et al., 2002).

As demonstrated by Liu et al. (2002) *D*-mannoheptulose is a primary photosynthetic product in avocado, hence, these authors postulated that this C7 sugar acts as an energy source, which is stored in the form of sucrose and/ or starch. As the plant utilizes the stored carbohydrates, these are broken down into simpler units, normally glucose, which is readily available for energy generation. Since *D*-mannoheptulose was found to be a primary

photosynthetic product, this heptose might be catalysed by transaldolases existing within the Calvin Cycle, to form the C7 storage product, perseitol. As the protein extract from seed as well as mesocarp tissues was able to convert *D*-mannoheptulose into perseitol and *vice versa*, these reactions might have occurred via an existing aldolase enzyme of the Calvin Cycle, possibly transaldolase.

Both heptoses are transported in the phloem (Liu et al., 2002) and can therefore be moved from source to sink. Perseitol is a more likely source of energy, at least for the young, fast growing seedling than *D*-mannoheptulose, as under light exclusion, and therefore without any additional energy input via photosynthesis, it is easier converted into *D*-mannoheptulose than *D*-mannoheptulose is into perseitol (Figure 2A). Perseitol, on the other hand, is predominantly occurring in the cotyledons, a typical feature of a storage carbohydrate. Additionally, etiolated seedlings can derive their energy only from the seed (Kazama et al., 1978), further substantiating our hypothesis that perseitol is the likely storage product in avocado. However, this is in contradiction to the postulation by Liu et al. (1999) that *D*-mannoheptulose is the energy source sustaining seedling growth.

Furthermore, the seed supplies energy from its reserve carbohydrates to maintain viability and to form compounds as enrgy sources for the germination process. Avocado seed predominantly stores perseitol and, to a lesser degree, sucrose as soluble non-structural carbohydrates as well as starch as an insoluble, non-structural carbohydrate. Perseitol may be a readily available energy source that can be converted to *D*-mannoheptulose as an energy-providing compound as well as a transport carbohydrate. Perseitol might therefore be related to an increased ‘de novo’ synthesis of C7 sugars particularly in the dry seed, further strengthening the proposal that perseitol is a storage CHO. However, these aldolases presence

in seed protein extracts indicates that this enzyme is not only translocated from the mesocarp, but also manufactured 'de novo' in the seed (Figure 7). The condensation of dehydroxyacetone phosphate and erythrose 4-phosphate to sedoheptulose 1, 7-bisphosphate, is catalysed by a transaldolase (Liu et al., 2002).

The present study provides clear evidence that an aldolase enzyme plays a role in the interconversion/ formation of the heptoses *D*-mannoheptulose and perseitol. Such an enzyme, probably transaldolase, is produced in a variety of tissues of avocado (Figures 6, 7), indicating the important roles heptoses in avocado. However, further research is needed to identify, and, if novel, characterize this enzyme in order to further elucidate heptose carbohydrate metabolism in avocado.

**Acknowledgements**

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Table 1

Absolute and relative concentrations of soluble, non-structural carbohydrates from avocado branch exudates. The branch exudates are fresh if collected immediately after girdling (while it is still wet)

| Exudates | Branch | Sugars (mg $mL^{-1}$) | | | % | | |
|---|---|---|---|---|---|---|---|
| | | Sucrose | Mannoheptulose | Perseitol | Sucrose | Mannoheptulose | Perseitol |
| Fresh | 1 | 0.106 | 0.403 | 0.184 | 15.31 | 58.12 | 26.57 |
| | 2 | 0.168 | 0.450 | 0.212 | 20.20 | 54.19 | 25.61 |
| | 3 | 0.158 | 0.451 | 0.209 | 19.31 | 55.17 | 25.52 |
| Dry | 1 | nd | nd | 7.713 | 0 | 0 | 100 |
| | 2 | nd | nd | 7.689 | 0 | 0 | 100 |
| | 3 | nd | nd | 7.349 | 0 | 0 | 100 |

Note: nd = not detected

Fig. 1. HPLC chromatograms of water-soluble carbohydrate standards detected isocratically using HPLC/RID-10A (A); substrate-enzymatic reaction products (B); branch exudate collected immediately after girdling (C); dry exudates collected 7 days after girdling (D). Standards: 1. Sucrose (9.2 min), 2. Glucose (11.0 min), 3. D-mannoheptulose (12.7 min), 4. Fructose (13.6 min), 5. Volemitol (16.9 min), 6. Perseitol (19.3-20.3 min).

Fig. 2A. Enzyme activities of crude desalted protein extracts used to catalyze the conversion of perseitol into *D*-mannoheptulose, NADP was a co-factor added into the reaction mixture. The spectrophotometric quantification of NADPH as a byproduct was at wavelength 340 nm.. The more spectrophotometric absorbance implies higher enzyme activity, more NADPH and finally higher conversion percentage (%). $LSD_{(0.05)} = 0.01$ (n =5).

Fig. 2B. Enzyme activities of crude desalted protein extracts used to catalyze the conversion of *D*-mannoheptulose into perseitol, NADPH was a co-factor added into the reaction mixture. The spectrophotometric quantification of NADP as a byproduct was at wavelength 340 nm. The more spectrophotometric absorbance implies higher enzyme activity, more NADP and finally higher conversion percentage (%). $LSD_{(0.05)} = 0.038$ (n =5).

Fig. 3. Comparison of *D*-mannoheptulose and perseitol concentrations of avocado seeds; dry seeds versus cotyledons germinated under light and exclusion of light ‘etiolated’. Bars are standard error (SE) of different means (n=5).

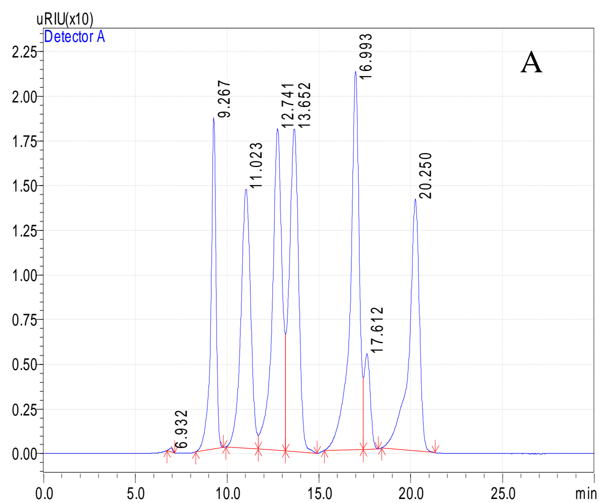
Fig. 4A. Percentage (%) of *D*-mannoheptulose conversion to perseitol by crude mesocarp enzyme extracts (250 µl).

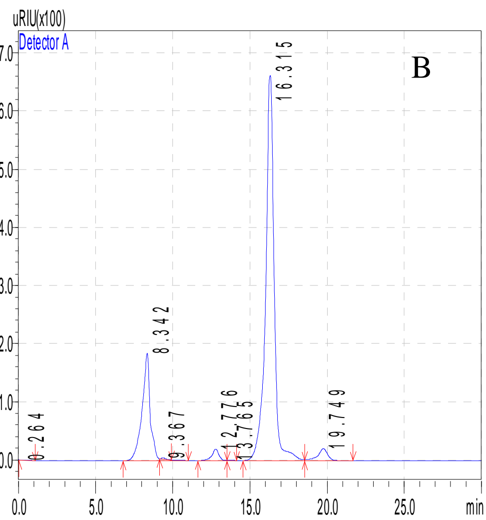
Fig. 4B. Pecentage (%) of perseitol conversion to *D*-mannoheptulose by crude mesocarp enzyme extracts (250 µl).

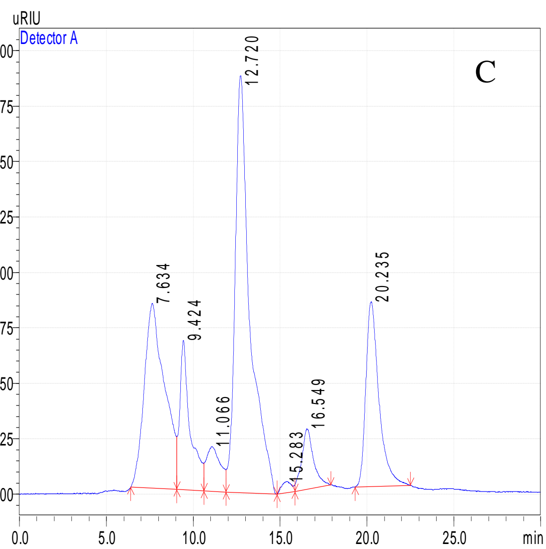
Fig. 5. Sugar concentrations of avocado branch exudates, collected two times, immediately after girdling and seven days later from the same branches. Bars are standard errors (SE) of different means (n=3).

Fig. 6. 12 % SDS–PAGE, of crude protein extract of mesocarp tissues. Gel A, all lanes are circled to indicate proposed mescarp aldolases aligned to crude spinach aldolase standard (Sigma) (Lane 1). Gel B, proposed mesocarp aldolases spiked with spinach aldolase (Sigma), thick bands are aldolases (sample and spinach aldolase mix). The protein amount loaded in each lane was 25-50 µg.

Fig. 7. 12 % SDS-PAGE was performed to identify aldolase in various avocado tissues. Lane 1: aldolase standard; Lane 2: mature dry seed; Lane 3: cotyledons of light grown seedlings seed; Lane 4: cotyledons of dark-grown seedlings The aldolase has molecular weight of 40 kD.



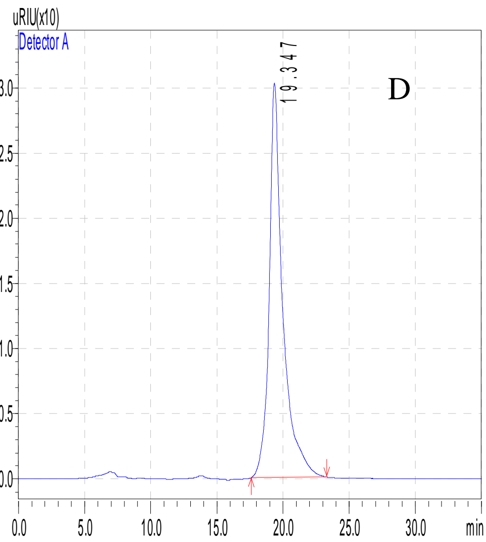
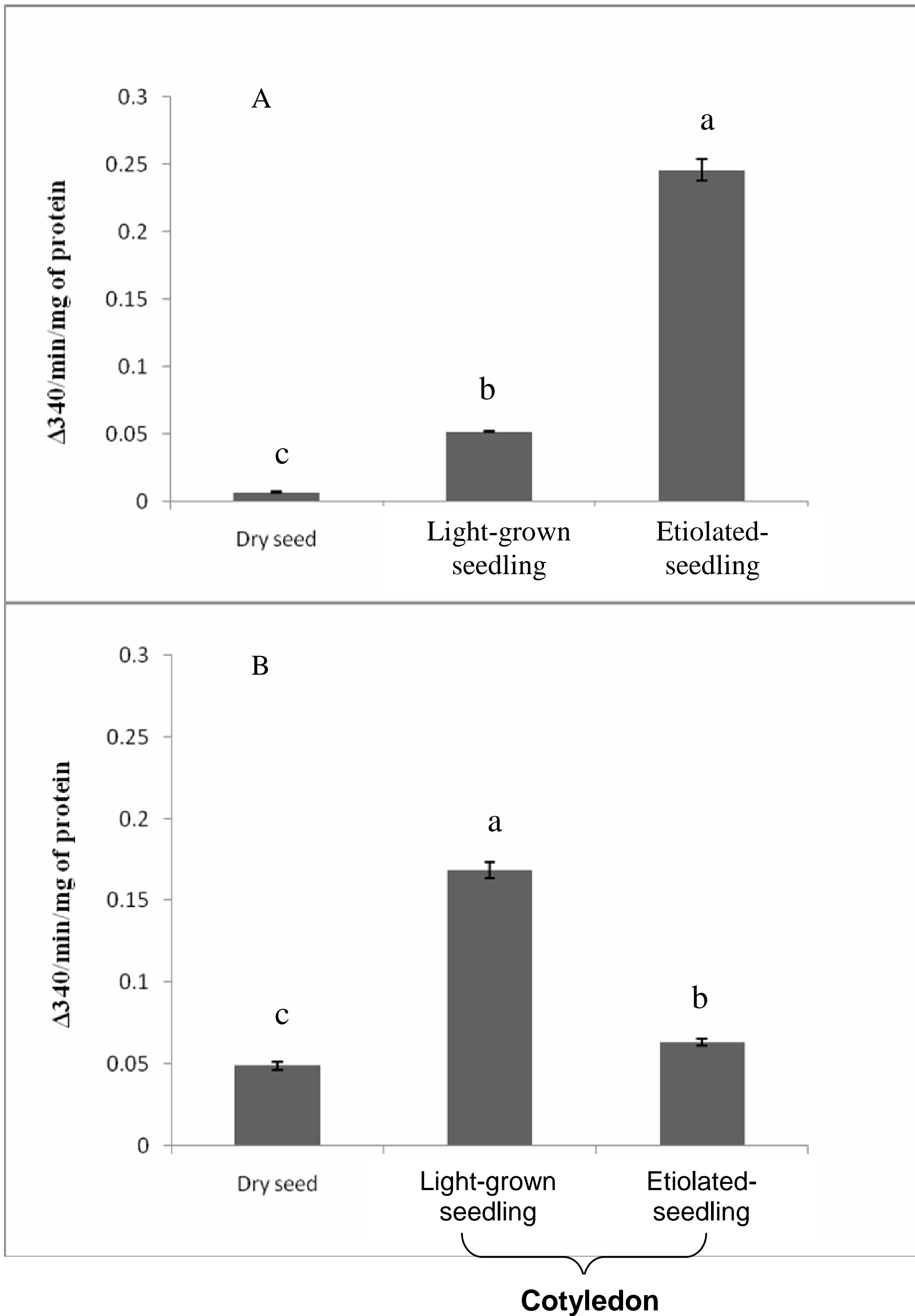




Fig.1.

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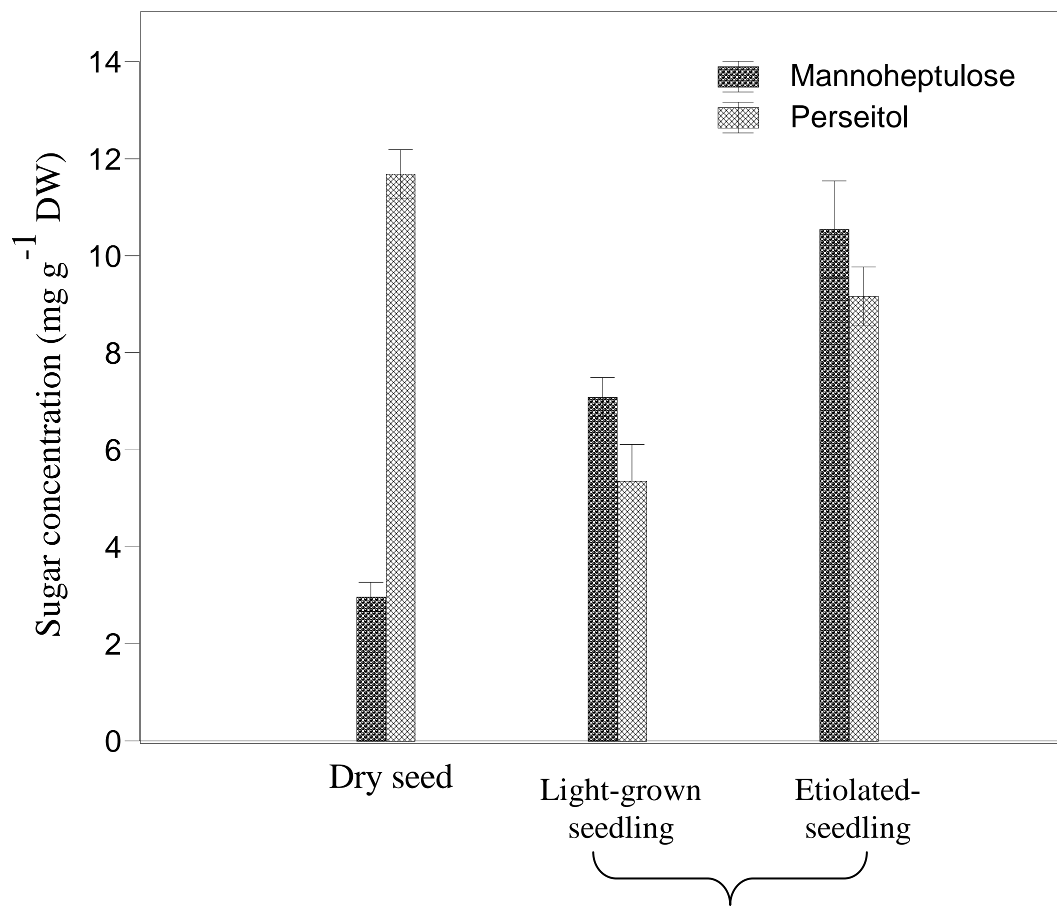



Fig 3.

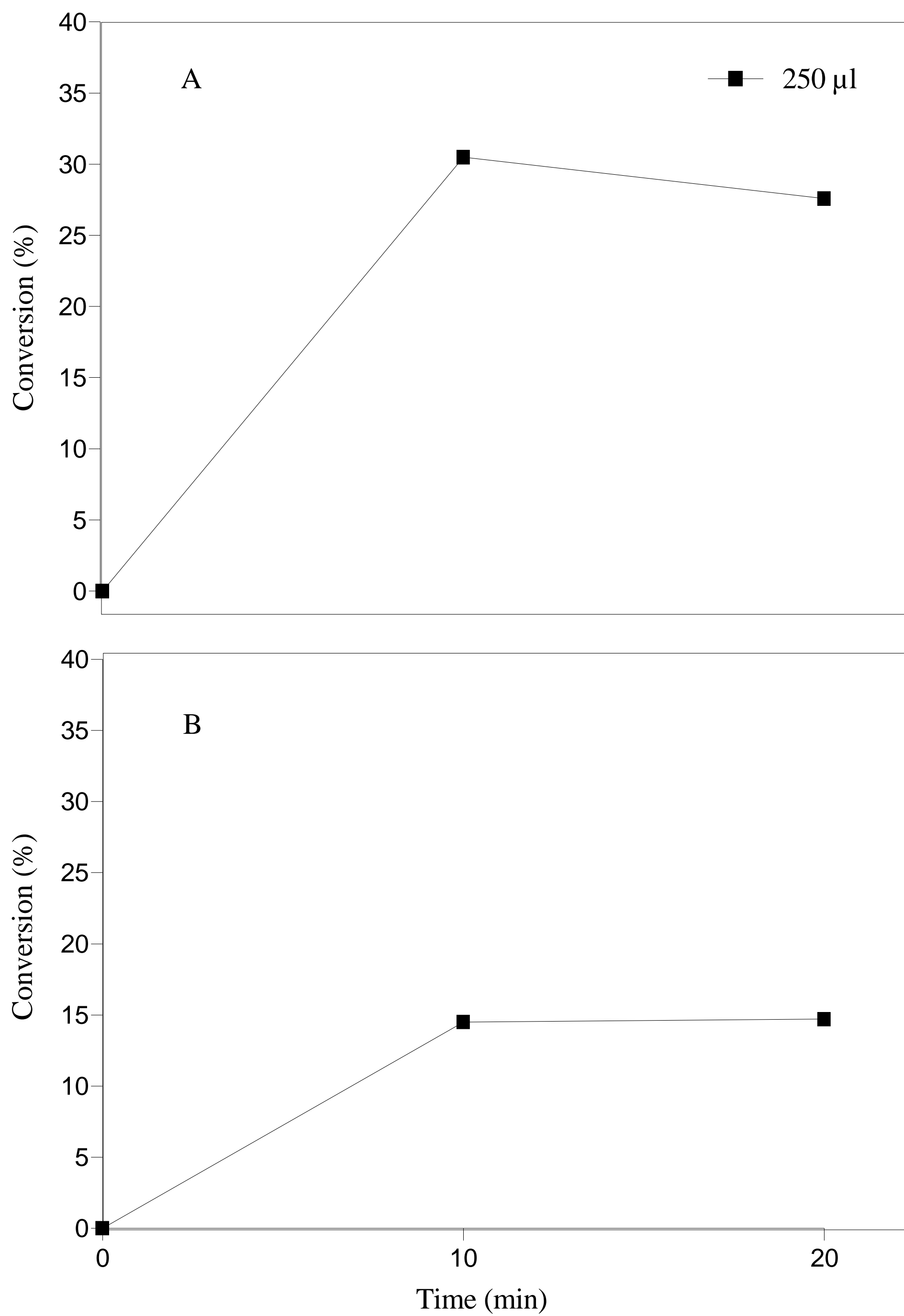



Fig. 4

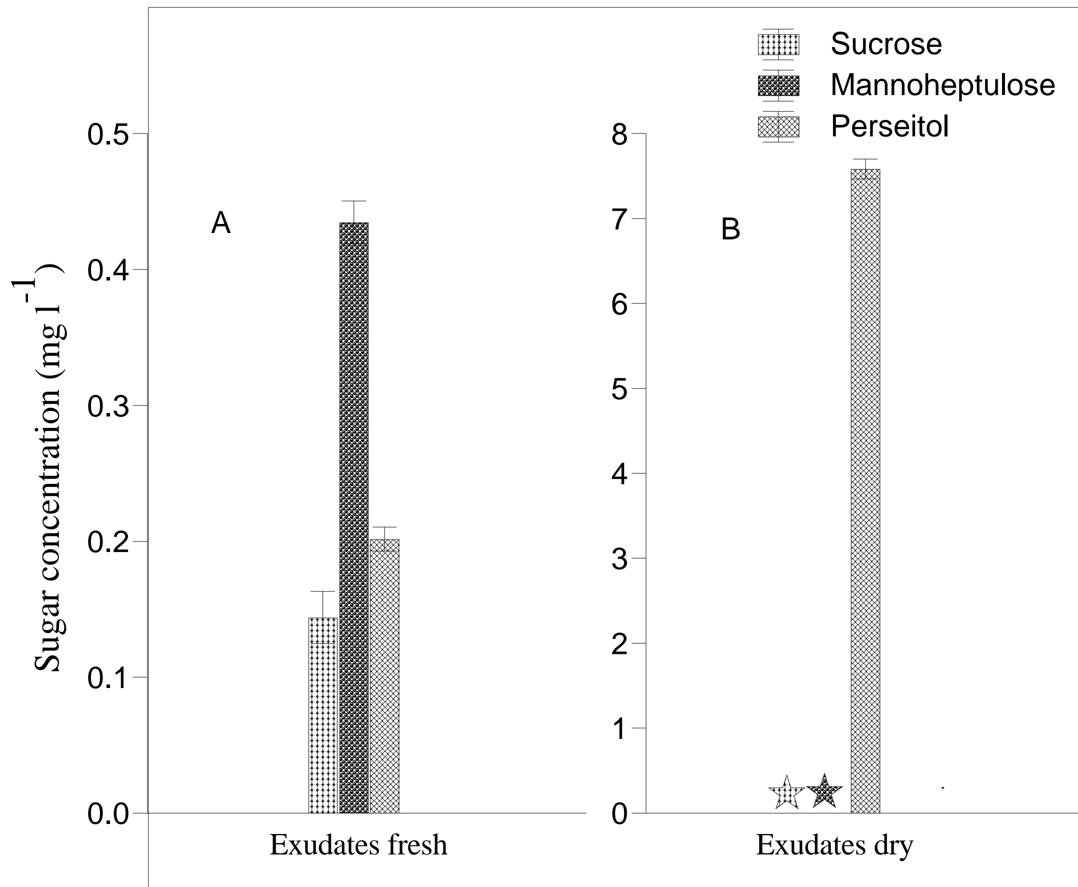
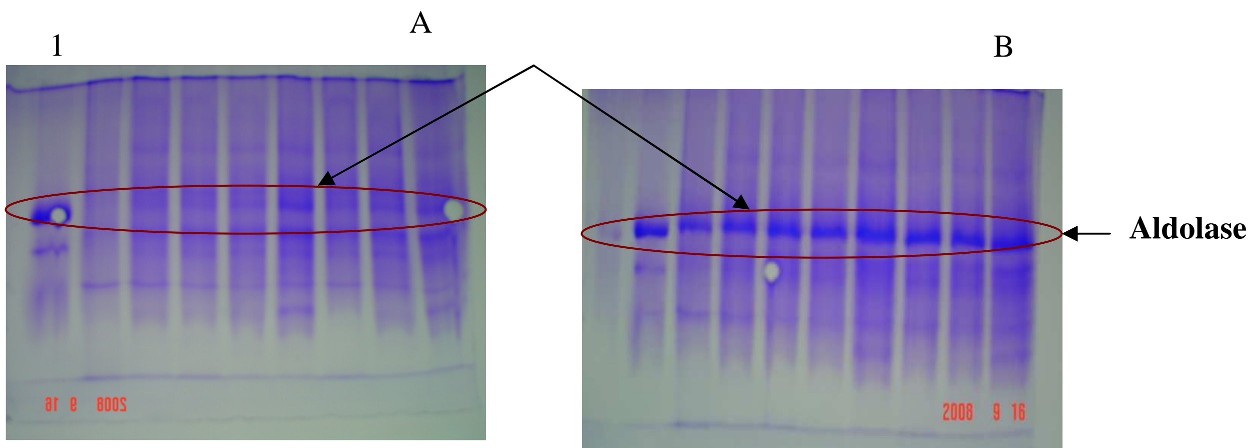



Fig 5

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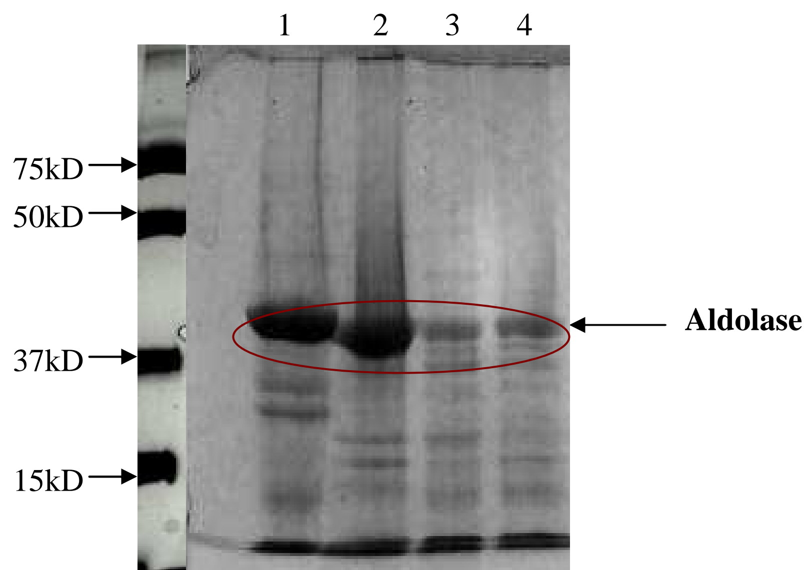


Fig. 7.

# CHAPTER 4

# LEVELS OF ANTI-OXIDANTS IN VARIOUS TISSUES DURING MATURATION OF 'HASS' AVOCADO (*Persea americana* MILL.)

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## SUMMARY

Avocado is a fruit high in polyunsaturated fatty acids, with a high mineral content. However, no reports are available on how valuable avocado could be as a source of anti-oxidants, depending upon its stage of maturity. Such a feature might become an important future marketing tool. To evaluate the presence of anti-oxidant systems throughout avocado fruit development, various tissues were analysed for their total and specific anti-oxidant compositions. Total anti-oxidant levels were found to be higher in the exocarp and in seed tissue than in the mesocarp, a possible reason for the susceptibility of avocado to post-harvest mesocarp disorders. While seed tissues contained predominantly ascorbic acid (AsA) and total phenolics (TP), the anti-oxidant composition of the mesocarp was characterised by the C7 sugar, *D*-mannoheptulose. Among the anti-oxidant enzymes assayed, peroxidase (POX) and catalase (CAT) were present in higher concentrations than superoxide dismutase (SOD) in mesocarp tissue. The low concentration of anti-oxidants in the mesocarp might be a major reason for the susceptibility of the fruit to physiological disorders.

Avocado fruit (*Persea americana* Mill.) is well-known for its high nutritive value (Bergh, 1992), high ratio of unsaturated to saturated fatty acids (14.3:1.0) (Slater *et al*., 1975), and relatively high contents of iron and potassium (Wolstenholme, 1990). Avocado fruit are also high in fat-soluble substances with anti-oxidant activity, such as *β*-carotene (Human, 1987) and *α*-tocopherol (Terasawa *et al*., 2006). Water-soluble vitamins, such as ascorbic acid and the B-complex vitamins, have been reported to exist in considerable amounts in avocado fruit (Slater *et al*., 1975). Moreover, avocado has certain anti-tumour properties, possibly arising from high concentrations of the C7 sugar, *D*-mannoheptulose (Board *et al*., 1995). In addition, Slater *et al*. (1975) reported that avocado contained varying

levels of anti-oxidants, depending on the stage of fruit maturity; however, little is known about the anti-oxidant systems present in various fruit tissues, although the presence of ascorbic acid and phenolics has been reported. Besides these low molecular weight anti-oxidants, anti-oxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX), which play an important role in scavenging reactive oxygen species (ROS) could exist in avocado.

Apart from such anti-oxidant systems, avocados also contain large amounts of the C7 sugar, *D*-mannoheptulose and its reduced polyol form, perseitol (Liu *et al.*, 1999). Such polyols are also efficient scavengers of free radicals (Jennings *et al*., 1998).

Seasonal alterations in anti-oxidant levels, and their relative composition, as well as levels of the radical scavenging enzymes, SOD, CAT, and POX, have not yet been reported during avocado fruit growth and development. Identifying these changes could become a useful tool with which to determine the optimal harvesting date, when such a determination was combined with other commonly used harvest indicators such as oil or water contents of the fruit. Understanding the composition of the anti-oxidant systems present, and the changes that occur in the pool of anti-oxidants in avocado may also permit future manipulation of anti-oxidant levels in avocado tissues to increase fruit quality and to enhance the ‘health food’ image of the fruit.

## MATERIALS AND METHODS

### *Materials*

Fruit from ten healthy, mature ‘Hass’ avocado trees located in the KwaZulu-Natal Midlands, South Africa (30°16´E, 29°28´S) were used for all experiments. Sampling of fruit

commenced 16 weeks after full bloom (WAFB; in January) and continued, on a monthly basis, from the exponential to the linear part of the sigmoid growth curve (Scora *et al*., 2002; Figure 1C), up to June, when the fruit had reached commercial maturity (62 -67% mesocarp moisture content, equivalent to 18 - 22% oil). Each month, 20 fruit (two per tree) were separated into exocarp, mesocarp, and seed, and the fractions were freeze-dried, ground, and stored at -20°C until further analysis.

### *Lipid concentrations*

Mesocarp lipid concentrations were determined according to Meyer and Terry (2008). The recovered oil was weighed and the percentage oil content was [% (w/w)] calculated.

### *Measurement of anti-oxidants*

Anti-oxidant levels were first determined as “total anti-oxidant capacity” (TAOC) using the FRAP (ferric reducing ability of plasma) assay (Benzie and Strain, 1996) and expressed as μmol $FeSO_47H_2O$ $g^{-1}$ DW equivalents. Second, total anti-oxidant activity (TAOA) was also determined using the ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) assay according to Re *et al.* (1999), in order to gauge the presence of hydrophilic as well as lipophilic anti-oxidants.

### *Determination of ascorbic acid (AsA) concentrations*

Ascorbic acid concentrations were determined according to Böhm *et al*. (2006) by comparing the absorbance of fruit tissue extracts at 520 nm with values obtained using an L-ascorbic acid standard curve. Results were expressed as mg AsA $g^{-1}$ DW.

### *Determination of total phenolics (TP) concentrations*

The simultaneous extraction of free and bound phenolic compounds was performed according to Böhm *et al*. (2006). TP concentrations were determined spectrophotometrically at 750 nm by adding Folin-Ciocalteu reagent and expressing the results as 'gallic acid equivalents' (GAE).

### *Determination of sugar concentrations*

Sugar concentrations were determined according to Liu *et al*. (1999) using an isocratic HPLC system (LC – 20AT, Shimadzu Corporation, Kyoto, Japan) equipped with a refractive index detector (RID-10A, Shimadzu Corporation, Kyoto, Japan) and a Rezex RCM–Monosaccharide column (300 mm x 7.8 mm) (8 micron pore size; Phenomenex®, Torrance, CA, USA). The concentration of individual sugars was determined by comparison with *D*-mannoheptulose and perseitol sugar standards (Glycoteam GmbH, Hamburg, Germany).

### *Total soluble protein extraction*

Total soluble proteins were extracted according to Kanellis and Kalaitzis (1992) from 1 g DW of frozen mesocarp. The extract was allowed to stand on ice for 15 min, was centrifuged at 20,000 x *g* for 20 min at 4°C, and the supernatant was used for enzyme assays after being passed through Miracloth®-quick filtration material for gelatinous grindates (20 - 25 µm pore size; Calbiochem, San Diego, CA, USA).

### *Total protein assay for determinations of specific enzyme activity*

The Bradford microassay was used to determine the protein content of the samples (Bradford, 1976). After addition of the reagent, samples were read spectrophotometrically at 595 nm and protein concentrations were determined by comparing the results with a standard curve for bovine serum albumin.

### *Assay for catalase (CAT) activity*

CAT (E.C.1.11.1.6) activity was determined as described by Beers and Sizer (1952) where 1 Unit of CAT activity was defined as the amount of protein that decomposed 1 µmol $H_2O_2$ within 1 min.

### *Assay for superoxide dismutase (SOD) activity*

SOD (E.C. 1.15.1.1) activity was determined by measuring the ability of the protein extract to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT), as described by Giannopolitis and Ries (1977). One Unit of SOD activity was defined as the amount of enzyme inhibiting 50% of NBT photo-reduction. The results were expressed as: SOD Units $mg^{-1}$ protein = 1,000 x $\mu g^{-1}$ enzyme extract resulting in 50% inhibition of NBT reduction.

### *Assay for peroxidase (POX) activity*

Peroxidase (E.C. 1.11.1.7) activity was assayed according to Reuveni *et al*. (1992) by measuring the oxidation of guaiacol as the increase in absorbance at 470 nm from 0 - 3 min. Activity was expressed as: POX Units $mg^{-1}$ protein = ($\Delta A_{470}$ $min^{-1}$ x 1,000 x 26.6 x mg enzyme $ml^{-1}$ reaction mixture).

*Statistical analysis*

Analyses of variance between tissues and between stages of maturation, and a correlation analyses among tissue parameters were performed using GenStat version 9.1 (VSN International, Hemel Hempstead, UK). Standard deviation values were calculated where a significant difference was found at $P < 0.05$ between individual values.

## RESULTS

*Total anti-oxidant capacity (TAOC) and total anti-oxidant activity (TAOA)*

TAOC differed significantly between tissues**:** Throughout the season, the TAOC of mesocarp tissue was ≤ 10% of the TAOC of exocarp or seed tissues (Table I). The TAOC values of the exocarp and the seed were initially similar (January, February); thereafter, the TAOC of seed exceeded the TAOC of the exocarp. Throughout the experimental period, the TAOC of mesocarp remained at levels lower than that of the exo- and mesocarp. The trends observed for TAOC were similar to those for TAOA (Figure 1).

*Ascorbic acid (AsA) concentrations*

As for TAOC, AsA concentrations were lowest in the mesocarp (Table I) and highest in exocarp tissue. After February, the exocarp consistently contained the highest concentrations of AsA for all tissues (Figure 2).

*Total phenolics (TP) concentrations*

TP concentrations in mesocarp tissue followed a similar trend to that for TAOC and AsA concentrations, being significantly lower in the mesocarp (Figure 3; Table I). TP exhibited a peak in seed tissue in April. Tissue type and stage of maturity, as well as an interaction between these factors, had a highly significant effect on TP concentration (Table I).

### *C7 sugars*

*D*-mannoheptulose concentrations were higher in the exocarp and mesocarp than in the seed (Table I). While concentrations of this sugar varied little in the exocarp over the growing season, a significant decline was observed in the mesocarp after March (Figure 4). As fruit approached maturity (June), *D*-mannoheptulose levels in the mesocarp dropped below those of the exocarp. In the seed, *D*-mannoheptulose concentrations followed a declining trend, similar to that in the mesocarp, to only a quarter of the January level by June (Figure 4). *D*-mannoheptulose concentrations were significantly affected by the type of tissue, as well as by stage of development, with a strong tissue x fruit maturity interaction.

Perseitol concentrations were similar in range to *D*-mannoheptulose concentrations, with the exception of the high January level in the mesocarp. Throughout the observed period the exocarp consistently contained less perseitol than the mesocarp and the seed (Figure 5). Seed perseitol concentrations declined with maturity, similar to *D*-mannoheptulose concentrations, with a tendency to higher perseitol than *D*-mannoheptulose concentrations in the seed tissue. Tissue perseitol concentrations were significantly affected by tissue type as well as fruit maturity, with a minor tissue x fruit maturity interaction (Table I).

*Superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) activities in mesocarp tissue*

Due to the low protein concentrations in the exocarp and seed ($\leq$ 3.3 µg 100 $\mu l^{-1}$ crude protein extract) compared with the mesocarp tissue (60.5 µg 100 $\mu l^{-1}$ crude protein extract), SOD, CAT, and POX activities were only analysed in the mesocarp tissue. SOD activity remained at a lower level than CAT, and POX activities throughout the experimental period (Figure 6). While SOD activity declined towards fruit maturity (May - June), CAT, and POX activities showed no differences throughout the observed period.

Altogether, the presence of various anti-oxidant fractions varied between tissues and stages of fruit development. Ascorbic acid and phenolics were, predominantly, found in the exocarp and the seed, with exocarp levels remaining high throughout the observed period, while those in the seed peaked during March and April. The anti-oxidant level of the mesocarp were, however, lower throughout the observed growth period. While heptoses do not greatly contribute to the TAOC of the exocarp and the seed, they constitute one of the major anti-oxidant fractions of the mesocarp. Comparing the strength of anti-oxidants by reacting standards in the TAOC assay (Figure 7) revealed that gallic acid and AsA react strongest in the FRAP assay, while similar catechin and epi-catechin concentrations show lower TAOC values. *D*-mannoheptulose shows only a weak reaction as anti-oxidant in the FRAP assay (Figure 7); however, at concentrations significantly higher than any other anti-oxidant, *D*-mannoheptulose can significantly contribute to anti-oxidant capacity. Perseitol, on the other hand, did not show activity in the FRAP assay ($A_{593nm}$ of 5 mg standard $ml^{-1} \leq 0.01$).

## DISCUSSION

The development and ripening patterns of avocado fruit are different to other fruit. Besides unique anatomical characteristics such as an extended cell division, continuing into fruit maturity (Moore-Gordon *et al*., 1998), physiologically the fruit are also unique in containing high amounts of the drought stress-related hormone, abscisic acid (ABA), which increases with fruit maturity (Cutting and Bower, 1987). Furthermore, the avocado fruit requires a developmental period of six to more than twelve months from flowering to maturity (Scora *et al*., 2002). Hence, it is likely, that during this extended period avocado fruit are exposed to a high amount of environmental stress. In order to protect the fruit and, more importantly, from an evolutionary point of view, the seed, from an environment of oxidative stress, tropical and sub-tropical fruit are equipped with a variety of ROS scavenger systems (Soong and Barlow, 2004). Therefore, anti-oxidants are more prevalent in stress-exposed tissue, such as the exocarp and the seed than in mesocarp tissue (Figure 1).

During fruit growth phase III, when fruit growth slows in avocados (Scora *et al*., 2002), grown in this climatic region, an increase in anti-oxidants in the exocarp and, to a lesser degree, in mesocarp tissue was evident. This phase of development is characterised by the accumulation of oil in the mesocarp (Kotzé, 1979), which makes the protection of these polyunsaturated compounds through anti-oxidants of paramount importance. Barlow (2002) hypothesizes that avocado seed dispersal used to occur via several extinct species, but in more recent time mainly mammals act as dispersers. This makes conserving the mesocarp taste, possibly by reducing oxidation of oil through the anti-oxidant, a vital feature for successful fruit dispersal. The anti-oxidants of the seed, phenols, however, could possibly as feeding deterrents to their fruit-dispersers. These astringent anti-oxidants provide a “taste-barrier” between the edible mesocarp and the inedible seed tissue. As the seed starts germinating,

phenolics loose this function resulting in reduced phenolic levels at the final stages of fruit maturity (Figure 3).

Trends in ascorbic acid and anti-oxidant enzymes (Figure 2; Figure 6) also support the assumption that the decline in anti-oxidant levels towards the end of fruit development is aligned to the means of avocado seed dispersal, with TAOC constituents which make the fruit less palatable for mammals, like ascorbic acid and total phenolics, decreasing significantly (Figures 1-3), resulting in a less astringent mesocarp and relatively high sugar concentration (Figure 4; Figure 5).

Different anti-oxidant systems seem to be dominant within the various fruit tissues. Tissues displaying the highest anti-oxidant capacity (seed and exocarp) also had the highest ascorbic acid and phenolic concentrations (Table I). The TAOC in mesocarp tissue was more closely aligned to the C7 sugars than the other analysed anti-oxidants (Table I). Hence, the seasonal patterns of TAOC observed were mainly due to fluctuations of AsA and TP (Figure 2; Figure 3). The decline in phenolics from March onwards, when fruit growth has surpassed the exponential stage (Figure 1), is in line with results by Montero *et al*. (1996) in strawberry fruit, of decreasing phenolics midway during fruit development.

In seed and rind the best correlation was found between TAOC and AsA (Table I), indicating the importance of anti-oxidant in these tissues. TP were also positively correlated with TAOC in seed. A positive correlation between TP and TAOC has been reported for a variety of plants, ranging from onion (*Allium cepa* L.) to lettuce (*Lactuca sativa* L.), and pomegranate (*Punica granatum)* (Li *et al*., 2006).

Of the three anti-oxidant enzymes examined in avocado mesocarp, POX and CAT had higher activities than SOD throughout the late fruit growth period (March - June; Figure

6). Similarly, Abassi *et al*. (1998) reported higher CAT and POX than SOD activities in immature as well as in mature apple fruit cortex tissues. As SOD and CAT are sensitive to oxidation (Davidson *et al*., 1986; Hossain and Asada, 1984), counteracting the inactivation of these enzymes by ROS by anti-oxidant molecules becomes even more important. The decrease in TAOC in the mesocarp towards the end of fruit maturity was most likely due to the decrease in *D*-mannoheptulose concentration in the tissue (Figure 4), as the other anti-oxidant, AsA does not decrease significantly during the experimental period (Figure 2). Furthermore, perseitol varied little in the tissues analysed within the examined period.

The various fruit tissues seem to contain specific anti-oxidant systems. AsA and TP seem to be aligned, as tissues with high concentrations of these anti-oxidants generally have a high TAOC and are low in C7 sugars. *D*-mannoheptulose declined as fruit approached harvesting maturity, strengthening previous assumptions (Bertling and Bower, 2006) of the importance of this C7 sugar in fruit quality. As lipophilic anti-oxidants do not largely contribute to the total anti-oxidant pool of the mesocarp pre-harvest (TAOC vs. TAOA results were similar), *D*-mannoheptulose and ascorbic acid are likely to be the major mesocarp anti-oxidants. Although the anti-oxidant capacity of *D*-mannoheptulose is only one hundred's of ascorbic acid, this sugar could, together with ascorbic acid, provide protection to the mesocarp tissue from oxidative stress. If *D*-mannoheptulose, as the major carbohydrate, additionally, acts in this tissue actins as the major respiratory substrate post-harvest, the reduction in this sugar during the ripening period will affect the of ability of the mesocarp to withstand stress, which could play an important role in the development of post-harvest mesocarp disorders avocado seems to be prone to.

Our investigation has demonstrated the importance of the C7 sugars, particularly *D*-mannoheptulose, in mesocarp tissue. The concentration of this sugar could play a role as stress-protection agent. The deterioration of post-harvest quality in 'Hass' avocado might, at least partially, be due to the lack of *D*-mannoheptulose in the mesocarp tissue. This declining trend of anti-oxidants in the mesocarp tissue as the season progresses could cause the occurrence of oxidation products and cell damage which would become visible as various post-harvest browning disorders. This implies the need to search for means to increase the pre-harvest level of anti-oxidants in general, and *D*-mannoheptulose in particular, to allow longer post-harvest storage of fruit. As anti-oxidants are produced as a stress response application of minor stresses in the pre-harvest period could impact positively on anti-oxidant accumulation in the mesocarp. Future research should therefore focus on pre- as well as post-harvest treatments which are able to increase or maintain the anti-oxidant capacity of the mesocarp, possibly by increasing the *D*-mannoheptulose pool. This could produce fruit with a better internal quality; furthermore, avocado could be marketed as an anti-oxidant-rich fruit, further adding to the already existing image of a 'healthy fruit'.

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TABLE I

*Total anti-oxidant capacity (TAOC), total anti-oxidant activity (TAOA), and concentrations of ascorbic acid (AsA), total phenols (TP), D-mannoheptulose, and perseitol in the mesocarp, seed, and exocarp of 'Hass' avocado (Persea americana* Mill.*) averaged over the 6 month experimental period*

| Tissue | TAOC ($\mu$moles $g^{-1}$ DW) | TAOA ($\mu$moles $g^{-1}$ DW) | Ascorbic acid (mg $g^{-1}$ DW) | Total phenolics ($\mu$g GAE $g^{-1}$ DW) | Mannoheptulose (mg $g^{-1}$ DW) | Perseito (mg $g^{-1}$ D |
|---|---|---|---|---|---|---|
| Mesocarp | 67.3 ± 27.8c | 784 ± 477b | 0.41 ± 0.1c | 17.80 ± 7.1c | 24.77 ± 9.4a | 21.30 ± 23 |
| TAOC[d] | 1.0 | - | 0.16 | 0.63* | 0.71* | 0.71* |
| AsA[d] | 0.16 | - | 1.0 | - | - | - |
| TP[d] | 0.63* | - | - | 1.0 | - | - |
| Seed | 1131.2 ± 393.3b | 2593 ± 245a | 1.84 ± 0.8b | 227.32 ± 111.8a | 10.32 ± 8.2b | 17.10 ± 1 |
| TAOC[d] | 1.0 | - | 0.88* | 0.81* | - | - |
| AsA[d] | 0.88* | - | 1.0 | 0.78* | - | - |
| TP[d] | 0.81* | - | 0.78* | 1.0 | - | - |
| Exocarp | 1457.2 ± 305.1a | 2649 ± 57a | 2.77 ± 0.6a | 174.85 ± 48.5b | 25.07 ± 6.0a | 7.50 ± 6. |
| TAOC[d] | 1.0 | - | 0.75* | 0.75* | - | - |
| AsA[d] | 0.75* | - | 1.0 | 0.78* | - | - |
| TP[d] | 0.75* | - | 0.78* | 1.0 | - | - |
| [b]F Prob. (5%) | < 0.001** | < 0.001** | < 0.001** | < 0.001** | < 0.001** | < 0.001* |
| [c]LSD (5%) | 148.7 | 177.1 | 0.28 | 26.23 | 3.11 | 5.76 |

[a] Values are means ± SD. (n = 5)
[b] ** Significant differences between means at P = 0.05.
[c] Values followed by a different lower-case letter within a column are significantly different at P = 0.05.
[d] Correlation coefficient (r), * indicates significance at P < 0.05.
(-) indicates correlation 'not determined'.

FIG. 1 Seasonal changes in total anti-oxidant activity (TAOA; ($ABTS^{\cdot+}$assay; Panel A) and in total anti-oxidant capacity (TAOC; (FRAP assay; Panel B) the mesocarp, exocarp, and seed of 'Hass' avocado fruit. Fresh weight (FW; Panel C) and oil content (%; Panel D) were also determined as fruit maturity indices with which to compare changes in seasonal total anti-oxidant with fruit phenology. $LSD_{0.05}$ = 182.2 (FRAP assay); $LSD_{0.05}$ = 419.4 ($ABTS^{\cdot+}$assay); $LSD_{0.05}$ = 6.5 (Lipid Content); Vertical bars represent ± SE. (n=5)

FIG. 2 Seasonal changes in mean AsA concentrations in the mesocarp, exocarp, and seeds of 'Hass' avocado fruit. $LSD_{0.05}$ = 0.3, Vertical bars represent ± SE. (n=5)

FIG. 3 Seasonal changes in mean total phenolics (TP) concentrations in the mesocarp, exocarp, and seeds of 'Hass' avocado fruit. $LSD_{0.05}$ = 32.1, Vertical bars represent ± SE. (n=5)

FIG. 4 Seasonal changes in mean *D*-mannoheptulose concentrations in the mesocarp, exocarp, and seeds of 'Hass' avocado fruit. $LSD_{0.05}$ = 3.8, Vertical bars represent ± SE. (n=5)

FIG. 5 Seasonal changes in mean perseitol concentrations in the mesocarp, exocarp, and seeds of 'Hass' avocado fruit. $LSD_{0.05}$ = 7.05, Vertical bars represent ± SE. (n=5)

FIG. 6 Superoxide dismutase (SOD), catalase (CAT) (Panel A) and peroxidase (POX) (Panel B) activities of mesocarp tissue during the rapid fruit expansion phase. Vertical bars represent ± SE. (n=5)

FIG. 7 Total antioxidant capacity (TAOC) of specific anti-oxidant standard compounds phenols (gallic acid, catechin, and epicatechin) and AsA as determined using the Ferric Reducing Ability of Plasma (FRAP) Assay. Insert *D*-mannoheptulose had lower anti-oxidant capacity.

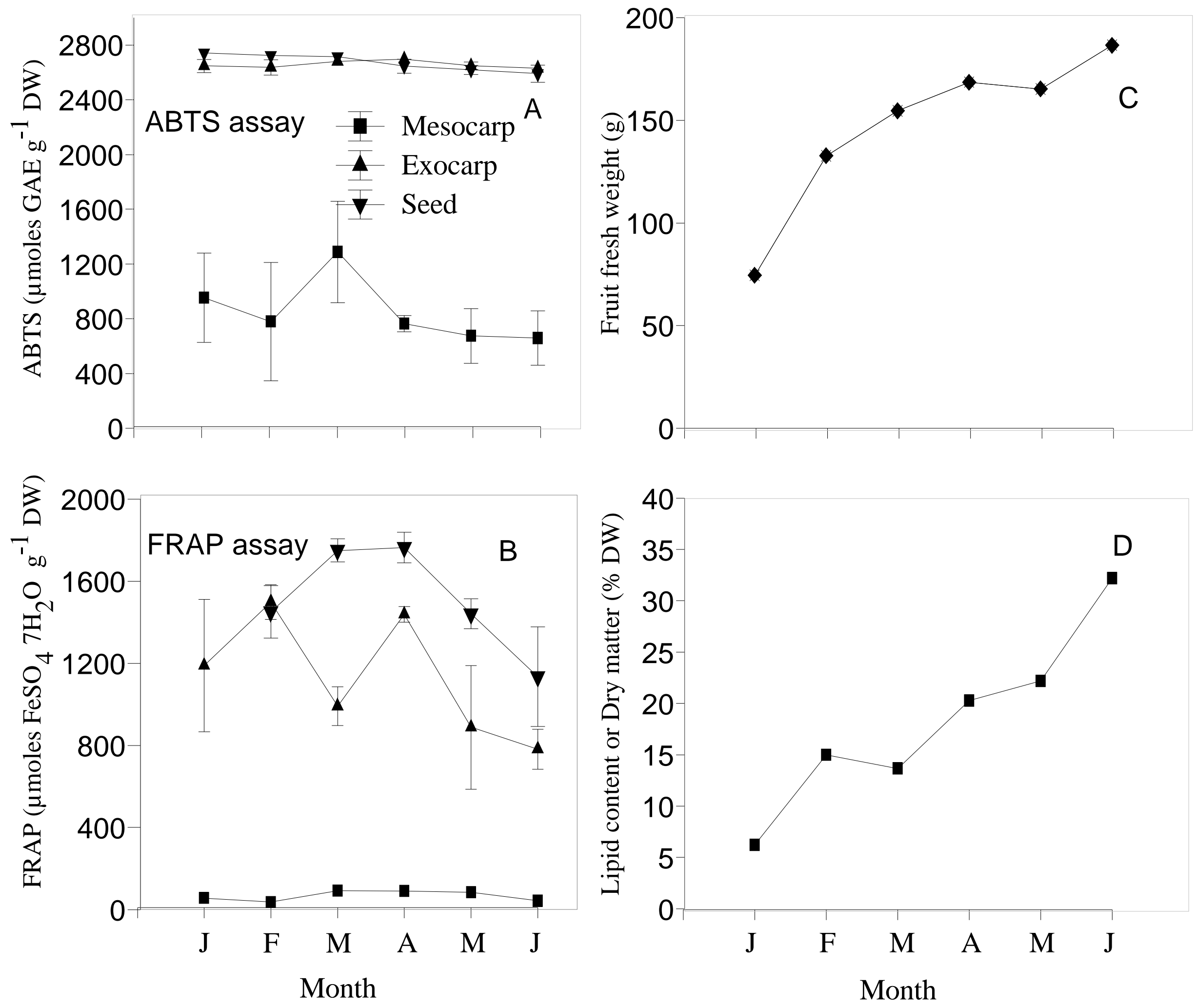


FIG. 1

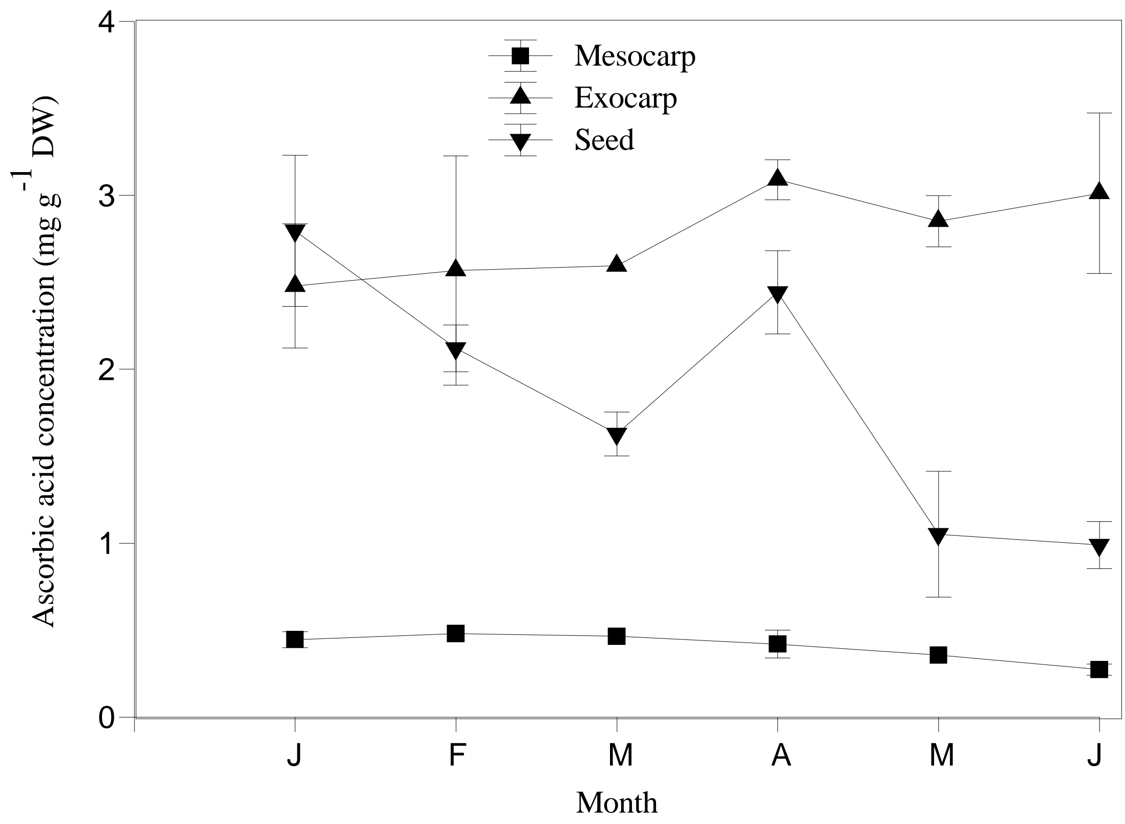


FIG. 2

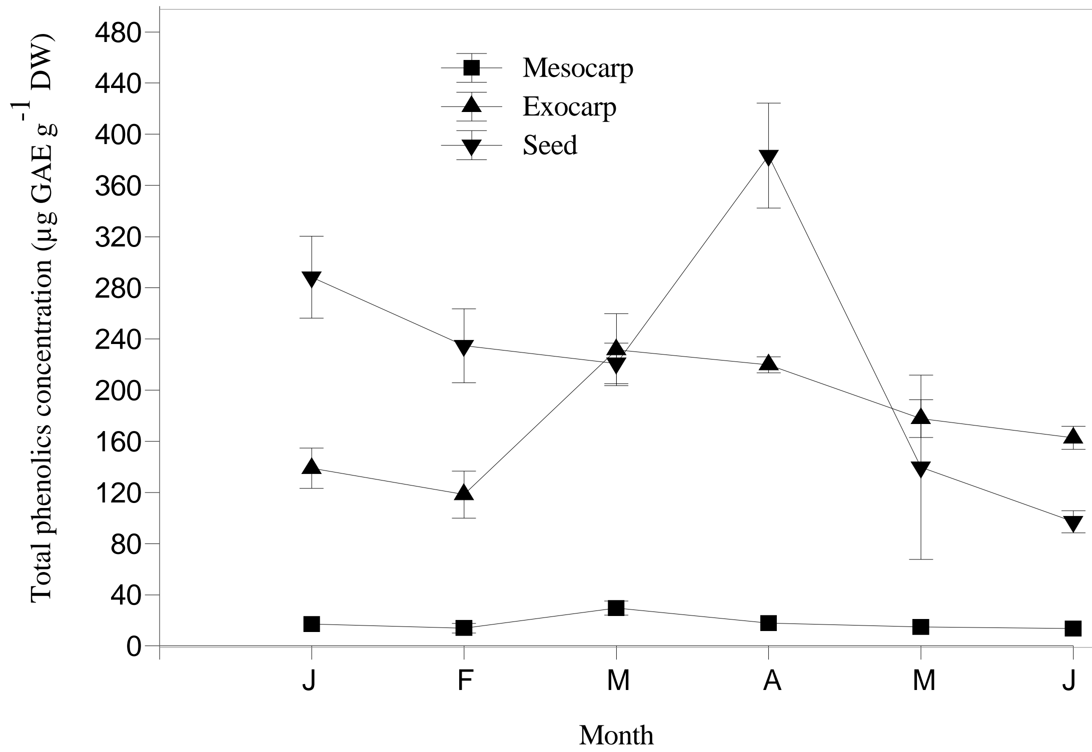


FIG. 3

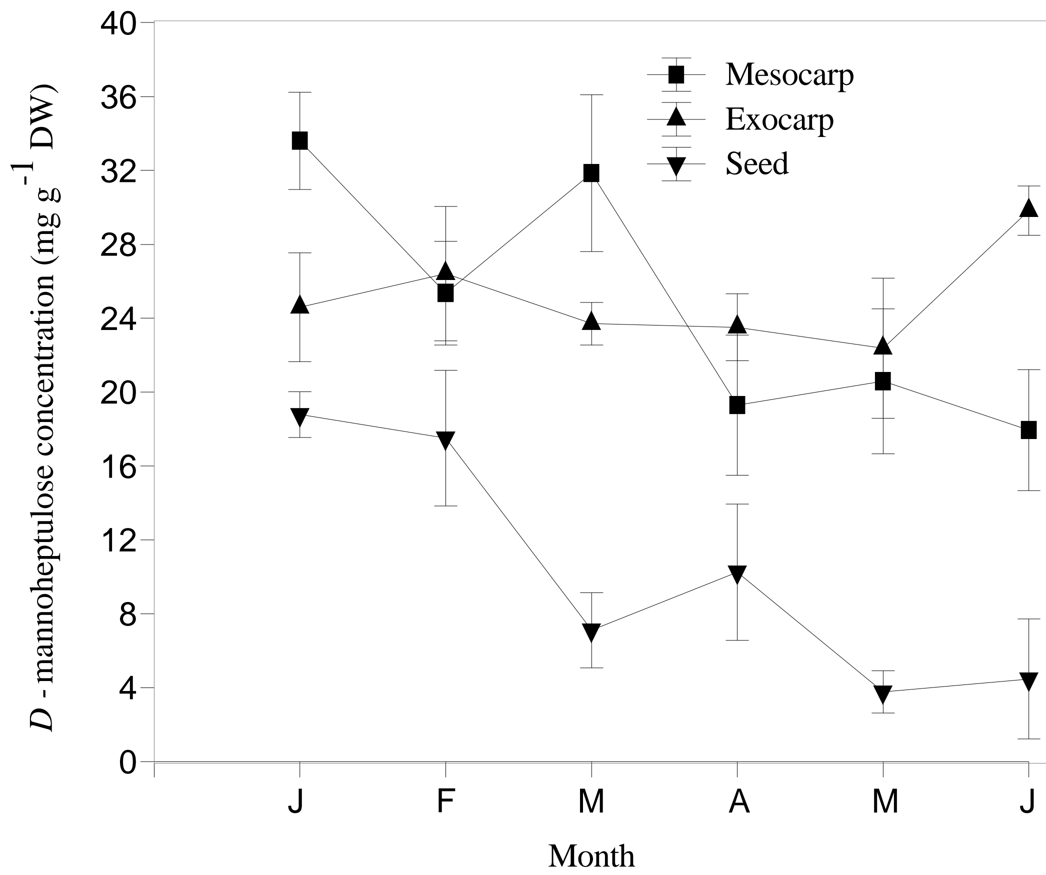


FIG. 4

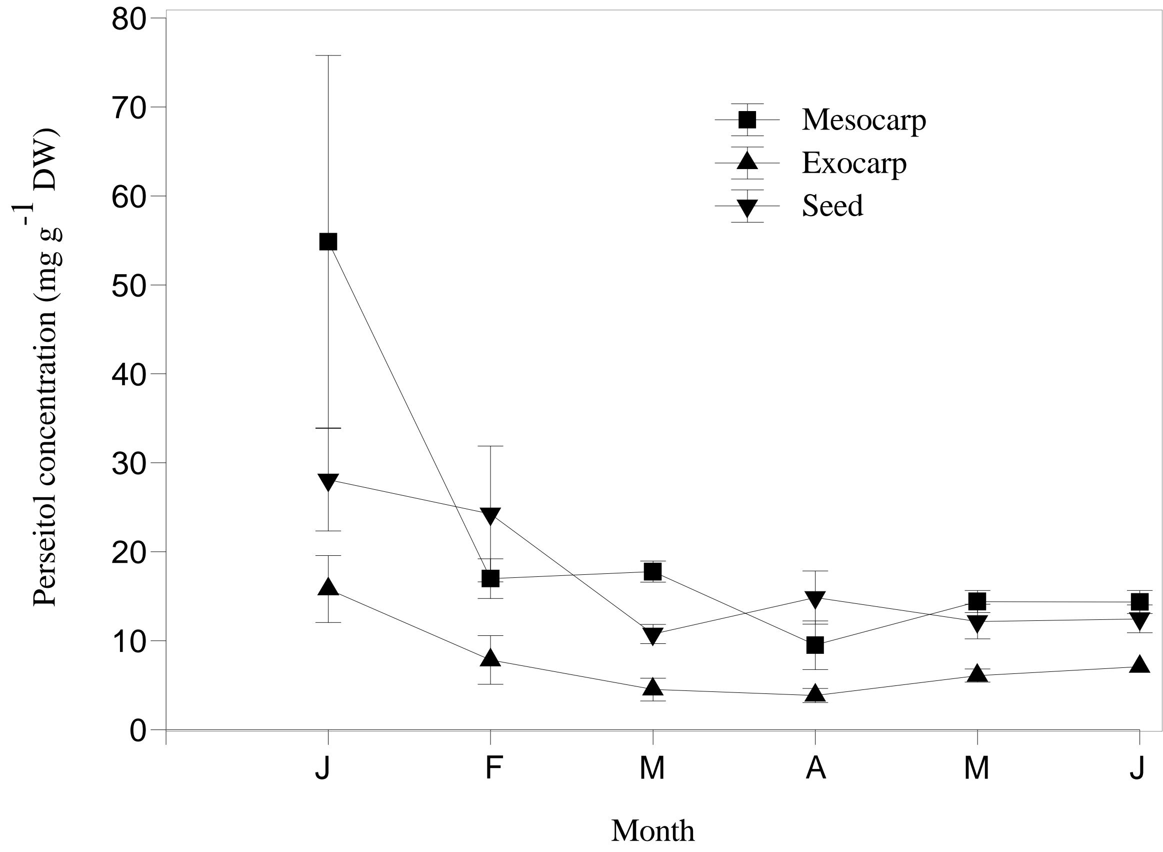


FIG. 5

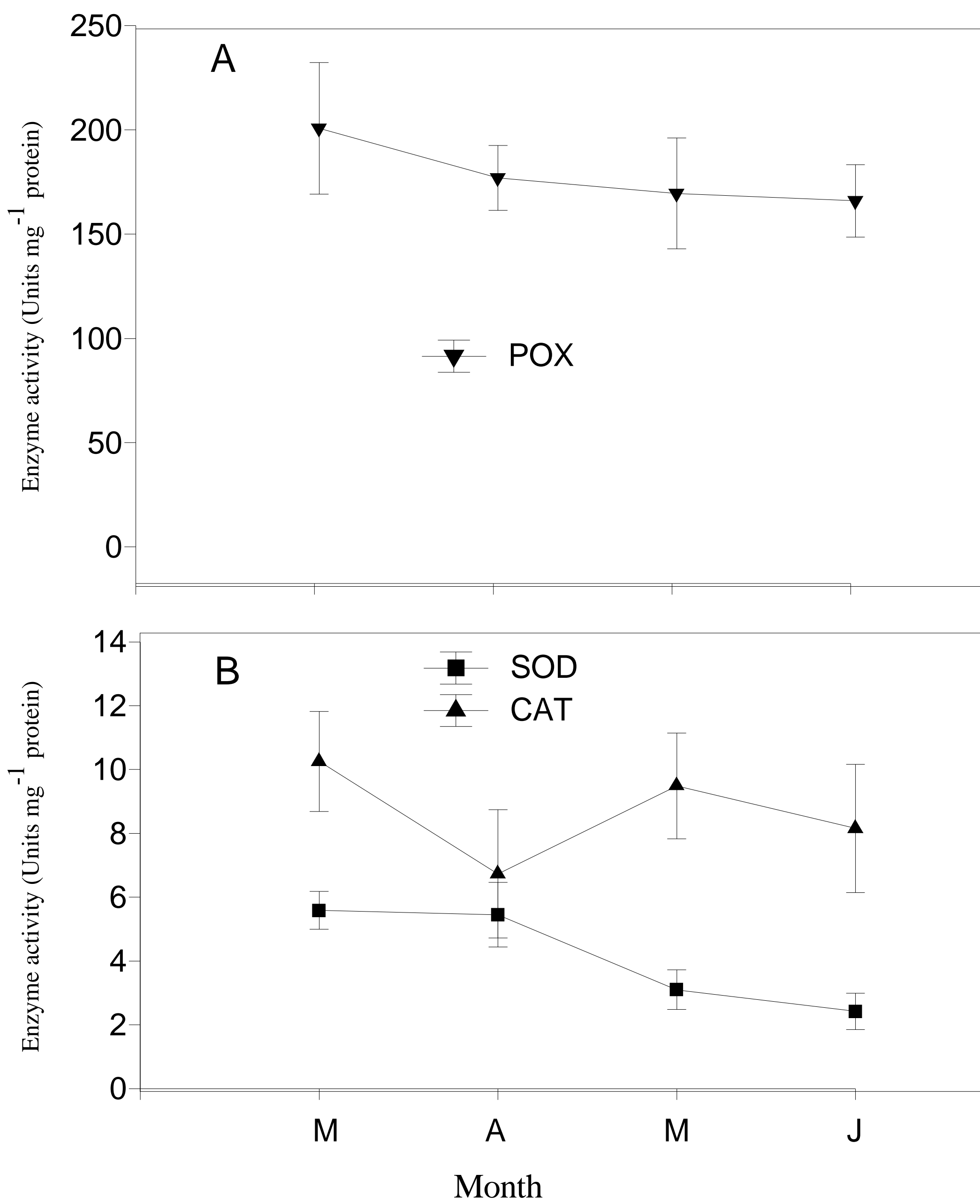


FIG. 6

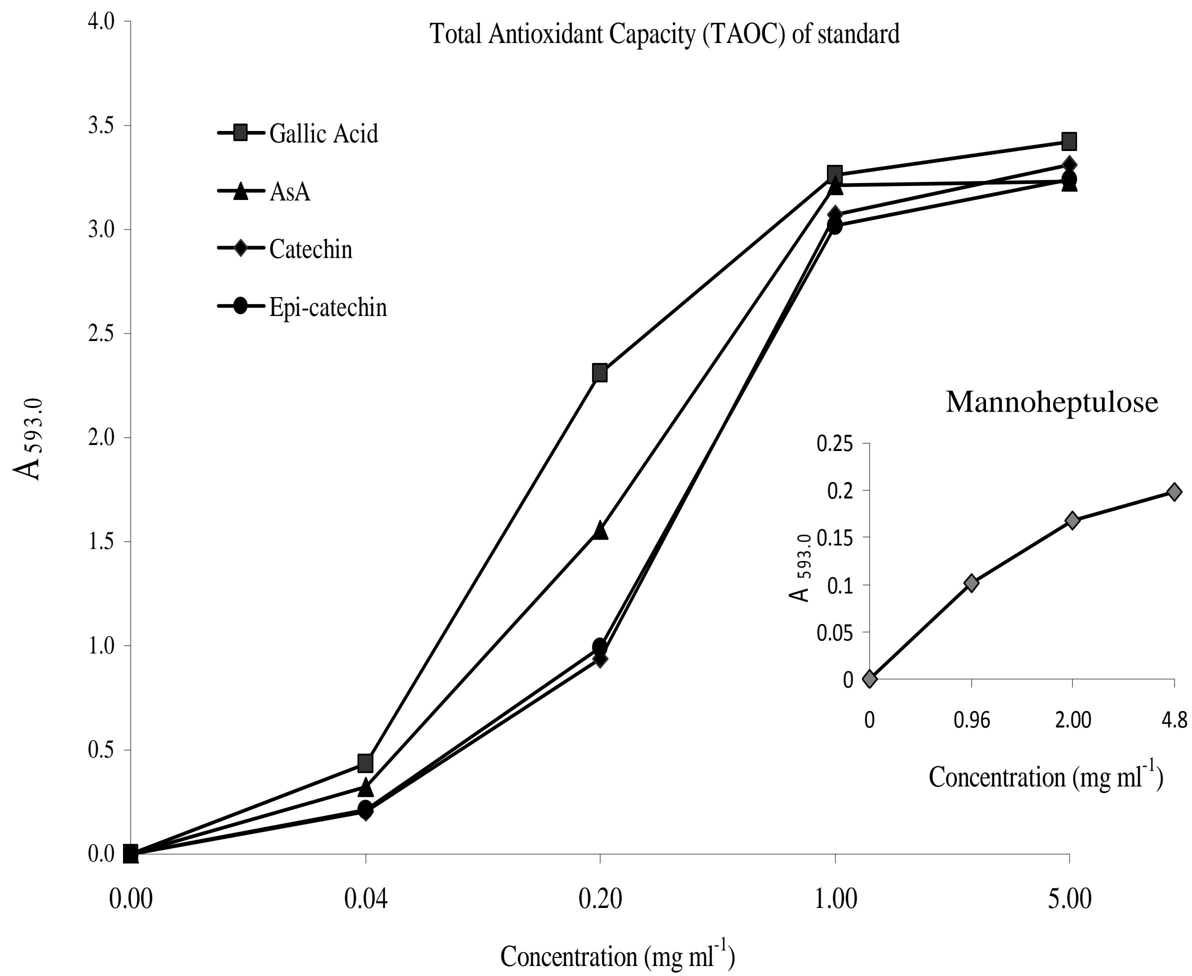


FIG. 7

# CHAPTER 5

# SEASONAL TRENDS OF SPECIFIC PHENOLS IN 'HASS' AVOCADO TISSUES

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## ABSTRACT

In avocado the polyphenols catechin and epicatechin are known for their anti-oxidant properties. However, they can also act as substrates of polyphenol oxidase (PPO) and contribute to browning. In order to be able to manipulate the availability of free polyphenols and their ability to act as anti-oxidants, an in-depth understanding of preharvest production and tissue distribution of these polyphenols is required. However, reports on the catechin and epicatechin tissue concentration preharvest are scarce. Thus, catechin and epicatechin concentrations in fruit tissue were determined using HPLC. The seed and the exocarp had higher concentrations of free catechin and epicatechin, while the mesocarp tissue showed higher concentrations of the conjugated forms of these phenols. As phenolics are able to participate in the induction/repression of genes as well as the activation-deactivation of enzymes of key metabolic pathways, their presence could increase the ability of fruit to better withstand stressful environmental conditions and, hence, increase fruit quality.

**Keywords:** avocado, catechin, epicatechin, phenolics

## INTRODUCTION

Plant phenolic compounds are ubiquitous and collectively make up several thousand different chemical structures, all characterized by hydroxylated aromatic rings. They are secondary metabolites and represent one of the most abundant groups of natural metabolites forming an important part of both, human and animal diets. Flavonoids are one of the groups of naturally occurring polyphenolic compounds found in vegetables, fruits and cereals (Amoros et al., 1992). Catechin is a flavonoid mainly found in green and black tea as well as in red wine (Manach et al., 2004). It belongs to the flavanols subclass, along with epicatechin.

Catechin and epicatechin are substrates for melanin - an undesirable brown pigment in fresh fruit, which becomes visible following bruising, cutting or storage (Lidster et al., 1986). Polyphenol oxidase (PPO) catalyses the reaction of endogenous phenolics into quinones, which then polymerize into melanin. In some instances, PPO enzymatic activity has been identified as the main factor in browning whereas in others the phenolic content has been highlighted as a key factor in the development of the undesirable color. (Lattanzio et al., 1994). Catechin and epicatechin, therefore have a significant but apparently contradictory role for the avocado industry because their anti-oxidant properties as well as potential to contribute to browning of avocado mesocarp tissue.

## MATERIALS AND METHODS

### Preharvest stage

Preharvest free and conjugated forms of catechin and epicatechin were determined using HPLC. Sampling of fruit commenced on the 16$^{th}$ week after full bloom (January) and continued on a monthly basis to June when fruit had reached commercial maturity (62-67 % moisture content) equivalent to 18-22 % oil in mesocarp (dry matter basis). Each month twenty fruit (two fruit per tree) were sampled. Fruit tissues (mesocarp, exocarp and seed) were freeze-dried, ground and subsequently stored at -20 °C until analysis. Additionally, the general profile of organic compounds was investigated at a wavelength of 280 nm (Fig. 1).

### Determination of total phenolics (TP) concentrations

The simultaneous extraction of free and bound phenolic compounds in fruit tissue was performed according to Böhm et al. (2006). TP concentrations were determined

spectrophotometrically at 750 nm by adding Folin-Ciocalteu reagent and expressing the results as ‘gallic acid equivalents’ (GAE).

## Determination of individual phenols by HPLC

Phenols were determined according to Hertog et al. (1992), with slight modifications. Briefly, freeze-dried tissue (1.0 g) was mixed with 10 mL 99.8 % (v/v) methanol and vortexed for 30 s. The mixture was then shaken overnight at room temperature to extract the free phenols. Subsequently the mixture was centrifuged and the supernatant filtered through Whatman® no. 1 filter paper and the pellet repeatedly rinsed with 10 mL of solvent until the rinsing solvent was clear. The pooled filtrates were taken to dryness under $N_2$ gas at 35 ºC. Dried samples were re-suspended in 99.8 % (v/v) methanol, filtered through a 0.45 µm nylon filter and analyzed using an isocratic HPLC system equipped with a photodiode array detector (PDA-100) on a Luna®-C18 column. Phenol quantification was made by comparison with external standards of catechin and epicatechin. Cell wall-bound phenols were released from the remaining plant residue by acid hydrolysis. A 10 mL portion of acidified (2 M hydrochloric acid) 60 % (v/v) aqueous methanol was added to each sample (pellet), the sample incubated at 90 ºC for exactly 90 min. Test tubes were allowed to cool before the supernatant was filtered through a 0.45 µm nylon filter before HPLC analysis.

## Statistical analysis

Analyses of variance between tissues and between stages of maturation, and correlation analyses among tissue parameters were performed using GenStat version 9.1

(VSN International, Hemel Hempstead, UK). Standard deviation values were calculated where a significant difference was found at $P < 0.05$ between individual values.

## RESULTS AND DISCUSSION

A higher production of total phenols (Fig. 2) as well as catechin and epicatechin, was found, in the exocarp and seed than in the mesocarp, while the highest concentration of free epicatechin was found in the exocarp (Table 1.). This is probably associated with the exposure of the exocarp to various stress factors. Vattem et al. (2005) reported that phenolics are ubiquitous in plants and that seeds and skins are especially rich sources of phenolics because of their role in protecting the fruit and the seed to ensure healthy propagation of the species. The production of free epicatechin is regulated by stress factors and most likely, favoured over catechin due to mild sensitivity and activity as anti-oxidants. This could explain why epicatechin is slightly more abundant than catechin, a trend also reported in seeds of red grape cultivars (Hatzidimitriou et al., 2007). This indicates that the production of free polyphenols might be stimulated by either/or external biotic factors (pathogenic), or abiotic (UV light) origin.

## CONCLUSION

Understanding the nature and distribution of the two major phenols, increasing the free forms of phenolic compounds in fruit mesocarp as they are regulated by biotic and/or abiotic factors, the postharvest fruit quality of avocados could be improved.

## ACKNOWLEDGMENTS

Provision of fruit material by Mr Rusty Roodt and Mr Rob Earl as well as financial support by the South African Avocado Growers' Association (SAAGA) for this research is kindly acknowledged.

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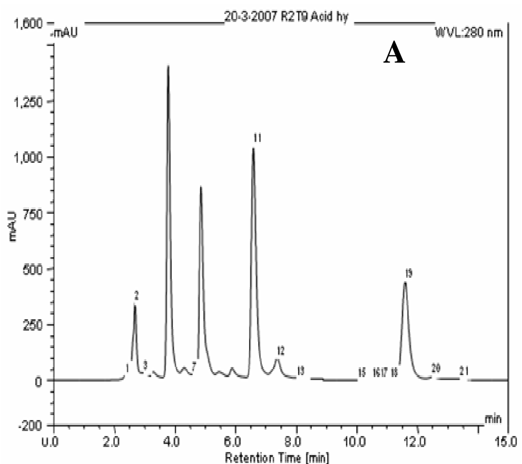
Table 1. Biosynthesis of free and conjugated forms of catechin and epicatechin in 'Hass' avocado fruit tissues at key stages of fruit developmental.

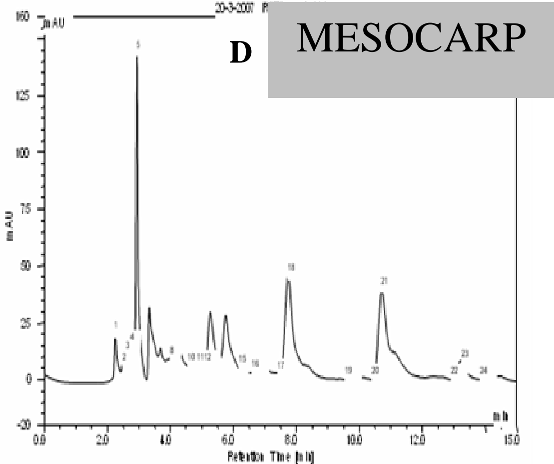
| Time<br>Tissue | | Catechin | | | Epicatechin | | |
|---|---|---|---|---|---|---|---|
| | | μg g $^{-1}$ | | | | | |
| | | **Free** | **Conjugated** | **Mean[1]** | **Free** | **Conjugated** | **Mean[3]** |
| Exocarp | Feb | 365.92 | 20.46 | 193.19bcd | 1101.77 | 12.82 | 557.3a |
| | March | 191.45 | 15.11 | 103.28def | 1078.76 | 20.31 | 549.54a |
| | April | 317.81 | 27.01 | 172.41bcd | 1111.01 | 58.64 | 584.83a |
| | May | 36.62 | 11.47 | 24.045f | 819.84 | 29.06 | 424.45a |
| | June | 200.6 | 12.49 | 106.55cdef | 966.28 | 28.98 | 497.63a |
| | Mean | 222.48b | 17.31d[2] | | 1015.53a | 29.96c[4] | |
| Mesocarp | Feb | 4.05 | 284.14 | 144.1cde | 5.93 | 175.6 | 90.77bcd |
| | March | 4.5 | 74.14 | 39.32ef | 3.38 | 28.8 | 16.09cd |
| | April | 3.25 | 50.96 | 27.105f | 0.71 | 16.98 | 8.85d |
| | May | 0.94 | 24.07 | 12.505f | 0.9 | 1.84 | 1.37d |
| | June | 0.65 | 97.83 | 49.24ef | 1.1 | 0.71 | 0.91d |
| | Mean | 2.68d | 106.23c | | 2.4c | 44.79c | |
| Seed | Feb. | 1047.84 | 3.55 | 525.7a | 211.22 | 0.58 | 105.9bcd |
| | March | 434.49 | 3.42 | 218.96bc | 465.91 | 0.47 | 233.19bc |
| | April | 467.87 | 5.34 | 236.61b | 418.41 | 13.86 | 216.14bcd |
| | May | 197.89 | 1.13 | 99.51def | 378.55 | 0.62 | 189.59bcd |
| | June | 292.12 | 0.36 | 146.24cde | 483.62 | 0.95 | 242.29b |
| | Mean | 488.04a | 2.76d | | 391.54b | 3.3c | |
| | | F pr. | **LSD(0.05)** | | F pr. | **LSD(0.05)** | |
| Tissue × Time | | 0.003 | 108.74[1] | | 0.839 | 224.1[3] | |
| Tissue × Forms | | < .001 | 68.772[2] | | < .001 | 141.73[4] | |

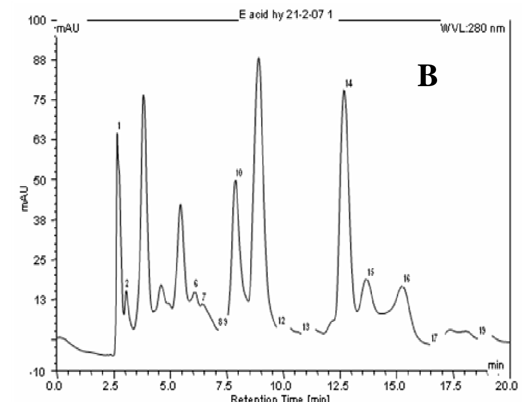
1. Values followed by a different lower-case letter within a mean column ([1, 3]) and row ([2, 4]) are significantly different at P = 0.05.

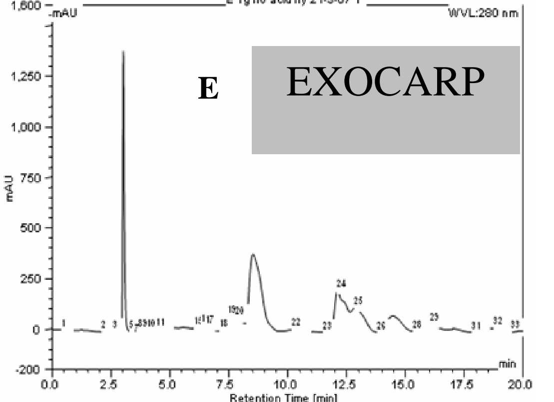
**Membrane- Bound phenols**

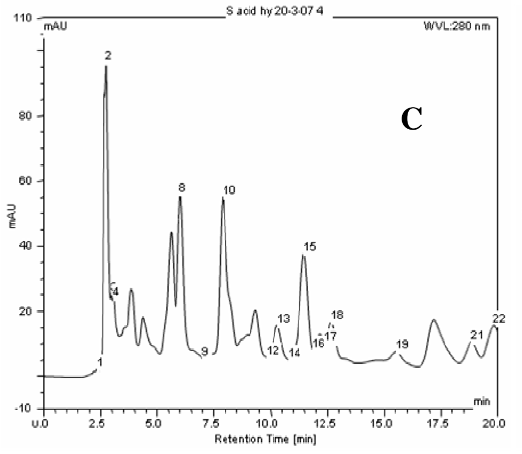
**Free phenols**



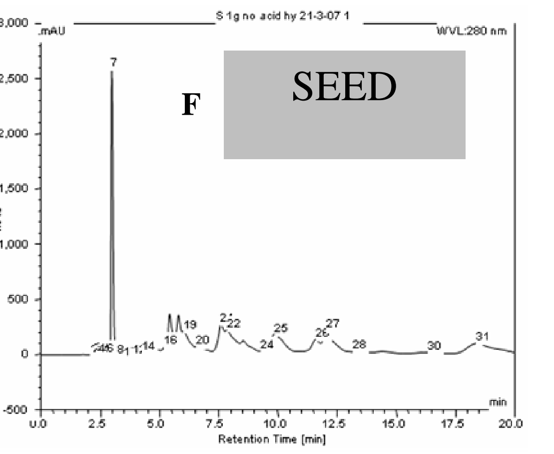
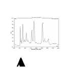




Fig. 1. Chromatographic presentation on distribution of different concentrations of free and conjugated forms of organic acids in ‘Hass’ avocado fruit tissues (mesocarp, exocarp and seed).





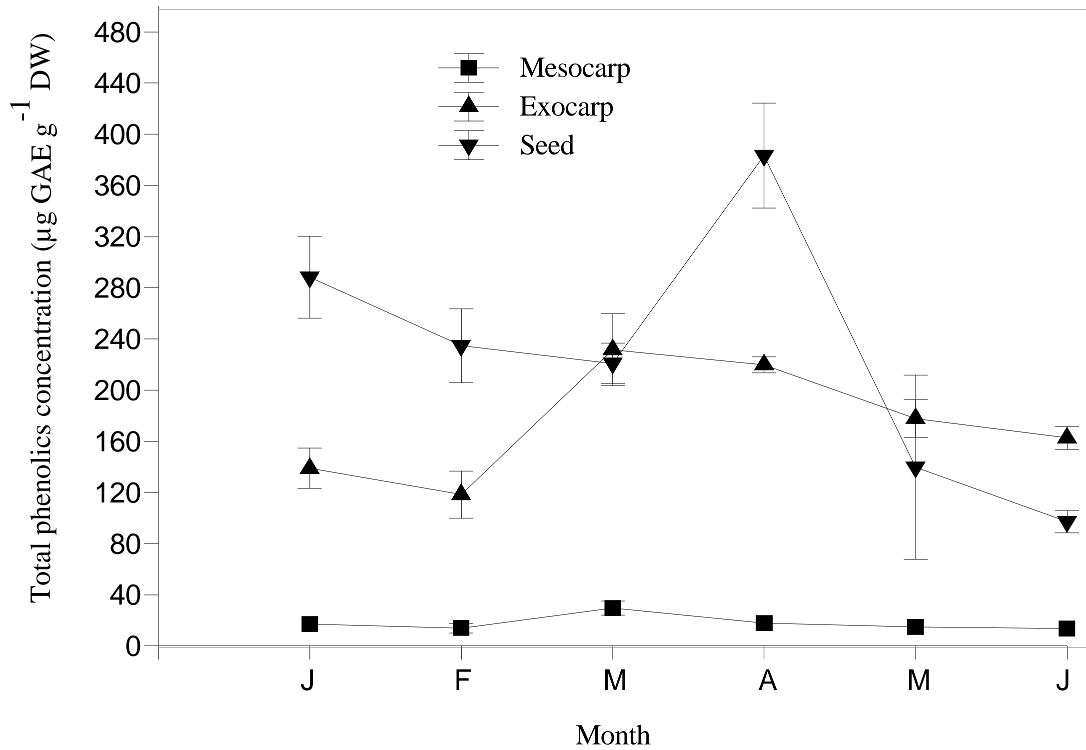



Fig. 2. Seasonal changes in mean total phenolics (TP) concentrations in the mesocarp, exocarp, and seeds of 'Hass' avocado fruit. $LSD_{0.05}$ = 32.1, Vertical bars represent ± SE. (n=5).

# CHAPTER 6

# FREE AND CONJUGATED FORMS OF CATECHIN AND EPICATECHIN IN 'HASS' AVOCADO FRUIT AND EFFECTS OF POTASSIUM SILICATE APPLICATION

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## ABSTRACT

The efficacy of Silicon (Si) to increase the pool of phenols in avocado mesocarp and, thereby, improve postharvest avocado fruit quality was determined. Catechin and epicatechin, the two major free and conjugated phenols in avocado fruit tissue were analysed using HPLC. The expression of catalase, the major enzyme with anti-oxidant activity which has been found to be increased by Si application, was determined by immunoblotting. Postharvest potassium silicate (KSil) applications had neither an effect on fruit firmness and weight nor on respiration rate. In contrast, mesocarp electrolyte conductivity (EC), total phenol concentration, lipid peroxidation, as well as polyphenol oxidase and catalase activity responded positively to the KSil treatments. Silicon might function as a major elicitor increasing free polyphenol concentrations. As phenolics participate in the induction and/or the repression of genes as well as the activation or deactivation of enzymes of key metabolic pathways, they might be able to increase the fruit's ability to better withstand stressful environmental conditions. Therefore Si application could increase the pool of phenols in the mesocarp and thereby increase fruit quality.

**Keywords:** avocado, catechin, epicatechin, phenolics, silicon.

### 1. Introduction

Phenolic acids can occur in free or bound forms (Régnier and Macheix, 1996; Renger and Steinhart, 2000). Several plant phenolics exist in conjugated forms, either as glycosides or as conjugate products with other moieties. Such a conjugation occurs via the hydroxyl groups of the phenolic. This reduces the ability of phenolic compounds to function as a strong anti-oxidant since availability of free hydroxyl groups on the phenolic ring is important for

neutralizing free radical accumulation. Therefore, if free phenolics are released from their, predominantly, sugar moeity, the anti-oxidant capacity of a tissue will be enhanced. Enzymatic hydrolysis of these phenolic glycosides appears to be an attractive means of increasing the concentration of free phenolic acids in food products, such as fruit juice and wines, to enrich taste, flavour and aroma, while also potentially increasing functional value for health (Zheng and Shetty, 2000; Vattem and Shetty, 2002, 2003).

Silicon plays numerous roles in plant growth and development, which include physiologically related effects. Various authors have reported that silicon partially offsets the negative impacts of NaCl stress. Silicon has also been reported to increase the tolerance of tomato plants to NaCl salinity by raising the activity of enzymatic anti-oxidants, such as superoxide dismutase (SOD) and catalase (CAT), as well as the chlorophyll content and the photochemical efficiency of photosystem II (Al-Aghabary et al., 2004). Furthermore, silicon was found to significantly increase SOD activity while decreasing malondialdehyde (MDA) concentrations in leaves of salt-treated barley (Liang, 1999). The ability of silicon to alleviate manganese toxicity of rice plants, especially through enhancing peroxidase activity, has also been reported (Horiguchi, 1988). Heath and Stumpf (1986) suggested that high levels of cell wall-associated phenolics in "silicon-depleted" tissue result in faster inhibition of fungal enzymes involved in the peg formation.

The objective of this research was therefore to investigate the effect of postharvest application of silicon on catechin and epicatechin concentrations in mesocarp tissue, as well as on fruit quality. Other physiological parameters related to fruit quality were also determined to investigate the basis of the silicon treatment.

## 2. Materials and methods

*2.1. Materials*

All chemicals were obtained from ab**cam**®, Amersham®, Fluka®, Saarchem® or Sigma-Aldrich®.

Fruit were obtained from ten mature 'Hass' avocado trees located from orchards in the KwaZulu-Natal Midlands (30°16′E and 29°28′S, South Africa). Fruit were collected in June, when they had reached commercial maturity. Eighty fruit were washed with distilled water, and left to air dry before being treated with KSil. Fruit were soaked in different KSil (K2550® potassium silicate (20.5-20.9% $SiO_2$, 8.0-8.15% $K_2O$), PQ Corporation, SA) concentrations (0, 5, 13 or 25 x $10^3$ mg/L) for 25 min (pH ≤ 9). Twenty fruit were used for each KSil concentration with five fruit used as a replication, with four replications per treatment. The fruit were placed into cold storage (5.5 ºC) for 17 days, followed by five days at room temperature (25-30 ºC) to imitate commercial shipping conditions.

*2.2. Determination of individual phenols by HPLC*

Phenols were determined according to Hertog et al. (1992), with slight modifications. Briefly, freeze-dried tissue (1.0 g) was mixed with 10 mL 99.8% (v/v) methanol and vortexed for 30 s. The mixture was then shaken overnight at room temperature to extract the free phenols. Subsequently the mixture was centrifuged and the supernatant filtered through Whatman® no. 1 filter paper and the pellet repeatedly rinsed with 10 mL solvent until colour was no longer released. The pooled filtrates were taken to dryness under $N_2$ gas at 35 ºC. Dried samples were re-suspended in 99.8% (v/v) methanol, filtered through a 0.45 µm nylon filter and analyzed using an isocratic HPLC system equipped with a photodiode array detector on a Luna-C18® column and peaks evaluated using Chromeleon® software. Phenols were

identified at 280 nm by comparison of elution times of compounds with catechin and epicatechin standards.

Cell wall-bound phenols were released from the remaining plant residue by acid hydrolysis. A 10 mL portion of acidified (2 M hydrochloric acid) 60% (v/v) aqueous methanol was added to each sample and incubated at 90 °C for exactly 90 min. Samples were allowed to cool before the supernatant was filtered and analysed.

Fruit firmness and carbon dioxide production ($CO_2$) were measured according to Van Rooyen (2005).

*2.3. Fruit firmness*

Fruit firmness was determined every four days using a hand-held firmness tester (Bareiss, Oberdischingen, Germany) during the postharvest storage period. Two readings on a scale of 100 (hard, unripe; ≡ 8.06 N) to < 60 (ready to eat; 5.05 N) were taken at the equatorial region of the fruit on opposite sides. Fruit firmness (N) was calculated using a formula: $N = 0.0751 x + 0.5491$ (according to Standard ISO 7619, International Organisation for Standardization).

*2.4. Fruit $CO_2$ Production*

Fruit $CO_2$ Production was measured with an environmental gas monitor (EGM-1, PP Systems, Hitchin, Hertfordshire, UK) every four days, after fruit were removed from the 5.5°C cold room. Each fruit was sealed in a 1 L jar for 10 min, after which the headspace $CO_2$ concentration ($\mu L\ L^{-1}$) was determined and the results calculated as the rate of $CO_2$ production ($mL\ kg^{-1}\ FM\ h^{-1}$), taking into account the fruit mass (FM), headspace and the ambient room $CO_2$ concentration.

*2.5. Determination of soluble silicon using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-OES)*

Plant soluble silicon was determined according to Wang et al. (2004), with slight modifications. Briefly, 1.0 g of fresh mesocarp tissue was flash-frozen in liquid nitrogen and ground using mortar and pestle. The samples were then homogenised in 10 mL ultra pure water using an ultrasonic cell disrupter (VirSonic 100, VirTis, NY, USA) for 1 min. The homogenate was shaken overnight, centrifuged twice at 20,000 × *g* for 10 min and filtered through Whatman® no. 1 filter paper. The amount of soluble silicon was determined using ICP-OES (Varian 720-ES, Varian, CA, USA) and pure silicon as a standard. [Working conditions of the ICP-OES were: RF Power 0.7–1.5 kW (1.2–1.3 kW for Axial); Plasma gas flow rate (argon) 10.5–15 L $min^{-1}$; Auxiliary gas flow rate (argon) 1.5 L $min^{-1}$; Viewing height 5–12 mm; Nebulizer flow rate 0.75 L $min^{-1}$; Copy and reading time 1–5 s (max 60 s); Stabilization delay 15 s; Pump rate 15 rpm].

*2.6. Transmission Electron Microscopy (TEM) analysis of mesocarp tissue*

Specimens for TEM were prepared from mesocarp tissue. Analysis of fruit mesocarp Si was undertaken according to Keeping et al. (2009).

*2.7. Electrolyte Conductivity*

The leakage of electrolytes from mesocarp tissue was determined by measuring the electrical conductance of cell leakages using a modified technique of Venkatarayappa et al. (1984). A mesocarp plug (1 cm diameter) was taken from the cut-half of each fruit at the distal end, between the seed and the mesocarp. A disc of 13 mm thickness (2.7-3.0 g) was cut from this plug and rinsed three times with distilled water before being placed in a boiling tube containing 25 mL distilled water. The tubes were then shaken for 3 h and the electrical

conductivity (EC) measured using a multi-range conductivity meter (HI 9033, Hanna instruments, Johannesburg, RSA). Subsequently, tubes were placed in a boiling water bath for 20 min, removed and allowed to cool. The EC of each tube was again recorded and the electrolyte leakage calculated as Δ EC.

*2.8. Total soluble protein extraction*

Total soluble proteins were extracted according to Kanellis and Kalaitzis (1992), with slight modifications. Freeze-dried, milled mesocarp tissue (1.0 g DM) was extracted in 5 mL 50 mM Tris-HCl buffer (pH 7.4) containing 0.2 M NaCl, 20 mM $MgSO_4$, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.5 mM PMSF, 10 mM leupeptin, and 10% (v/v) glycerol. The samples were then homogenised using an ultrasonic cell disrupter to extract both, free and membrane bound proteins. Subsquently, the mixture was allowed to stand on ice for 15 min and centrifuged at 20,000 *x g* for 20 min. The supernatant was used for enzyme assays after being filtered through Miracloth®.

*2.9. Total protein quantification*

The protein concentration of the samples was quantified by Bradford microassay (Bradford, 1976). Bradford dye reagent was prepared by diluting the dye concentrate with distilled water at a ratio of 1:4. The diluted dye (1 mL) was added to test tubes containing 20 µL sample extract, mixed by inversion three times and incubated at room temperature for 5 min. Samples were then read spectrophotometrically at $A_{595}$ nm and the protein concentration determined by comparing results with a standard curve constructed using bovine serum albumin (BSA).

*2.10. Assay for lipid peroxidation*

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) reacted with thiobarbituric acid (TBA) to form a TBA–MDA complex (Chong et al., 2005). An amount 1 mL supernatant from the crude protein extract was added to a test tube containing 1 mL 20% (w/v) TCA, 0.01% (w/v) BHT and 0.65% (w/v) TBA. Samples were mixed vigorously, incubated at 95 °C for 30 min, cooled on ice and centrifuged at 3000 × *g* for 10 min. Absorbance was read at 532 and 600 nm using a UV–Visible spectrophotometer (DU800, Beckman Coulter, CA, USA). Total MDA equivalents were calculated according to Heath and Packer (1968) as:

Total MDA (nmol $g^{-1}$ DW) = (Amount of extraction buffer (mL) × amount of supernatant (mL) × [(Abs 532 – Abs 600)/155] × $10^3$)* Amount of sample $(g)^{-1}$

*2.11. Assay for total polyphenol oxidase activity*

Total polyphenol oxidase (PPO; EC 1.14.18.1) was assayed as described by Van Lelyveld et al. (1984) with slight modification. Crude enzyme extract (100 μL) was added to a mixture of 1.450 mL 10 mM acetate buffer (pH 5.0) and 1.450 mL 20 mM 4-methyl-catechol. PPO activity was expressed as the change in optical density (OD) at 420 nm $min^{-1}$ $mg^{-1}$ protein.

*2.12. Assay for catalase activity*

A method originally described by Beers and Sizer (1952) was used with slight modifications to determine catalase (CAT; EC 1.11.1.6) activity. The reaction solution (3 mL) contained 0.05 M potassium phosphate (pH 7.0), 0.059 M hydrogen peroxide, 0.1 mL enzyme extract and 1.9 mL distilled water. This mixture was incubated for 4-5 min to achieve temperature equilibration and to establish a blank rate. To this mixture 0.1 mL diluted enzyme

was added and the disappearance of $H_2O_2$ was followed spectrophotometrically by recording the decrease in absorbance at 240 nm every 20 s for 3 min. The change in absorbance ($\Delta A_{240}$ nm $min^{-1}$) from the initial (20 s) linear portion of the curve was calculated. One unit of CAT activity was defined as the amount of enzyme extract that decomposes one µmol $H_2O_2$. Enzyme activity was reported as Units $mg^{-1}$ protein using the following equation:

Units/mg protein = ($\Delta$240/min*1000)* (43.6* mg enzyme/mL of reaction mixture)$^{-1}$

*2.13. SDS-PAGE and Western Blotting*

This was done according to Coetzer et al. (1993) with slight modifications. One hundred micro-gram (100 µg) protein was loaded per lane onto a 12% acrylamide running gel and a 5% acrylamide stacking gel, and the proteins were separated by SDS-PAGE using a tetra cell (Mini-PROTEAN®, Bio-Rad, CA, USA) at 200 V. 'Precision Plus Protein™' standard (Bio-Rad, CA, USA) was used for protein identification. Following electrophoresis, western blotting was performed. Proteins were electroblotted (200 mA) overnight onto a nitrocellulose membrane (0.45 µm) using a Bio-Rad Mini Trans-Blot® electrophoretic transfer cell containing transfer buffer (50 mM Tris, 200 mM glycine , and 20% methanol). The nitrocellulose membrane was briefly stained in Ponceau S (0.1% Ponceau S in 1% (v/v) glacial acetic acid) to ensure that the transfer was successful. The nitrocellulose membrane was blocked by 5% non-fat milk in TBS (Tris Buffered Saline) (Tris buffer solution containing 20 mM Tris (pH 7.4) for 2 h and 200 mM NaCl). The membrane was incubated overnight with primary antibody of rabbit polyclonal catalase-peroxisome marker (ab1877) (diluted 1:1000) in a medium of 5% non-fat milk in TBS with at 4 ºC. Afterwards the membrane was washed with 0.1% Tween in TBS three times for 15 min, and followed by incubating it for 8 h at 4 ºC with secondary antibody Goat anti-Rabbit IgG (H+L) conjugated

in alkaline phosphatase (AP) and diluted in TBS 1:2000. The membrane was then washed three more times with TBS. Finally, the membrane was developed using premixed BCIP/NBT solution.

*2.14. Measurement of anti-oxidants*

*2.14.1. Total anti-oxidant capacity*

Total anti-oxidant capacity (TAOC) was determined according to Benzie and Strain (1996) with slight modifications. These authors developed the FRAP assay which is based on the reduction of the ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) complex by a reductant, therefore determining the combined anti-oxidant capacity of anti-oxidant molecules present in the tissue under investigation. Fresh FRAP reagent solution (300 mM sodium acetate buffer pH 3.6, 10 mM Fe(II)-TPTZ prepared in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ (10:1:1)) was prepared prior to measurement. An aliquot of the sample (30 µL) was mixed with 900 µL FRAP reagent solution and the absorbance was measured at 593 nm after 10 min.

*2.14.2. Total anti-oxidant activity*

Total anti-oxidant activity (TAOA) was determined using the ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) assay according to Re et al. (1999), with slight modifications, in order to be able to measure the hydrophilic as well as the lipophilic anti-oxidant fraction. ABTS was prepared as a 7 mM solution in water or ethanol, for measuring hydrophilic and lipophilic anti-oxidant fractions respectively. The ABTS radical cation ($ABTS^{\cdot+}$) was 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 3 to 6 h.

Thereafter, 1.0 mL activated ABTS solution ($A_{734}$ nm = 0.700 ± 0.5) was added to 10 μL sample solution from extracts of freeze-dried material in acetate buffer (pH 4.0). The decrease in absorbance at 734 nm was recorded after 6 min.

*2.14.3. Oxygen radical absorbance capacity*

Oxygen radical absorbance capacity (ORAC) was determined according to Huang et al. (2002), with slight modifications. Briefly, 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH) was daily dissolved in 10 mL of 75 mM phosphate buffer (pH 7.4) to a final concentration of 153 mM. A fluorescein stock solution (4.0 μM) was made in 75 mM phosphate buffer and stored in dark place. To all experimental microplate wells, 150 μL of working sodium fluorescein solution were added. Standard wells received 25 μL of gallic acid, ferullic acid, catechin and epicatechin of the same concentration (1.25 mg $mL^{-1}$). Reactions were initiated by the addition of 25 μL of AAPH solution and 25 μL ammonium persulphate. Working conditions of the optima BMG labtech fluorescence (FLUOstar Optima, BMG Labtech, Offenburg, Germany) were: excitation filter 485 nm, emission filter 520 nm, gain 516, 100 cycles, cycle time 166 s, measuring start time 0.0 s, number of flashes 10, target temperature 37 °C.

*2.15. Statistical analysis*

Analyses of variance were performed using GenStat version 9.1 (VSN International, Hemel Hempstead, UK). Standard deviation values were calculated and differences among treatments were separated by the least significant difference at $P < 0.05$ level.

## 3. Results

*3.1 Effect of silicon treatments on fruit silicon concentration*

The silicon concentration of the mesocarp following all silicon treatments was significantly higher than that of control fruit (Fig. 2A).

*3.2. Fruit quality parameters*

Fruit mass loss (%) was significantly decreased by silicon application. The decrease in fruit mass loss was greater with the increase in silicon concentration applied (Fig. 1A; Table 1). Fruit firmness, on the other hand was not significantly increased by the silicon application (Fig. 1B). Fruit respiration rate was expectedly low during the cold storage period but increased rapidly when fruit were transferred to room temperature (Fig. 1C). Respiration rate was not affected by silicon treatments. The fruit quality attributes electrolyte conductivity (EC), lipid peroxidation, PPO and CAT activity were significantly ($P < 0.05$) affected by silicon treatments (Fig. 2).

*3.3. Internal fruit quality attributes*

Fruit treated with 25 $x10^3$ mg/L silicon had a significantly higher electrolyte leakage, expressed as electric conductivity (EC) than the two lower silicon treatments. There were no significant differences between the control and the silicon applications (Fig. 2B).

The fruit lipid peroxidation was significantly different among treatments. The control treatment exhibited the highest peroxidation, followed by 13 and lastly by 5 and 25 $x10^3$ mg Si /L, respectively (Fig. 2E).

The fruit treated with 5 ($x10^3$) mg Si/L had significantly higher PPO activity than the 13 and 25 ($x10^3$) mg/L treatments. However, there were no significant differences between the control and the 13 or 25 ($x10^3$) mg/L silicon treatments. It was observed that the PPO activity decreased as the silicon concentration applied increased (Fig. 2C).

Mesocarp CAT activity increased significantly with an increase in silicon application rate (Fig. 2D). Furthermore, immunoblotting showed that mesocarp catalase was expressed in response to silicon treatments (Figs. 6A, B; Lane 3-5).

### *3.4. Anti-oxidant capacity of phenol standards*

The anti-oxidant capacity of the phenol standards was in order of GA > FA > EC > C (Fig.4). Where GA=Gallic Acid; FA = Ferullic Acid; EC = Epicatechin; C = Catechin.

### *3.5. Polyphenols catechin and epicatechin*

There were only slight significant differences in conjugated catechin and epicatechin of fruit mesocarp tissue. However, the treatments had an effect on the total phenol concentration (data not presented). The control fruit tissues had a lower concentration of both phenols compared with fruit tissues treated with the three levels (5, 13 and 25 ($x10^3$) mg/L) of silicon solutions (Fig. 3). Whereas silicon treatments had a significant effect ($P < 0.05$) on the free forms of catechin and epicatechin, the highest concentration of these phenols was recorded for the $5 \times 10^3$ mg/L treatment (Fig. 3).

### *3.6. Transmission Electron Microscopy (TEM)*

Deposition of silicon was observed on the exocarp of treated fruit (Fig. 5). As a result the cell membrane was pushed away from the cell wall, creating a gap between cell wall and cell membrane, seemingly impregnated with silicon.

## 4. Discussion

Integrated fruit quality management (IFQM) is a pivotal step to ensure postharvest fruit quality by the integration of preharvest cultural practices, which improve fruit quality

with the production of secondary metabolites (such as phenolics), and postharvest practices, that maximize membrane integrity (Arapia, 2004). Fruit subjected to optimal pre- and postharvest management practices can, therefore, maintain fruit quality over a longer storage period, thus increasing consumer confidence in a certain commodity.

A more direct role of phenolic phytochemicals in fruit quality is the ability of phenols to modulate cellular physiology at the biochemical, physiological and the molecular level via the anti-oxidant enzyme response pathway (glutathione, ascorbate, superoxide dismutase, catalase and glutathione-transferase interface) (Block et al., 1992; Serdula, 1996). Due to their structural similarities with several key biological effectors and signal molecules, phenols are able to participate in the induction or repression of genes or the activation or deactivation of proteins, enzymes and transcription factors of key metabolic pathways. Phenols can also play a role in modulating cellular homeostasis because of their physiochemical properties (Vattem et al., 2005).

Nilsen and Orcutt (1996) suggested that plants utilize energy to maintain cell metabolism and the amount of energy used could be estimated by the rate of $CO_2$ production. The fruit stored at 5.5 ºC had a similar trend of $CO_2$ production for each treatment (Fig. 1). Although the respiration rate increased after cold storage, the trend remained the same. Fruit firmness also showed a similar trend in all treatments over time. Furthermore, $CO_2$ production rate and firmness were negatively related after removal of fruit from storage. Fruit mass, measured over time, showed no significant differences among the treatments. However, the main effect of treatments had a significant effect on mass loss. The fruit treated with silicon solution had weighed more than control ones. Therefore, silicon possibly played a role in maintaining fruit moisture content. Similarly, Gong et al. (2003) reported that silicon treated

wheat plants could maintain a higher water status and dry matter content compared with non-silicon treated plants under preharvest drought conditions.

Fruit mesocarp tissue was able to absorb silicon from the treatment solution. Additionally, silicon deposition between the cell wall and cell membrane were visualized by TEM. This deposition of silicon causes impregnation of the intercellular parts of fruit exocarp and also partially filled fruit stomates, reduced fruit respiration, and concomitantly resulted in decreasing weight loss, in agreement with Hammash and El Assi (2007). Silicon treatments, therefore, could positively be associated with delaying fruit weight loss by maintaining fruit moisture.

Furthermore, silicon deposition could be related to silicon effects on membrane integrity. As electrolyte leakage is related to the breakdown of cell membrane integrity, with increased electrolyte leakage indicative of decreased membrane integrity (Thompson, 1988). Silicon treated fruit had lower electrolyte leakage compared with the control (Fig. 2B), possibly due to silicon deposition between cellwall and cell membrane, maintaining a barrier against solute leakage.

Vaughn et al. (1988) reported that PPO activity is substrate dependent. Therefore, PPO activity depends on concentrations of individual phenolics (e.g., catechin and epicatechin). Although silicon has been reported to increase PPO activity in maize and sorghum leaves by triggering defense processes in plants through acting as an elicitor, probably through production of phenols (Roncatto, 1998), PPO can cause browning of fruit tissue, by catalyzing the conversion of phenols to melanin (a by-product visible as brownish spots in fruit tissues.). Silicon may play a significant role in removing the necessary oxygen for oxidation of phenols. The control of tissue browning is one of the most important factors

for the food industry, as colour is a significant attribute of food influencing consumer decision since brown fruits are considered spoiled. Several methods can be applied to avoid enzymatic browning, based on inactivating the enzyme (heat) or by removing essential components (most often oxygen) from the product (FAO, 2009).

Bower and Dennison (2005) reported that pre-heating of cut avocado fruit prevents and/or limits mesocarp browning due to reaction in PPO activity. The mesocarp pre-heating may denature the enzyme, allowing leaked membrane-bound phenols to function as anti-oxidants, thereby reducing the browning effect. As a result, the cut fruit could be kept fresh for longer time. Alternatively, silicon functions to bind cellular oxygen, as it has a powerful affinity for oxygen (Řezanka and Sigler. 2008). The oxidation of silicon yields a solid silicon dioxide that forms a lattice in which each silicon atom is surrounded by four oxygen molecules.

Silicon application affected mesocarp catalase activity. Immunoblotting results showed increased catalase expression in mesocarp tissue in response to silicon application (Fig. 6). This result is in agreement with Gong et al. (2005), who reported that silicon increased CAT activity, thereby reducing $H_2O_2$ concentration in wheat leaves under drought. Application of silicon can also alleviate lipid peroxidation of wheat under drought stress. In strawberry, it was also observed that foliar application of silicate increased the ratio between poly-unsaturated to mono-unsaturated fatty acids in glycolipids and phospholipids and elevated the amounts of membrane lipids (Wang and Galletta, 1998). In salt-stressed barley and cucumber, addition of silicon decreased membrane permeability and content of the thiobarbituric acid reactive substances (TBARS), the end product of membrane lipid peroxidation. The results suggest that application of silicon could limit lipid peroxidation and

maintain membrane integrity under stress condition as found in the avocado mesocarp (Fig. 2E)

Although there was a high production of catechin and epicatechin in the exocarp and seed, the highest concentration of free phenolics was in the exocarp (Tesfay et al., 2010). This is probably as a result of the higher exposure of the exocarp to various stress factors. Vattem et al. (2005) reported that phenolics are ubiquitous in plants seeds and that outer fruit layers are especially rich sources of phenolics. These authors attributed this distribution pattern of phenols to their role in protecting fruit and seed to ensure healthy propagation of the species. The production of free epicatechin is regulated by stress factors and favoured over that of catechin due to higher sensitivity and activity as an anti-oxidant (Fig. 4; FRAP, ABTS, ORAC). Therefore the production of free polyphenols can be stimulated by biotic (pathogenic) or abiotic (UV light) factors or postharvest silicon fruit treatments.

Silicon positively affected free and conjugated phenols of mesocarp tissue postharvest, with a tendency towards higher epicatechin than catechin concentrations (Fig. 3). Phenolics play an important role in plant resistance and in defense against microbial infections, which are intimately connected with the appearance of reactive oxygen species (ROS). Free phenols also contribute to the overall fitness of fruit by providing protection against pathogens (Beckman, 2000).

Generally, fruit quality of ‘Hass’ avocado was improved following silicon treatments probably due to a reduction in electrolyte leakage and lipid peroxidation and an increase in catalase expression and activity. Results confirm the potential of potassium silicate to significantly improve fruit quality through an increased anti-oxidant pool in fruit. In mesocarp

tissue, the increase in free phenols released from the conjugated form, could be a major factor for improving postharvest fruit quality.

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Table 1 Main effects of KSil (0, $5 \times 10^3$, $13 \times 10^3$, $25 \times 10^3$ mg/L Si) on fruit weight, firmness and respiration rate during fruit storage (5.5 °C)

| Treatments | Fruit mass loss (%) | Fruit firmness (N) | Respiration rate (ml $kg^{-1}$ $h^{-1}$) |
|---|---|---|---|
| Control | 5.6 | 6.47 | 28.60 |
| $5 \times 10^3$ mg/L | 0.5 | 6.65 | 31.60 |
| $13 \times 10^3$ mg/L | 3.5 | 6.56 | 30.90 |
| $25 \times 10^3$ mg/L | 0.2 | 6.67 | 26.10 |
| F.Pr $_{(0.05)}$ | < 0.001** | < 0.008 | 0.670 |

- N= newton

FIG. 1. Postharvest quality attributes (fruit weight loss, Panel A; fruit firmness, Panel B; respiration rate; Panel C) in response to KSil treatments: Response of fruit to different concentrations of potassium silicate (0, 5, 13, 25 x $10^3$ mg/L) over 22 days of storage.

FIG. 2. Biochemical analyses of mesocarp tissues in response to different concentrations of potassium silicate (0, 5, 13, 25 x $10^3$ mg/L) at the 'eat-ripe' stage. $LSD_{(0.05)}$ values: 0.016 (ICP, Panel A); 0.656 (EC, Panel B); 0.031 (PPO, Panel C); 3.721 (CAT, Panel D); 7.45 x $10^{-3}$ (Lipid peroxidation, Panel E).

FIG. 3. Free and conjugated forms of catechin and epicatechin in fruit following potassium silicate treatments. $LSD_{(0.05)}$ values: 8.48 (Free catechin, Panel A); 10.86 (Conjugated catechin, Panel B); 20.00 (Free epicatechin, Panel C); 27.68 (Conjugated epicatechin, Panel D).

FIG. 4. Evaluation of anti-oxidant capacity of four phenol standards (Gallic acid, Ferullic acid, Catechin and Epicatechin) using FRAP, ABTS and ORAC assays.

FIG. 5. Transmission electron microscopy (TEM) displays the structural changes on cellular components of avocado peel, comparisons between before (control) versus after silicon treatments of fruit.

FIG. 6A. SDS–PAGE was performed on 12% homogeneous gels using the Bio-Rad system (Bio-Rad Laboratories, CA, USA). Lane 1, molecular marker; lane 2, control; lane 3, $5 \times 10^3$ ppm Si; lane 4, $13 \times 10^3$ mg/L Si; lane 5, $25 \times 10^3$ mg/L Si.

FIG. 6B. Immunoblotting of catalase expressed in response to treatment with silicon containing solutions. Lane 2, control; lane 3, $5 \times 10^3$ mg/L; lane 3, $13 \times 10^3$ mg/L; lane 4; $25 \times 10^3$ mg/L. Each lane contained 100 μg of protein.

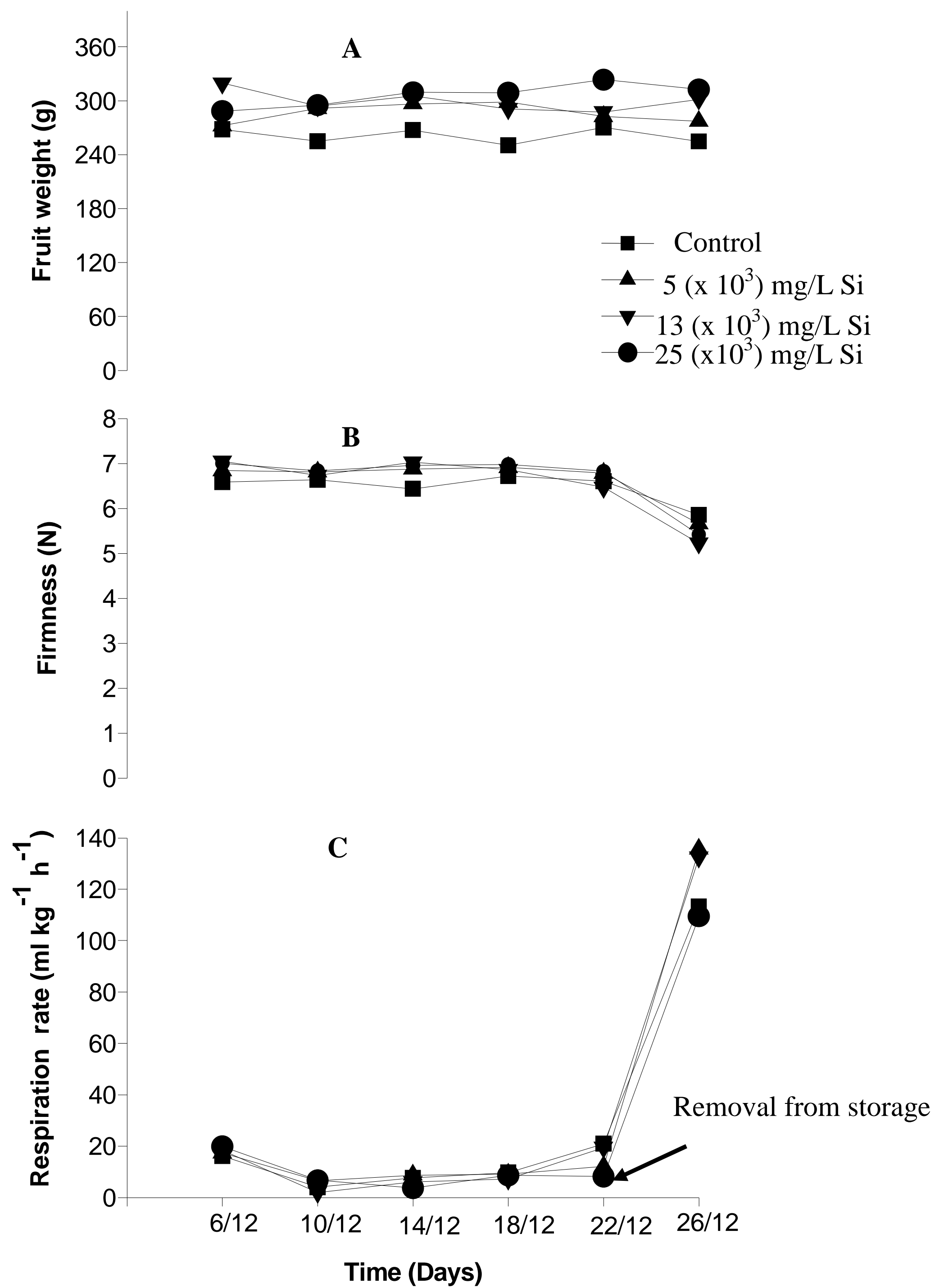



FIG. 1.

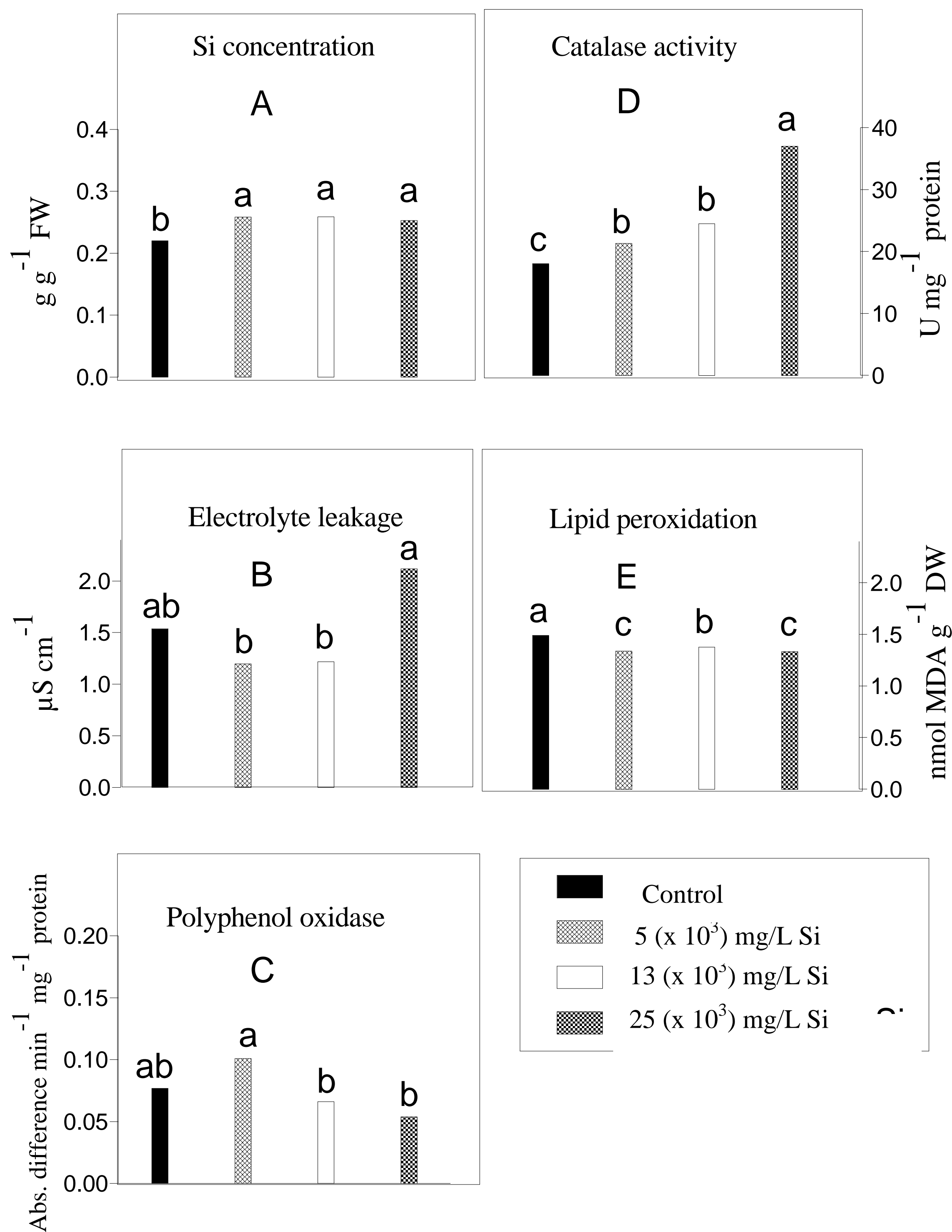



FIG. 2.

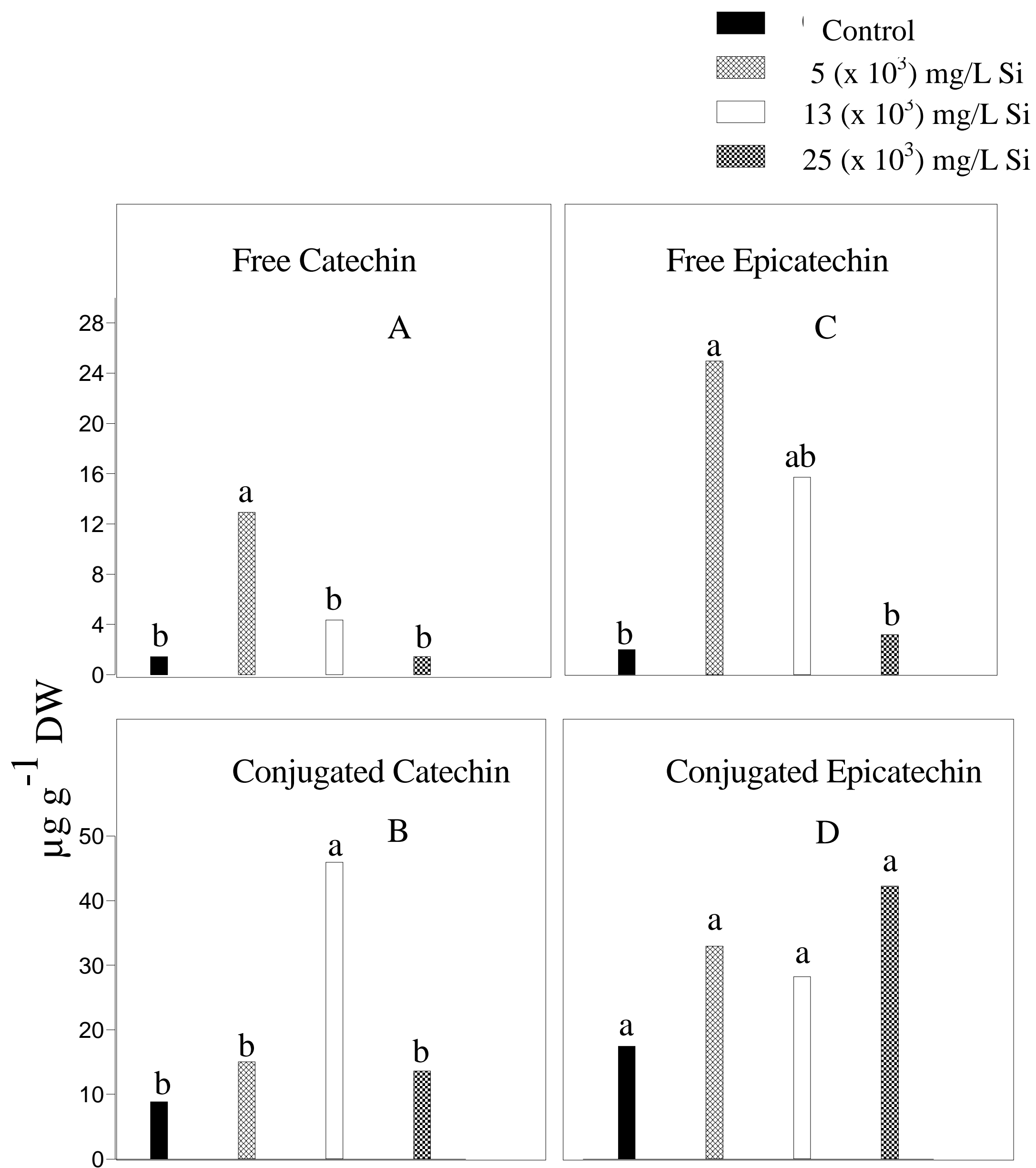
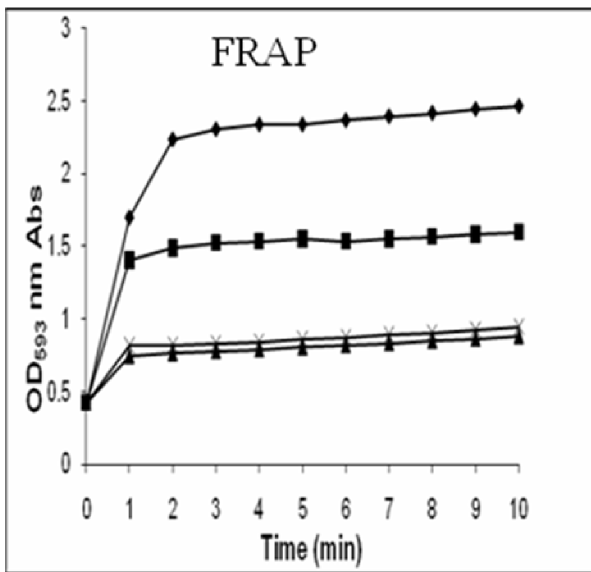
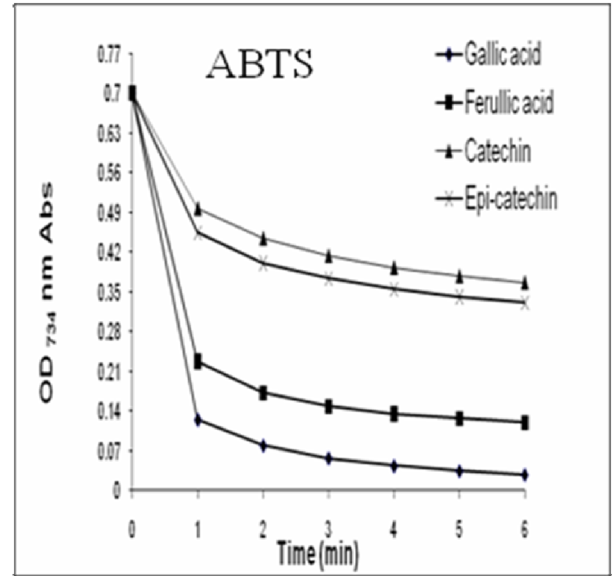



FIG. 3.



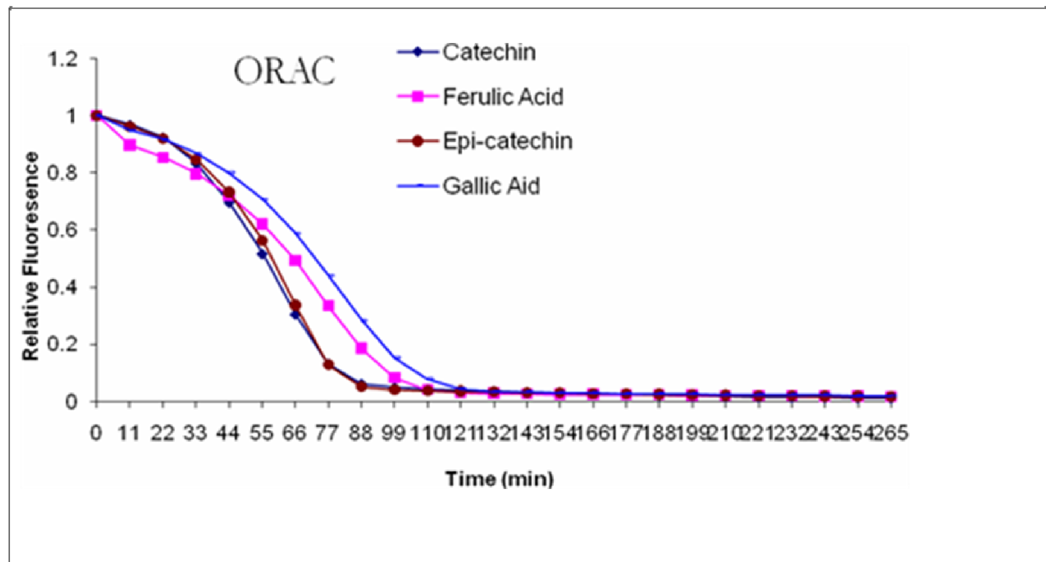




FIG. 4.

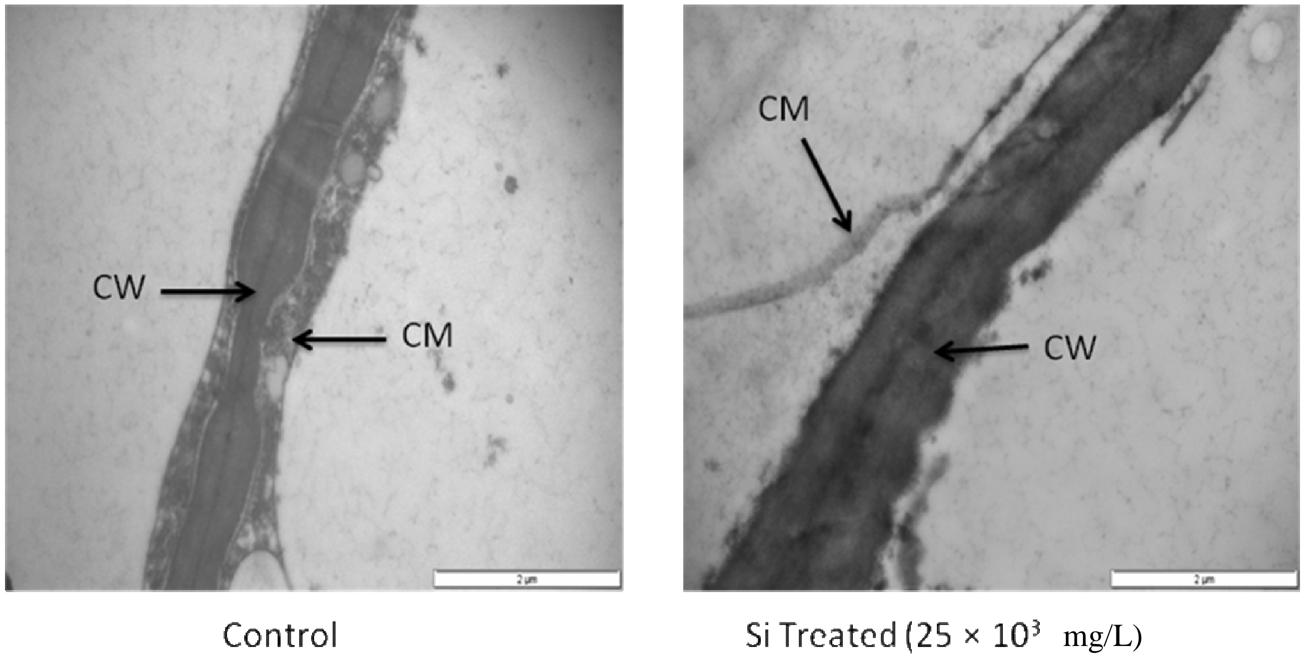



FIG. 5.

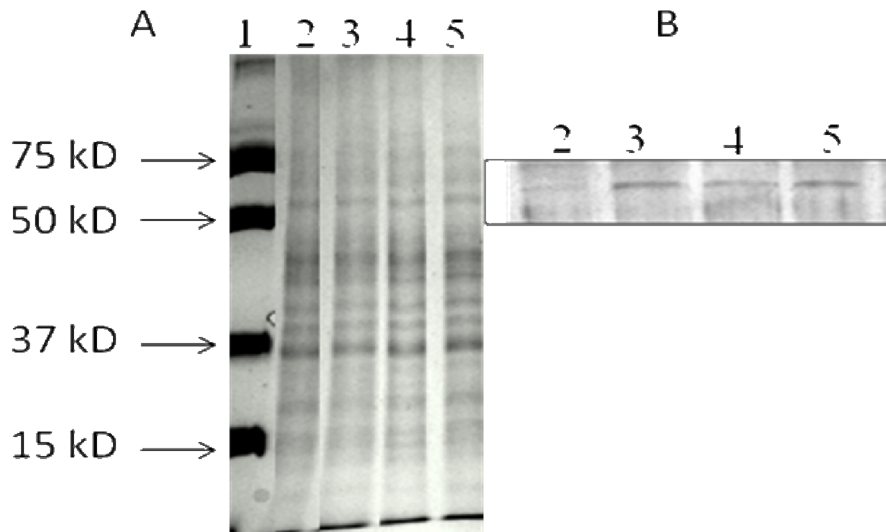



FIG. 6.

# CHAPTER 7

# GENERAL DISCUSSION, CONCLUSION AND OUTLOOK

Various experiments have been conducted in the search of the role of the C7 sugars *D*-mannoheptulose and perseitol abundant in avocado. These carbohydrates have been thought to act as 'ripening inhibitors' (Adato and Gazit, 1974; Liu et al., 2002), while other authors (Bertling and Bower, 2005, 2006) reported the possibility of C7 sugars to act as an indicator of sink strength for fruit. Cowan (2004) and Bertling et al. (2007) proposed that these sugars have a variety of important functions, amongst them protection of certain key enzymes essential for fruit growth and development against ROS. Fruit tissues exposed to different abiotic factors express defense mechanisms, including the biosynthesis of anti-oxidants. The research presented confirms that C7 sugars in avocado do form part of a pool of anti-oxidants. Of the two major C7 sugars, *D*-mannoheptulose is the compound that not only protects the mesocarp against damage, but also carries health benefits for the consumer, a function that could become an important factor marketing avocado fruit.

The ontogenic study of avocado revealed that seeds predominantly contain starch, sucrose and perseitol as non-structural storage carbohydrates. As the seed germinates, the embryo produces almost equivalent proportions of *D*-mannoheptulose plus perseitol compared with hexoses. This implies that the avocado seedling uses carbohydrates derived from two groups of storage compounds: hexoses from starch and *D*-mannoheptulose from perseitol. This argument is supported by Lewis and Smith (1967), who postulated that plant polyols play a very important role as storage compounds in growth and development. Furthermore, avocado produces hexoses and C7 sugars from an early stage of fruit developmental onwards. While hexoses are produced in substantial amounts in the early stages of fruit development, these sugars decline as the fruit approaches maturity, while C7

sugars, particularly perseitol, increase. In postharvest fruit *D*-mannoheptulose decreases simultaneously with the accumulation of perseitol in the seed. It is, therefore, assumed that a switch from hexoses to heptoses, aligned to a change in ontogenic development of avocado fruit, exists.

Apart from the evidence provided that C7 sugars are important anti-oxidants, the question of why avocado uses C6 and C7 sugars was addressed. Avocado carbohydrates in fruit and leaf tissues show significant variations in their distribution and production at different phenological stages (Chapter 2). Although the plant produces certain concentrations of hexoses during fruit growth and development, the fruit predominantly contains C7 sugars, especially in mesocarp and exocarp tissue towards fruit maturation. While *D*-mannoheptulose acts as an anti-oxidant of the mesocarp, it could also act as a 'ripening inhibiting factor' as originally proposed by Adato and Gazit (1974). The role of the avocado-characteristic sugar perseitol (Liu et al., 2002) as a major contributor to the carbon balance of the avocado tree has been further verified. However, this sugar acts also as a storage compound, as it is present in all vegetative storage sinks. Increasing levels of this sugar in seeds postharvest, indicate that this sugar acts as a storage sugar in seeds, as well as a minor transport sugar, possibly transporting carbon and energy into the seed (Duffus and Duffus, 1984).

The two heptoses *D*-mannoheptulose and perseitol were collected in considerable amounts from girdled branches as well as in phloem and xylem sap from leaves and seedlings. The interconversion of these two heptoses is probably responsive to environmental conditions.

The C7 sugar concentrations could also play an important role as a stress-protection agent not only during early fruit development, but also postharvest. The deterioration of

postharvest quality in 'Hass' avocado could be aligned to the lack of anti-oxidants in mesocarp tissue, therefore, the occurrence of oxidation products would result in cell damage, which would become visible as various postharvest browning disorders.

Avocado phenols also play an essential role in fruit quality. While their production as anti-oxidants is of significant importance, they can also be responsible for poor fruit quality, when oxidised by polyphenoloxidase (PPO), a reaction resulting in tissue browning due to melanin production (Mayer and Harel, 1979; Lidster et al., 1986). Phenols are found in free and conjugated forms in different fruit tissues. Fruit pre-treated with KSil and stored under low temperature showed reduced electrolyte leakage and lipid peroxidation and increased catalase expression and activity. These reactions are associated with an increased resistance to cell disintegration and breakdown. Therefore, potassium silicate has the potential to significantly improve fruit quality through an increase in the size of the antioxidant pool in fruit and in mesocarp tissue. In particular, silicate can increase free phenols and the expression of the anti-oxidant enzyme catalase. Postharvest silicon treatments suggest that application of silicon can alleviate lipid peroxidation and maintain membrane integrity to maintain postharvest fruit quality.

Overall, this study has contributed to horticultural science by defining the role of the special avocado sugars *D*-mannoheptulose and perseitol in various biochemical and physiological reactions. The multifunctional role of theses C7 sugars has been demonstrated.

### *Future research and commercial implication*

Preharvest tree management practices have long been known to determine the fate of postharvest fruit quality, not only in avocado. In this fruit it is, however, pivotal to increase

the C7 pool during the preharvest stage through proper cultural practices, as fruit with high *D*-mannoheptulose concentrations have an advantage of longer storability. The better fruit quality in well-managed orchards is probably aligned to lower stress levels in avocado trees, which would result in higher C7 sugar pools.

The avocado fruit contains different anti-oxidants, distributed in variable compositions in different tissues depending on factors, such as phenological stages, seasons and fruit age. The anti-oxidant concentration declines as fruit approach maturity and the pool of *D*-mannoheptulose, the potential 'inhibitor of ripening', is drastically depleted. This could therefore be the ripening trigger; however, the point of initiation of this ripening process needs further investigation. It is postulated that the C7 sugar pool is related to storability and postharvest quality due to the anti-oxidant activity of these sugars, particularly *D*-mannoheptulose. Measurements of this pool would, therefore, provide the industry with a tool to determine whether fruit from a particular crop / orchard can be exposed to further stress during the extended shipping period. In addition, the composition of fruit stored either at 1 °C or 5.5 °C could give insights into whether the increased fruit quality at 1 °C is due to the fruit's ability to maintain a larger size pool of C7 sugars at this cooler temperature compared with 5.5 °C.

From a scientific viewpoint this research has posed a variety of new challenges. The enzyme able to convert *D*-mannoheptulose into perseitol and *vice versa* could be a novel enzyme, unless sedoheptulose reductase is capable of converting these two C7 sugars into one another. Furthermore, the C7 sugar volemitol was only found to be present in significant amounts in one occasion. The role of this sugar compound in relation to *D*-mannoheptulose and perseitol needs further investigation. Increasing the C7 sugar pool of fruit to a higher

level at the point of harvest would probably improve fruit quality. Thus, a means to improve the *D*-mannoheptulose pool preharvest should be investigated. Horticultural practices that reduce stress to the fruit preharvest will probably contribute to an increased pool of *D*-mannoheptulose. Post-harvest methods for increasing the concentration of this particular sugar in the mesocarp are likely to improve fruit quality and could enhance the post-harvest quality of avocado fruit, particularly when shipped over a long distance.

It was reported that “raffinoses (RFOs) accumulate in the late stages of soybean seed maturation and desiccation, indicating that they play a role in the desiccation tolerance of seeds as osmoprotectants” (Saravitz et al., 1987). In addition, they provide a “readily metabolizable carbohydrate source for energy generation during germination” (Obendorf, 1997). Similarly, perseitol also accumulates in avocado seeds in the late postharvest stages. Therefore, the question arises whether perseitol plays the same role as RFOs, functioning as osmoprotectants as well as providing of metabolizable carbohydrates. This question requires also attention in future research.

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# APPENDIX

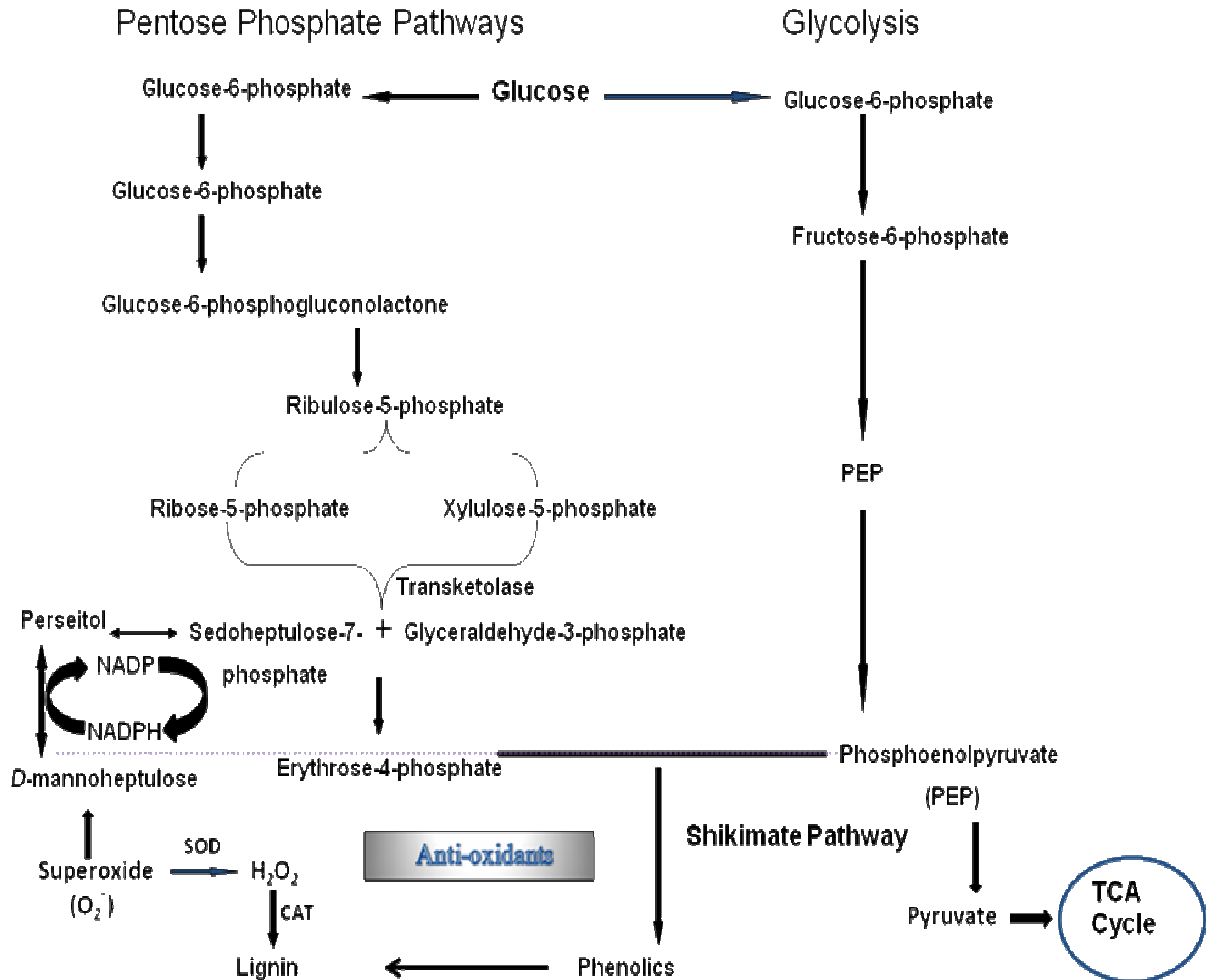


Figure 1 C7 sugars (D-mannoheptulose and perseitol), anti-oxidant compounds linked to pentose phosphate pathway.