POST-PHLOEM TRANSPORT AND METABOLISM OF SUCROSE IN AVOCADO

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ABSTRACT

In South Africa, and in several other sub/tropical countries, the avocado represents a commercially important crop. Very little is currently understood about the metabolism of sugars in this fruit. The variety 'Hass' is a popular cultivar that is grown extensively in South Africa. However this cultivar has a tendency to produce two distinct fruit phenotypes: a normal sized variant and a small, undersized variant. Current literature suggests that the small fruit phenotype is characterised by an elevated abscisic acid (ABA) to cytokinin ratio and altered isoprenoid metabolism. The results presented in the current investigation represent the findings from a detailed study into the metabolism and transport of sugars in 'Hass' fruit in an attempt to characterise solute allocation in developing avocado fruit. Furthermore, the activities of sugar metabolising enzymes, routes of solute movement and polyphenolic contents of normal, small and ABA-treated fruit were compared and contrasted to evaluate the potential role of ABA in the induction and expression of the small fruit phenotype.

The enzymes invertase, sucrose synthase (SSy) and sucrose phosphate synthase are involved in the metabolism of sucrose (Suc) and, hence, phloem unloading, post-sieve element transport and fruit growth. Although not the major sugar present, Suc was found in avocado phloem sap, and the enzymology for its metabolism was shown to exist in avocado fruit. It appears that sink strength is established during early fruit growth by high acid invertase activity, especially during the period of rapid cell division. As fruit growth progresses the activity of SSy and an enzyme responsible for the oxidation of perseitol (tentatively termed perseitol dehydrogenase) increases, suggesting that these enzymes play an important role in the supply of carbon during the linear phase of fruit growth. All Suc metabolising enzyme activity diminishes as the fruit approaches maturity.

With the exception of SSy (in the cleavage direction), all enzymes assayed showed a general increase in relative rates of activity in small and ABA-treated fruit. Similarly, ABA-treatment of seed coat discs *in vitro* resulted in the elevation of insoluble and soluble acid invertase, SSy (in the synthesis direction), and sucrose phosphate synthase activity. Furthermore, both small and ABA-treated fruit were characterised by elevated total soluble sugars, glucose and fructose levels. These observations suggest that altered sugar metabolism, as a consequence of changes in endogenous ABA levels, may contribute to the occurrence of the small fruit.

The seed coat represents an import link between the seed, the mesocarp and the parental plant tissues. Loss of seed coat and endosperm integrity accompanied fruit maturation and a reduction in the movement of solutes into the seed. An increase in polyphenolics in the seed coat tissue seemed critical in this reduced movement. Both the small and ABA-treated fruit were characterised by early senescence of the seed coat, which was accompanied by both a loss of transport into and out of the seed and premature maturation of the fruit. This premature seed coat senescence appeared similar to programmed cell death in tissues exposed to stress or elevated reactive oxygen species, stimuli that are often accompanied by elevated ABA levels. Callose was localised to the plasmodesmata and is proposed to play a role in the gating of, and hence movement through, these pores. Small fruit were characterised by a loss of symplastic continuity, as represented by fewer plasmodesmata, and reduced callose degradation. Comparison of callose content and rates of synthesis suggest that ABA-treatment, similarly, reduces callose catabolism. The association of ABA with both the premature senescence of the seed coat and a reduction in symplastic continuity, and, hence, a reduction in solute transport, further cements the potential role of ABA in the occurrence of the small fruit phenotype.

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DECLARATION

I hereby declare that the research reported in this dissertation is the result of my own investigation, except where acknowledged.

Signed: _____

Ryan F. Cripps

I certify the above statement is correct.

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ABBREVIATIONS

7C = seven carbon
ABA = abscisic acid
ADP = adenosine diphosphate
AI = acid invertase
AlkI = alkaline invertase
AO = aldehyde oxidase
APO = apoplastic
ATP = adenosine triphosphate
BDH = British Drug Houses Ltd
BSA = bovine serum albumin
CC = companion cell
CI = cytoplasmic invertase
CK = cytokinin
CPD = critical point drying
CW = cell wall
D = amorphous deposit
DAFB = days after full bloom
DAT = days after treatment
DDSA = dodecyl succinic anhydride
DER 736 = diglycidyl ether of polypropylene glycol
DHAP = dihydroxyacetone-phosphate
DM = dry mass (g)
DMAE = dimethylamino-ethanol
DMP-30 = 2,4,6-tri(dimethyl-aminomethyl) phenol
DMSO = dimethyl sulfoxide
DTT = 1,4-dithiothreitol
E = exocarp
EDTA = Ethylene diamine tetra-acetic acid
ER = endoplasmic reticulum
ERL 4206 = vinyl cyclohexene dioxide
FK = fructokinase
Fru = fructose
FW = fresh weight
G3P = glyceraldehyde-3-phosphate
GA = Gibberellic acid
GC = gas chromatography
GLC = gas liquid chromatography
Glu = glucose
GTP = guanosine triphosphate
h = hour(s)
Hepes = 4-(2-Hydroxyethyl)-1-piperazine ethanesulfoninc acid
Hex-H^+ Sym = membrane-localised hexose/H^+ symporter
HMG-CoA = 3-hydroxy-3-methylglutaryl coenzyme A
HMGR = 3-hydroxy-3-methylglutary coenzyme A reductase
HPLC = high performance liquid chromatography
HR = hypersensitive response
HXK = hexokinase
I = idioblast
IAA = indoleacetic acid
IAI = insoluble (extracellular) acid invertase
iP = isopentenyl adenine
K_m = Michaelis constant
KPO_4 = potassium phosphate
LB = lipid body
$LSD_{0.05}$ = least significant (95%) difference
M = mesocarp
MC = median cavity
MDH = mannitol dehydrogenase
Mes = 4-morpholine ethanesulfonic acid
m-Hep = *manno*-heptulose
Mi = mitochondria
min = minute(s)
MoCo = molybdenum cofactor
Mops = 4-morpholinepropane-sulfonic acid
MVA = mevalonic acid
NAD = nicotinamide adenine dinucleotide
NADH = reduced NAD
NADP = NAD phosphate
ND = not detected
NI = neutral invertase
NMR = nuclear magnetic resonance
NR = nitrate reductase
NSA = nonenyl succinic anhydride
P = phosphate
P_i = phosphate ion
Pa = pachychalaza
PC = parenchyma cell
PCD = programmed cell death
PDH = perseitol dehydrogenase
Pd = plasmodesmata
PEG = polyethylene glycol
Ph = phenolics
Pm = plasma membrane
Po = pore-like structure
PVP = Polyvinylpirolidone
$r_{18:0}$ = relative retention time compared to that of stearic acid (18:0)
ROS = reactive oxygen species
Rt = retention time
SAI = soluble (vacuolar) acid invertase
SD = standard deviation
SDH = sorbitol dehydrogenase
SDS = sodium dodecyl sulfate
SE = sieve element
sec = second(s)
SEL = size exclusion limit
SEM = scanning electron microscopy
SG = starch grain
SnRK1 = sucrose non-fermenting -1-related protein kinase
SPP = sucrose phosphate phosphatase
SPS = sucrose phosphate synthase
SSy = sucrose synthase
Suc = sucrose
SUT = sucrose uptake transporter
SYM = symplastic
T = tannin
Te = testa
TE = tracheary elements
TEM = transmission electron microscopy
TEMED = N,N,N',N'-tetra-methylethylene-diamine
TES = N-tris[hydroxymethyl]-methyl-2-aminoethane sulfonic acid
TMAH = tetramethylammonium hydroxide
To = tonoplast
Tris = 2-amino-2-(hydroxy-methyl)-1,3-propandiol
TSS = total soluble sugars
UDP = uridine diphosphate
UTP = uridine triphosphate
UV = ultra-violet
V = vesicle-like body
VT = vascular tissue
Vvht1 = *Vitis vinifera* hexose transporter 1
XDH = xanthine dehydrogenase

CHAPTER 1

GENERAL INTRODUCTION

1.1 PROLOGUE ~ THE FRUIT AND THE MOTIVATION

The avocado fruit is well known for its high nutritive value, favourable unsaturated:saturated fatty acid ratio and numerous vitamins (discussed in: Biale and Young 1971; Slater *et al.* 1975; Wostenholme 1990) and has been consumed by the people of central America for several thousand years (Bergh 1975a). Today the avocado represents a significantly important commercial crop in several countries, such as the United States of America (especially California), Brazil, Venezuela, Colombia, Mexico, South Africa, Israel and Australia. In South Africa, the avocado industry generated revenue in excess of 160 million rand in the 1999/2000 season (Anon. 2001).

The avocado is one of the few commercially important members of the genus *Persea* and belongs to the family Lauraceae. Of commercial interest are three 'horticultural races' of *Persea americana* Mill. viz. Mexican, Guatemalan and West Indian (Popenoe 1974), which have more recently been classified botanically as the varieties *drymifolia*, *guatemalensis* and *americana* respectively (Bergh and Ellstrand 1986). The original classification was based on the ecological origin of the three varieties, with the Mexican race originating in the mountains of Mexico and Central America, the Guatemalan race from the highlands of Central America, and the West Indian race native to the lowlands of Central America and northern South America (Biale and Young 1971; Ahmed and Barmore 1980). Horticulturally and physiologically these races differ in characteristics such as fruit size and appearance, fruit oil content and flavour, maturation date and storage characteristics, and disease and temperature (especially cold) tolerance (Ahmed and Barmore 1980). However, the three races are cross-compatible, share many physiological and chemical characteristics (Scora and Bergh 1990) and readily hybridise to form a variety of genotypes that suit different climatic conditions (Whiley and Schaffer 1994) and display different developmental and phenotypical fruit characteristics. Some important commercial varieties include 'Fuerte', which is a Guatemalan×Mexican hybrid, and 'Hass', which originated from a Guatemalan seedling (Seymour and Tucker 1993) and is believed to be 85-90% Guatemalan and 10-15% Mexican (Bergh and Ellstrand 1986).

'Hass' avocado (*Persea americana* Mill. var *guatemalensis*) is a commercially important cultivar in South Africa, believed by many to produce a better quality, richer tasting fruit. 'Hass' is a late

maturing cultivar and can be left to hang on the tree after it has reached physiological maturity (Whiley *et al.* 1996), allowing the grower a greater ability to 'play the markets'. One of the criteria used to determine avocado fruit price is size, with larger fruit often receiving better prices. 'Hass', like most fruits of Guatemalan origin (Bergh and Ellstrand 1986), has a tendency to produce medium and below-sized fruit (Chandler 1958), irrelevant of health status of the tree (Kremer-Köhne and Köhne 1995). It is understood that the availability, supply and composition of photoassimilates, such as sucrose (as well as plant hormones, minerals and water) to the fruit, and the subsequent ability of the fruit to metabolise these transported solutes, plays a crucial role in fruit development, and thus final fruit size. In 'Hass' the availability of assimilates does not appear to be limiting as under- and normal-sized fruit occur side by side with no pattern with respect to distribution (Cowan 1997), and thus the ability of the fruit to compete, import and metabolise assimilates seems to be of primary importance. Although the composition of avocados, especially after maturity, is well documented, very little is understood about the transport and metabolism of sugars in this fruit. Thus the aim of this research was to establish a basic understanding of transport and metabolism of sucrose in 'Hass' fruit in relation to final fruit size.

1.2 SUCROSE ~ THE UBIQUITOUS TRANSPORTED ASSIMILATE

Photosynthesis defines the process by which light energy is converted into chemical energy, and is used to produce carbohydrates. These carbohydrates form the backbone of plant life and are the primary and essential component, either directly or during synthesis, of nearly every metabolic, structural or messenger substrate/molecule in the plant. Sucrose (Suc; α-D-glucopyranosyl β-D-fructofuranoside), a non-reducing disaccharide of glucose and fructose linked by an *O*-glycosidic bond, is synthesised within the cytosol, transiently stored in the vacuole and exported via the phloem to photosynthetically inactive organs (Stitt and Steup 1985; Tymowska-Lalanne and Kreis 1998). Suc is generally accepted as the principle product of carbon fixation and the major and preferred form in which carbohydrate is transported from photosynthetic organs to the rest of the plant (Avigad 1982; ap Rees 1987; Lucas and Madore 1988; Sung *et al.* 1989; Sonnewald *et al.* 1991; Miller and Chourey 1992; Sturm and Tang 1999; Tauberger *et al.* 1999; Barker *et al.* 2000). Hence Suc is considered essential for the supply of carbon, energy and maintenance turgor necessary for plant metabolism, growth, storage and reproduction.

Suc has several properties that allow it to function effectively as a transport molecule. These include an unreactive chemical structure that prevents futile synthesis/degradation cycling during

transport, and a high free energy of hydrolysis (±30 kJ mol^{-1}) allowing it to be easily hydrolysed in enzyme-catalysed reactions (Lucas and Madore 1988).

For many plants Suc is the most abundant compound found in the phloem sap (Sheen *et al.* 1999). However, in some species sugar alcohols and oligosaccharides of the raffinose series are the principle forms in which carbon is transported (Dey 1990; Lunn and Furbank 1999). Examples of these include the phloem-transported sugar alcohols, mannitol, which predominates in celery (*Apium graveolens* L.)(Pharr *et al.* 1995; Greutert *et al.* 1998) and is found in olive (*Olea europaea* L.)(Flora and Madore 1993), sorbitol, which predominates in peach (*Prunus persica* L.)(Lo Bianco *et al.* 1999) and apple (*Malus domestica* L. Borkh.)(Archbold 1999), and the sugars raffinose and stachyose that are transported in muskmelon (*Cucumis melo* L.)(Hubbard *et al.* 1989), summer squash (*Cucurbita pepo* L.)(Richardson *et al.* 1982) and olive (Flora and Madore 1993). However, where these sugars prevail, Suc is always present and none of them completely replace Suc (Lunn and Furbank 1999). The ratio and metabolic importance of these sugars may also change developmentally, as in the peach which accumulates Suc with maturation (Vizzotto *et al.* 1996), in response to stress, e.g. the elevation of mannitol in response to osmotic stress in celery (Stoop and Pharr 1994), or in different tissues and sinks, as seen in the different sugar compositions in olive leaf, phloem and sink tissues (Flora and Madore 1993) and the different rates of sorbitol and Suc metabolism seen in reproductive and vegetative tissue in peach (Lo Bianco *et al.* 1999).

Not only does Suc serve as a compound for the long-distance transport of metabolites and to drive osmotic solute movement, but, via the Suc sensing pathway, it plays a role in the activation/repression of many genes and hence the modulation of transport activity and assimilate partitioning (Koch 1996; Chiou and Bush 1998; Smeekens 2000). Suc therefore plays an important role in communication within the plant and between the plant and the environment.

In 'Hass' avocado, the seven carbon (7C) sugar D-*manno*-heptulose and its polyhydroxy derivative, the sugar alcohol perseitol, were found to be the predominant soluble sugars in stem, trunk, root and fruit peel, flesh and seed (Liu *et al.* 1999a; 1999b). The ubiquitous distribution of these sugars allowed the authors to propose an unconfirmed essential role in both reproductive and vegetative development. However, the occurrence of these sugars in the phloem or their role in assimilate transport was not conclusively shown, and though one assumes they are of great significance, the potential importance of Suc in transport and metabolism of carbohydrates in avocado cannot be overlooked.

1.3 ASSIMILATE SUPPLY

1.3.1 THE PHLOEM ~ THE PATHWAY OF ASSIMILATE IMPORT

The term phloem relates to the vascular tissue that serves principally to allow long-distance translocation of sugars, nutrients and a diverse range of macromolecules, including proteins, systemic wound signals, RNAs and pathogens throughout the plant (reviewed in: Thompson and Schulz 1999; Oparka and Santa Cruz 2000). Phloem tissue consists of sieve elements (SE) and a variety of sclerenchyma and parenchyma cells, an example of the latter being companion cells (CC). The SE are the most highly specialized cells in the phloem, consisting fundamentally of modified protoplasts with restricted metabolic activity that are linked to adjacent SE by large pores in the end walls known as sieve plates. This arrangement allows for the formation of a functional continuum of cells forming a conduit that permits the long-distance movement of solutes and macromolecules from source (net carbon exporting) tissues to sink (net carbon importing) tissues. The 'driving force' of this long-distance transport by mass flow is generally attributed to the differences in pressure gradient between the source and the sink (Oparka and Santa Cruz 2000; and the references therein), as described by Münch's (1930) pressure-flow hypothesis, and appears to be unhindered by the sieve plates (Knoblauch and van Bel 1998). The associated sclerenchyma and parenchyma cells provide support, function as storage tissues, and later assist in the radial distribution of translocated substances (Esau 1977).

It is generally accepted that most Suc is synthesized in mature leaves, primarily by the combined action of the enzymes sucrose phosphate synthase (SPS; EC 2.4.1.14) and sucrose phosphate phosphatase (SPP; EC 3.1.3.24), and transported to the sink tissues via the phloem. However, the phloem also transports a variety of other sugars, including the monosaccharides glucose (Glu) and fructose (Fru), the oligosaccharides raffinose, stachyose and verascose, the sugar alcohols sorbitol and mannitol, and a variety of other compounds (van Bel 1993; and the references therein). Plasmolysis and high-resolution autoradiographic studies indicate that the concentration of these sugars is substantially higher in the phloem than in leaf tissue (Giaquinta 1983) and thus the transport of these sugars through the phloem depends in part on the accumulation of sugars in the phloem and the subsequent removal of these sugars from the phloem at the sink i.e. the transport and distribution of phloem solutes is determined by the transport and metabolic processes occurring at the end of the source-phloem-sink system (Patrick 1990).

1.3.2 PHLOEM UNLOADING ~ PHOTOASSIMILATE ARRIVAL IN THE FRUIT

As much as 80% of carbon acquired by photosynthesis is transported in the plant vascular system of the plant to sink tissue (Chiou and Bush 1998), with up to 65% of total photosynthate going into the fruit (Bieleski 2000). Phloem unloading occurs along the entire length of the phloem (Thorpe and Minchin 1996), and involves the removal of solutes, macromolecules and water from the SE. Two possible pathways exist for phloem unloading, namely the symplastic and the apoplastic routes (Figure 1.1).

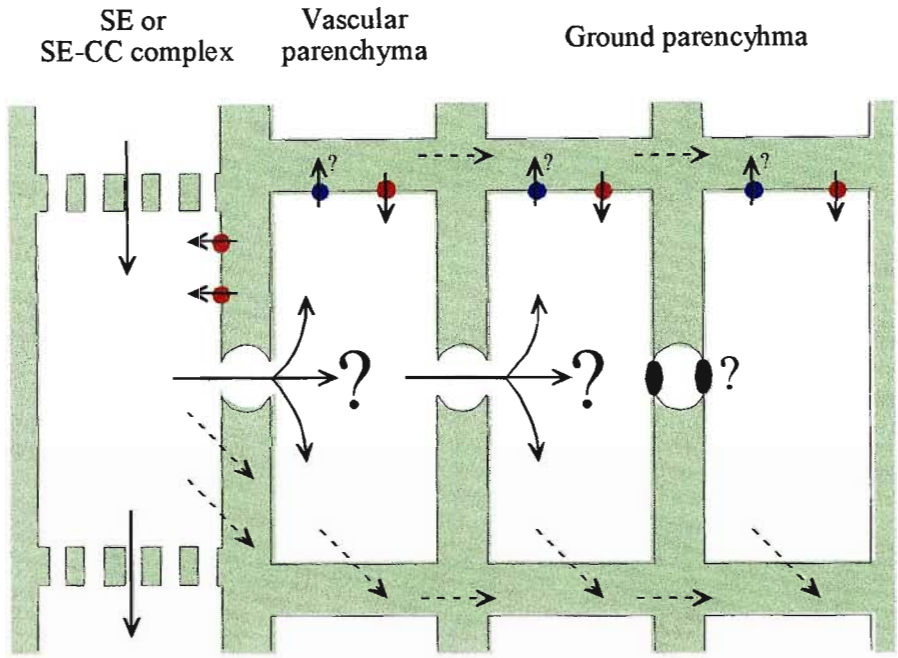


Figure 1.1. Cellular pathway of phloem unloading and subsequent post-SE transport through the symplast by bulk flow (→), with possible regulation of flux through plasmodesmata by altered conductivity or occlusion (●), and the apoplast by diffusion (⇢). Solutes may move out of the symplast via turgor-sensitive and -insensitive porters or pores in the plasma membrane (●), or into the symplast via proton symporters (●). The apoplast appears light green, the symplast white, and the two are delimited by a black line representing the plasma membrane. Adapted from Patrick (1997).

The symplast is the continuum of cell protoplasts permitted by the presence and conductivity of plasmodesmata (Pd), or, as in the case of the phloem SE, by the presence of pores in the sieve plate. Symplastic transport is the movement of water and small molecules from cell-to-cell via the Pd. These molecules are, with the exception of viruses, usually <1 kD in size. During phloem

unloading symplastic flux occurs by two means: diffusion and bulk flow (possibly assisted by cytoplasmic streaming).

Diffusion is the movement of molecules/ions from areas of high concentration to areas of low concentration. Thus assimilates will move from the SE to the neighbouring CC or parenchyma cells which have lower assimilate concentrations. Diffusion allows for sink determination of solutes unloaded through compartmentation and metabolism. The rate of diffusion depends on concentration differences, and the nature and number of the Pd between the SE and the sink cell, as illustrated by Fick's law of diffusion (after Patrick 1997):

$$m = nP\left(\frac{DA\Delta Ct}{L}\right) \qquad (1)$$

where;

m = mass of solute

n = number of Pd

P = Pd conductivity

D = diffusion constant of solute

A = cross sectional area of Pd

ΔC = concentration difference at the ends of the Pd

t = time

L = length of Pd

Adequate differences in solute concentration may exist for unloading by diffusion (Patrick 1997; and references therein). However, for terminal, sugar-accumulating sinks, unloading by diffusion will retard water movement out of the phloem at the sink and so hinder phloem transport. This situation may be avoided if unloading occurs by bulk flow (Murphy 1986).

Bulk (convective) flow involves the overall movement of phloem solution induced by differences in water potential (i.e. pressure). Thus high solute concentrations, i.e. a low water potential, in the SE at the source will cause water to enter the phloem by osmosis. The simultaneous movement of water out of the sink will cause the phloem solution to move from the source to the sink, and from the SE to the CC or parenchyma, thereby passively unloading the phloem. The rate of bulk flow, as for diffusion, depends on the nature and frequency of the Pd, and the differences in concentration between the phloem and CC or sink parenchyma, as illustrated by Poiseuille's law:

$$v = nP\left(\frac{\pi r^4 (P_{SE} - P_{SINK})t}{8\eta L}\right) \quad (2)$$

where;

v = volume of phloem solution

n = number of Pd

P = Pd conductivity

r = radius of Pd

P_{SE} = hydrostatic pressure (water potential) of SE, as determined by the solute concentration (osmotic potential)

P_{SINK} = hydrostatic pressure (water potential) of sink cell, as determined by the solute concentration (osmotic potential)

t = time

η = viscosity of phloem solution, as determined by the solute concentration

L = length of the Pd

Patrick (1990; 1997; and references therein) suggested that bulk flow can, in certain instances, account for phloem unloading and the dissipation of phloem-imported water, and so prevent the build up of pressure in the SE, especially in situations where Pd have a high conductance (large size exclusion limit (SEL)). Unloading from the phloem normally occurs symplastically, either into the SE-CC complex or directly into the surrounding vascular parenchyma (Patrick 1990), most likely by a combination of both diffusion and mass flow.

The number of Pd connections in the post phloem pathway may thus be assumed to be a rate-limiting step for symplastic phloem unloading. However, Oparka and Santa Cruz (2000) suggest that Pd in sink tissue may have higher size exclusion limits (SEL), >10kD as opposed to the universally accepted Pd SEL of <1kD (Fisher and Oparka 1996; Patrick 1997) which can be observed in leaf Pd (Goodwin 1983; Terry and Robards 1987), and so accommodate sufficient Suc/solute flux rates needed to sustain development. Interestingly, Pd between SE and CC have been observed to have SELs of 10-25 kD (Kempers and van Bel 1997; Imlau *et al.* 1999) suggesting an increased capacity to assist in the unloading of the phloem.

The apoplast is the continuum of non-protoplasmic matter, such as the cell wall and intercellular material. Hence apoplastic unloading of the phloem involves transport across the plasma membrane (Pm) and subsequent movement of solutes in the apoplast. Apoplastic unloading may

occur by simple diffusion or be facilitated by membrane porters or channels (Patrick 1997), and is, in part, determined by the surface area of the Pm (Patrick 1990). The pump-leak system may be used to describe apoplastic unloading of the SE-CC complexes. Solutes diffuse or passively leak out of the SE-CC complex symplast and into the apoplast, an area of low solute concentration. It has been suggested apoplastic loading may also be, in part, driven by turgor-sensitive and -insensitive porters, including those driven by NADH redox systems (Patrick 1990) and H^+-ATPases (DeWitt and Sussman 1995; Ruan and Patrick 1995). De Jong and co-workers (1996) hypothesized the existence of pore-forming proteins which may also facilitate the diffusion of Suc across the Pm. Solutes then travel in the apoplast and are subsequently actively pumped back into the symplast. This 'retrieval' into the symplast is facilitated by Pm bound proton symporters (van Bel and Patrick 1984; Aloni *et al.* 1986; Secor 1987). In pea (*Pisum sativum* L.) sieve element unloading is achieved against a transmembrane concentration gradient and is facilitated by Suc/H^+ symporters located in the Pm of transfer cells symplastically connected to the vascular tissue (Tegeder *et al.* 1999). The net apoplastic efflux from the SE-CC complex is governed by the difference between the rates of diffusion into, and the rate of solute removal from, the apoplast. The passive diffusion flux is illustrated by the equation proposed by Patrick (1990):

$$F_d = M\left(C_{SE} - C_{APO}\right) \qquad (3)$$

where; F_d = passive diffusion flux

M = membrane permeability coefficient

C_{SE} = solute concentration in SE

C_{APO} = solute concentration in apoplast, as determined by subsequent solute-proton symport activity and apoplastic metabolism of the solute

In most cases unloading in the SE-CC complex involves the simultaneous transport of assimilates through the apoplastic and symplastic routes; however, the emphasis on these routes may change developmentally. For example, in young growing tissues or meristematic regions, vascular unloading frequently occurs via the Pd (Turgeon and Webb 1976), but in older leaf tissue, unloading is thought to occur across the Pm and into the apoplast of the sink tissue (Turgeon 1984). However, even where symplastic transport dominates, SE unloading normally involves some apoplastic contribution, the extent of which depends on the relative conductances of the apoplastic and symplastic routes.

1.3.3 POST-SIEVE ELEMENT/COMPANION CELL TRANSPORT ~ PHOTOASSIMILATE DISTRIBUTION IN THE FRUIT

Subsequent transport of solutes in the sink parenchyma will follow a similar route to the unloading route of the phloem; however, it is influenced by a wide range of developmental, morphological and metabolic factors. Three primary cellular pathways are recognized: symplastic, apoplastic and symplastic interrupted by apoplastic (Patrick and Offler 1996).

Symplastic transport allows for greater photoassimilate flux (Patrick and Offler 1995; Wang *et al.* 1995), reduces the requirement for an extensive vascular system (Patrick 1990), dissipates phloem-imported water (Murphy 1989), and efficiently utilizes transcellular concentration and osmotic gradients (Warmbrodt 1987; Fisher and Wang 1993) to drive solute movement. Possibly as a consequence of these factors, symplastic transport is considered to be the primary route of movement for simple sugars, small proteins and some nucleic acids during normal vegetative development. In terminal sink tissues there appears to be a functional symplastic continuum between storage parenchyma cells (Oparka and Prior 1987; Oparka and Prior 1988; Ruan and Patrick 1995; Moore-Gordon *et al.* 1998). In fruits and seeds the rate of transpiration and, hence, water flow in the sink tissue is very low (Fisher and Oparka 1996), and therefore a large proportion of post-phloem solute movement must occur by diffusion. In sink tissues, cytoplasmic streaming is predicted to facilitate this largely passive symplastic transport (Gunning and Overall 1983; Lucas *et al.* 1993). Cytoplasmic streaming (cyclosis) is the movement of the cytoplasm within cells and is believed to augment diffusion and so facilitate movement through the Pd. The rate of cytoplasmic streaming is, in part, determined by the viscosity of the cytoplasm; which , in turn, is dependant on the solute concentration and the temperature. Thus the solute levels between contiguous cells and within the cytoplasm will help determine the rates of symplastic solute transport in sink tissues. However, post-sieve element Pd often have large SELs indicating that they support high fluxes of photoassimilates, and the high levels of solute accumulation in some sink tissues suggests that bulk flow must also contribute to post-phloem symplastic transport. This bulk flow must thus be determined by sink metabolism and compartmentation of solutes (such as Suc). The latter refers to both compartmentation within the cell and to the isolation of cells with high solute concentrations by the occlusion of Pd. Fisher and Wang (1995) found a linear Suc concentration gradient in developing wheat (*Triticum aestivum* L.) grains that was adequate to supply Suc by diffusion alone. However, they postulated that if Suc were to be transported convectively by a turgor and/or osmotic gradient, it would require excessive volumes of water to move into the endosperm. Though this efflux of water from the phloem would theoretically

increase the movement of solutes into the sink cells (Lang and Thorpe 1986) and the turgor would decrease with cell expansion (Stitt 1996), this would be insufficient to account for all the water imported. Thus the question of diffusive vs. convective symplastic transport in the sink remains unresolved.

Apoplastic movement in the post-SE pathway follows a similar mechanism to that in phloem unloading, involving a Pm bound sugar/proton symport mechanism (Patrick and Offler 1996), driven by an H^+-ATPase (Ruan and Patrick 1995). H^+-ATPases are enzymes that couple the hydrolysis or synthesis of ATP to a transmembrane movement of protons (Palmgren 1990) allowing the unidirectional flux of ions or solutes across a membrane. Recently, Weise and co-workers (2000) reported a new subfamily of Suc transporters, SUT4. Although primarily associated with phloem loading, this low affinity/high capacity system was also expressed in sink tissues of *Arabidopsis* and tomato, and may facilitate high rates of Suc uptake from the apoplast back into sink cells or *vice versa.* Other members of this proton-coupled Suc uptake transporter (SUT) family have been identified and associated with both the loading/unloading of sieve elements or with Suc uptake/transport in sink tissues (Kühn *et al.* 1997; 1999; Weber *et al.* 1997a; Tegeder *et al.* 1999). In addition, extracellular invertases maintain concentration differences across the Pm (Damon *et al.* 1988; Weber *et al.* 1995). In seed coat parenchyma cells of pea (*Pisum sativum* L.) De Jong and co-workers (1996) showed that a small Suc gradient across the Pm is sufficient to result in a net Suc efflux from the seed coat to maintain embryo development, and hypothesized that the Pm permeability can be increased by pore forming proteins to facilitate diffusion across the Pm. In cases where apoplastic invertase is present, subsequent sugar transport can occur via hexose transport systems (Frommer *et al.* 1996), either apoplastically or by returning the hexose to the symplast for further metabolism, compartmentation or symplastic transport (reviewed in Sauer and Tanner 1993). Apoplastic transport is normally only a secondary route (Patrick and Offler 1996) due to the comparatively low rate of flux. Typically, dominant apoplastic transport is restricted to situations where movement through the symplast is not compatible with sink function (Patrick 1990). For example, sinks that accumulate high levels of solutes, e.g. maturing fruit, or tissues of different genomes that exhibit symplastic discontinuity, e.g. the interface of seed (filial) and maternal tissue (Patrick 1997) where Pd are absent altogether, or at the interface between a host plant and a biotroph (Patrick 1989). In these situations, loss of symplastic continuity may arise from the permanent or reversible closure/occlusion of Pd. Indeed, in higher plants all the cells in the embryo are connected into a single symplastic domain (Mansfield and Briarty 1991), and during development down-regulation of Pd results in isolated symplastic zones.

Switches between symplastic and apoplastic pathways may accompany fruit development. Offler and Horder (1992) observed that in young tomato fruit symplastic solute movement dominates, and there are sufficient Pd to sustain this. However, at the onset of sugar accumulation, transport in the symplast is discontinued and subsequent sugar movement is apoplastic. This allows for solute accumulation to occur against, and isolates transport from, solute and osmotic gradients caused by sugar accumulation. This phenomenon also highlights the important role of Pd in the regulation of phloem unloading and subsequent sugar transport (Stitt 1996). In some instances photoassimilates have been observed to move against a pressure gradient via the Pd and it is thought that this transport is partly 'active' and requires metabolic energy to sustain it (Trębacz *et al.* 1988).

1.4 SINK DEMAND ~ THE ASSURANCE OF CONTINUED ASSIMILATE SUPPLY

The term 'sink' refers to a site where demand exists for a particular substrate, created by utilization, compartmentation and/or storage. Sink strength can thus be defined as the competitive ability of the sink organ to import assimilates (Herbers and Sonnewald 1998). Münch's mass flow hypothesis and the principle of diffusion are based on the theory that sugar concentration in the sink is lower than that of the source. Equations 1, 2 and 3 (above), which illustrate phloem unloading flux in the symplast and apoplast, all show the need for solute concentration differences between the phloem and the parenchyma. These equations, when applied to post-sieve element transport, similarly show the requirement for a solute gradient between adjacent cells in sink tissues. Thus it is evident that both phloem unloading and post-phloem transport are controlled, in part, by sugar metabolism and compartmentation (between the apoplast, cytoplasm and the vacuole) in sink tissue. Similarly, differences in solute concentration between the symplast and apoplast will impact on cell turgor. The unloading flux into the apoplast is very sensitive to turgor (Giaquinta 1983; Patrick *et al.* 1986). Changes in cell turgor have been suggested to effect the efflux of photoassimilates across the Pm (Patrick 1993), regulate the activity of Pm bound H^+-ATPases and so effect symporter activity (Wyse *et al.* 1986), and impact on Pd conductivity (Barklay and Fensom 1984; Oparka and Prior 1992). Hence control of phloem unloading is subject to control by solute levels (Patrick 1990).

As Suc is generally accepted as the main phloem transported sugar in plants (Avigad 1982; Sung *et al.* 1989), high levels of Suc in the fruit will reduce the concentration gradient of this transport sugar from source to sink and so impact negatively on the rate of carbon import (Klann *et al.*

1993). Thus the metabolism (Ho 1988; Sung *et al.* 1989; Cano-Medrano and Darnell 1997; Gao *et al.* 1999) or partitioning (Patrick and Offler 1995) of the solutes, especially Suc, at the sink are equally as important as the continued intensive loading of sugars in the source (Gifford and Evans 1981) to assure the maintenance of assimilate influx. Similarly, the removal of water from the phloem will increase the osmotic gradient between the source and the sink. It is postulated that continuous passive leakage and active retrieval ('pump and leak' system), involving a possible sugar/proton co-transporter (Giaquinta 1983), along the length of the phloem allows for the maintenance of pressure flow over long distances (Thorne 1985).

The partitioning of Suc is important in the continued maintenance of a solute gradient and sink strength, both within the sink tissue and the source-phloem-sink system. Plants allocate carbon to regions of growth, reproduction and storage, and partition carbon among metabolic fractions that support growth and maintenance (e.g. amino acids, organic acids, lipids, pigments, proteins, lignin and hemicelluloses, and residue), defence (e.g. phenolic glycosides, tannins and phenols), storage (e.g. sugars and starch) (Kleiner *et al.* 1999), and respiration. Whole plant carbon partitioning and Suc/hexose compartmentation within the sink is, in part, hormonally controlled (Pérez and Gómez 2000). For example, hormones like abscisic acid (ABA) and indoleacetic acid (IAA) are known to directly affect carbohydrate metabolism and transport (Bangerth 1989; Brenner and Cheikh 1995; Ofosu-Anim *et al.* 1996; and the references therein). It is important to remember that assimilate partitioning is regulated by both sink-located transfer and transport processes, and solute metabolism (Weber *et al.* 1997b; and the references therein). Many processes controlling assimilate import require respiratory energy (Ho 1988) and thus in some fruits, such as persimmon (Nakano *et al.* 1998), high respiration rates are important in maintaining sink strength. This is facilitated by the creation/maintenance of a solute gradient between the source and the sink by the utilization of sugars. Thus it can be seen that the regulation of net flow of photoassimilates in the whole plant, into and within the sink, between compartments within each individual cell, and the partitioning into the various metabolic pools is an integrated and finely controlled process.

1.5 THE PROVISION OF CARBON ~ SUCROSE CLEAVAGE

Suc cleavage gives a reasonable biochemical measurement of sink strength (Sung *et al.* 1989). Not only is the cleavage of Suc important for the creation and maintenance of sink strength, but it is also essential for the metabolism of non-photosynthetic cells. Provision of carbon and energy is only achieved following the cleavage of Suc (Hawker 1985; Hedley *et al.* 2000), and thus cleavage of Suc into hexoses and the subsequent conversion of hexose to hexose-phosphate can be

considered to be the starting point of fruit metabolism (ap Rees 1988) and carbohydrate partitioning. The enzymes invertase, sucrose synthase (SSy) and SPS are involved in the cleavage/synthesis of Suc and hence phloem unloading, post-SE transport, and fruit growth.

1.5.1 INVERTASE

Plant invertases (β-D-fructofuranosidase; EC 3.2.1.26) represent a family of enzymes that catalyse the irreversible hydrolysis of Suc into its monosaccharide constituents Glu and Fru. Invertases have been purified and characterised from a number of plants (see review by Tymowska-Lalanne and Kreis 1998) and exist in three isoforms, each consisting of numerous different isozymes. The three isoforms can be classified by both their solubility, pH optima and spatial location in the cell, viz. extracellular (cell wall or apoplastic), vacuolar and cytoplasmic (Table 1.1).

Table 1.1 Invertase isoforms and properties.

Location	Solubility	pH optimum	Common term
Extracellular	Insoluble	4.5-5.0	Acid
Vacuolar	Soluble	4.5-5.0	Acid
Cytoplasmic	Soluble	6.5-8.0	Alkaline /Neutral

The physiological role of invertases is diverse and still not fully understood. The discrete subcellular locations of the isoforms, differential expression with development and key position in Suc metabolism of invertases allows these enzymes to play a crucial role in the control of biochemical pathways (Sturm *et al.* 1999), source-sink relations and metabolic fluxes, downstream sugar partitioning, sugar-modulated gene expression (reviewed by: Sheen 1994; Koch 1996; Jang and Sheen 1997; Smeekens and Rook 1997; Lalonde *et al.* 1999; Yu 1999; Smeekens 2000), and subsequent phenotypic responses (Cheng *et al.* 1999), including growth, development and reproduction.

1.5.1.1 Acid invertase

The invertase isoforms that function optimally in the acid pH range (4.5-5.0) are collocatedly termed acid invertase (AI). AI is a β-fructofuranosidase, hydrolysing Suc and other β-Fru-containing oligosaccharides from the Fru residue (Sturm 1999). Thus, though AI preferentially

attacks the disaccharide Suc, it will also act on other substrates such as the trisaccharide raffinose and the tetrasaccharide stachyose.

Extracellular AIs are bound to the cell wall by ionic bonds (Little and Edelman 1973; Sturm 1999), giving them the appearance of being insoluble in aqueous solutions and thus they are termed insoluble AIs (IAI). These IAI, depending on the isozyme, can be released into solution by high-molarity salt solution or chelating agents. Cell-wall invertases are characterised by a high pI (Glasziou and Gaylor 1972). Vacuolar invertases accumulate as soluble polypeptides in the vacuole (Sturm 1996) and are thus termed soluble AIs (SAI). These are characterised by a low pI. In muskmelon (*Cucurbita pepo*)(Ranwala *et al.* 1991) and Jerusalem artichoke (*Helianthus tuberosus*) (Glasziou and Gaylor 1972), certain IAIs released from the cell wall were found to have similar properties to SAI, with respect to pH optima, substrate specificity, K_m values for Suc, and effects of metal ions, and were thus proposed to be the soluble form bound to the cell wall. Indeed, invertase elution patterns, immunological recognition (Weil and Rausch 1990; Isla *et al.* 1999), and amino acid sequences of the soluble and insoluble isoforms of AI indicate these two isoforms are closely related and may originate from the same enzyme, sharing several highly conserved motifs (Unger *et al.* 1994), and appear to show evolutionary relatedness to invertases from yeast and bacteria (Sturm and Chrispeels 1990). Differences in properties are possibly a consequence of the enzyme microenvironment or binding to the cell wall (Isla *et al.* 1999).

AI appears to be inhibited by Glu and Fru, Hg^{2+} and Ag^{2+} ions (Ranwala *et al.* 1991), $CaCl_2$ (Doehlert and Felker 1987) and by an endogenous proteinaceous inhibitor (Isla *et al.* 1999). Glu inhibits AI in a non-competitive manner (Isla *et al.* 1991). Fru, on the other hand, was found to inhibit AI in a complex non-linear competitive manner (Isla *et al.* 1995), producing a two site competitive inhibition with interaction between the two sites (Isla *et al.* 1999). Isla and co-workers (1995) classified the SAIs from rice (*Oryza sativa*) into two groups: that inhibited by Fru in a simple competitive fashion, and that in which SAI was inhibited in a complex competive manner. Inhibition in the first case could be suppressed by invertase complexing with a protein. AI is a N-linked glycoprotein (Doehlert and Felker 1987; Wagner and Wiemken 1987; Sturm 1991; Walker *et al.* 1997), and the formation of a protein-glycoprotein complex by invertase appears to stimulate SAI *in vitro* (Isla *et al.* 1995). Interestingly, a small polypeptide of 17 kD that inhibits IAI activity, by co-localising with the enzyme in the cell wall in a pH-dependent manner, maximally at pH 4.5, was identified by Weil and co-workers (1994). A similar apoplastically located proteinaceous inhibitor has been proposed to also interact with both SAI and IAI (Isla *et al.* 1999).

Calculations based on extractable invertase activity in sink leaves in oat (*Avena sativa*) (Greenland and Lewis 1981) and poison rye grass (*Lolium temulentum* L.)(Walker *et al.* 1997) indicate that there is sufficient invertase activity to cleave all leaf Suc in 45-60 minutes. Thus, AI activity in the leaf must be modulated, possibly by end product inhibition by Fru, and/or Suc compartmentalisation in the cell. A decrease in apoplastic pH induced by salinity was shown to cause a loss in IAI activity with an associated increase in Suc in the symplast (Balibrea *et al.* 1999), clearly illustrating the co-ordinated role of SAI and IAI in the compartmentation of Suc in the cell. Cheng and co-workers (1999) showed that IAI in maize (*Zea mays* L.) is encoded by the cell-wall invertase gene *Incw*1. This gene has been shown to be regulated at both the transcriptional and post-transcriptional levels by metabolisable sugars (such as Suc and Glu) and by non-metabolisable sugars (such as the Glu analogues 3-*O*-methylglucose and 2-deoxyglucose), in a hexokinase (HXK; EC 2.7.1.1, 2.7.1.2, 2.7.1.4, 2.7.1.7) independent manner. Thus the expression of *Incw*1 may provide a link between carbon sensing and the regulation of sugar metabolism at the molecular level. Furthermore, in transgenic potato (*Solanum tuberosum* L.) tubers with an over expressed yeast-derived invertase, Glu levels were found to be exceptionally high (Tauberger *et al.* 1999) suggesting an induced inability to regulate Suc cleavage and sugar composition in the sink. Similarly, the introduction of an antisense cauliflower mosaic virus 35S promoter gene into tomato (*Lycopersicon esculentum* L.), which suppresses AI in both the soluble and insoluble fractions, caused an increase in Suc content and a concomitant decrease in hexoses (Ohyama *et al.* 1995). This suggests that AI plays an important role in the determination of sugar composition in tomato fruit. In tomato, IAI isozymes are encoded by a *Lycopersicon* invertase gene family comprising of four members: *Lin*5, *Lin*6, *Lin*7, and *Lin8* (Godt and Roitsch 1997). These genes are expressed differentially during development and carbohydrate demand. *Lin*5, *Lin*6 and *Lin*7 are expressed during early development, permitting the establishment and maintenance of sink metabolism, with *Lin6* mRNA levels peaking during periods of high carbohydrate demand. Similarly, potato also displays more than one gene coding for IAI and these genes are expressed differentially in different tissues at different stages of vegetative and reproductive development (Maddison *et al.* 1999). AI therefore appears to have the ability to modulate its own activity by product feedback and the regulation of gene expression, whilst simultaneously directing carbon demand, compartmentation and composition within the sink. The effects of altered Suc metabolism by AI are far-reaching, and may play a direct role in the morphology of the tissue, organ or plant. Transgenic plants with altered AI have shown a wide range of phenotypic alterations during early development, including disturbed growth habits in tobacco (*Nicotiana tabacum*)(von Schaewen *et al.* 1990), stunted hypocotyls and roots, and the inability of cotyledons to develop normally in carrot (*Daucus carota* L.)(Tang *et al.* 1999).

A primary action of IAI is the control of phloem unloading and the creation of sink strength. Cleavage of Suc at the site of phloem unloading is the key step in generating sink strength. IAI has been found to be highly localised to the vasculature of developing leaves in pea (*Pisum sativum* L.)(Zhang *et al.* 1996), barley (*Hordeum vulgare* L.)(Kingston-Smith and Pollock 1996), and carrot (Ramloch-Lorenz *et al.* 1993), within the phloem of potato (Hedley *et al.* 2000), and to sites of phloem unloading and active Suc import, such as in the basal endosperm and pedicel tissue in maize (Doehlert and Felker 1987; Miller and Chourey 1992). At the site of phloem unloading IAI cleaves transported Suc into hexose sugars in the apoplast. The effect of this is threefold: firstly, phloem unloading is facilitated and regulated (Cheng *et al.* 1999; Miyamoto *et al.* 2000); secondly, sink strength is generated in a controlled manner (Eschrich 1980); and thirdly, hexose sugars are introduced into the apoplast. These hexose sugars then move throughout the apoplast and are unloaded into the sink cells via hexose-mediated proteins (Roitsch and Tanner 1996) where they are used to maintain subsequent sink metabolism and growth. In young fruit such as tomato, this extracellular cleavage of Suc and subsequent hexose transport across the Pm is considered to be essential for normal fruit development (Damon *et al.* 1988; Godt and Roitsch 1997). In green bean (*Phaseolus vulgaris*)(Estruch and Beltrán 1991) and lima bean (*P. lunatus*)(Xu *et al.* 1989), ovary and embryo growth is accompanied by high levels of IAI. In legumes the embryo is isolated from the maternal seed coat by the apoplast, and hexose levels in the cotyledons and endosperm apoplasm correlate with IAI levels in the seed coat (Weber *et al.* 1995). The hexoses generated by the IAI are loaded into the embryo symplast by hexose-proton co-transporters and used for growth and cell division (Weber *et al.* 1996). At the onset of maturity, IAI activity appears to be replaced by an active Suc transport system in the embryo epidermis. Thus it appears that the developing immature legume embryo relies solely on IAI for the establishment of sink strength and assimilate import in the seed. In both tomato and legumes the presence of an IAI and a hexose-transporter suggests that extracellular invertase plays a role in the partitioning of assimilates among different metabolic fractions, permitting both the generation of a solute gradient into the sink and an element of control over subsequent sink metabolism.

The expression and activity of IAI seems to follow a distinctive trend during fruit/seed growth. IAI appears to be high in young, rapidly growing cells in a variety of fruit including *Cucumis sp.*, blueberry (*Vaccinium ashei*), tomato, pea, grape (*Vitis vinifera* L.) and muskmelon; and declines (often rapidly) with maturation and the onset of sugar accumulation (Chin and Weston 1973; Lingle and Dunlap 1987; Schaffer *et al.* 1987a; Hubbard *et al.* 1989; Estruch and Beltrán 1991; Ranwala *et al.* 1991; Stommel 1992: Klann *et al.* 1993; Cano-Medrano and Darnell 1997; Balibrea *et al.* 1999; Famiani *et al.* 2000). In some fruits, IAI activity persists throughout fruit ontogeny but

at greatly reduced levels, possibly ensuring a continuous sugar influx into the fruit from the source. IAI thus seems important for the sugar uptake in plant cells (Komor *et al.* 1981) of rapidly growing tissues (Glasziou and Gaylor, 1972).

Vacuolar invertase is believed to play an important role in osmoregulation (Wyse *et al.* 1986; Sturm *et al.* 1995), cell enlargement, control of sugar composition in fruits and storage organs, storage organ response to cold (Sturm and Tang 1999), gravitropism (Wu *et al.* 1993a), and plant response to pathogens and wounding (Sturm and Chrispeels 1990). Of primary interest, though, is the role of SAI on Suc import, compartmentation and metabolism. In higher plants, the main intracellular site of Suc storage is the vacuole (Willenbrink 1982; Lunn and Furbank 1999). It is thought that cytosolic Suc, arising from SSy or SPS activity, starch degradation, or symplastic transport, may enter the vacuole by a tonoplast-bound Suc/H^+ antiport system (Getz 1991) or a similar energy-dependent transport system (Keller 1992). The driving force for this Suc transport is a H^+ electrochemical gradient across the tonoplast generated by the tonoplast-bound proton pumps, ATPase and phosphorylase (Rea and co-workers 1987; 1993). Suc may also enter the vacuole by facilitated diffusion (Milner 1995; Echeverria *et al.* 1997a). Once in the lumen of the vacuole, SAI hydrolyses the Suc to hexoses, the extent of which is in part determined by the vacuole volume and thus relative amount of SAI (Smith 1999). This removal of Suc from the cytoplasm and its subsequent compartmentation in the vacuole is believed to be essential for the maintenance of a physiological gradient of photosynthate in the sink tissue (Miller and Chourey 1992). The action of this enzyme will thus regulate the amount of Suc stored in the vacuole (Leigh *et al.* 1979; Lingle and Dunlap 1987; Huber 1989; Zhu *et al.* 1997) and determine sugar composition (Sturm 1996; Zrenner *et al.* 1996; Cheng *et al.* 1999) and metabolism in the sink tissue. The cleavage of Suc in the vacuole will further, though indirectly, regulate sink activities by affecting vacuolar and cell pressure (Hoffmann-Benning *et al.* 1997), so impacting on the key concepts of Münch's mass flow hypothesis, as well as bulk flow, Pd transport and Pm associated transport processes.

Unlike IAI, SAI distribution appears to be heterogeneous in fruit (Famiani *et al.* 2000), implicating a more universal role than the establishment of sink strength or localised phloem unloading. However, like IAI, SAI has been found to be relatively high in the developing fruits of tomato (Manning and Maw 1969), water melon (*Citrullus lanatus*)(Walker and Hawker 1976), sweet pepper (*Capsicum annuum*)(Walker and Hawker 1976; Nielsen *et al.* 1991), muskmelon (Hubbard *et al.* 1989; Iwatsubo *et al.* 1992), buttercup squash (*Cucurbita maxima*)(Irving *et al.* 1997), flowers of tomato (Russell and Morris 1982), leaves of green bean (Morris and Arthur 1984a) and

tuberising roots of potato (Ross *et al.* 1994). This activity normally declines during growth/maturation and suggests that hydrolysis of Suc by SAI may provide substrate needed for growth. In tomato (Klann *et al.* 1996) and carrot (Tang *et al.* 1999), changes in sugar composition as a consequence of SAI activity have been found to have a direct effect on fruit/root size. Whilst a decrease in SAI and an accompanying reduction in the levels of reducing sugars in maize was observed in maize ovaries that exhibited reduced growth (Zinselmeier *et al.* 1995), changes in SAI will also affect hexose accumulation. Some fruit, such as tomato (*L. esculentum*), grape and blueberry, accumulate hexoses at maturation. In tomato and blueberry SAI activity is closely associated with hexose accumulation in the maturing fruit (Klann *et al.* 1996; Cano-Medrana and Darnell 1997). The SAI cleavage of Suc in these situations has the advantage that it occurs within the confines of the vacuole and hence does not affect further allocation of assimilates to the sink. Interestingly, wild type tomatoes such as *L. peruvianum* and *L. chmielewskii* do not accumulate hexoses at ripening, but rather accumulate Suc. In these fruit, AI activity at ripening has been found to be very much reduced in comparison to *L. esculentum* (Stommel 1992; Klann *et al.* 1993).

AI can thus be seen to play a very important role in the development of young heterotrophic plant tissues and organs. IAI appears to have a crucial role in phloem unloading, the establishment of sink strength before it can be maintained by metabolic demand and effective carbohydrate partitioning, the apoplastic transport of hexoses between adjacent tissues that do not have any symplastic connections, and the compartmentation of sugars in the apoplast. Likewise, SAI appears to participate in the maintenance of sink strength, both directly, and through its effects on vacuolar and hence cellular turgor. Furthermore, SAI appears to play an instrumental role in determining cellular sugar composition and compartmentation and, hence, in the supply of carbon substrates to fuel growth and metabolism in rapidly growing tissues.

1.5.1.2 Cytoplasmic invertase

The third 'category' of invertase, by virtue of its recovery in the soluble fraction and pH optimum of 6.5-8.0, is localised in the cytoplasm (Tymowska-Lalanne and Kreis 1998), and is termed cytoplasmic invertase (CI). These invertases have been separated from AI by DEAE-cellulose chromatography and exhibit clear differences in pH optima, substrate specificity, K_m values for Suc, and inhibition by metal ions in comparison to their acidic counterparts (Ranwala *et al.* 1991). Similarly, CI is not N-glycosylated (Copeland 1990; Chen and Black 1992; Walker *et al.* 1997), shares no homologous polypeptide sequences with AI, and has been detected only in the genomes

of plants and photosynthetic bacteria (Sturm *et al.* 1999). These facts suggest CI evolved independently from other Suc-cleaving enzymes. Purification of CI to electrophoretic homogeneity in soybean (*Glycine max* L.)(Chen and Black 1992), faba bean (*Vicia faba*)(Ross *et al.* 1996) and chicory (*Cichorium intybush*)(Van den Ende and Van Laere 1995) suggests two different invertase isoforms exist in the cytoplasm: neutral invertase (NI), with a pH optima of 6.5-7.0, and alkaline invertase (AlkI), which is most active in the slightly alkaline (7.0-8.0) pH range.

NI is an octamer, which, in carrot, consists of eight subunits of 57 kD (Lee and Sturm 1996). Contrary to the definition of invertases, recent studies suggest NI is not a β-fructofuranosidase and Suc is its sole substrate (Sturm *et al.* 1999). NI is inhibited by the nucleotides ATP, GTP and UTP (Lee and Sturm 1996) and heavy metal ions (Sturm 1999), displaying total inhibition by Hg^{2+} and Ag^{2+} and partial inhibition by Cu^{2+} and Zn^{2+} (Ranwala *et al.* 1991). AlkI is a tetramer, consisting of four subunits, normally with a molecular weight of 54-65 kD (Sturm 1999), but has been observed weighing up to 126 kD (Lee and Sturm 1996). AlkI appears not to be Suc specific, and has a low affinity for raffinose and stachyose. AlkI is inhibited by $CaCl_2$, $MgCl_2$, $MnCl_2$ (Lee and Sturm 1996) and Tris (Sturm 1999). Both NI and AlkI appear to be completely inhibited by Fru and non-competitively inhibited by Glu (Sturm 1999). These differences in substrate specificity and inhibition, and the fact that the native polypeptides in these enzymes have different masses and pH optima, suggests that NI and AlkI may be products of different genes. However, they appear immunologically related and it is thus possible that NI is generated by the differential splicing or proteolytic cleavage of AlkI (Lee and Sturm 1996).

Most literature fails to distinguish between NI and AlkI, colloquially describing them as CI or NI, but at present it is apparent they play a similar role in Suc metabolism in the plant. For the sake of simplicity, both NI and AlkI activity will be discussed under the collective term CI.

Suc is stored in the vacuole (Avigad 1982) and during initial development is broken down into hexoses by SAI. The hexoses then move into the cytosol where they are converted to hexose phosphates by HXKs and enter the metabolic cycle. Thus the ability of a tissue to store appreciable amounts of Suc is determined by the levels of SAI. It has already been established that maturation of fruit is accompanied by a decrease in AI, thus hexoses needed to fuel metabolism must be derived from cytoplasmic sucrolysis. Furthermore, a general trend of CI activity that supports this supposition emerges in plants which accumulate Suc at maturity. In crops such as sugar cane (*Saccharum officinarum* L.)(Hatch and Glasziou 1963), sugar beet (*Beta vulgaris* L.) (Masuda *et al.* 1987; 1988), citrus (Kato and Kubota 1978), blueberry (Cano-Medrana and Darnell 1997), and

carrot (Ricardo and ap Rees 1970) CI was found to be low in immature fruit/roots and to increase with the accumulation of Suc. In carrot, CI has been found in all tissues, at all stages of development (Sturm *et al.* 1999), albeit at different levels both developmentally and spatially in the plant. Although significant, CI activity appears to be confined to mature storage organs (Masuda *et al.* 1987), and slow-growing tissues such as tuberous roots (Van den Ende and Van Laere 1995). CIs (especially AlkIs) are believed to be present in most, if not all, living cells in higher plants (ap Rees 1974; Avigad 1982). Therefore, although functions of CIs are largely unknown, they are presumed to play a role in channelling Suc into general metabolism (Stommel and Simon 1990; Chen and Black 1992; Godt and Roitsch 1997; Sturm and Tang 1999).

Expression of cytosolic yeast invertase in transgenic tobacco plants resulted in the accumulation of starch and soluble sugars in both sink and source leaves, and a change in growth pattern indicating more rapid cell expansion or division on the upper leaf surface (Sonnewald *et al.* 1991). Similarly, it is thought that CI activities are associated with pea pod elongation (Estruch and Beltrán 1991). Furthermore, in contrast to the other fruits described already, CI activity is substantial in immature muskmelon and declines in parallel with AI in developing fruit (Ranwala *et al.* 1991; Lingle and Dunlap 1987). Thus it is also possible that CI plays a role in providing some of the substrates needed for the control of tissue growth. In some plants, such as tomato, CI activity increases during the rapid growth phase (Balibrea *et al.* 1999), after sink establishment and prior to maturation. During this phase it is believed that the fruit are largely dependant on symplastic unloading of Suc, driven by cytoplasmic sucrolysis. Further evidence to support this opinion comes from research on the tomato by Balibrea and co-workers (1999). They found that when Suc accumulation in the cytoplasm is elevated as a consequence of salinity stress, cytoplasmic cleavage (both CI and SSy) of Suc is induced. It was thus proposed that CI plays a role in the control of assimilate import, dry matter accumulation and fruit growth.

CI therefore appears to play an essential role in controlling assimilate import in symplastically unloaded sinks and in the provision of substrates for metabolism. The universal distribution within the plant, unique biochemical properties and cytoplasmic location suggests that CI plays a role in the independent control of Suc metabolism, translocation and storage within the cell (Chen and Black 1992) and may have a more general, possibly growth related, function than simply channelling Suc into metabolism.

1.5.2 SUCROSE SYNTHASE

Sucrose synthase (SSy; UDP-D-glucose: D-fructose 2-α-glucosyltransferase; EC 2.4.1.13) is a glycosyl transferase that, in the presence of uridine diphosphate (UDP), converts Suc into UDP-Glu and Fru. This conversion of Suc and UDP into UDP-Glu and Fru is reversible, and SSy catalyses both reactions, although it is thought that enzyme activity preferentially occurs in the cleavage direction (Geigenberger and Stitt 1993; Heim *et al.* 1993). SSy is thought to be a key enzyme in Suc synthesis/metabolism, especially in non-photosynthetic tissues. Due to its UDP-transferase activity, SSy has an energetic advantage over AI, with respect to sucrolysis, as UDP-Glu is formed and thus does not require ATP-dependent phosphorylation before entry into metabolism. Hence only the Fru moiety needs to be phosphorylated. SSy is a homotetramer (Chourey *et al.* 1991; Ross and Davies 1992), located primarily in the cytoplasm. In some cases, however, SSy may associate with membranes as part of a membrane-bound biosynthetic enzyme complex (Ruan *et al.* 1997) at the Pm (Amor *et al.* 1995; Carlson and Chourey 1996) or actin cytoskeleton (Winter *et al.* 1998). SSy has been shown to function with other diphosphate nucleotides such as ADP or thymidine 5′-diphosphate (Nguyen-Quoc *et al.* 1990), but the V_{max} with UDP is four times higher than with ADP (Ross and Davies 1992). SSy is competitively inhibited in the cleavage direction by Fru (Ross and Davies 1992; Déjardin *et al.* 1997; Schaffer and Petreikov 1997b), and appears to be inhibited by Mg^{2+} (Morell and Copeland 1985).

SSy is believed to have the potential to mobilize Suc into a variety of diverse pathways that are important for structural, storage and metabolic functions in plant cells (Chen and Chourey 1989). SSy is a good indicator of sink strength (Claussen *et al.* 1985; Sung *et al.* 1989; Sun *et al.* 1992), and plays a central role in phloem loading and unloading, and the metabolic interplay of Suc, hexoses and starch (Smith 1999) in the sink. The derivatives of UDP-Glu generated by SSy are believed to play key roles in the biosynthesis of cell wall polysaccharides, amino acids, lipids etc., and in starch accumulation, root development (storage sink) and storage of sugars (Feingold and Avigad 1980; Sturm 1996).

SSy involvement with the supply and establishment of sink strength appears to be multi-faceted. Firstly, SSy has been found in high concentrations in the bundle sheath cell of C_4 plants (Bucke and Oliver 1975), in companion cells of the SE-CC complex (Nolte and Koch 1993; Kühn *et al.* 1997) and associated with vascular tissue (Martin *et al.* 1993; Fu and Park 1995), where it is believed to play a role in the catabolism of Suc to generate ATP for phloem loading (Lerchl *et al.* 1995). Studies done on detached eggplant (*Solanum melongena* L.) leaves showed that Suc content

and SSy in the leaves are closely related, and suggested that SSy may play a role in regulating Suc content in conducting tissue (Claussen *et al.* 1985). Thus it appears that SSy plays an important role in loading the phloem and hence in the creation of a Suc gradient between the phloem and the source. Similarly, in the sink, the cleavage of Suc by SSy will contribute to the maintenance of a Suc gradient into the sink. SSy activity has been observed to be highest in growing (sink) leaves of eggplant, cassava (*Manihot esculenta* Crantz), grape and sugar cane, and decrease with development, suggesting a close relationship exists between SSy and Suc import in young tissues (Claussen *et al.* 1985) and the determination of import capacity in young fruit (N'tchobo *et al.* 1999). In pea, SSy activity is in excess of the rate of Suc cleavage throughout development, whilst that of invertase is much lower, thus SSy is assumed to contribute the most to Suc cleavage (Edwards and ap Rees 1986). Initial cleavage of Suc in potatoes occurs largely via SSy, which is low in small tubers and increases during growth to a peak before the end of the growing season and then declines (Tsay and Kuo 1980). A similar trend is seen in tomato fruit, where SSy increases to a maximum 20-25 days after anthesis (when fruit is about 2 cm in diameter) and then decreases (Demnitz-King *et al.* 1997; Schaffer and Petreikov 1997a). Interestingly, in transgenic tomato plants expressing an antisense fragment of the fruit specific SSy RNA (*TOMSSF*), D'Aoust and co-workers (1999) found that Suc unloading was substantially reduced in 7 day old fruit, but not reduced in 23 day old fruit. SSy antisense fruit also had a slower initial growth rate. These observations led the authors to conclude that in tomato SSy only controls the capacity of very young fruit to metabolise Suc, and that AI must play an essential role in import of Suc into older fruit, especially once the vacuoles are sufficiently developed (Yelle *et al.* 1991). The SSy developmental profile in tomato was found to occur in parallel to that of sink strength (N'tchobo 1999), starch content (Wang *et al.* 1993; Schaffer and Petreikov 1997a), dry weight (Demnitz-King *et al.* 1997) and fresh weight (Stommel 1992). The decline in SSy activity in tomato is believed to occur once Suc import switches from symplastic to apoplastic (Patrick 1997). SSy is assumed to have particular importance in the determination of sink strength in tissues where the phloem is unloaded symplastically via the Pd (Sung *et al.* 1989; Zrenner *et al.* 1995). This is because the influx of Suc into the tissue depends directly on the Suc content within the cytoplasm, and the removal of cytoplasmic Suc is vital for the maintenance of a concentration gradient between the cells and the phloem. Suc in the symplasm can be reduced in two ways. Firstly, it can be transported into the vacuoles against a Suc gradient in an energy-requiring, carrier-mediated manner (Getz 1991). Secondly, Suc can be cleaved by cytoplasmic SSy or CI. The activity of SSy is not limited to the mediation of Suc cleavage and sink strength in developing tissues, but plays a role, via its Suc synthesis ability, in the storage of Suc. The importance of cytoplasmic resynthesis of Suc can be envisaged in mature fruit that are importing sugars apoplastically. These sugars enter

the cytosol as hexoses, but need to be converted to Suc prior to accumulation within the vacuole. In maturing tissues, such as sugar beet (Giaquinta 1977), melon (Lingle and Dunlap 1987), peach and strawberry (Hubbard *et al.* 1991) SSy activity has been seen to accompany Suc storage. Similarly, SSy has also been shown to contribute to Suc synthesis in heterotrophic *Chenopodium rubrum* cell-suspension cultures, developing potato tubers, and germinating castor bean (*Ricinus communis*) cotyledons (Geigenberger and Stitt 1993). The effect of this resynthesis of Suc and compartmentation within the vacuole is important as it maintains a hexose gradient between the apoplast and the symplast, a concentration gradient that is subsequently transposed to that of Suc between the phloem and apoplast, helping to ensure continued influx of sugars into the sink whilst still permitting Suc accumulation.

Starch is the most abundant and important reserve polysaccharide in plants. The incorporation of ADP-Glu, by a series of enzymatic steps involving the enzymes ADP-Glu pyrophosphorylase (EC 2.7.7.27), starch synthase (EC 2.4.1.21) and the branching enzyme, *Q* enzyme (EC 2.4.1.21), is believed to be the primary route of starch synthesis in plants (Preiss 1988). Before entry into the starch biosynthetic pathway, Glu needs to be phosphorylated by the enzyme phosphorylase (EC 2.4.1.1), and subsequently converted into Glu-1-phosphate by UDP-Glu pyrophosphorylase (EC 2.7.7.9) which is converted to ADP-Glu and incorporated into starch. It is thought that SSy plays an important role in the partitioning of UDP-Glu into starch (and/or cellulose) biosynthesis. The distribution of SSy mRNA, revealed by *in situ* hybridisation, closely paralleled starch distribution in young fruit (Wang *et al.* 1994). Similarly, in tomato, a reduction in SSy due to a brief heat treatment, was accompanied by a reduction of [^{14}C]-labelled Suc incorporation into starch (Wang *et al.* 1993). SSy gene expression was shown to be positively correlated with starch accumulation in tomato fruit (Wang *et al.* 1993; 1994), maize endosperm (Chen and Chourey 1989; Heinlein and Starlinger 1989), banana (do Nascimento *et al.* 2000), and faba bean cotyledons (Heim *et al.* 1993). Similarly, in potato (Zrenner *et al.* 1995) and maize (Chourey and Nelson 1976; Cheng *et al.* 1996) a reduction in SSy activity has been associated with a reduction in starch content and dry weight. Starch accumulation in fruit often follows a distinct trend. In young fruit, starch accumulates and is broken down during growth and maturation to provide Suc. In seeds, tubers and some fruits, starch accumulation persists throughout development or a second peak is often observed prior to maturation, during which starch is synthesised and stored. Examples of such tissues include banana (*Musa paradisiaca*) and potato (Ross *et al.* 1994; Cordenunsi and Lajolo 1995), and it is apparent in these tissues that SSy levels follow a similar developmental pattern. However, using antisense inhibition of SSy it has been shown that a reduction in SSy of 98-99% in young tomato fruit (Chengappa *et al.* 1999; D'Aoust *et al.* 1999) and up to 80% in potato tubers

(Zrenner *et al.* 1995) did not significantly affect starch accumulation. Similarly, the absence of SS1 (one of the two SSy isozymes in maize) did not affect starch levels in maize (Chourey and Nelson 1976). Interestingly N'tchobo and co-workers (1999) found, using [^{3}H]-labelled uptake experiments, that up to 25% of imported Suc is incorporated into starch independently of SSy. Thus it is possible that AI may contribute significantly to starch synthesis, especially when SSy is present at low levels, or absent. In spite of this, not only may SSy play a role in providing substrates for starch biosynthesis, but it may also contribute to the regulation of starch levels, firstly by inducing starch degradation and, secondly, by partitioning Suc into starch synthesis. A reduction in SSy may lead to a demand for hexose phosphates in the cytosol and hence stimulate starch degradation. Fru levels in the cytoplasm are dictated by a combination of invertase, SSy and fructokinase (FK; EC 2.7.1.4). FK is responsible for the phosphorylation of Fru (Renz *et al.* 1993). However, all these enzymes are inhibited by Fru, FK exhibiting both substrate inhibition by Fru (Schaffer and Petreikov 1997b) and product inhibition by Fru-6-phosphate (Renz and Stitt 1993). Schaffer and Petreikov (1997b) observed that in young tomato fruit Fru levels are sufficient to inhibit both SSy and FK, and they proposed that this SSy-FK regulation of Suc metabolism might play a potential role in the control of the conversion of Suc to starch.

The association of SSy with cell membranes (Ruan *et al.* 1997) is believed to be responsible for providing substrates for the biosynthesis of cell wall polysaccharides, such as cellulose and callose (Amor *et al.* 1995). In cotton (*Gossypium hirsutum L.*) seed SSy appears to play no role in starch synthesis, but rather contributes to the synthesis of the cellulose for the fibres (Ruan *et al.* 1997). SSy localisation and [^{14}C] incorporation studies in cotton showed that SSy is localised to the cotton fibre and its products are rapidly incorporated into cellulose, suggesting SSy is important in controlling cellulose biosynthesis. SSy may also supply the nucleotide sugars for cellulose biosynthesis in rapidly expanding fruit. In addition, maize mutants deficient in SSy showed early cell degeneration characterised by a brittle cell wall (Chen and Chourey 1989).

In monocotyledons, such as maize (Chourey *et al.* 1998), and dicotyledons, such as tomato (Chengappa *et al.* 1998), potato (Fu and Park 1995) and *Arabidopsis* (Martin *et al.* 1993), there appear to be at least two differentially expressed SSy genes. These SSy isoforms usually have highly homologous amino acid sequences and similar biochemical properties (Sturm and Tang 1999), and are believed to play a role in partitioning UDP-Glu into different metabolic pools. Chourey *et al.* (1998) proposed an isoform-specific role for the two SSy enzymes found in maize. The first, coded for by the *Sh*1 gene, was responsible for the production of precursors for cellulose biosynthesis, and the second, coded for by the *Sus*1 gene, provided the substrates for starch

biosynthesis. In *Arabidopsis*, Déjardin and co-workers (1999) found the SSy-encoding genes (*Sus1* and *Sus2*) to be profoundly and differentially regulated in leaves exposed to environmental stresses (cold, drought, O_2 deficiency). *Sus1* was induced by cold and drought, and was associated with a decrease in leaf osmotic potential and an increase in Suc accumulation. *Sus2* was induced by O_2 deficiency and was associated with nearly complete depletion in endogenous sugars. The differential expression of these genes under different circumstances suggests that the SSy genes may be independently regulated by different signal transduction mechanisms and play an important role in plant developmental programmes and plant responses to environmental stimuli.

Thus it is apparent that SSy is expressed temporally and spatially in developing fruit, playing an important role in the maintenance of sink strength in symplastic unloading in fruit, the production of precursors for both starch synthesis and cell wall polysaccharide synthesis, and the partitioning of Suc into various metabolic pools within the sink. All these would clearly impact significantly on fruit growth. These functions occur concurrently with, and are affected by, invertase activity and thus the relative importance of SSy and invertase cleavage of Suc would be dependant on a complex inter-relationship between fruit age, plant species and environmental stimuli.

1.6 SUCROSE RESYNTHESIS

Suc import into the sink and subsequent compartmentation, partitioning and metabolism in the sink, is strongly affected by its resynthesis. In heterotrophic sink tissue this is achieved by the action of SSy (in the synthesis direction) or SPS (Geigenberger and Stitt 1993). The actions and implications of Suc resynthesis have already been discussed (Section 1.5.2).

SPS (UDP-glucose: D-fructose-6-phosphate α-D-glucosyl transferase; EC 2.4.1.14) catalyses the reversible synthesis of Suc-phosphate from Fru-6-phosphate and UDP-Glu. This transglycosylation reaction is regulated by phosphorylation, and SPS activity is thus both activated, and inhibited, by distinct phosphorylation reactions (Huber and Huber 1996; Toroser and Huber 1997; MacKintosh 1998). SPS activity is also believed to be elevated by Glu-6-phosphate (Trethewey *et al.* 1999). SPS is believed to form a multi-enzyme complex with SPP, leading to the creation of a metabolite channel. A metabolite channel allows for the reaction product of one enzyme (SPS) to be immediately transferred to the next enzyme in the reaction sequence (SPP) without completely equilibrating with the cytoplasmic contents (Ovardi 1991). This permits the rapid channelling of intermediates and micro-compartmentation of metabolites within the cell. SPP catalyses the conversion of Suc-6-phosphate to Suc, increases the maximal

catalytic activity of SPS (Echeverria *et al.* 1997b) and partially relieves the inhibitory effects of phosphates (Salerno *et al.* 1996) on SPS activity. The rapid removal of Suc-6-phosphate by SPP keeps the cytosolic levels of Suc-phosphate low and hence effectively renders the SPS reaction irreversible (Huber and Huber 1996). Both enzymes have been localised to the cytosol of photosynthetic and storage cells (Winter *et al.* 1982; Echeverria 1995).

The physiological importance of SPS may lie in the regulation of carbon partitioning (MacKintosh 1998). In blueberry (Cano-Medrano and Darnell 1997), muskmelon and buttercup squash (Lingle and Dunlap, 1987; Hubbard *et al.* 1989; 1991), banana (Hubbard *et al.* 1990), tomato (Dali *et al.* 1992) peach, strawberry, kiwi and mango (Hubbard *et al.* 1991), SPS activity appears to increase coincidently with Suc accumulation and ripening (Miron and Schaffer 1991; Dali *et al.* 1992). The significance of this elevated activity is that it allows for continued apoplastic import of sugars simultaneously with Suc accumulation and permits UDP-Glu from stored starch to be reincorporated into the Suc pool. The amount of Suc unloaded into tomato fruits significantly increased (70%) in plants displaying SPS over-expression (Nguyen-Quoc *et al.* 1999), demonstrating that SPS over-expression increases sink strength. It is understood that Suc storage is usually a consequence of Suc hydrolysis in the apoplast by IAI and subsequent resynthesis by SPS in the cytosol. This Suc is then transported to, and stored in, the vacuole. Hence SPS activity will play an important role in determining the rate of Suc accumulation and maintaining the hexose gradient between the apoplast and cytosol (Miron and Schaffer 1991). However, under normal circumstances, the ability of SPS to contribute to Suc accumulation will depend on the relative activities of SAS, IAS and SSy (cleavage direction), i.e. a balance of Suc synthesis and Suc cleavage (Zhu *et al.* 1997), and the creation of symplastic isolation from neighbouring cell systems of lower Suc concentration. In cases where there is an imbalance (or rather a balance of these enzymes at the incorrect stage of development), Suc may undergo futile degradation-resynthesis cycling in the sink (ap Rees and Hill 1994; Geigenberger and Stitt 1993). It has been proposed that this seemingly futile cycling of Suc by SPS (and SSy) may act as a fine control mechanism in the uptake of Suc (Herbers and Sonnewald 1998).

1.7 OVERVIEW

Suc transport and metabolism in the sink is briefly summarised in Figure 1.2. Suc flows to the fruit in the phloem primarily by bulk flow, driven in part by the Suc gradient between the source and the sink created by a combination of carbon metabolism, compartmentation and partitioning. At the sink the phloem is unloaded either symplastically, via the Pd, or apoplastically. The apoplastic

unloading of the phloem involves a combination of passive leaking/diffusion into the extracellular space, the action of turgor sensitive and insensitive porters located at the Pm, such as NADH redox and H+-ATPases, and the possible role of proteins inducing pore formation in the Pm. In the SE adjacent tissues, which may consist of CC, transfer cells or sink parenchyma, movement of Suc in the symplast is by diffusion, facilitated by cyclosis, and is driven by the constant flux of Suc out of the 'system' and hence the creation/maintenance of a concentration gradient in the sink tissues. This flux of Suc is achieved by the separation of the carbon pool into apoplast and symplast, mono- and disaccharides, and other metabolic pools. This is achieved by the combined action of the enzymes IAI, SAI, NI, AlkI, SSy (in both the synthesis and cleavage directions), SPS, SPP,

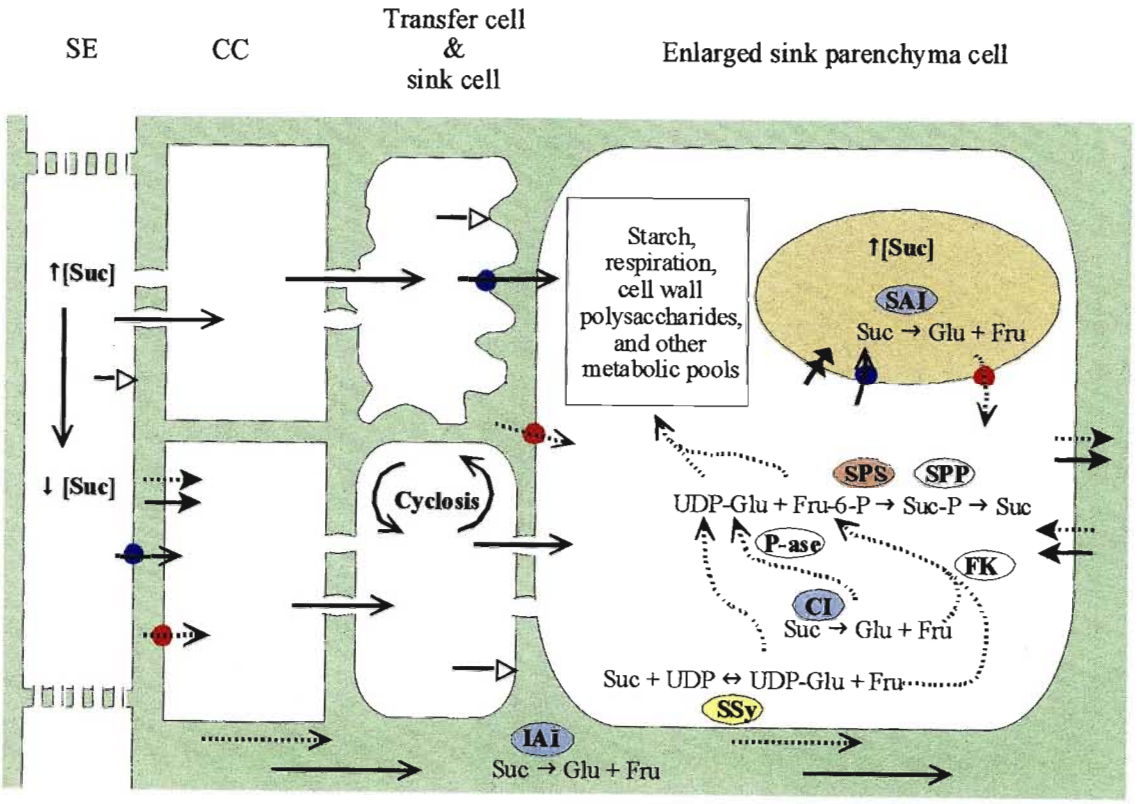


Figure 1.2 Carbohydrate movement within the sink tissue. Suc flux is represented by a solid arrow (→), hexose movement by a broken arrow (⇢), general flux direction represented by 'empty' arrowheads (→). Suc may cross the Pm by Suc/H^+ symporters or other turgor in/sensitive porters (●), via pores in the Pm produced by pore forming proteins (—▷), diffusion (—➤), or facilitated diffusion (—⏩). Hexoses may enter (and leave) the symplast by Pm bound hexose/proton symporters (●), or diffusion. Diffusion within cells is facilitated by cyclosis (↻). The light green area represents the apoplast, the vacuole appears light brown, the symplast white, and the black lines represent a membrane system that delimits the symplast. P = phosphate; P-ase = phosphorylase.

HXK and enzymes involved in the phosphorylation and subsequent utilization of Glu, UDP-Glu and Fru-6-phosphate.

The primary control of Suc levels, however, can be directly related to the activity of the invertases, SSy and SPS. The differential, spatial and temporal expression of these enzymes thus plays an important role in the control of sink strength, sugar composition and the supply of substrates needed for growth and respiration in all heterotrophic sink tissues. This intrinsic association of these enzymes in many developmental processes in the sink makes them a prime target for hormonal and sugar-mediated regulation, and thus in signal perception, parent plant-sink relations and stress responses. In most developing sink tissues, the expression of these three enzymes appears to a follow a similar trend. Initial rapid fruit growth is characterised by high levels of IAI, which declines rapidly with sugar accumulation. Young developing fruits show high levels of both SAI and SSy (in the cleavage direction). At the onset of maturation, there is a switch from symplastic to apoplastic unloading and IAI, SAI, and SSy all decrease in comparison to their earlier levels. CI is expressed throughout development, but is especially prevalent in symplastically unloaded sink tissues. The relative levels of these enzymes varies with maturation depending on whether the tissue accumulates Suc, hexoses or starch at ripening. In Suc accumulating fruit IAI, SSy (in the synthesis direction), CI and SPS (and SPP) are all high. In hexose accumulating fruit SAI is high, whilst in starch accumulating tissues SSy (in the cleavage direction) and CI are high. These trends indicate the importance of IAI in the creation of sink strength and the continued import of sugars in situations of apoplastic isolation, SAI in the control of sugar composition, CI in the provision of hexoses for metabolism, SSy in the maintenance of sink strength, the provision of nucleotide sugars for further carbon partitioning and in the control of cytoplasmic sugar composition, SPS (together with SPP) in the control of sugar composition and Fru levels, and all of the enzymes in the united contribution to the maintenance of sink strength and fruit growth.

1.8 EPILOGUE ~ CARBOHYDRATE METABOLISM AND FRUIT GROWTH IN DEVELOPING 'HASS' AVOCADO FRUIT

Growth of avocado fruit follows a single sigmoidal curve (Schroeder 1958, Valmayor 1967), consisting of a ±10 week lag phase (phase I), a ±30 week linear growth phase (phase II), and a maturation phase (phase III), the duration of which is cultivar- and environment-dependant. In avocado fruit development, cell division is not restricted to phase I but continues in the mesocarp throughout fruit development (Schroeder 1953, 1958), although at a reduced rate near maturity.

The avocado fruit stores lipids and starch in the mesocarp and seed respectively at maturity. The lipids are stored in oleosomes that take up much of the space in mature avocado mesocarp cells (Platt-Aloia and Thompson 1980). Glu-1-phosphate, derived from Suc, is believed to be the precursor to fatty acid synthesis (Stumpf 1980), whilst glycerol-3-phosphate (G3P), the precursor for triacyglycerol synthesis (Gurr 1980), is thought to arise as an intermediate of the glycolytic pathway, indicating that Suc is the primary source of carbon in lipid biosynthesis. Thus both starch and lipids in avocado may be products of Suc metabolism, and both are compartmentalised (and simultaneously partitioned out of the soluble sugar pool) at maturation. These observations suggest that Suc metabolism in avocado fruit may follow trends similar to those observed in other plants, involving the cleavage of Suc during early rapid fruit growth, and during the extended period of reduced growth. Furthermore, the hydrolysis and compartmentation of Suc and hexoses by invertase, SSy and SPS, and the interaction of these carbohydrates with plant growth regulators, are likely to dictate the timing and occurrence of phenomena such as phloem unloading, resource allocation and utilisation, cell division and enlargement, and ultimately fruit growth.

Much research has been done on avocado fruit development (for more detailed discussion see: Biale and Young 1971; Ahmed and Barmore 1980; Seymour and Tucker 1993) and the principal morphological/anatomical changes, patterns of lipid and sugar accumulation, and the cycling of carbohydrates between the shoots and trunk during fruit growth and maturation are well-documented (Schroeder 1958; Valmayor 1967; Platt-Aloia and Thompson 1980; Shaw *et al.* 1980 Platt-Aloia and Thompson 1981; Platt-Aloia *et al.* 1983; Scholefield *et al.* 1985; Finazzo and Davenport 1987; Zilkah and Klein 1987; Wolstenholme *et al.* 1990; Whiley 1994; Whiley *et al.* 1991; Kaiser 1993; Steyn *et al.* 1993; Whiley *et al.* 1996; Liu *et al.* 1999a; 1999b). However, there is currently very little available information on solute transport and metabolism in the avocado, and how these two processes impact on fruit growth.

The marked preference for larger fruit by consumers has made fruit size an important quality parameter in avocado production. Hence, fruit size has become as important as yield in determining the profitability of growing avocados. Intrinsically, organ size is determined by cell number and thus the control of cell proliferation during organogenesis will play an important role in the determination of fruit size (Mizukami and Fischer 2000). In avocado, fruit size has been shown to be limited by cell number (Schroeder 1953; Cowan *et al.* 1997a). Thus the maintenance of meristematic competence is essential to continued fruit growth. The final size of avocado fruit is, therefore, determined by the rate of cell division (especially in the mesocarp), and hence the control of fruit size requires the maximization of cell division and expansion during the

developmental programme (Coombe 1976). Cell number and size will similarly influence the capacity of the fruit to act as a sink and so contribute to assimilate import (Bohner and Bangerth 1988). The availability, supply and composition of photoassimilate, plant growth substances, minerals and water play a crucial role in fruit growth. Of these factors it has long been understood that plant hormones, and more recently, sugars exert multiple controls on fruit growth by directly and/or indirectly altering gene expression and co-ordinating/synchronizing many developmental programmes and physiological processes. Cowan and co-workers (Cowan *et al.* 1997a; Moore-Gordon *et al.* 1998; Campbell *et al.* 2000; Richings *et al.* 2000; Cowan *et al.* 2001) have proposed a model for the metabolic control of 'Hass' fruit size (Figure 1.3).

In this model, cell proliferation is modulated by the CK-ABA ratio and directly linked to the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; EC 1.1.1.34), the enzyme responsible for the irreversible conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonic acid (MVA), which is considered to be the committed step in isoprenoid, and hence ABA and CK, biosynthesis. In this model it is proposed that sugar content and composition in sink tissue impacts on the expression of sugar-metabolising enzymes, HMGR and molybdenum cofactor (MoCo)-containing enzymes, affecting sugar metabolism, hormone homeostasis and subsequently fruit growth. MoCo is required by both AO and XDH for the catalysis of the final steps of ABA, CK and IAA biosynthesis. This interaction between endogenous sugars and enzyme activity is mediated by the sugar sensing systems Suc non-fermenting-1-related protein kinase (SnRK1) and HXK. Whilst this model highlights the importance of sugar content and composition in 'Hass' avocado fruit growth, it also necessitates the need for a more detailed understanding of solute transport and metabolism in the fruit.

The activities of invertase, SSy and SPS represent the first step in the metabolism of Suc, and are strongly correlated with sink function, solute import, cell division and carbon storage. Indeed, a change in activity of these enzymes can thus be expected to impact on the sugar content and composition in fruit. Changes in sugar content and composition, especially hexoses (particularly Glu) will induce corresponding changes in feedback mechanisms, signal response coupling and gene expression. All of these will, in turn, affect subsequent physiological processes, including solute transport and metabolism, the provision of basic structural molecules, plant growth substance synthesis, catabolism and sensitivity, Pd formation, integrity and conductivity, cell cycle activity, and ultimately fruit growth. Thus it is necessary to examine the factors affecting solute allocation in 'Hass' fruit. For these reasons and the lack of information pertaining to sugar

metabolism on a whole in the avocado fruit, the activities of invertase, SSy and SPS, and the partitioning of carbon to various metabolic pools were investigated in 'Hass' avocado.

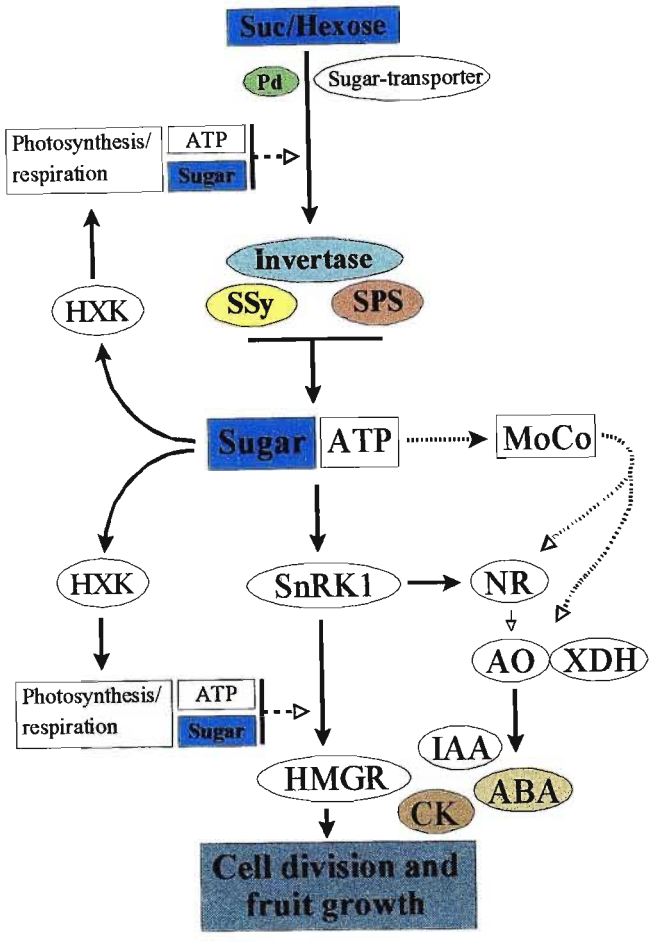


Figure 1.3 Hypothetical scheme illustrating the relationship between sugar sensing and the activity of invertase, SSy, SPS, HMGR and hormones in sink cells of developing avocado fruit. Alterations in sugar metabolism, content and composition, coupled with changes in adenylate status, impact on SnRK1 and/or HXK activity, and MoCo biosynthesis, so altering isoprenoid metabolism and ensuring sugar/hormone homeostasis, cell division, sink strength and fruit growth are maintained. Redrawn from Cowan *et al.* (2001). AO = aldehyde oxidase (EC 1.2.3.1), the enzyme responsible for catalysing the final steps in ABA and IAA synthesis, oxidising abscisic-aldehyde and indole-acetaldehyde to ABA and IAA respectively; NR = nitrate reductase (EC 1.6.6.1), an enzyme essential for the assimilation of nitrogen into organic nitrogen compounds including amino acids, reducing nitrate to nitrite; XDH = xanthine dehydrogenase (EC 1.2.1.37), a key enzyme in the oxidative catabolism of purines that plays an important role in CK metabolism.

1.9 OBJECTIVES

The objectives of this research are:

1. To examine the potential routes of solute transport within 'Hass' avocado fruit.
2. To investigate Suc metabolism in the 'Hass' avocado fruit by examining aspects of its primary cleavage and resynthesis by the enzymes invertase, SSy and SPS.
3. To explore the partitioning of carbon to metabolic pools that may be associated with the inhibition of solute transport and with carbon storage, namely starch, lipids and polyphenolics.
4. To determine how the above three facets of solute transport and metabolism are affected by ABA and how, together with ABA, they affect fruit growth and ultimately final fruit size in 'Hass' avocado fruit.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS

2.1.1 RADIOCHEMICALS

D-[1-^{14}C]-Glucose (55.0 mCi mmol^{-1}), [U-^{14}C]-Sucrose (626 mCi mmol^{-1}) and uridine diphosphate-D-[U-^{14}C]-Glucose (296 mCi mmol^{-1}) were purchased from Amersham International plc, Buckinghamshire, England.

2.1.2 GROWTH REGULATORS

(±)-2-*cis*-4-*trans*-abscisic acid (ABA) and 6(γ,γ-dimethylallyl-amino)-purine (isopentenyl adenine; iP) were purchased from Sigma, St. Louis, U.S.A.

2.1.3 COLORIMETRIC REAGENTS, STAINS, FIXATIVES AND RESINS

Aniline Blue (water soluble) was purchased either from Edward Gurr Ltd, London, England or Merck, Darmstadt, Germany. Basic fuchsin, bromophenol blue, Congo red, iodine, oil of cloves, potassium iodide and Ruthenium red were purchased from the British Drug Houses Ltd (BDH), Poole, England. Anthrone, brilliant blue G (Serva blue G dye), brilliant blue R (Coomassie blue R-250), methyl cellosolve (ethylene glycol monomethyl ether) and phosphotungstic acid were purchased from Sigma, St. Louis, U.S.A. Formaldehyde (37% formalin solution), nitroblue tetrazolium and phenazine methosulphate were purchased from Sigma-Aldrich Chemie, Steinheim, Germany. Amido black, Canada balsam, Folin-Ciocalteu's phenol reagent, hexamethylenetetramine, phenol and tertiary butanol were purchased from Merck, Darmstadt, Germany. Resorcinol blue, safranin and Sudan IV was purchased from Edward Gurr Ltd., London, England. Fast green was purchased from Hopkin and Williams, Essex, England. Ammonium oxalate was purchased from Albright and Wilson Ltd., London, England. Crystal violet and paraffin wax (57-60°C) was purchased from SAARChem, Krugersdorp, South Africa. Osmium tetroxide was purchased from Electron Microscopy Sciences, Washington, U.S.A. Diglycidyl ether of polypropylene glycol (DER 736), vinyl cyclohexene dioxide (ERL 4206), glutaraldehyde (25%

EM grade), nonenyl succinic anhydride (NSA), dimethylaminoethanol (S-1 DMAE), 1,2-epoxypropane (propylene oxide) and dodecyl succinic anhydride (DDSA) were purchased from Agar Scientific, Stansted, U.K. LR White medium grade acrylic resin was purchased from London Resin Co. Ltd., Reading, England. Paraformaldehyde EM, epoxide equiv. Formvar, TAAB 812 resin (Epon) and 2,4,6-tri(dimethylaminomethyl) phenol (DMP-30) were purchased from TAAB, Berkshire, England. Araldite resin (grade 502, CY212) was purchased from Polyscience Inc., Warrington, England.

2.1.4 ELECTROPHORETIC REAGENTS

Agarose was purchased from Associated Chemical Enterprises, Reuven, South Africa. Acrylamide was purchased from Boehringer Mannheim, Indianopolis, U.S.A. Premixed protein molecular weight markers, low range (14 – 97 kD) and sodium dodecyl sulfate (SDS) were purchased from Boehringer Mannheim, Germany. Ammonium persulfate was purchased from Biosolve B.V., Valkensuraard, Netherlands. NN[1]-methylenebisacrylamide (*bis*-acrylamide) was purchased from ICN, Aurora, U.S.A. 2-Mercaptoethanol and silver nitrate were purchased from BDH, Poole, England. N-tris[hydroxymethyl]-methylgylcine (tricine) and N,N,N',N'-tetramethylethylene-diamine (TEMED) were purchased from Sigma, St. Louis, U.S.A.

2.1.5 ENZYMES

Cellulase (*Trichoderma viride*) was purchased from Boehringer Mannheim, W. Germany. Hemicellulase (*Aspergillus niger*) was purchased from Sigma Chemical Co., St. Louis, U.S.A. Pectinase (*Aspergillus niger*) was purchased from Sigma-Aldrich Chemie, Steinheim, Germany.

2.1.6 CARBOHYDRATES, PHOSPHORYLATED SUGARS AND NUCLEOSIDE PHOSPHATES

D(-)-Fru, glycerol, D(-)-mannitol, and Suc were purchased from Associated Chemical Enterprises, Reuven, South Africa. Fru-6-phosphate, Glu-6-phosphate uridine diphosphate (UDP) and UDP-Glu were purchased from Boehringer Mannheim, Germany. D(+)-Glu was purchased from BDH Chemicals Ltd, Poole, England. Pachyman (*Poria cocus*) was purchased from Calbiochem, La Jolla, U.S.A. Perseitol (from avocado), sedoheptulose, D-*manno*-heptulose, galactose, α-lactose, β-D(+)-Glu and soluble potato starch were purchased from Sigma, St. Louis, U.S.A. L(+)-Arabinose, cellobiose, *meso*-erythritol, *meso*-inositol, maltose, D-mannose, raffinose, D(+)-rhamnose, D(-)-ribose, 2-deoxy-D-ribose, D(-)-sorbitol, L(-)-sorbose, trehalose and D(+)-xylose were purchased

from Merck, Darmstadt, Germany. Gas liquid chromatography (GLC) fatty acid model reference standards were purchased from Nu-Chek Prep. Inc., Elysian, U.S.A.

2.1.7 GENERAL CHEMICALS

ε-Amino-n-caproic acid, acetic anhydride, benzamidine, bovine serum albumin (BSA), Dextran T-500 (*Leuconostoc mesenteroides*), dimethyl sulfoxide (DMSO), 3,4,5-trihydroxybenzoic acid (gallic acid), polyethylene glycol (PEG 3350), pyridine, Sephadex-G25 and tetramethylammonium hydroxide (TMAH) were purchased from Sigma, St. Louis, U.S.A. Cellobiose and Extran MA03 were purchased from Merck, Darmstadt, Germany. Complete™ protease inhibitor cocktail tablets and 1,4-dithiothreitol (DTT) were purchased from Boehringer Mannheim, Germany. Ethylene diamine tetra-acetic acid disodium salt (EDTA) was purchased from UnivAR®, SAARChem, Krugersdorp, R.S.A. Complete™ EDTA-free protease inhibitor cocktail tablets were purchased from Roche, Mannheim, Germany. Digitonin was purchased from Fluka BioChemika, Switzerland. Acetic anhydride, polyvinylpirolidone (insoluble PVP, Polyclar SB100), pyridine, Tween® 20 and Triton X-100 were purchased from BDH, Poole, England. G127 manual X-Ray developer and G334c manual X-Ray fixer were purchased from Agfa. JIK® (3.5% (w/v) sodium hypochlorite) was purchased from Rickitt and Colman, Elandsfontein, South Africa. STOX™ (Oxime-internal standard reagent) and Tri-Sil® Z were purchased from Pierce, Rockford, U.S.A.

2.1.8 SOLVENTS

Acetone was purchased from NT Laboratories, Johannesburg, South Africa. Ethanol, hexane, methanol and petroleum ether, all of analytical grade, were purchased from either Associated Chemical Enterprises, Glenvista, South Africa or BDH, Poole, England. Diethyl ether, propanol, toluene and xylene were purchased from BDH, Poole, England. HPLC grade acetonitrile, chloroform, methanol and propanol was purchased from Burdick and Jackson, Muskegon, U.S.A.

2.1.9 SCINTILLANTS

2,5-diphenyloxazole (PPO) was purchased from Packard, Groningen, Netherlands. Pico-Fluor™ 40 was purchased from Packard Instrument Co., Meridon, U.S.A. Ultima Gold™ was purchased from Packard Bioscience B.V., Groningen, The Netherlands.

2.1.10 BUFFERS

4-(2-Hydroxyethyl)-1-piperazine ethane sulfonic acid (Hepes), 4-morpholine ethane sulfonic acid (Mes), 4-morpholine propane sulfonic acid (Mops) and 2-amino-2-(hydroxymethyl)-1,3-propandiol (Tris) were purchased from Boehringer Mannheim, Germany. Sodium cacodylate was purchased from Agar Aids, Stansted, U.K. Sodium Acetate was purchased from May and Baker Ltd., Dagenham, England. N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid (TES) was purchased from Sigma, Steinheim, Germany.

2.2 CHROMATOGRAPHIC MEDIA

A Rezex 8μ 8% Ca monosaccharide high performance liquid chromatography (HPLC) column (300×7.80 mm DIM) for the separation of sugars was purchased from Phenomenex®, Torrance, U.S.A. Solid phase extraction cartridges (C_{18}, 6 mL volume) were purchased from Sorbent, Mid-Glamorgan, U.K. or Waters Chromatography Division, Millipore Corp., Milford, U.S.A. A fused silica capillary SP-2330 liquid gas liquid chromatography (GLC) column (30 m, 0.25 mm ID, 0.2 μm film thickness) for the separation of fatty acids was purchased from SupelCo. Inc., Bellefonte, U.S.A. An OV-17 glass gas chromatography (GC) column for the separation of sugars was purchased from Langet Laboratories, Durban, South Africa.

2.3 STUDY SITE

Fruit was collected from two study sites, Everdon Estates and Bounty Farm.

During the period 1997 - 1999 fruit were treated and collected from the experimental orchard on Everdon Estates in the KwaZulu-Natal midlands. This location (30°16'E and 29°28'S) has a mean summer temperature of 23°C and winter temperature of 11°C, mean annual precipitation of 1030 mm, is at about 1060 m above sea level[1], with a summer rainfall pattern and is situated in Phillips' Bioclimatic region 3 (Phillips 1973), which is characterised by cool mesic conditions. Orchards were irrigated by micro-jet scheduled to tensiometer readings (minimum -40 kPa).

[1]Climatic data supplied by the Computing Centre for Water Research, University of Natal, Pietermaritzburg, South Africa.

During the 1999/2000 season the study site was at Bounty Farm, Winterskloof in the KwaZulu-Natal midlands. This location (30°17'E and 29°34'S) has a mean summer temperature of 23°C and winter temperature of 10°C, precipitation of 1047 mm, is at about 1181 m above sea level[1], with a summer rainfall pattern and is also situated in Phillips Bioclimatic region 3. Orchards were cultivated dry land, relying on rainfall for irrigation.

Both sites are categorized as cool subtropical regions (annual heat index of 2500 to 3500) and are believed to achieve the optimum climatic requirements for avocado (Guatemalan) cultivation (Wolstenholme 1977).

2.4 PLANT MATERIAL

In both orchards, fruit was randomly selected from a minimum of four different 'Hass' trees on 'Duke 7' rootstocks. The trees in Everdon Estate were nine years old at the commencement of the trial (1997), while those at Bounty Farm were twelve years old at the time of the study.

In 1997 and 1998, 20 fruit were treated *in vivo* with either ABA, iP or a combination of these two plant growth regulators. This was done by injecting 20 μg of the plant growth regulator in 20 μL Tween® 20:acetone:water (1:1:8, v/v) into the pedicel of the fruit whilst attached to the tree, during the stage of rapid (linear) growth (Cowan *et al.* 1997a), approximately 163 and 87 days after full bloom (DAFB; based on the average date when maximum anthesis occurred) for 1997 and 1998 respectively. This was done using a 5μL 22° point Hamilton syringe 7105 KH (Hamilton Company, Reno, U.S.A.) and the injection site was sealed with silicone grease. Fruit were harvested 62 days and 120 days after treatment (DAT) for the 1997 and 1998 seasons respectively. 40 untreated small- and 40 normal-fruit phenotypes were also harvested.

In 2000, 60 fruit were treated *in vivo* with ABA. This was done by injecting 30 μg of ABA in 20 μL 7.5% (v/v) DMSO through the base and into the testal region of the seed coat, approximately 66 days after full bloom. This was done for three reasons: firstly, to overcome the difficulty associated with inserting a needle into the secondary thickened vasculature of the pedicel; secondly, based on the first experiments in 1997/8, it appeared it was questionable if sufficient compound entered the fruit when they were injected through the pedicel; thirdly, the initiation and occurrence of small fruit was believed to take place earlier during fruit development, and thus it was hoped that treatment of younger fruit with ABA would initiate a more pronounced response. Treated fruit were harvested 40 DAT. 20 untreated small- and 20 normal-fruit were harvested 66,

92, 106 and 120 DAFB.

2.5 MEASUREMENT OF FRUIT GROWTH

All fruit were tagged, including untreated small and normal fruit. Fruit length was measured, using digital callipers (Mitutoyo-500, Tokyo, Japan), at regular intervals until the fruit was harvested. In the 2000 season, fruit length and width, seed length and width, seed coat thickness at the pedicel end and fruit fresh and dry mass was recorded at each harvest interval and data was presented as a percentage change relative to initial value.

2.6 MEASUREMENT OF RESPIRATION RATES

In 1998, small- and normal-fruit, still attached to approximately 20 cm of branch, were collected and immediately transported to the laboratory for analysis. The fruit were placed in glass jars, flushed with ambient air, incubated in a water bath at 25°C and gaseous samples taken every 15 sec. CO_2 evolution was measured by infrared gas analysis (Series 225 Gas Analyser, The Analytical Development Co. Ltd., Hoddesdon, England), and quantified against a CO_2 standard of 320 ppm CO_2 in nitrogen (Afrox, South Africa).

In 2000, fruit were harvested as described above. The fruit were then placed in glass flasks, flushed with ambient air, and maintained at room temperature. Evolution of CO_2 was measured at 5 min intervals using an EGM-1 Environmental Gas Monitor (PP Systems, Stotford, United Kingdom).

Photosynthetic rates were not determined and therefore net CO_2 production was taken as the respiration rate.

2.7 [^{14}C] TRANSPORT STUDIES

Fruit (treated and untreated) with long pedicels and a 15-20 cm portion of branch were collected and placed in a dark cooler box at ambient temperature. The pedicels were cut under water to prevent embolism formation, placed in a pony vial containing 5 μCi of either D-[1-^{14}C]-Glu or [U-^{14}C]-Suc in 200 μL water. Once this solution had been taken up, but whilst the cut end of the pedicel was still covered by a meniscus of the solution, a chase of 200 μL equimolar (0.32 mM) nonlabelled Suc or Glu solution was added to the vial. After uptake, the pedicel was immersed for

12, 24 or 48 h in a clean vial of the chasing solution and the fruit and left at room temperature. Fruit was taken for autoradiography or separated into the component tissues, freeze-dried, milled and extracted in 80% (v/v) ethanol at 80 °C for 1 h and 4°C for an additional 24 h. Radioactivity in the extract was determined after addition of Pico-Fluor™ using a 1500 Tri-Carb® Liquid Scintillation Analyser (Packard®, Downers Grove, U.S.A.). The extracts were dried, resuspended in water and separated by HPLC into the component sugars. Peaks corresponding to authentic sugars were collected, dried, resuspended in 100 μL water and amount of [^{14}C] determined.

Distribution of [^{14}C]-label within the fruit was determined by autoradiography. Fruit were treated as described above, cut longitudinally, and placed on photographic film (MI-NH blue base medical imaging film or New RX NIF X-Ray film both from Fujifilm), wrapped in tissue and aluminium foil, sealed in a black bag and kept at -18°C for 28 days (after the methods of Narváez-Vásquez *et al.* 1995). All manipulations were carried out under safe light. The film was developed for 4 min in developer solution, rinsed in water for 1 min, fixed for 3 min, rinsed in water for 10 min and allowed to air dry.

2.8 LIGHT MICROSCOPY

Light microscopy was used in combination with electron microscopy in an attempt to:

1. Investigate the association of callose with the plasmodesmata;
2. Identify the dark, apparently amorphous material in the seed coat of small- and ABA-treated fruit.

2.8.1 SAMPLE PREPARATION

Fruit tissue was either hand sectioned or embedded in paraffin wax and sectioned, depending on the staining technique used.

Fruit tissue was sectioned into 4×4×4 mm blocks and placed in F.A.A. solution for at least 24 h at 4°C. Fixed material was dehydrated in an ethanol:tertiary butanol series and embedded in paraffin wax (details described in Appendix A.1). Embedded plant material was sectioned to give an even continuous ribbon of sections approximately 17 μm thick on a Lipshaw 45 Rotary Microtome (Lipshaw Mfg. Co., Detroit, U.S.A.) using a Reichert-Jung microtome blade (Reichert Scientific, Buffalo, U.S.A.). Sections were mounted with Haupt's adhesive, dewaxed with 100% (v/v) xylene

and rehydrated in alcohol to either 70% (v/v) (alcoholic stage) or 0% (v/v) (aqueous stage) ethanol (Appendix A.2) depending on the staining protocol used.

All light microscopy was viewed on a Ziess Axiophot Photomicroscope (Carl Ziess, Germany) using Fujicolor Superia 400 film (Fuji Photo Film Co. Ltd., Tokyo, Japan).

F.A.A. was prepared by mixing 70% (v/v) ethanol, glacial acetic acid and 37% (m/v) formalin in the ratio 18:1:1. Haupt's adhesive was prepared after the method of Johansen (1940). 1 g gelatine was dissolved in 100 mL ultra-pure water close to boiling point. 2 g phenol and 15 mL glycerine were then added and the solution was stored at room temperature.

2.8.2 HISTOCHEMICAL TECHNIQUES

2.8.2.1 Callose

Staining of callose with aniline blue for light microscopy was done after the methods of Brundrett *et al.* (1988). Fruit tissue was hand sectioned, stained in 0.5% (w/v) aniline blue for 20 min, rinsed with distilled water, mounted in water and viewed immediately under ultra-violet light (BP 395-400, FT 460, LP 470). 'Sirofluor', an impurity present in aniline blue, fluoresces bright yellow-white with callose (Hough *et al.* 1985). Precautions were taken to capture fluorescent tissue and avoid fading.

In an attempt to get thin, even sections for staining for callose, wax embedded sections were dewaxed and taken through a graded alcohol series to water, stained with 0.5% (w/v) aniline blue and viewed under UV light. However, no fluorescence was seen. Wax sections were then dewaxed, taken to 70% (v/v) alcohol and stained with 0.5% (w/v) aniline blue in lactophenol (which is normally used under bright field to stain for cellulose) and viewed under UV light. This was prepared by mixing equal volumes of phenol crystals, lactic acid, glycerine and distilled water. No fluorescence was seen although callose was expected. Thus, wax embedded sections were not used for callose localization using aniline blue.

Resorcin blue was used to stain callose, after the methods of Wittich and Graven (1989). Hand sectioned fruit or wax microtome section processed to the aqueous stage were stained with 1.5% (w/v) resorcin blue in 0.5% (v/v) ammonium hydroxide for 30 min. The sections were rinsed with running water, and viewed. Callose stains blue.

2.8.2.2 Carbohydrates

Periodic acid-Schiff's reagent was used to stain carbohydrates, after the methods of Jensen (1962) and Locquin and Langeron (1983). Hand sectioned fruit were oxidized in 0.5% (w/w) periodic acid for 10 min. The sections were then rinsed in water for 10 min, stained for 30 min in Schiff's reagent washed under running water, and viewed. Carbohydrates stain pink/red.

Schiff's reagent was prepared by dissolving 0.5 g basic fuchsin and 0.5 g potassium metabisulfate in 100 mL 0.15 N HCl. This mixture was stirred for 2-3 h until completely dissolved and then filtered through an activated-charcoal column under gravity at 4 °C. The clear solution obtained was tested by monitoring its reaction with starch, which turns red-pink when the reagent is successfully prepared.

Attempts to localize sugars with Periodic acid-Schiff's reagent were not successful on wax embedded tissue.

2.8.2.3 Cellulose

Congo red was used to stain for cellulose, after the methods of Locquin and Langeron (1983). Hand sectioned fruit or wax sections dewaxed to the aqueous stage were stained with 2.5% (w/v) congo red for 30 min, rinsed with running water, and viewed. Cellulose stains red.

2.8.2.4 Cutin, suberin and fats

Sudan IV was used to stain cutin, suberin and fats, after the methods of Clark (1981). 0.02 g Sudan IV was dissolved in 10 mL 70% (v/v) ethanol mixed with 10 mL acetone. Mounted sections, dewaxed to 70% (v/v) ethanol, were stained with the mix for 5 min, rinsed quickly in 70% (v/v) ethanol, transferred to distilled water and viewed. Typically, Sudan IV stains nuclei blue, fats red/orange and cholesterol brilliant red. Fatty acids are unstained.

2.8.2.5 Lignin

Safranin and fast green were used to stain for lignification and cutinised walls, after the methods of Johansen (1940). Sections were dewaxed and taken to 70% (v/v) alcohol, stained with 1% (w/v) safranin in methyl cellosolve:ethanol (1:1, v/v) for 2 h and washed twice in water for 30 sec. The

sections were then dehydrated and differentiated by emersion in 0.5% (w/v) picric acid in 95% (v/v) ethanol for 5 sec. The action of the acid was stopped by placing the slide in 95% (v/v) ethanol containing 4-5 drops ammonia per 100 mL for 2 min. The sections were then placed in 100% (v/v) ethanol for 10 min to complete dehydration. The sections were counter-stained with fast green in clove oil for 15 sec, rinsed in clean clove oil for 10 sec, placed in clove oil:xylene:ethanol (2:1:1) for 5 sec, and given three 5 sec washes in 100% xylene. Sections were mounted with Canada balsam and viewed by bright field light microscopy. Nuclei, chromosomes, lignified and cutinised walls stain bright red, cellulose walls and the cytoplasm stain green. Fast green was prepared by making a nearly saturated solution of fast green in equal parts of methyl cellosolve and ethanol and adding this mix to a mixture of clove oil:ethanol (75:25, v/v). The amount of fast green solution added to the oil:ethanol mixed depends on the intensity of staining required, but did not exceed 0.5% (m/v) fast green. This reagent was prepared several days before use.

2.8.2.6 Pectin

Ruthenium red was used to stain for pectic substances, after the methods of Jensen (1962). Hand sectioned fruit or wax sections dewaxed to the aqueous stage were stained with 0.02% (w/v) ruthenium red until the walls turned red, rinsed with running water, covered and viewed. Pectic substances appear pink.

2.8.2.7 Proteins

Amido black was used to stain for proteins, after the methods of Clark (1981). Hand sectioned fruit or tissue dewaxed to the aqueous stage were rinsed with water, stained with 1% (w/v) Amido black in 7% (v/v) acetic acid for 10 min, rinsed in 7% (v/v) acetic acid, mounted in water, covered and viewed. Amino acids, mitochondria, plastids, nuclei and protein bodies stain black.

2.8.2.8 Invertase

Histochemical staining of invertase was done after the methods of Miller and Chourey (1992). Fruit tissue was hand sectioned and fixed in 4% (m/v) formalin (pH 7.0) for 30 min. The fixed sections were rinsed in five changes of water over several h at 4 °C to remove endogenous sugars and incubated in the dark at 25 °C for 30 min in a reaction mixture comprised of equal volumes of 0.56 mg mL^{-1} phenazine methosulfate, 0.96 mg mL^{-1} nitroblue tetrazolium, 0.067 mL mL^{-1} Glu oxidase and 20 mg mL^{-1} Suc, all made up in 0.38 M sodium phosphate (pH 6.0), 0.38 M Tris-HCl (pH 6.8)

or 0.38 M sodium acetate buffer (pH 4.5). The reaction was terminated by briefly rinsing with distilled water and post-fixation for 15 min in 4% (m/v) formalin, followed by five 30 sec rinses in water. The sections were mounted in 15% (v/v) ethanol and viewed by bright field light microscopy. Invertase activity is shown by the formation of an insoluble blue fomazan precipitate. Using this method invertase (pH 6.8) was observed in the cytoplasm of seed and mesocarp tissue.

2.9 ELECTRON MICROSCOPY

All fruit tissues for electron microscopy were harvested, placed on ice, and transported immediately to the laboratory for fixation and processing.

2.9.1 SAMPLE PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY (TEM)

The fruit tissue was separated into the respective tissues and cut into 1×2×3 mm blocks and fixed in either 3% (m/v) glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) or 0.05 M sodium cacodylate buffer (pH 7.2) containing 4% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde for 24-56 h. Fixed plant material was washed twice for 30 min, post fixed with 2% (w/v) osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2) for 1 h, block stained in freshly prepared 2% (m/v) uranyl acetate in 2% (v/v) ethanol for 45 min and embedded in either Epon-Araldite, Spurr's or LR White resin and polymerised at 70°C for 48 h; 70°C for 16 h; or 50°C for 24 h respectively (full details are provided in Appendices A.3-A.5).

Small resin blocks were then mounted on perspex stubs, sectioned (LKB Ultratome III, Stockholm, Sweden) with a knife clearance angle of 5° to produce gold sections (60-80 nm thick) using a tungsten-coated glass knife or a diamond knife (6° Micro Star Diamond Knife) and collected on formvar coated 200 mesh copper grids. Sections were stained with 2% (m/v) lead citrate (to stain nucleic acids, proteins, phospholipids, glycogen and fats) in the presence of sodium hydroxide for 10 min, rinsed with a direct stream of double-distilled water, air dried and viewed on either a JEM-100CX (JEOL, Tokyo, Japan) or CM120 Biotwin (Phillips, Eindhoven, Holland) transmission electron microscope with an accelerating voltage of 80 kV. Photographs were taken on Kodak EM film with Estar thick base (Eastman Kodak Co., Rochester, U.S.A.).

To stain for callose the tissue was embedded as described above but post fixation with osmium, block staining with uranyl acetate, and post staining of sections with lead citrate were all omitted. Sections were collected on 200 mesh nickel grids and processed according to section 2.9.3.

0.05 M sodium cacodylate buffer (pH 7.2) containing 4% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde was prepared just before use to prevent polymerisation of formaldehyde with itself. 0.8 g paraformaldehyde was dissolved slowly in 10 mL water at 60°C. To this 0.04 mL 25% glutaraldehyde and 5 mL 0.2 M sodium cacodylate buffer (pH 7.2) were added. If necessary the pH was then adjusted to 7.2 with 0.01 N HCl or NaOH, and the solution made up to 20 mL. Epon-Araldite resin was prepared by mixing Epon, Araldite and DDSA in the ratio 1:1:3 respectively. Spurr's resin was prepared by mixing 10 g ERL 4206, 6 g DER 736, 26 g NSA and 0.6 g S-1 DMAE gently for 45 min in the fume cupboard.

Glass knives were cut from 6 mm thick glass sheets (Ultramicrotome glass, Agar Scientific Ltd, Stansted, England) using an LKB knifemaker (Type 7801 B, Bromma, Sweden). The glass knives were then placed on a stage tilted at 8° and coated with tungsten using an Edwards E306A High Vacuum Evaporator (Edwards, Crawley, England) for 2 min (Bandu 1991). Grids were coated with formvar by floating off a formvar film (0.25 g formvar in 100 mL chloroform) from a clean glass slide on water. The grids were placed gently on this floating film, picked with a wire mesh pad and air dried. Lead citrate was prepared by dissolving 1.33 g $Pb(NO_3)$ and 1.76 g $Na_2(C_6H_5O_7).2H_2O$ in 30 mL freshly boiled (to remove gas) double-distilled water. This solution was shaken intermittently for 30 min and then added to 8 mL 1 N NaOH to produce a clear solution, that was then made up to 50 mL with freshly boiled double-distilled water. During staining precautions were taken to minimize contact with CO_2 by including KOH pellets in the staining dish.

2.9.2 Sample preparation for scanning electron microscopy (SEM)

2.9.2.1 Critical Point drying (CPD)

Fruit tissue was cut into 5×5×2 mm blocks and fixed in 3% (m/v) glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) for at least 24 h. The fixed plant material was washed twice for 30 min in 0.05 M sodium cacodylate buffer (pH 7.2), dehydrated with a graded ethanol series and critically point dried (Hitachi Critical Point Dryer HCP-2, Hitachi Koki Ltd., Tokyo, Japan)(Appendix A.6). Samples were mounted under a dissecting microscope on brass stubs using two-sided tape and DAG 580 colloidal graphite in alcohol (Wirsam Scientific, Ashwood, South Africa) for support. Following coating with gold-palladium (SEM Coating Unit E51000, Polaron Equipment Limited, England)(Appendix A.7), specimens were viewed on a Hitachi S-570 scanning electron microscope (Hitachi, Tokyo, Japan), and photographed using Agfapan APX 100, 120 black and white film (Agfa, Germany).

Avocado tissue was found to exude a crystalline substance, believed to be *manno*-heptulose or perseitol, that covered the cut surfaces of fixed tissue. Specimens with this crystalline substance did coat well and charged when exposed to the electron beam (Figure 2.1). To avoid this problem avocado fruit sections were cryo fractured after CPD. This was done by immersing the dried tissue in liquid nitrogen, and using a prechilled razor blade and a hammer, tissues were fractured to reveal a clean surface for viewing. However, seed coat tissue normally separated into the two layers that constitute the seed coat during cryo-fracturing.

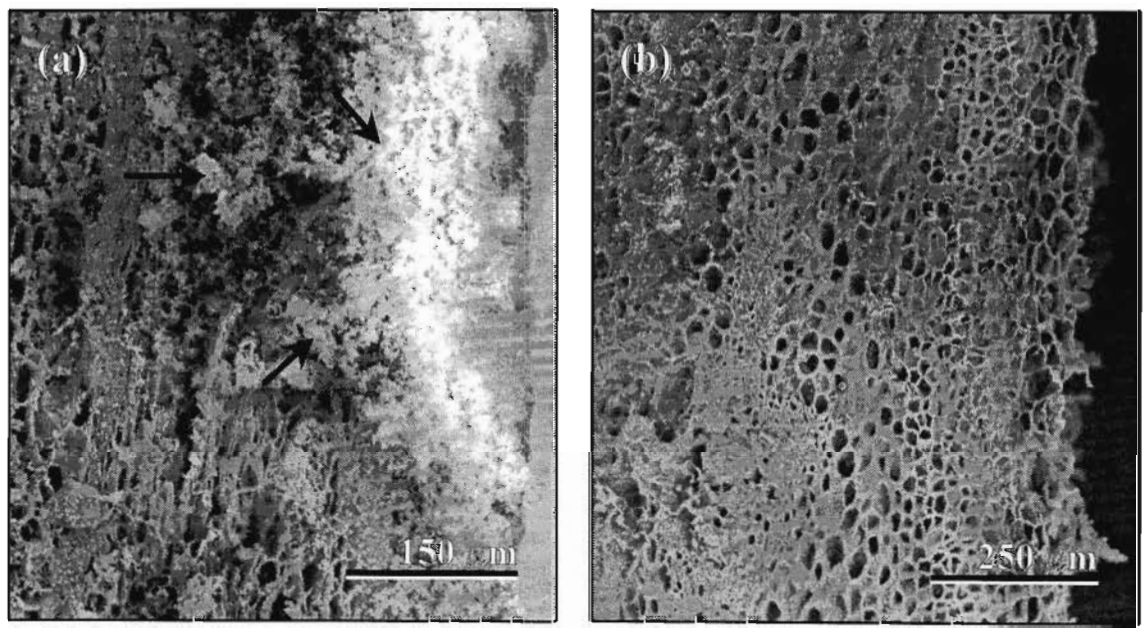


Figure 2.1 SEM electron micrographs of seed coat tissue (in cross section) fractured (**a**) prior and (**b**) subsequent to dehydration. Arrows indicate crystalline deposits. Images are representative of 6 observations.

2.9.2.2 Cryo

Fresh 2×2×2 mm blocks of tissue were mounted on copper stubs with Tissue-Tek® (Miles Scientific, Naperville, U.S.A.). The samples were then placed into an EMScope SP 2000 cryo unit (Ashford, England), frozen in liquid nitrogen slush and sublimed in the chamber at -70°C for 10 min. The samples were coated *in situ* with gold-palladium for 6 min, and viewed at liquid nitrogen temperatures in a Hitachi S-570 SEM.

2.9.2.3 Freeze-drying

Fresh 2×2×2 mm tissue samples were placed on the stage of an Emitech K775 Freeze Drier/Carbon Coater (Emitech, England), immersed in liquid nitrogen and the freeze drier was programmed to remain at -120°C for 4 h. Over the period of 1 h the temperature was increased to -75°C, at which it was maintained for 4 h. This was followed an increase in temperature to -50° for over a period of 1 h, at which the sample was maintained for 4 h. The tissue sample was then brought to 25°C over a period of 1 h and maintained at this temperature for about 3 h. Samples were mounted, sputter coated with gold-palladium and viewed as described in 2.9.2.1.

2.9.2.4 Fresh tissue

Fresh tissue was cut into 2×4×4 mm blocks, mounted on aluminium stubs using double-sided carbon tape and viewed on a Phillips XL30 Environmental Scanning Electron Microscope (ESEM) (Phillips, Eindhoven, Holland).

2.9.3 STAINING OF CALLOSE FOR TRANSMISSION ELECTRON MICROSCOPY

Staining of callose for electron microscopy was done after the methods of Brander and Wattendorff (1987) and Hayat (1989). Unstained Spurr's or LR White sections were mounted on nickel 200 mesh grids. Grids were placed face down on droplets on a waxed (parafilm) surface, except when incubating in the oven, during which the grids were totally immersed in about 2 mL solution to prevent drying out.

The sections were oxidized for 30 min on 1% (w/v) potassium permanganate at room temperature. The sections were washed twice with distilled water and placed on 1% periodic acid for 15 min. The grids were then immersed in staining solution for 1.5 h at 55-60°. This was followed by treatment with 5% (w/v) sodium thiosulphate for 1 min. After which the samples were rinsed thoroughly with water and viewed on a JEOL JEM-100CX transmission electron microscope at 80 kV.

The staining solution was prepared by mixing a solution of 1 mL of 5% (w/v) silver nitrate and 20 mL of 3% (w/v) hexamethylenetetramine with a solution of 2 mL of 1.44% (w/v) boric acid and 20 mL of 1.9% (w/v) borax. The resultant mixture was filtered through Whatman No. 2 filter paper.

2.10 TISSUE PREPARATION

2.10.1 TISSUE STORAGE

Unless otherwise stated, harvested fruit was separated into seed, endosperm, seed coat (pachychalaza) and mesocarp, diced, frozen in liquid nitrogen and freeze-dried (Freeze Drier, Science Workshop, University of Natal, South Africa). Freeze-dried tissue was milled (Analysenmühle A10, Janke and Jackson, Staufen, Germany) into a homogenous powder and stored in sealed vials at -18°C until used, for a maximum of 6 months.

2.10.2 *IN VITRO* EXPERIMENTS WITH SEED COAT

To determine the *in vitro* effect of the various sugars and ABA on tissue physiology, seed coat tissue was treated with Glu, Fru and Suc in the ratio 2:3:2 respectively (determined by Richings *et al.* (2000) to be the ratio of these sugars in avocado seed coat). The procedure used was adapted from De Jong *et al.* (1996). 80 Normal-fruit were collected and kept on ice. The seed coats were removed, punched into 1 cm disks and placed in 2 mM Mes-KOH buffer (pH 5.5) containing 0.5 mM $CaCl_2$ at 4°C. The tissue was then placed in the same buffer containing either 2.85 mM Glu, 2.85 mM Suc or 4.275 mM Fru, or a combination of the three, and the total osmolarity was adjusted to 400 mM with mannitol. Seed coat tissue prepared the same way was treated with 500 μM ABA; the control consisted of buffer containing 400 mM mannitol. The tissue was vacuum infiltrated for 5 min at -1800 kPa (-5 Psi) at 25°C. The tissue was then incubated in the dark at 21°C for 0, 1, 2, 4, 8 and 24 h. After incubation the tissue was stored in liquid nitrogen until assayed.

2.10.3 PROTOPLAST ISOLATION

Protoplast isolation was performed after a modification of the methods of Mitchell (2000). Untreated 'Hass' fruit were harvested 272 DAFB and kept on ice in the dark until returned to the laboratory. The fruit were scrubbed with Extran and sterilized in 50% (v/v) JIK® for 15 min. The skin (exocarp) and the green layer of the mesocarp closest to the skin were removed and ±5 g of the yellow part of the mesocarp grated with an autoclaved domestic grater into Petri dishes containing 30 mM $CaCl_2.2H_2O$, 20 mM KH_2PO_4, 100 mM KNO_3, 100 mM $MgSO_4.7H_2O$, 100 μM KI, 10 μM $CuSO_4.5H_2O$, 1 mM DTT, 0.5 M mannitol and 5 g L^{-1} BSA at 4°C. The mixture was covered with foil and left for 45 min at 25°C. A 15 mL aliquot of the above solution containing 0.5% (w/v) hemicellulase, 0.5% (w/v) cellulase and 0.005% (v/v) pectinase was added, and the tissue left to

digest in the dark for 16 h at 25°C. After this period the remaining tissue was cut into small pieces using a sterile razor and gently rolled into a smooth paste using a glass rod. The paste was filtered through a 150 μm nylon sieve and centrifuged at 100*g* for 10 min at 4°C. The upper layer, containing the protoplasts, was collected, washed with enzyme-free buffer and centrifuged at 100*g* for 10 min at 4°C. This was repeated until the top layer of protoplasts was clean, as determined by light microscopy. The protoplasts were removed, resuspended in the same buffer and used to prepare plasma membrane fractions.

2.10.4 PREPARATION OF PLASMA MEMBRANE FRACTIONS FOR GEL ELECTROPHORESIS

2.10.4.1 Isolation of cellular membranes by ultra centrifugation

Membrane rich fractions, from both fresh mesocarp tissue and isolated protoplasts, were prepared by ultra centrifugation according to the methods of Larsson *et al.* (1987).

Fresh avocado mesocarp tissue (40-70 g) from normal-fruit 262 DAFB was homogenised on ice in 100 mL of 10 mM Tris-Mops buffer (pH 7.0) containing 0.5 M Suc, 1 mM benzamidine and 5 mM amino caproic acid in the presence of 10% (w/w) PVP using an Ultra-Turrax T25 (Janke and Jackson, Staufen, Germany). The homogenate was filtered through two layers of miracloth® (Calbiochem, La Jolla, U.S.A.) and centrifuged at 12 000*g* for 15 min at 4°C. The supernatant was mixed with 0.1489 g EDTA and allowed to stand on ice for 30 min. This mixture was centrifuged (Beckman L8-M Ultracentrifuge, Beckman Instruments Inc., Palo Alto, U.S.A., rotor type: Beckman SW 40Ti) at 120 000*g* for 60 min at 4°C. The supernatant was discarded and the pellet resuspended in 10 mL 10 mM Tris-Mops buffer (pH 7.0) containing 0.33 M Suc, 10 mM KCl and 10 mM $MgCl_2$ and centrifuged at 2 500*g* for 5 min at 4°C. The supernatant was collected, diluted to 20 mL with the same buffer and centrifuged at 120 000g for 60 min at 4°C. The resultant supernatant was discarded and the pellet resuspended in 2 mL of the same buffer, frozen in liquid nitrogen and stored at -70°C (SS-CDF0751 Freezer, Specht Scientific, Johannesburg, South Africa) prior to further purification.

Clean protoplast extracts were ultra-sonicated (VirSonic 100, The Virtis Company Inc., Gardiner, U.S.A.) to lyse the protoplasts. The membrane-containing solution was centrifuged at 10 000*g* at 4°C for 15 min. The supernatant was centrifuged at 90 000*g* at 4°C for 60 min and the pellet resuspended in 1 mL of buffer, frozen in liquid nitrogen and stored at -70°C for not more than 3 weeks prior to further purification.

2.10.4.2 Membrane purification

The membrane-rich fractions produced from protoplasts and fresh tissue were purified, after the methods of Larsson and co-workers (1987), to give two fractions, one containing plasma membrane fractions and the other containing membranes from the endoplasmic reticulum and chloroplasts.

To prepare the phase-system, 93 g of 20% (w/w) dextran T-500, 46.5 g of 40% (w/w) PEG 3350, 33.89 g Suc, 7.5 mL of 0.2 M potassium phosphate (KPO_4) buffer (pH 7.8) and 0.45 mL of 2 M KCl were mixed together in a separating funnel and made to a final weight of 300 g with distilled water. The solution was allowed to equilibrate at 4°C and then vigorously inverted and allowed to settle overnight at 4°C. The upper and lower phases were then collected and stored separately at 4°C.

The phase-mixture was prepared by mixing 11.16 g of 20% (w/w) dextran T-500, 5.58 g of 40% (w/w) PEG 3350, 3.05 g Suc, 0.675 mL of 0.2 M KPO_4 buffer (pH 7.8) and 0.0.41 mL of 2 M KCl, and the solution was made up to a final weight of 27 g with distilled water. The mixture was shaken vigorously and immediately separated equally into 4 centrifuge tubes, capped, and allowed to settle overnight at 4°C.

A 2 mL aliquot of membrane rich fractions was mixed with the phase-mixture by 20-30 inversions of the centrifuge tube and centrifuged at 1 500*g* for 5 min at 4°C. 90% of the upper phase (excluding the interface) was collected with a Pasteur pipette and partitioned twice against 9 mL of the lower phase-system using clean centrifuge tubes each time, and centrifuged at 1 500*g*. The three upper phases were combined, and diluted three-fold with 10 mM Tris-Mops buffer (pH 7.0) containing 0.33 M mannitol, 10 mM KCl, and 2 mM $MgCl_2$ and then centrifuged at 120 000*g* for 60 min at 4°C. The supernatant was discarded and the pellet resuspended in 0.5 mL of the same buffer. Protein content was determined using the dye-binding method (Bradford 1976) and the mixture diluted or re-centrifuged and re-diluted to give a protein concentration of ±1 μg per μL. The resultant mix, containing the plasma membrane fraction, was stored as 50 μL aliquots, frozen in liquid nitrogen and stored at -70°C, for approximately 4 weeks.

The lower phase from the original phase-mixture extraction was partitioned against upper phase system mixture in a similar manner to yield a fraction containing endoplasmic reticulum and chloroplast membranes.

2.10.5 ENZYME EXTRACTION

Fresh or freeze-dried tissue was homogenised on ice for 3-5 15 sec bursts using an Ultra-Turrax in 50 mM Hepes-NaOH buffer (pH 7.0) containing 0.5 mg mL^{-1} BSA and 0.005% (v/v) Triton X-100 or 50 mM Tris-HCl buffer (pH 7.2) containing 100 mM glycerol and Complete™ EDTA free (according to the manufacturers requirements: 1 tablet per 50 mL), in addition to 1 mM EDTA, 2 mM DTT, 5 mM $MgCl_2$ and 10% (w/w) insoluble PVP. The homogenate was stood on ice for 30 min, filtered through two layers of miracloth and centrifuged (Himag CR20B2 Centrifuge, Hitachi Koki Co. Ltd., Tokyo, Japan; or Beckman J2-HS Centrifuge, Beckman Instruments, Palo Alto, U.S.A.) at 2 500*g* for 30 min at 4°C. The supernatant was filtered through glass wool and used as a source of soluble enzyme. The pellet was washed 3 times in the extraction buffer containing 0.005% (v/v) Trition X-100. The final pellet was resuspended in 10 mL extraction buffer and used as a source of insoluble enzyme. For both soluble and insoluble enzyme extracts, samples were decanted into 1 mL portions, frozen with liquid nitrogen, and stored at -70°C for not more than 6 months. Three separate extractions were made for each treatment, each using at least three fruit.

Freeze-drying of tissue reduced enzyme activity (Figures 2.2 and 2.3). The extraction of enzymes from fresh tissue and subsequent storage at -70°C in a buffer containing Complete™ protease inhibitors and glycerol was found to be the best method of preserving enzyme activity (Figure 2.3) and was subsequently used for all enzyme extractions.

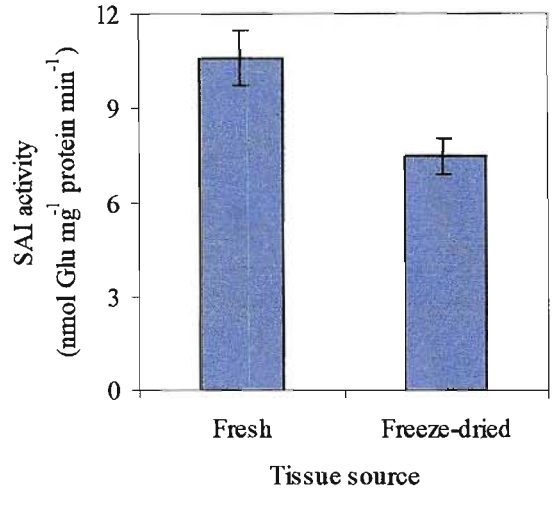


Figure 2.2 SAI activity in small-fruit mesocarp tissue (180 DAFB) assayed from fresh and freeze-dried tissue, both extracted in Hepes-NaOH buffer (pH 7.0). $n = 4$. Error bars represent standard deviation (SD). $LSD_{0.05}$: 2.46.

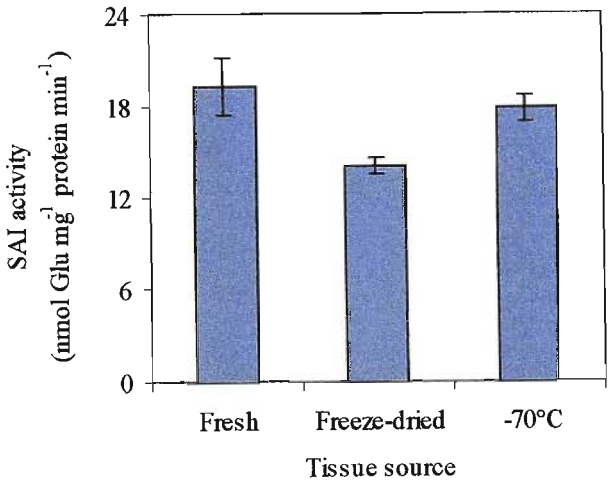


Figure 2.3 SAI activity in normal-fruit mesocarp tissue (120 DAFB) assayed from fresh and freeze-dried tissue and enzyme extracts stored at -70°C with protease inhibitors and glycerol. Freeze dried tissue and frozen extracts were stored for 8 weeks before assaying. All extractions were with Tris-HCL buffer (pH 7.2). $n = 3$. Error bars represent SD. $LSD_{0.05}$: 3.10.

2.11 ENZYME ASSAYS

Unless otherwise stated, enzymes were extracted as described in section 2.10.5.

The soluble fraction was passed through Sephadex-G25 microcentrifuge desalting columns prepared after the methods of Helmerhorst and Stokes (1980). Initially the use of Sephadex-G25 columns for SAI resulted in highly variable results, as indicated by the unacceptably large standard deviations (Table 2.1). However, desalting was found to be more reliable if the Sephadex-G25 was swollen with Tris-HCl buffer (pH 7.2), and the columns were packed to a bed volume of 2 mL and only 1 mL enzyme extract passed through the column without further washing through of the column. Enzyme extracts passed through Sephadex-G25 micro-centrifuge columns prepared in this way were found to have SAI and NI activity approximately ten times higher than that in crude enzyme extracts (Figure 2.4). The activity of α-amylase was also enhanced by Sephadex columns (data not shown). This may be attributed to, amongst other factors, the inhibitory affects of *manno*-heptulose and/or perseitol. This was supported by the results of reducing sugars in the column exudates which indicated a marked reduction in reducing sugar levels (Figure 2.5). Thus all subsequent samples for enzyme analysis were passed through Sephadex columns. Other difficulties may have resulted from interactions between the different buffers used for column preparation and enzyme extraction, or from interactions with the sodium azide used to keep the column free from fungi.

Table 2.1 SAI activity of extracts passed through Sephadex-G25 micro-centrifuge desalting columns prepared with KPO_4 buffer and stored with sodium azide compared with that of crude enzyme extracts.

Preparation	Tissue	SAI activity (nmol Glu mg^{-1} protein min^{-1})	
		Mean (n = 3)	SD
Passed through Sephadex	Mesocarp	9.84	11.47
	Seed Coat	ND	-
	Seed	45.22	75.66
Crude extract	Mesocarp	30.93	1.86
	Seed Coat	44.41	3.76
	Seed	7.64	0.17

ND = Not detected; activity lower than 0.005 nmol Glu mg^{-1} protein min^{-1}
SD = Standard deviation

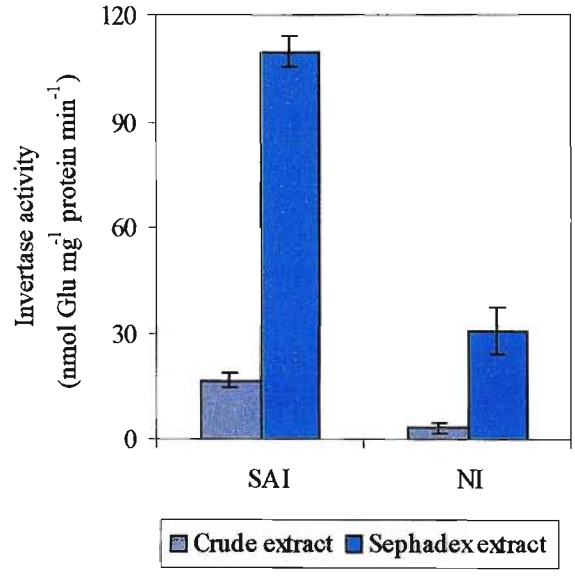


Figure 2.4 SAI and NI activity in the seed tissue from normal-fruit 120 DAFB, assayed from either crude enzyme extracts or extracts passed through Sephadex-G25 prepared using 50 mM Tris-HCl buffer (pH 7.2). Enzyme assays were conducted at pH 4.55 and pH 6.80 for SAI and NI, respectively. n = 3. Error bars represent SD. $LSD_{0.05}$: SAI = 24.53; NI = 12.40.

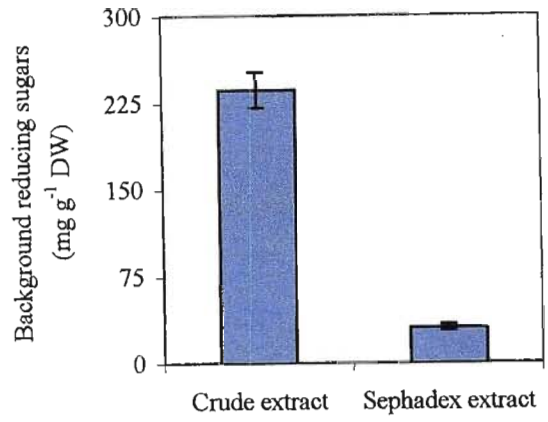


Figure 2.5 Reducing sugar levels from the soluble phase of seed tissue from normal-fruit 120 DAFB in crude and desalted enzyme extracts. $n = 5$. Error bars represent SD. $LSD_{0.05}$: 90.28.

2.11.1 INVERTASE

Invertase was assayed after the methods of Zinselmeier *et al.* (1995) and the amount of Glu produced measured colorimetrically (Nelson 1944), with a few adaptions. Volumes greater than 50-60 μL of avocado extract resulted in precipitate formation (between the copper reagent and the reducing sugars) which interfered with the colorimetric technique. The protocol was thus modified and 30 μL enzyme extract was incubated at 30°C for 30 min with 70 μL 0.1 M Suc in 0.1 M sodium acetate buffer (pH 4.50 or 4.55) or 0.1 Hepes-NaOH buffer (pH 6.80). The reaction was terminated by boiling for 10 min with 1 mL copper reagent, and then allowed to cool. 1 mL of arsenomolybdate was added, the solution mixed, and colour allowed to develop for 10 min. The solution was vortexed (Vortex-2-Genie, Scientific Industries inc., Bohemia, U.S.A.) and the absorbance read at 560 nm (Beckman DU®-65 Spectrophotometer, Beckman RIIC Ltd., Glenrothes, Scotland; or Anthelie Advanced spectrophotometer, Secomam CE, Dormont, France) and compared to a Glu standard curve at 0, 20, 40, 60, 80 and 100 μg mL^{-1}. Background sugars and activity were subtracted by assaying samples without substrate and without enzyme extract, respectively. Protein was determined using Bradford's method (section 2.12.4.1).

Copper reagent was prepared by dissolving 24 g Na_2CO_3 and 12 g $KNaC_4H_4O_6.4H_2O$ in 250 mL water. Separately, 4 g $CuSO_4$ was dissolved in 40 mL of water and this solution added to the first. To the resultant solution, 15 g $NaHCO_3$ was added. Separately, 180 g anhydrous Na_2SO_4 was dissolved in 500 mL water, boiled and allowed to cool. After cooling, the two solutions were mixed together, made up to 1 L and stored in darkness at 37°C for at least 48 h before use.

Arsenomolybdate reagent was prepared by dissolving 25 g $(NH_4)_6Mo_7O_{24}.4H_2O$ in 450 mL water and adding 21 mL concentrated H_2SO_4. Separately, 3 g $Na_2HAsO_4.7H_2O$ was dissolved in 25 mL of water. The two solutions were mixed and stored in darkness at 37°C for at least 48 h before use.

2.11.2 Sucrose Synthase

SSy activity was assayed using a combination of the methods of Klann *et al.* (1993) and Sowokinos *et al.* (1985). 30 μL enzyme extract was incubated with 70 μL 50 mM Tris-HCl (pH 7.2) containing 1 mM EDTA, 2 mM DTT and, for synthesis studies, containing 25 mM Fru and 25 mM UDP-Glu; alternatively, to assay for cleavage of Suc, containing 25 mM Suc and 25 mM UDP. After 1 h at 37°C, the reaction was terminated by boiling for 10 min with 100 μL 30% (w/v) KOH, and allowed to cool. 1 mL anthrone reagent was added and the assay mixture incubated for 20 min at 40°C. The absorbance was read at 620 nm and values compared to a Suc standard curve at 0, 20, 40, 60, 80 and 100 μg mL^{-1}. Background sugars and activity were subtracted by assaying samples without substrate, and without enzyme extract, respectively. Protein content was determined using Bradford's (1976) method.

Anthrone reagent was prepared after the method of van Handel (1968). 76 mL concentrated H_2SO_4 was slowly added to 30 mL 26 mM anthrone solution. The mixture was allowed to cool and stored in the dark at 4°C until use.

2.11.3 Sucrose Phosphate Synthase

SPS activity was assayed as SSy activity using 50 mM Tris-HCl (pH 7.2) containing 1 mM EDTA, 2 mM DTT, 25 mM Fru-6-phosphate, 25 mM Glu-6-phosphate and 25 mM UDP-Glu as the substrate.

2.11.4 1,3-β-Glucan Synthase

1,3-β-glucan synthase enzyme extract were prepared by homogenizing 100 mg freeze-dried tissue with 3×20 sec pulses, on ice, in 100 mM TES-NaOH buffer (pH 7.0) containing 1 mM DTT and Complete™ protease inhibitor cocktail (according to manufacturer's instructions: 1 tablet per 50 mL). The extract was centrifuged for 480*g* for 5 min at 4°C, the supernatant collected and kept on ice until used.

1,3-β-Glucan Synthase activity was assayed after the methods of Kauss *et al.* (1983) and Köhle *et al.* (1985). 50 μL enzyme extract was mixed with 55 μL 50 mM TES-NaOH buffer (pH 7.0) containing 20 mM cellobiose, 16% (w/v) glycerol, 10 mM $MgCl_2$, 0.04% (w/v) digitonin, 4 mM EDTA, 3.6 mM $CaCl_2$ and 0.03 μCi UDP-D-[U-^{14}C]-Glu. The mixture was incubated at 25°C for 15 min and the reaction terminated by boiling for 5 min. Thereafter 50 μL of the incubation mixture was transferred to 0.5×1.5 cm Whatman No. 3 paper and allowed to air dry. The paper strips were washed twice for 1 h in 0.25 M ammonium acetate in 21 % (v/v) ethanol adjusted to pH 3.6 with acetic acid and allowed to air dry. The paper was then cut into small strips, placed in 5 mL 0.5% (m/v) PPO in toluene, and counted using a Packard® 1500 Tri-Carb® Liquid Scintillation Analyser.

2.11.5 α-AMYLASE

α-Amylase was assayed after the methods of Abe *et al.* (1996), Choi *et al.* (1996) and Kaur *et al.* (1998). Soluble enzyme extract was heated to 70°C for 15 min to inactivate β-amylase. 20 μL enzyme extract was incubated at 35°C for 20 min with 100 μL starch substrate. The reaction was terminated by boiling with 1 mL copper reagent and α-amylase activity determined colorimetrically by Nelson's method, as described for invertase activity (Section 2.11.1).

The starch substrate was prepared by boiling 2 g soluble potato starch in 100 mL 0.1 M sodium acetate buffer (pH 5.5) containing 10 mM $CaCl_2$. This solution was allowed to cool and centrifuged at 5000*g* for 10 min; the supernatant was used as the substrate.

2.12 MOLECULAR ANALYSIS

2.12.1 CARBOHYDRATES

2.12.1.1 Colorimetric determination of starch and total soluble sugars

Starch and total soluble carbohydrates were assayed after a modification of the methods of Buysse and Merckx (1993). 0.05 g freeze-dried tissue was homogenised in 4 mL 80% (v/v) ethanol and centrifuged at 1 500*g* for 20 min. The supernatant was collected and the pellet resuspended in 4 mL 80% (v/v) ethanol and centrifuged at 1 500*g* for 20 min. This was repeated again. The 3 supernatants were combined and used to assay for soluble sugars. The pellet was boiled in 10 mL 3% (w/v) HCl for 3 h and used to determine starch.

400 μL extract was mixed with 400 μL 28% (w/w) phenol in 80% (v/v) ethanol. To this 2 mL of concentrated H_2SO_4 was rapidly added, directing the stream onto the liquid surface. The resultant mixture was vortexed, allowed to stand for 15 min for colour development, and the absorbance read at 490 nm.

This process was repeated using 22% and 34% (w/w) phenol to allow for the regressive determination of relative Glu, Fru and Suc content. Although workable regressions/relationships were obtained with the sugar standards, fruit extracts gave erroneous results. This was attributed to interference of *manno*-heptulose, perseitol and other unidentified reducing sugars and perseitol with the reagents. However, the absorbances of Suc, Glu and Fru were found to intercept at approximately 28% (w/w) phenol (Figure 2.6) and this percentage was used to determine sugar content, using Glu as a standard to determine relative soluble sugars. Starch that had undergone 3 h hydrolysis in 3% (w/v) HCl was found to also intercept at approximately 28% (w/w) phenol (data not presented), and was used as the standard for starch (insoluble carbohydrate) quantification.

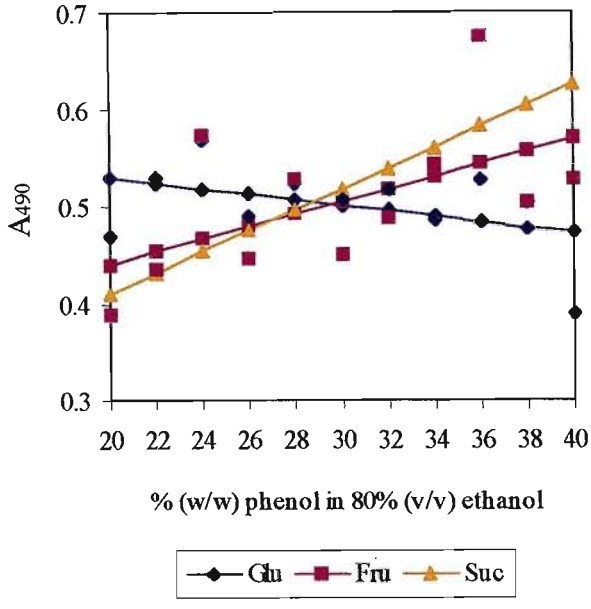


Figure 2.6 Absorbances (490 nm) of Glu, Fru and Suc solutions (0.06 g L^{-1} 80% (v/v) ethanol). Equation of best fit: Glu y = -0.013x + 0.581; Fru y = 0.014x + 0.410; Suc y = 0.025x + 0.352.

2.12.1.2 High performance liquid chromatography of soluble sugars

Freeze-dried tissue (0.1 g) was homogenised in 10 mL 80% (v/v) ethanol with 0.005 μCi [U-^{14}C]-Suc, incubated at 80°C for 1 h, and allowed to stand at 4°C for 24 h. The extracts were then centrifuged at 2000*g* for 10 min, filtered through glass wool, and dried overnight on a Savant SC 200 220 Vacuum Drier (Savant Instruments Inc., Farmingdale, U.S.A.). The dry extracts were made up to 2 mL with ultra-pure water, filtered through a 0.45 μm nylon syringe filter (Lida, Kenosha, U.S.A.) and 100 μL loaded onto a Rezex 8μ 8% Ca monosaccharide 300×7.80 mm Phenomenex® column, maintained at 75°C. Water was the mobile phase at a flow rate of 0.5 mL min^{-1}. Sugars were detected using either a Waters Differential Refractometer R401 (Waters Associates, U.S.A) or an Erma ECR-7515A RI Detector (Erma, Kawaguchi City, Japan) maintained at 40°C, and integrated with a SP4600 Data Jet Integrator (Thermoseparations, Spectra-Physics, Riviera Beach, U.S.A.)(Figure 2.7). Sugars were tentatively identified by co-elution with authentic sugar standards (Table 2.2).

Initially, the extracts were partitioned against an equal volume of hexane followed by the solid phase partitioning through C_{18} cartridges to remove excess lipids. However, this practice was discontinued as it was thought that glycosylated carbohydrates may have been removed.

The column was cleaned after every 10-12 sample injections by washing for 10 h with 5% (v/v) acetonitrile containing 500 mg L^{-1} Ca-EDTA at a flow rate of 0.5 mL min^{-1}. Standard curves were constructed for Suc, Glu, Fru, perseitol and *manno*-heptulose at 0, 10, 20, 30, 40 and 50 μg per 100 μL. The standard curve responses of the RI detector to all the sugars was found to be almost identical (r=1), thus the values were averaged and used to construct a standard curve to quantify unidentified sugar peaks.

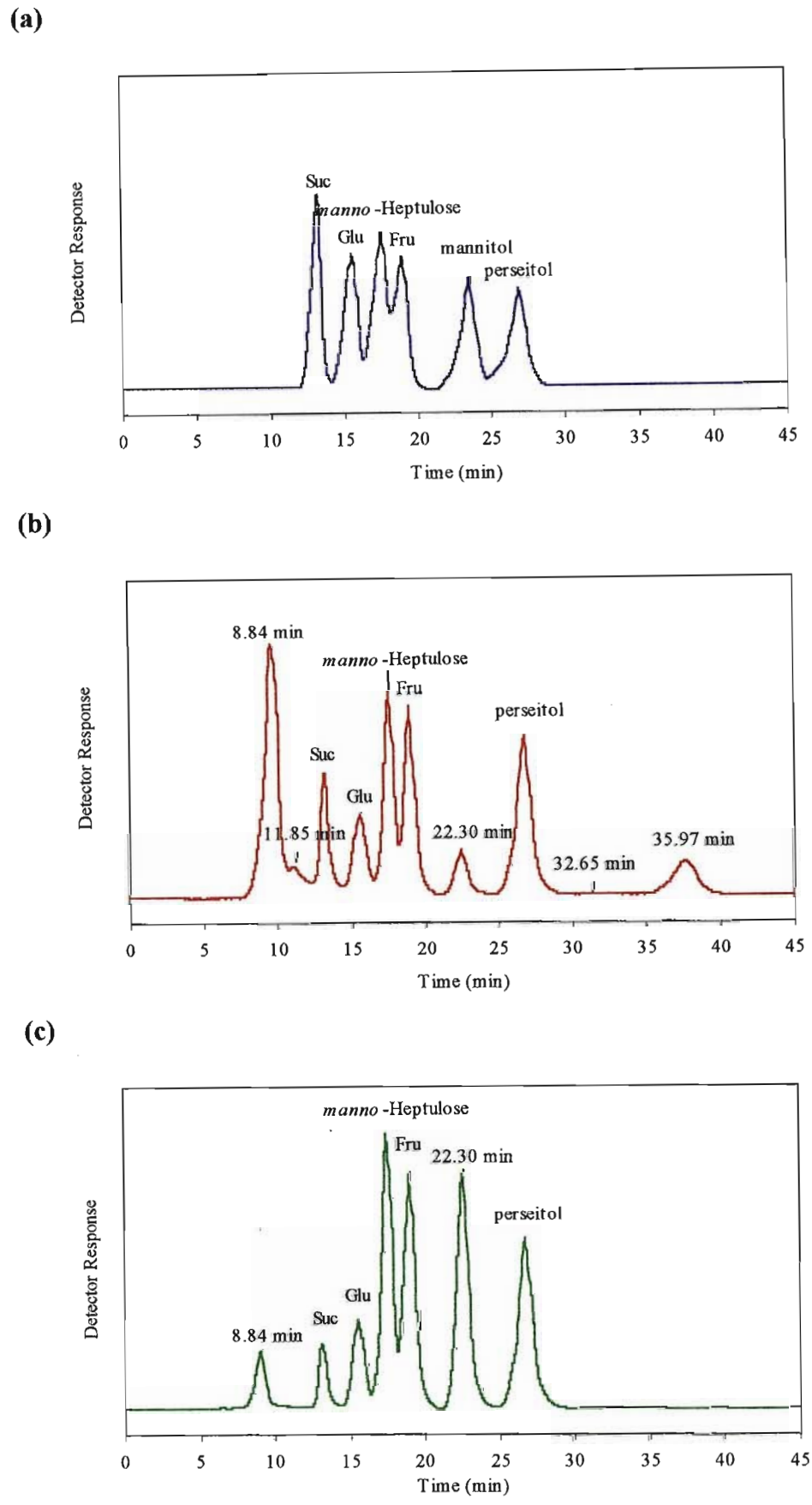


Figure 2.7 Representative HPLC chromatograms of (**a**) authentic sugar standards, and peaks obtained from samples of (**b**) seed and (**c**) mesocarp tissue. Unidentified sugars occur at 11.85, 22.30, 32.65 and 35.97 min.

Table 2.2 Retention, and relative retention, times of various sugar and sugar alcohol standards obtained by HPLC analysis.

Sugar	Rt (min)	r_{Suc}
Raffinose	11.79	0.88
Cellobiose	12.72	0.95
Trehalose	12.97	0.97
Maltose	13.08	0.98
Sucrose	13.36	1.00
α-Lactose	13.59	1.02
D(+)-Glucose	15.33	1.15
β-D(+)-Glucose	15.44	1.16
L(-)-Sorbose	16.78	1.26
D(+)-Xylose	16.84	1.26
D(+)-Galactose	16.91	1.27
D-*manno*-Heptulose	17.03	1.27
L(+)-Rhamnose	17.12	1.28
D(+)-Mannose	17.61	1.32
D(-)-Fructose	18.63	1.39
Sedoheptulose	18.68	1.40
L(+)-Arabinose	18.81	1.41
meso-Inositol	19.10	1.43
D(-)-Arabinose	19.26	1.44
2-deoxy-D-Ribose	19.61	1.47
Glycerol	21.51	1.61
meso-Erythritol	22.06	1.65
D(-)-Mannitol	23.84	1.78
D(-)-Sorbitol	27.87	2.09
Perseitol	28.93	2.17
D(-)-Ribose	29.06	2.18

r_{Suc} = relative retention time compared to Suc
Rt = Retention time (min)

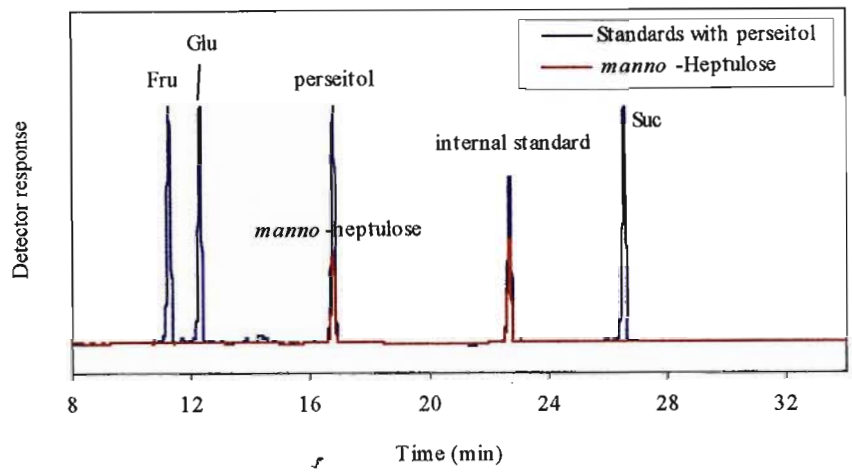
2.12.1.3 Gas chromatography of soluble sugars in 'Hass' avocado

Additional confirmation for the presence of the sugars Suc, Glu, Fru, perseitol and *manno*-heptulose in avocado tissue, was made by GC. Soluble sugars were extracted by the same method used for HPLC analysis (described in 2.12.1.2). The dry extracts were dissolved in 900 μL pyridine and 300 μL ultra-pure water. From this solution 200 μL was taken, mixed with 100 μL STOX™ and dried at 50°C under N_2. The dry oxime and sugar residue was silylated with 200 μL Tri-Sil® Z and the mixture left for 5 min at 50°C. 1 μL of this mixture containing the silylated sugars was analysed on a 2 m × 3 mm ID OV-17 packed glass column. The initial column temperature was 150°C for 3 min and the temperature then raised by 4°C min^{-1} to 270°C, and held at this temperature for 12 min. The carrier gas was N at a flow rate of 30 mL min^{-1}. Injector and detector temperatures were both 300°C. Detection was by Varian 3700 GC (Varian Instrument Group, Walnut Creek, U.S.A.) fitted with a flame ionisation detector. Integration was by a Hewlett Packard HP3394 A integrator (Avondale, U.S.A.).

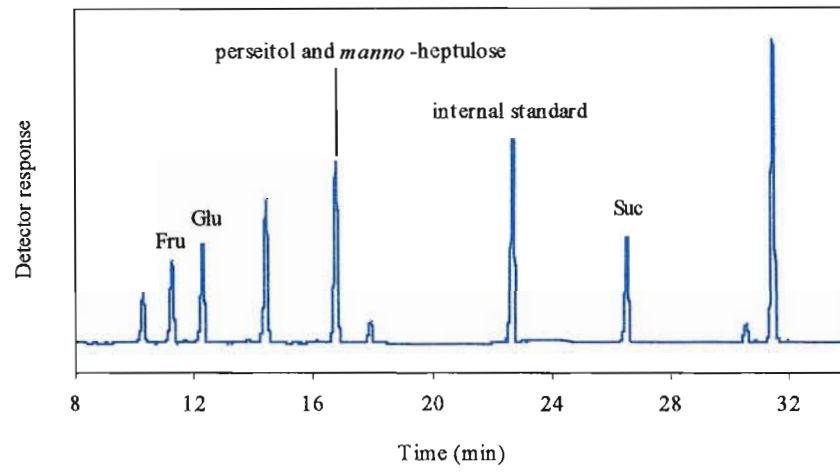
For the analysis of acylated sugars, soluble sugars were extracted by the same method used for HPLC analysis (described in 2.12.1.2). Sugar samples were acetylated by dissolving the dry extract in a 1:1 acetic anhydride:pyridine mixture at 80°C for 20 min. A 1 μL sample was injected into the same GC column using the following programme: 230°C for 3 min and the temperature then raised by 10°C min^{-1} to 270°C, and held at this temperature for 12 min. All other conditions were the same as those described for the GC analysis of silylated sugars.

Peaks were identified by comparison with authentic standards and phenyl-β-D-Glu was used as an internal standard. Silylation of sugars allowed for Suc, Glu and Fru to be positively identified (Figure 2.8). Although silylized perseitol and *manno*-heptulose emerged as a single peak, the acetylated derivatives of these two compounds gave baseline-to-baseline separations, which allowed for the confirmation of their identity (data not presented).

(a)



(b)



(c)

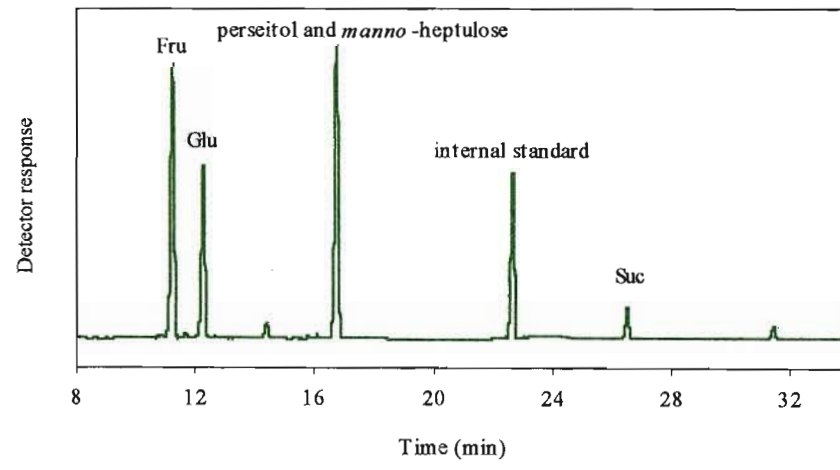


Figure 2.8 Representative GC chromatograms of trimethyl-silyl derivatives of (**a**) authentic sugar standards of Suc, Glu, Fru, perseitol and *manno*-heptulose, and peaks obtained from samples of (**b**) endosperm and (**c**) mesocarp tissue.

2.12.1.4 Callose quantification

Callose was quantified after a modification of the method of Köhle *et al.* (1985), using the principle that aniline blue fluoresces with callose. 0.05 g freeze-dried tissue was homogenised in ethanol and allowed to stand for 5 min. The mixture was centrifuged at 2 000*g* for 10 min, the supernatant discarded and the pellet vortexed in 1 N NaOH. This mix was incubated for 15 min at 80°C, centrifuged at 400*g* for 10 min and the supernatant collected. 10 μL supernatant was mixed with 190 μL 1 N NaOH, and to this the following were added, with vortexing between each addition: 200 μL 0.1% (w/v) aniline blue; 210 μL 1 N HCl; 590 μL 1 M glycine-NaOH buffer (pH 9.5). The mixture was then incubated in the dark at 50°C for 20 min. The mixture was observed to change colour with changes in pH, starting with a deep red when aniline blue was added, changing to deep blue after the addition of the acid. After incubation, the mixture was clear. The resultant mix was held at room temperature in the dark for 30 min before the fluorescence was determined using a Hitachi-2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), excitation 400 nm, emission 510, band pass EX 10 EM 10 and PM voltage 700. Samples were compared to a freshly made pachyman standard curve, which was linear between 0 to 2 μg per 200 μL (assay volume), and results expressed as pachyman-equivalents.

Avocado tissue, especially the seed and seed coat, was found to show very high auto-fluorescence. Attempts to remove this with methanol and ethanol failed, and for this reason very low volumes of sample were used (10 μL) and a correction for background fluorescence made by measuring fluorescence in samples treated with 200 μL water instead of aniline blue. The amounts of aniline blue used were also reduced from 400 μL to 200 μL as the larger volumes of aniline blue did not decolourise sufficiently to permit reading on the fluorimeter.

2.12.2 Lipids

2.12.2.1 Total oil content

Oil content was determined using a modification of the methods of Frick *et al.* (1994). 0.2 g freeze-dried tissue was homogenised for 5×45 sec pulses in 5 mL 100% (v/v) petroleum ether and allowed to stand overnight at 4°C. After filtering through two layers of Whatman No. 1 under vacuum, the residue was washed twice with 5 mL petroleum ether. The filtrate was partitioned twice with water, keeping the upper phase, which was dried on a rotary evaporator (Büchi Rotavapor R110, Flail,

Switzerland; or Bibby Rotary Evaporator RE 100, Stone Staffordshire, England) at -20°C. The mass of the remaining oils was determined.

2.12.2.2 Gas chromatography analysis of fatty acids

Fatty acids were extracted by homogenizing 0.2 g freeze-dried tissue in 5 mL methanol:chloroform (1:2, v/v). After standing for 12 h at 4°C, the extract was centrifuged at 500*g* for 10 min. The supernatant was partitioned twice against 2 mL 2 M KCl in 0.5 M KPO_4 buffer (pH 7.4) (Garbus solution; Garbus *et al.* 1963) and then twice with water, keeping the lower phase each time. The lower phase was centrifuged at 500*g* for 10 min, 2 mL collected and dried with a gentle stream N_2. The dry sample was flushed with N_2 and stored in a sealed glass container at -18°C until use (two weeks).

The oil extract was suspended in 2 mL diethyl ether, mixed with 0.5 mL 1 M TMAH in 100% (v/v) methanol and allowed to stand for 1 min. 1 mL distilled water was added and the solution allowed to separate. The upper (ether phase) was collected, dried with 0.5 g anhydrous Na_2SO_4 and 1 μL loaded onto a 30 m × 0.25 mm ID SP-2330 fused capillary SupelCo column, operated from 120°C to 220°C at 8°C min^{-1}, and held at this temperature for 10 min. The carrier gas was He at a flow rate of 1 mL min^{-1} (splitting 20:1). Injector and detector temperatures were 220°C and 300°C, respectively. Detection was by Varian 3300 GC (Varian Instrument Group, Walnut Creek, U.S.A.) fitted with a flame ionisation detector and linked to a Hewlett Packard HP3395 integrator (Avondale, U.S.A.).

Peaks were identified by comparison with premixed authentic standards (Nu-Chek Prep. Inc., U.S.A.) containing 18:0, 18:1, 18:2, 18:3 and 20:4, and soybean oil extract, extracted as described above and known to contain 16:0, 18:0, 18:1, 18:2 and 18:3 (Figure 2.9). Relative retention times (compared to 18:0) were used to tentatively identify the other fatty acids. Examples of GC separation of fatty acids are presented in Figure 2.9.

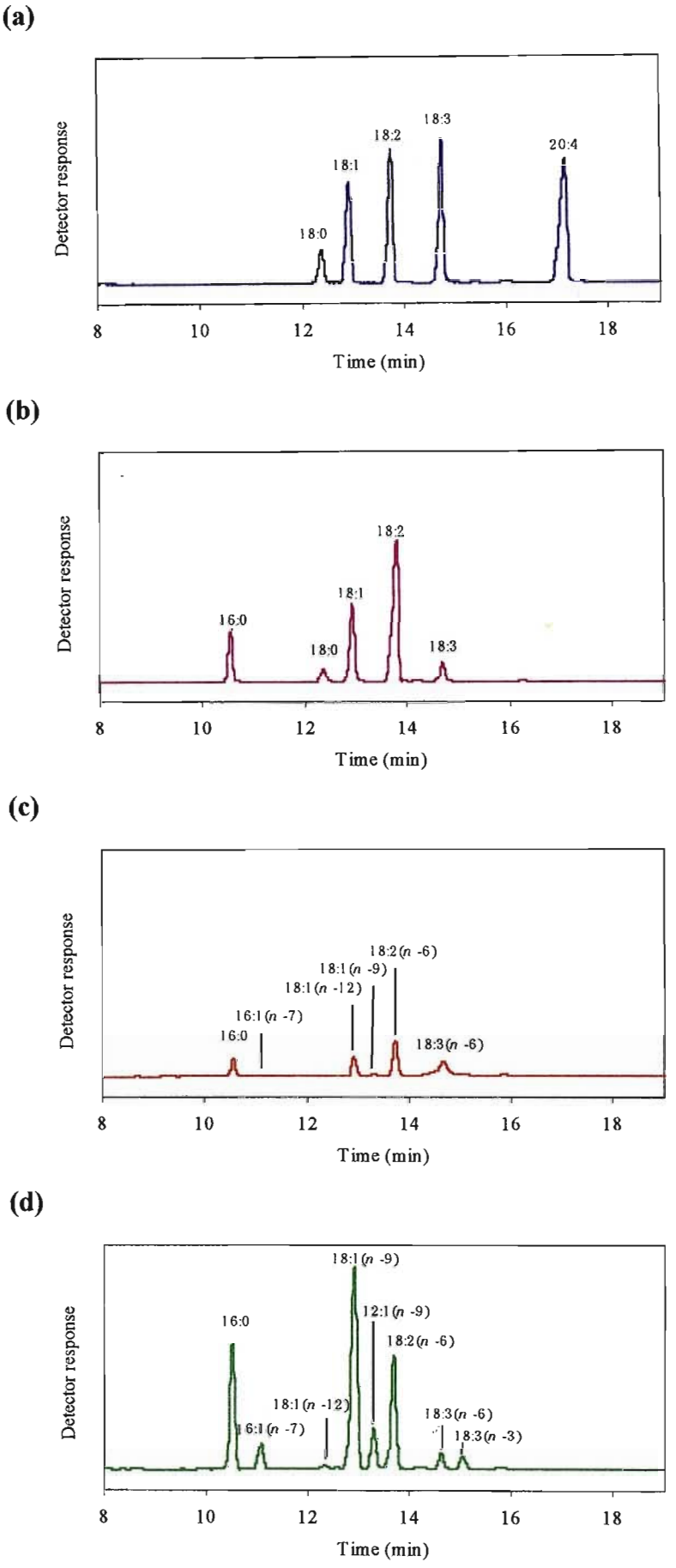


Figure 2.9 Representative GC chromatograms of (**a**) authentic fatty acid standards, and peaks obtained from samples of (**b**) soybean, (**c**) seed, and (**d**) mesocarp tissue.

2.12.3 QUANTIFICATION OF PHENOLIC-CONTAINING POLYMERS

The dark substance in the seed coat was found to exhibit some properties of phenolic-like compounds at both the light and electron microscope level. In an attempt to categorise the dark substance the seed coat was assayed for phenol-containing polymers.

2.12.3.1 Phenol Quantification

Soluble phenols were assayed after the methods of Osborne (1992). 0.01 g freeze-dried tissue was homogenised in 4 mL chloroform:hexane (1:1), and the tubes then sealed and shaken for 2 h. The solution was then centrifuged at 2 500*g* for 10 min, the supernatant discarded and 4 mL 100% methanol added to the pellet. The pellet was shaken in methanol for 2 h, filtered through Whatman GF/A filter paper and used to assay for soluble phenols.

An attempt was made to assay insoluble phenols by incubating the insoluble residue collected on the filter paper in 3 mL 1 M H_2SO_4 at 80°C for 12 h. The solution was then centrifuged at 1 000*g* for 10 min and the supernatant loaded onto an acidified PVP column. The column was washed three times with 5 mL water-NaOH (pH 8.0). The column was then washed twice with 3 mL 50% (v/v) acetonitrile adjusted to pH 5.0 with HCl, and the eluent collected, combined and used to assay for insoluble phenols.

20 μL of the filtrate (soluble phenols) or eluent (insoluble phenols) was then mixed with 1.5 mL water and 100 μL Folin-Ciocalteu's phenol reagent added, and, following mixing, the sample was allowed to stand for 8 min. 300 μL sodium carbonate, followed by 380 μL water were added, and the solution incubated at 50°C for 2 h. The absorbance was measured at 765 nm and compared to a gallic acid standard curve, which was linear between 0 and 400 μg mL^{-1}. Results were expressed as gallic acid-equivalents.

The acidified PVP column was prepared by washing 3 mL PVP three times with 0.1 M KH_2PO_4 adjusted to pH 5.0 with HCl.

2.12.3.2 Anthocyanin quantification

The reported anthocyanin pigments in avocado are cyanidin 3-galactoside and cyanidin 3,5-diglucoside acylated with ρ-coumaric acid (Prabha *et al.* 1980). Cyanidin has an average absorbance of 535 nm (Toldam-Anderson and Hansen 1997) and there is no anthocyanin absorbance above 650 nm (Lange *et al.* 1971), thus these wavelengths were used to quantify the apparent main anthocyanin in avocado, cyanidin. A molar extinction of 28800 and molecular weight of 595.2 (Wrolstad 1976) were used to calculate relative cyanidin amounts.

Anthocyanins were quantified after the methods of Lange *et al.* (1971). 0.05 g freeze-dried tissue was homogenised in 10 mL propanol:HCl:water (18:1:81) and immersed in boiling water for 1½ min, stood in the dark for 24 h, centrifuged at 5 000*g* for 40 min, the absorbance read at 535 nm and 650 nm and actual absorbance calculated using Raleigh's formula:

$$A_{535} = A_{535} - 2.2A_{650}$$

This method of analysis was confirmed using the methods of Cheng and Breen (1991). 0.05 g freeze-dried tissue was homogenised in 10 mL 0.1% (w/v) HCl (pH 0.2), centrifuged at 5 000*g* for 40 min and the supernatant divided into two 4 mL portions. The pH was adjusted by the addition of 0.1 M sodium acetate buffer at either pH 4.5 or pH 1.0 and the absorbance determined at 535 nm and 650 nm. The actual absorbance of the anthocyanins was calculated by the equation;

$$A = \left[\left(A_{535} - A_{650}\right)_{pH1.0} - \left(A_{535} - A_{650}\right)_{pH4.5}\right]$$

Since anthocyanin quantities were found to be very similar using either method (data not presented), the former, simpler method was used in subsequent determinations.

2.12.3.3 Tannin quantification

Bussotti *et al.* (1998) were able to observe an increase in phenolics, identified primarily as tannins, in the leaves of beech trees. The accumulation of these tannins in the vacuole and in droplets along the tonoplast resemble very closely the occurrence of the electron dense deposits observed in the seed coat of small 'Hass' fruit.

Condensed tannins were quantified after the methods of Resende *et al.* (1996), under low light intensity to avoid possible photodegradation of tannins. 0.05 g freeze-dried tissue was homogenised in 5 mL propanol:HCl:water (18:1:81) and centrifuged at 10 000*g* for 15 min. The supernatant was discarded and the pellet resuspended in 2 mL 2 M HCL, covered with aluminium foil and incubated at 95 °C for 1 h. Thereafter, 2 mL n-butanol was added and the extract incubated at 95 °C for 1 h. A fraction of the upper phase (butanolic phase) was collected and centrifuged at 3 000*g* for 5 min. The condensed tannin (procyanidin) content was determined by estimating the amount of cyanidin in the supernatant at 545 nm. The absorption coefficient of 150×10^3 for an average procyanidin was used to calculate the tannin concentration, using the formula;

$$\text{Tannin (mg g}^{-1}\text{ DW)} = (A_{545} \times \text{dilution}) \div (150 \times 10^3)$$

2.12.4 PROTEINS

2.12.4.1 Bradford's method for protein determination

For all enzyme assays, activity was expressed per mg protein. Protein concentration was determined colorimetrically after the method of Bradford (1976). 100 μL enzyme extract was mixed with 5 mL Bradford's solution and left to react for 5 min. The absorbance was determined at 595 nm and protein concentration interpolated from a standard curve of 0, 20, 40, 60, 80 and 100 μg BSA per 100 μL distilled water.

Bradford's solution was prepared by mixing 500 mg brilliant blue G with 250 mL 95% (v/v) ethanol and 500 mL 85% phosphoric acid. This solution was made up to 1 L, stirred for 24 h in the dark at 4 °C, and filtered through Whatman No. 1 filter paper under vacuum. The solution was stored in an amber bottle at 4 °C, and was stable for up to six months.

2.12.4.2 Gel electrophoresis

Membrane-rich fractions purified as described in 2.10.4.2 were used in an effort to identify membrane bound proteins in mesocarp tissue. Membrane fractions were thawed on ice and mixed with an equal volume of 3 M Tris-HCl buffer (pH 8.45) containing 4% (m/v) SDS and 20% (v/v) glycerol for non-reduced proteins, or an additional 10% (v/v) 2-mercaptoethanol for reduced proteins. To this solution 10 μL 0.1% (w/v) bromophenol blue was added as a marker dye and the solution boiled for 4 min. Samples were held on ice until loaded.

Polypeptides were separated in either a MGV-202 (CBS Scientific Co., Del Mar, U.S.A.) or a Mighty Small II SE 250 (Hoefer Scientific Instruments, San Franscisco, U.S.A.) vertical slab electrophoresis unit by SDS-PAGE using the tricine system, with 1 mm thick 5, 7.5 and 10 % polyacrylamide gels, or a gradient gel from 2% to 15% acrylamide. Approximately 10 μg protein was loaded per lane. Premixed low-range molecular weight markers (Boehringer, Germany) were phosphorylase B (97.4 kD), BSA (66.2 kD), aldolase (39.2 kD), triose phosphate isomerase (26.6 kD), trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD).

The gels were fixed overnight with 50% (v/v) methanol, 12% (v/v) acetic acid and 0.5% (v/v) formaldehyde and the proteins stained with silver (Appendix A.9).

The best separation of the bands was obtained with an acrylamide concentration of 7.5%, but the best resolution was at 10%. Gradient gels did not show superior separation. Gels were prepared as described in Appendix A.8.

CHAPTER 3

THE SOLUTE PATH IN DEVELOPING 'HASS' AVOCADO FRUIT

3.1 INTRODUCTION

Botanically, the avocado is a berry comprising of a single carpel and a single seed. The pericarp constitutes the majority of the carpel, and consists of the exocarp (rind), mesocarp (flesh) and a thin layer of cells adjacent to the seed coat, the endocarp. The exocarp constitutes the peel and the endocarp, which consists of a few layers of parenchyma cells, adheres to the seed coat. The mesocarp is the edible, oil accumulating portion of the avocado and is composed of largely uniform isodiametric parenchyma cells that are about 60 μm in diameter in mature fruit (Biale and Young 1971) (Figures 3.1a, 3.1b, 3.1c). The seed (embryo) develops from a single anatropous ovule (Tomer and Gottreich 1976) and consists of two fleshy cotyledons enclosing the embryonic axis, which ultimately differentiates into a plumule, hypocotyl and radicle. Starch is the main storage material in the large cotyledons (Figure 3.1d).

Initially, the young embryo is embedded in a yellow gelatinous endosperm (Blumenfeld and Gazit 1971)(Figure 3.1e) which disappears during the course of development (about 3 months after fruit set). Surrounding the seed and delimiting the endocarp is the seed coat. The seed coat consists of two cell layers that develop from the integuments, which envelop the embryo sac in the young ovule (Schroeder 1958; Blumenfeld and Gazit 1971). The majority of the mature seed coat is traversed by an extensive network of vascular tissue (Blumenfeld and Gazit 1974) (Figure 3.1f). The vascularised part of the seed coat represents the pachychalaza and arises from the basipetal, intercalary growth of the chalazal region below the place of attachment of the integuments. At the base of the seed coat is a small, colourless, unvascularised portion (Figure 3.1a) which represents the testa (Steyn *et al.* 1993).

Several studies have shown that, after the pedicel, the vasculature branches into well-developed strands that ramify through the entire mesocarp (Cummings and Schroeder 1942; Blumenfeld and Gazit 1971; Kaiser 1993; Moore-Gordon 1997). Subsequently the vascular tissue appears to converge, forming a single bundle that enters the seed coat at the testa. The vessels then again branch extensively into the pachychalaza. Some vascular tissue has been suggested to end blindly in the mesocarp (Cummings and Schroeder 1942), an observation refuted by Blumenfeld and Gazit

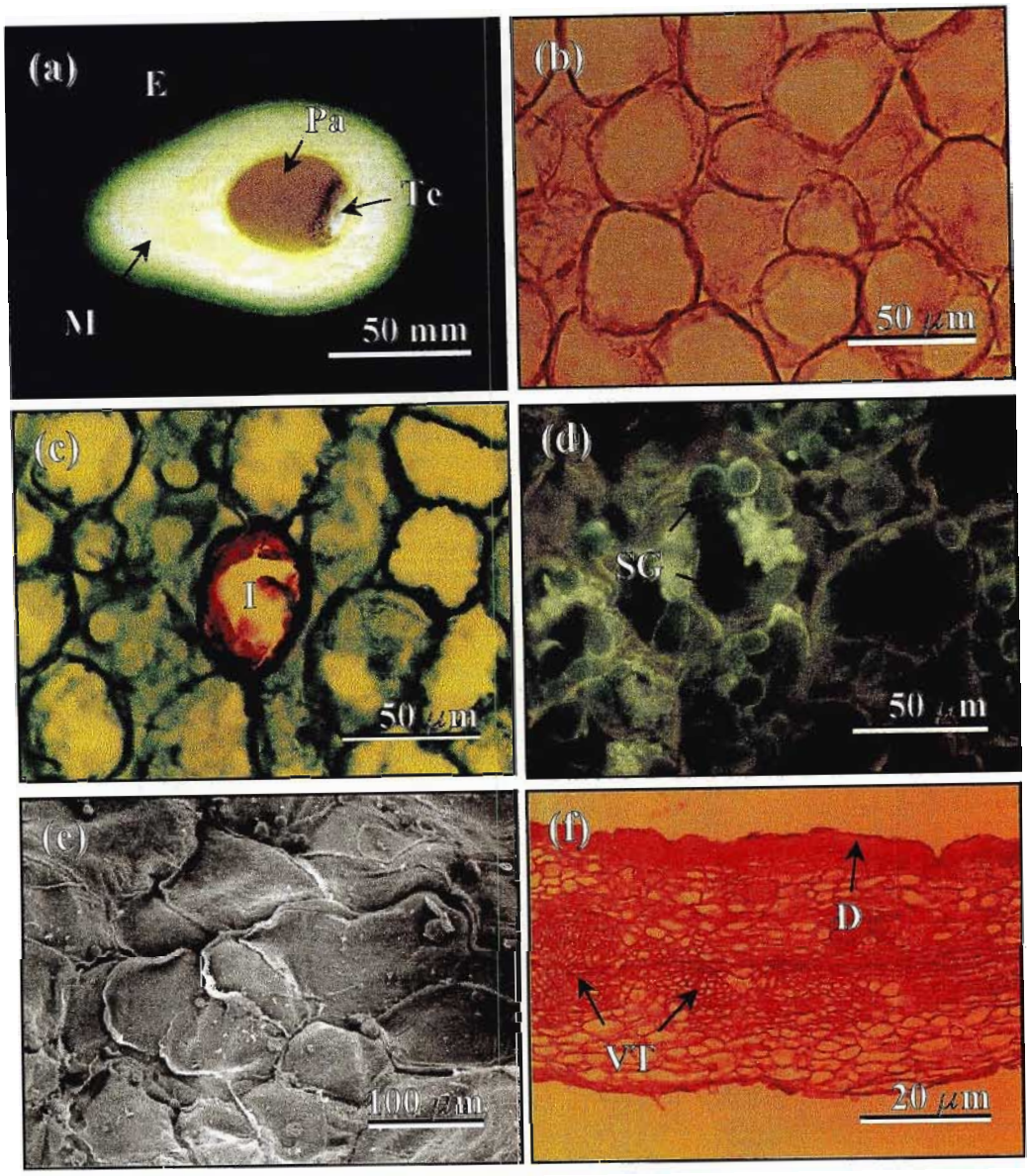


Figure 3.1 Light and SEM micrographs of 'Hass' fruit tissue. (**a**) Longitudinal section of a 'Hass' avocado fruit (180 DAFB) showing a well-developed seed coat with distinct vascularisation in the pachychalaza (Pa) and a white basal testal region (Te). M = mesocarp; E = exocarp. This was representative of more than 500 fruit examined over 4 seasons of cultivation. (**b**) Large isodiametric cells from mesocarp tissue, stained with ruthenium red; (**c**) Modified, lignified idioblastic cell (I) in mesocarp tissue that serve to store oil at maturity and increase in size with maturation, stained with safranin red and counter stained with fast green. (**d**) Seed cotyledon tissue with numerous starch grains (SG), stained with aniline blue in lactophenol and viewed under UV. (**e**) Large, loosely packed cells from the endosperm tissue. (**f**) Cross section of the pachychalazal portion of the seed coat stained with ruthenium red showing numerous vascular bundles (VT) and an amorphous layer (D) located on the inner (seed) side. Micrographs are representative of tissue (26-203 DAFB) prepared for light microscopy collected from 38 fruit and SEM from 24 fruit, over 3 seasons, respectively.

1971). Thinner bundles also extend to, and terminate within, various parts of the exocarp (Biale and Young 1971). During early fruit development, the ventral carpel vascular trace enters via the funiculus, branches and enters the chalaza region where an extensive vascular network is formed that does not enter the integuments (Steyn *et al.* 1993). This symplastic isolation of the embryo is maintained during development with the very branched vascular system in the seed coat having no direct connection with the vascular system of the embryo (Blumenfeld and Gazit 1971). It is generally accepted that there is little symplastic connection between filial and maternal tissues and that vascular tissues terminate in the maternal tissue and photoassimilate must move apoplastically to developing zygotic tissue (Thorne 1985). Unloading of phloem for seed development occurs in the testa of dicotyledonous plants or the placental-chalazal tissue or pericarp of monocotyledonous plants (Brenner and Cheikh 1995). Thus it is believed that the pachychalaza with its extensive vascular system supplies photoassimilate, mineral nutrients and water to the enlarging seed (Steyn *et al.* 1993) and that the seed coat is the only route of exchange between the developing embryo and the rest of the fruit and tree (Blumenfeld and Gazit 1971).

The term solute allocation refers to the distribution of assimilates from source tissue to sink tissue. Sink strength is determined by sink size, metabolic activity, and proximity to the source and other competing sinks (Zeevaart *et al.* 1979; Daie 1985; Finazza and Davenport 1987; Ho 1988; Ho 1996). In tomato, sink strength seems to be related to the route of sugar transport into cells during fruit development (Ho 1996). Thus fruit size may be limited by the nature of the solute path rather than sink metabolism and the supply of assimilate (source strength). The ability of the sink to import solutes, and hence solute allocation and tissue growth may, therefore, be controlled by altering the route of solute import. The programmed deterioration and/or occlusion/isolation of conducting tissues, such as phloem, the seed coat and Pd, will limit solute movement into an organ/tissue, alter the route of solute import and allow for switches between symplastic and apoplastic solute movement. Any such limitation might be expected to cause a reduction in the supply and/or availability of the basic units of metabolism and will ultimately be manifested as a reduction in fruit size, or may permit the accumulation of solutes against a concentration gradient and herald the onset of a different physiological state.

This chapter examines the path of solute flow in developing avocado fruit in an effort to identify potential barriers to symplastic and apoplastic solute transport that might impact on final fruit size. Emphasis is placed on the seed coat as it is believed to function as the major conduit of photoassimilate supply to the developing fruit (Cowan *et al.* 2001).

3.2 RESULTS

3.2.1 FRUIT GROWTH

To study the 'architecture' of the solute path in developing fruit it was necessary to establish that the component tissues are actively growing at a constant rate. The results in Figure 3.2 illustrate the progressive increase in seed and mesocarp tissue measured on a dry mass (DM) basis between 66 and 120 DAFB. The endosperm and seed coat tissue were the exceptions and showed a decrease in DM during the same period. The increase in mesocarp DM correlated with the rapid (linear) growth phase (phase II) (Schroeder 1958; Valmayor 1967).

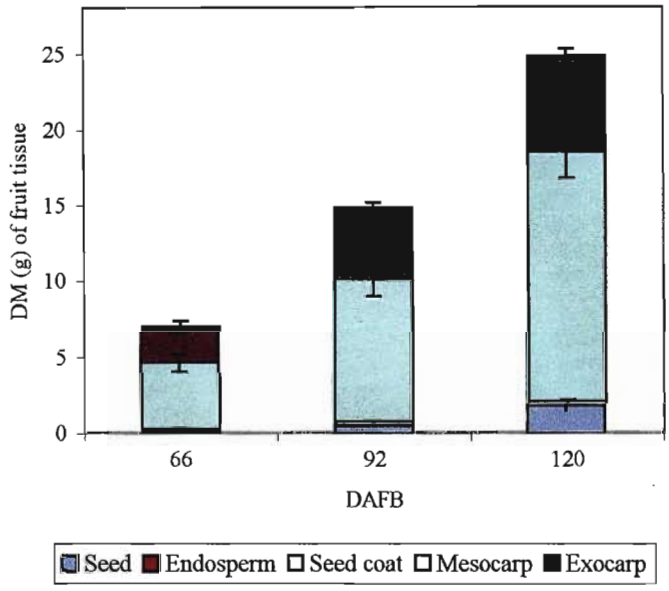


Figure 3.2 Change in tissue DM during the linear phase of rapid growth of 'Hass' fruit development (66-120 DAFB). n = 10. Error bars represent the standard deviation (SD). $LSD_{0.05}$ = 3.98.

3.2.2 DISTRIBUTION OF [^{14}C] IN DEVELOPING FRUIT AFTER APPLICATION OF [^{14}C]-SUCROSE

The distribution of [^{14}C], derived from [^{14}C]-Suc applied to intact fruit is shown in Figure 3.3. Label accumulated in the base of the fruit where the vasculature coalesces and enters the seed coat, and from where it distributes to the seed through the endosperm and into or through the testa to the embryo.

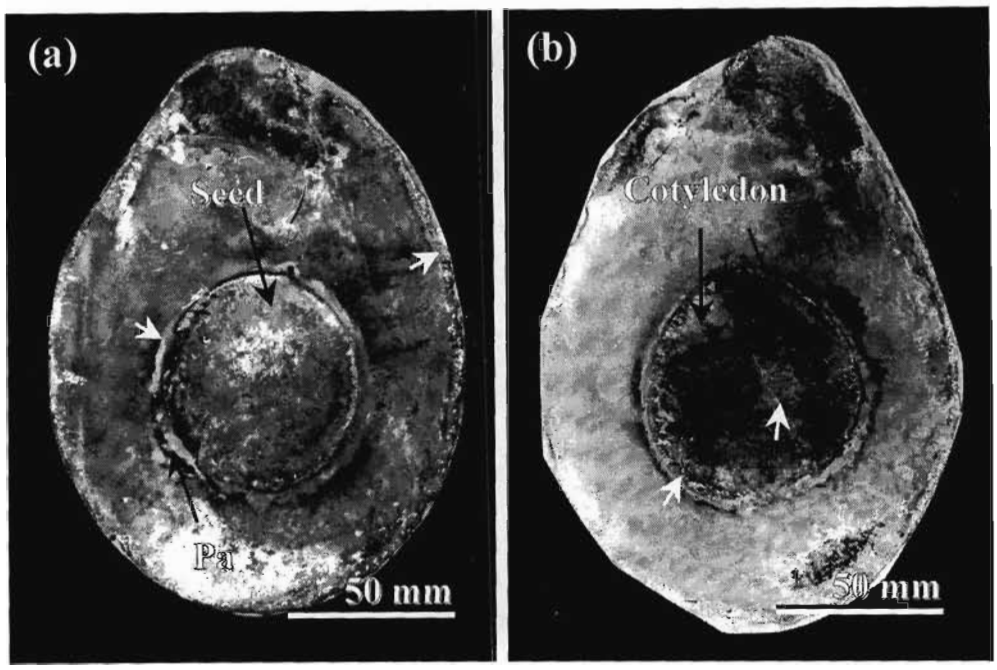
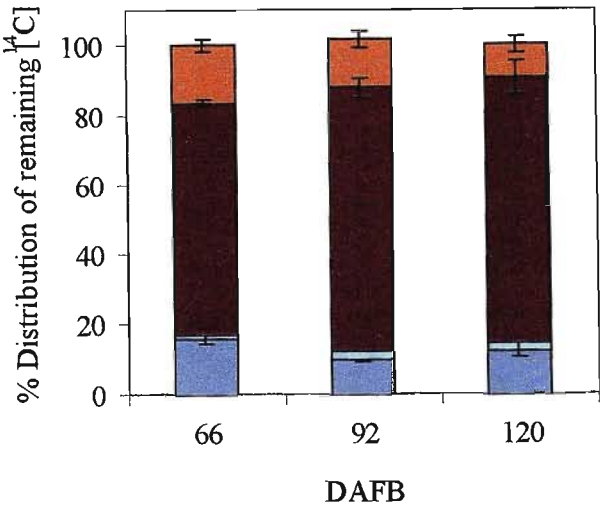


Figure 3.3 Autoradiographs showing the allocation of [^{14}C]-label in normal 'Hass' fruit harvested and treated 200 DAFB. Radioactivity is represented by regions of lighter colour (highlighted by white arrows); (**a**) [^{14}C] distribution throughout the fruit, present largely in the basal regions of the mesocarp, the exocarp and the pachychalazal (Pa) regions of the seed coat; (**b**) [^{14}C]-label distribution in the seed showing allocation of label to the cotyledons, the seed coat and between the two cotyledons. Images are representative of 20 fruit from two seasons.

Application of [^{14}C]-label via the pedicel allowed for the determination of the primary route of assimilate movement in the fruit and assimilate distribution to the various tissues. In untreated, normal 'Hass' fruit, label was predominately located in the mesocarp, irrespective of age of fruit and length of incubation (Figure 3.4). Of particular interest was that, as the fruit developed, less [^{14}C]-label remained in the pedicel or was allocated to the exocarp, and more label was observed in the seed and mesocarp. Though not illustrated in Figure 3.4, the relative amount of label in the seed coat decreased over the 12 h incubation period, whilst that in the seed showed a concurrent and significant ($P \leq 0.01$) increase.

(**a**) 12 h after treatment



(**b**) 24 h after treatment

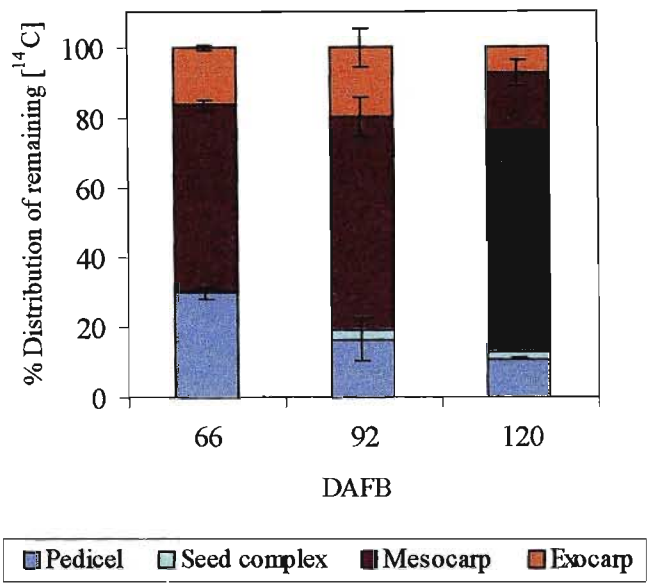


Figure 3.4 Percentage [^{14}C]-label (remaining in the fruit) distribution in normal 'Hass' fruit (**a**) 12 h; and (**b**) 24 h after pulsing with [^{14}C]-Suc. The seed complex in the legend refers to the seed, endosperm and seed coat tissue combined. $n = 3$. Error bars represent SD.

3.2.3 ULTRASTRUCTURE OF THE SOLUTE PATH IN DEVELOPING FRUIT

Moore-Gordon (1997) presented data to show that pedicel-fed eosin, a symplastically transported dye, remained in the vasculature of avocado fruit, suggesting the absence of lateral symplastic unloading from the phloem. The well-defined vascular pathway thus suggests that solute

movement into both the seed and the mesocarp must occur via the seed coat (Cummings and Schroeder 1942; Blumenfeld and Gazit 1971; Kaiser 1993; Moore-Gordon 1997). In an attempt to confirm and extend our understanding of the major solute pathway in avocado fruit ultrastructure, vasculature, and Pd structure-function were examined in detail.

3.2.3.1 Seed coat ultrastructure in developing 'Hass' fruit

Normally developing fruit were observed to have two distinct cell type layers in the pachychalazal region of the seed coat that were often separated by extensive vasculature. The inner (seed side) layer consisted of small, flattened cells containing numerous organelles including amyloplasts, mitochondria (Mi) and endoplasmic reticulum (ER) (Figure 3.5a). These cells also had limited vacuolation. The outer (mesocarp) layer consisted of larger, rounded cells containing fewer cytoplasmic organelles, with large vacuoles (Figure 3.5b) and numerous Mi (Table 3.1). These structural differences suggest there may be differences in function between the two layers.

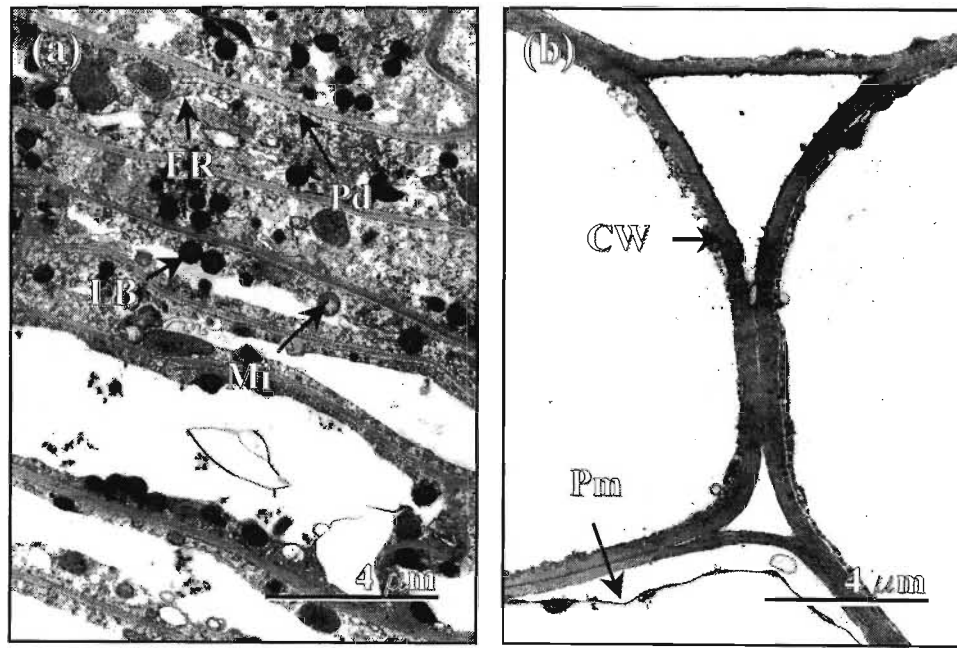


Figure 3.5 Electron micrographs showing examples of cells from the (**a**) inner and (**b**) outer cell layers observed in 'Hass' pachychalazal tissues. CW = Cell wall; ER = endoplasmic reticulum; LB = lipid body; Mi = mitochondria; Pd = plasmodesmata; Pm = plasma membrane. Images are representative of observations made in 12 fruit over two seasons.

The intact endosperm tissue (66 DAFB) consisted of large, loosely packed thin-walled cells (Figure 3.1e). Fixation of isolated endosperm tissue in glutaraldehyde resulted in the exudation of white crystalline material. Subsequent dehydration and critical point drying revealed the possibility of small pores located in the cell walls of the cells on the outer extremities of the endosperm. These pores had crystalline deposits surrounding them (Figure 3.6a). During maturation the endosperm appeared to dehydrate, showed a significant decrease in FW and was closely appressed against the seed coat forming a flattened, apparently dead layer that adheres firmly to the inner seed coat (Figure 3.6b), thereby forming a possible physical barrier to subsequent seed coat – seed solute transport.

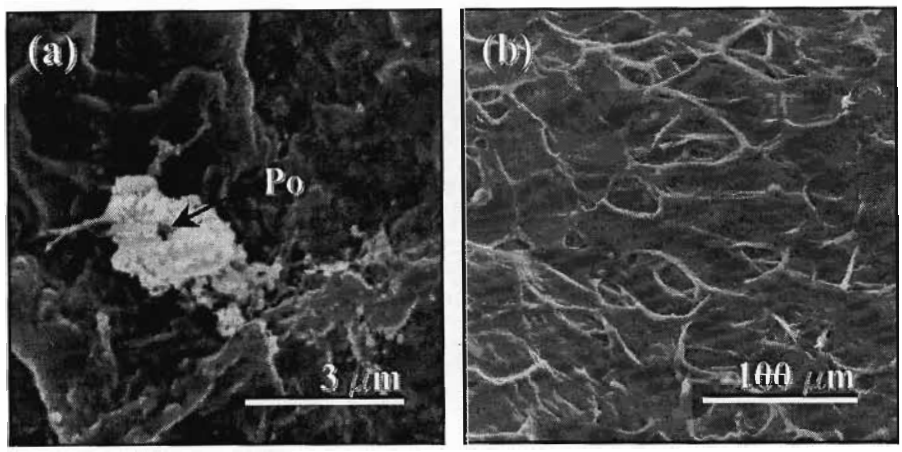


Figure 3.6 SEM micrographs of endosperm tissue from 'Hass' avocado fruit showing (**a**) crystalline deposits surrounding a pore-like structure (Po) located in the outer cells of the endosperm tissue, and (**b**) the dehydrated flattened endosperm tissue adhering to the inner seed coat of older, more mature fruit. Images are representative of tissue collected from 3 fruit at different stages of development.

3.2.3.2 Symplastic route of solute flow ~ plasmodesmata in 'Hass' fruit tissue

In all fruit examined there were numerous single and branched Pd, usually localised in pit fields. There was no major structural difference between the Pd of the seed and mesocarp tissue. Seed coat Pd appeared to be more numerous (Table 3.1) and occurred in larger pit fields (Figure 3.7), with up to 11 Pd connections in a single pit field in the inner layer of the seed coat. Pd were frequently spanned with ER, which was highly branched and often joined in the centre of the cell wall by a common median cavity (MC) (Figure 3.7b; c; 3.8b). Pd were often observed to be associated with a highly convoluted plasma membrane (Pm) (Figure 3.7a) and organelles such as

Mi (Figure 3.8c). Pd were numerous in the inner layer of the seed coat (Table 3.1) and were more numerous in the seed than the mesocarp. Mi were numerous in cells from the inner layer of the pachychalaza and the mesocarp (Table 3.1).

In several cases small vesicle-like structures were observed between the CW and Pm in cells from mesocarp tissue (Figure 3.8d), in a lomasome-like invagination of the Pm into the cytoplasm. The identity of these structures is not known; but it is presumed that they are associated with CW formation, or are a consequence of tissue preparation for TEM.

Table 3.1 Pd, pit field and Mi frequency in cells of the seed, seed coat (pachychalazal portion) and mesocarp of normal 'Hass' fruit. The values represent the mean values of counted structures in a 60-80 nm thick section from 30 randomly selected cells taken from several sections from at least 2 different fruit. Single Pd were not regarded as coming from a pit field. (mean±SD).

Tissue	Layer orientation	Cellular Structure		
		Pd	Pit field	Mitochondria
Seed		8±1	3±2	4±1
Seed coat	Inner	17±6	3±2	12±4
	Outer	6±3	2±1	4±2
Mesocarp		5±3	3±1	14±7

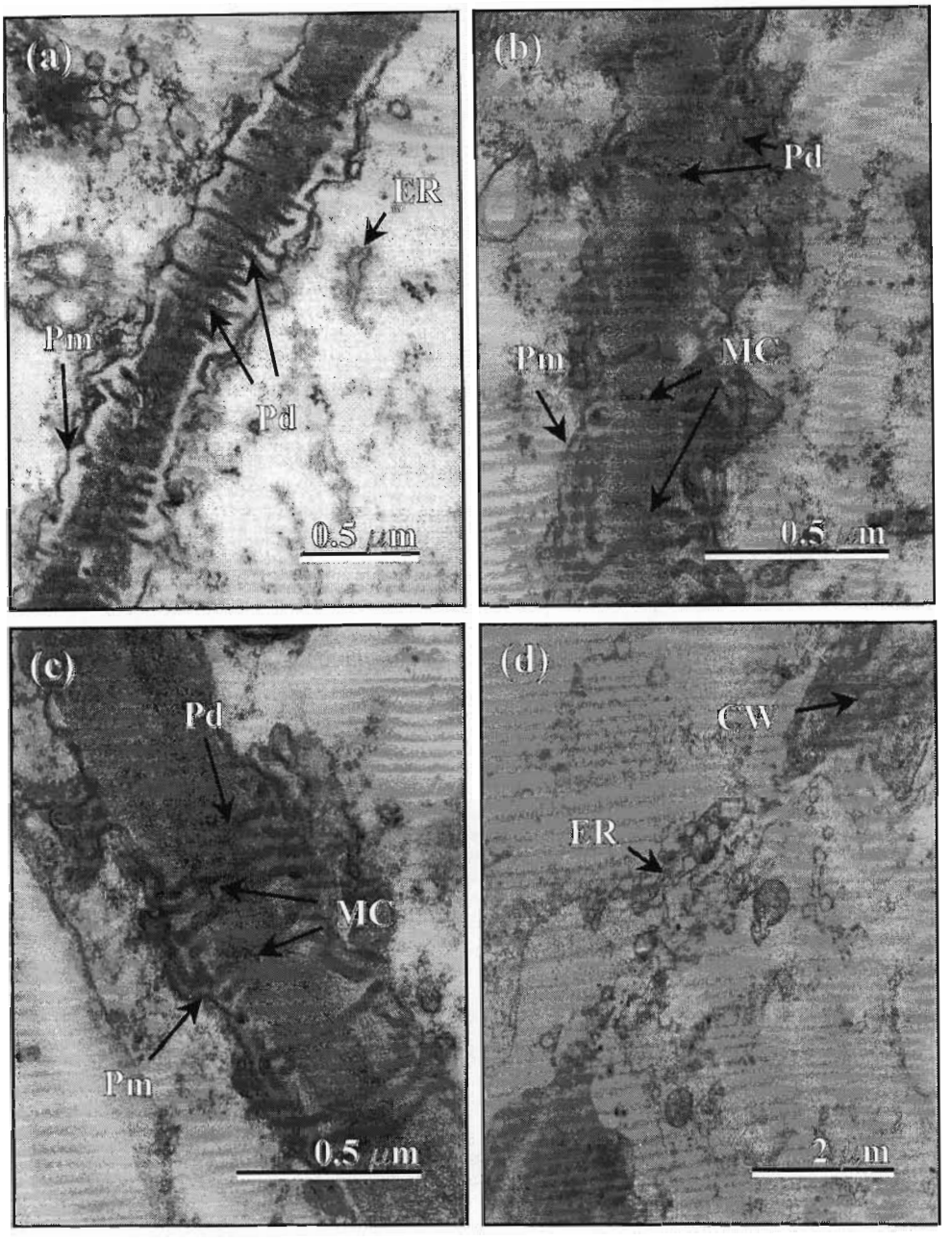


Figure 3.7 Pd in 'Hass' fruit: (**a**) numerous Pd in large pit field in the seed coat tissue, with a highly convoluted Pm in the pit field region; (**b** and **c**) Pd branching and fusing in the cell wall in MC; (**d**) thin non-uniform cell wall with large pit field with possible pore-like region in cell wall of endosperm tissue. Images are representative of observations made on 80 fruit over 3 seasons.

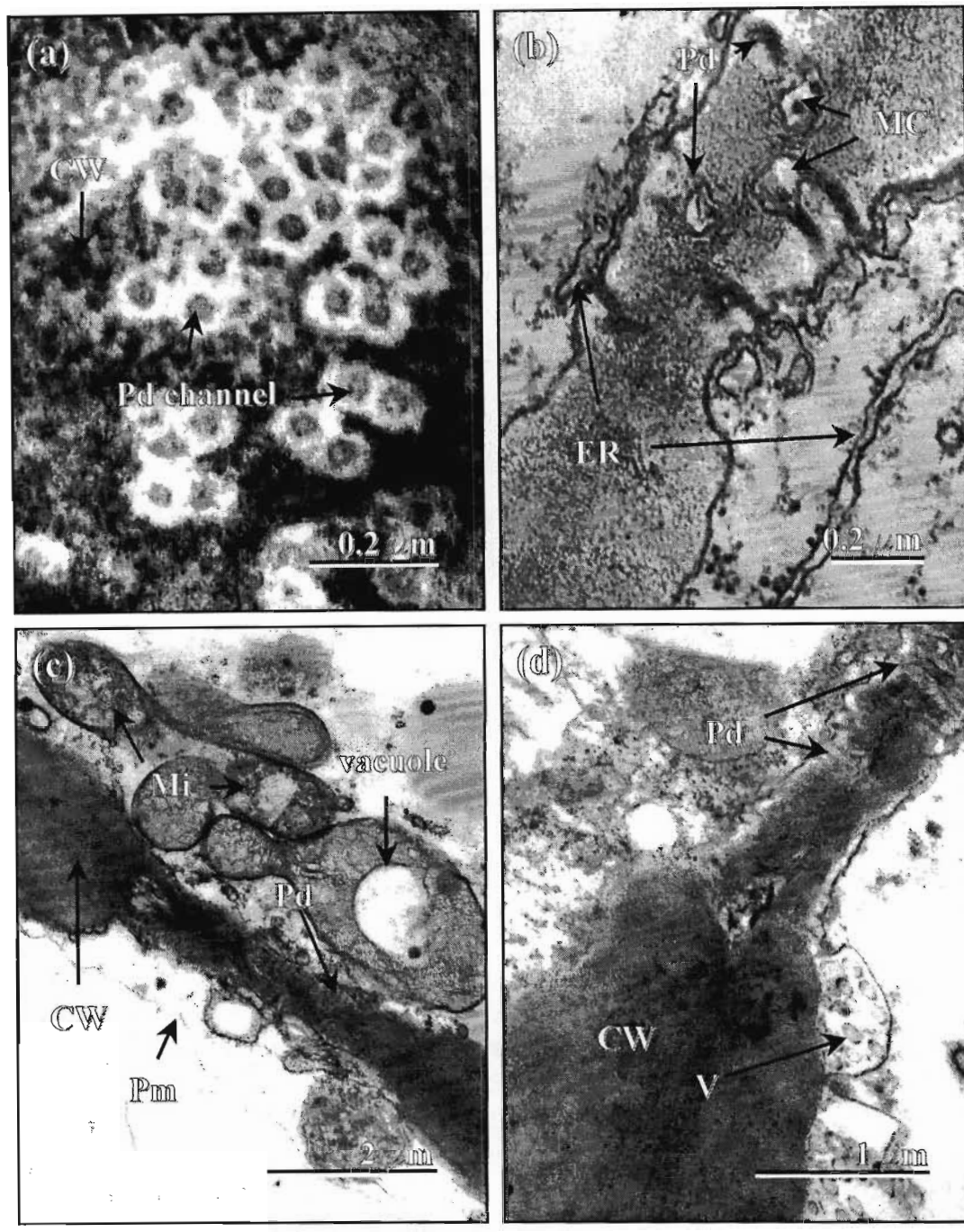


Figure 3.8 (**a**) Surface view of the Pd pit field; (**b**) ER associated with, and possibly spanning, Pd; (**c**) numerous organelles in close association with the pit field in the mesocarp, including an unidentified vacuole (vacuole) with protrusions extending to the Pd and Mi; and (**d**) unidentified vesicle-like structures (V) located between the CW and Pm. Images are representative of observations made on 80 fruit over 3 seasons.

3.2.3.3 Association of callose with the plasmodesmata

Callose is a structural polysaccharide consisting of spirally arranged glucose residues linked by β-(1→3) glycosidic bonds, which is frequently deposited in response to wounding or stress in plants. Callose has been localised to the Pd and/or the micro-domain that surrounds the Pd (Northcote *et al.* 1989; Turner *et al.* 1994; Kauss 1996; Enkerli *et al.* 1997; Roy *et al.* 1997), and has been suggested to play a role in the transient blocking/gating of Pd (Olesen and Robards 1990; Lucas *et al.* 1993). Such differential deposition of callose at the Pd could regulate assimilate movement and so control fruit morphogenesis, growth and maturation. In an attempt to determine whether callose was localised at Pd in avocado, and to elucidate a possible role for callose in Pd structure-function, the association of callose with the Pd was investigated during fruit development.

Limited callose association with the Pd was observed in seed tissue (Figure 3.9a) even though Pd were numerous (Table 3.1). On the other hand, a clear association of callose with the Pd was noted in the seed coat and mesocarp tissue (Figure 3.9b; c). The xylem tracheary elements (TE) (the dye components of aniline blue do not strictly bind to pure β-(1→3) glucans (Evans *et al.* 1984)) were also observed to fluoresce with aniline blue, showing distinctive spiral secondary thickening (Figure 3.9d) This observation is of interest as it allowed the visualisation of a ring of symplastically linked cells surrounding the vascular bundles (Figure 3.9e; f).

At the light microscope level, the precise association of callose with the Pd cannot be visualised. Callose is electron-lucent (Kauss 1996) and thus contrast staining (or immunolocalisation) must be used to observe (and the latter to positively correlate) any association of callose and Pd. Contrast staining revealed that callose is deposited around the Pd sleeve within the cell wall matrix (Figure 3.10). In some cases a ring-like structure can be seen surrounding the outer Pd (Figures 3.10b; c; d). Although this staining technique is adapted for the contrast staining of callose (Brander and Wattendorff 1987), slight variations in the technique allow silver methenamine to stain for sulphhydryl, disulphide and aldehyde groups (Hayat 1989). Thus it is possible, though unlikely, that these rings may be of proteinaceous and/or other polysaccharide origin.

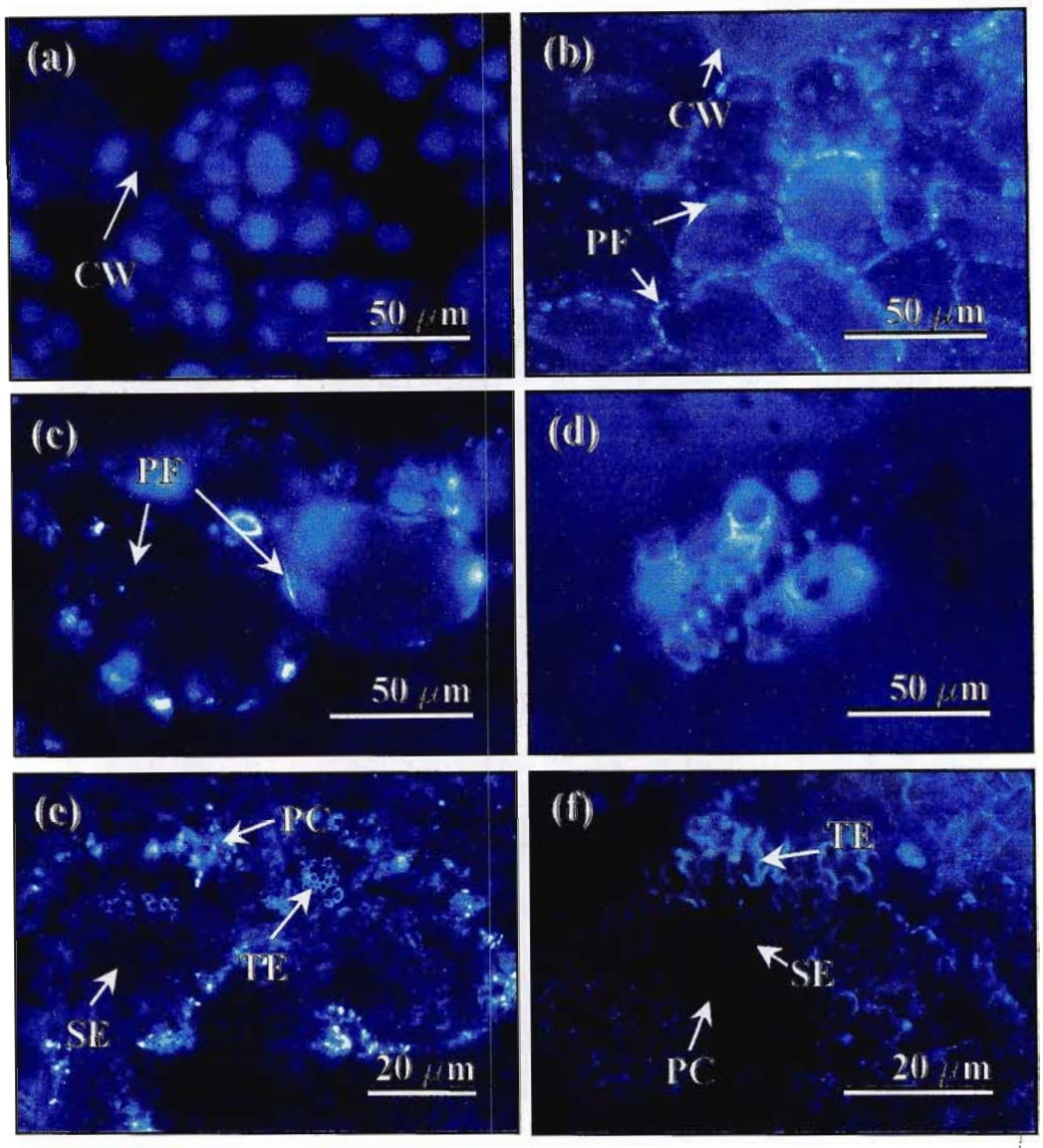


Figure 3.9 Light micrographs of (**a**) seed, (**b**) seed coat, (**c**) mesocarp, (**d**) xylem tracheary elements, and (**e** and **f**) vascular bundles in the mesocarp tissue. Sections stained with aniline blue and viewed under UV light. Callose fluoresces yellow-white under UV light and is localised to the Pd/pit fields (PF) in (**a**) seed coat and (**b**) mesocarp tissue. (**c**) In seed tissue there appears to be an absence of fluorescence in the cell wall. (**d**) The xylem cell wall shows a positive response to callose staining; and (**e** and **f**) there appears to be no florescence localised to the phloem, but a high degree of fluorescence in the cells (PC) immediately surrounding the vascular tissue. Micrographs represent data collected from 20 fruit, and 2 sampling intervals over 2 seasons.

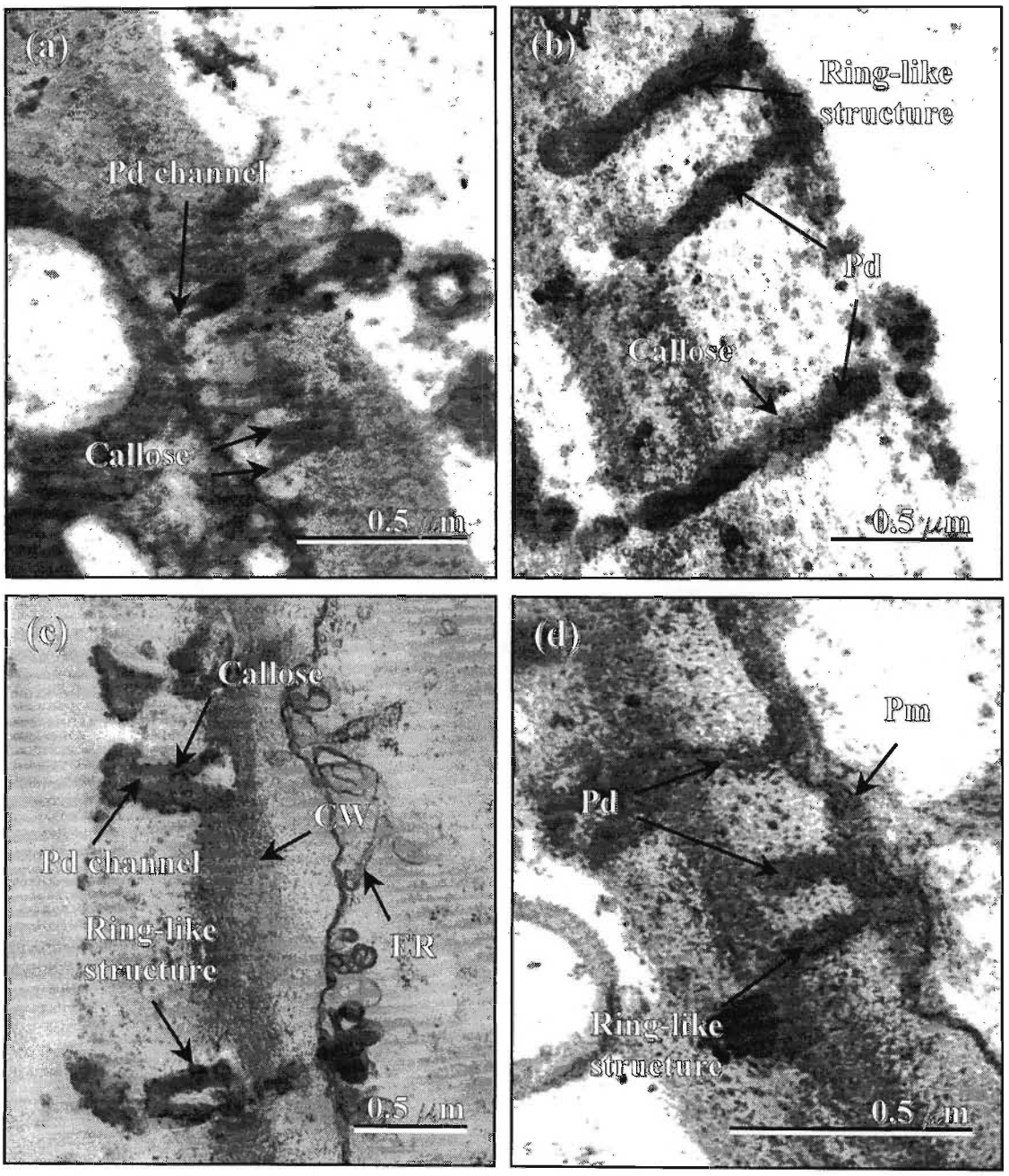


Figure 3.10 TEM micrograph of Pd from seed coat tissue of 'Hass' fruit stained with silver methenamine to contrast callose. Callose appears to surround the Pd channels (**a**, **b**, **c**, and **d**), often appearing to form ring-like structures (**b**, **c** and **d**). Micrographs are of representative images collected from 8 fruit.

3.2.4 FACTORS CONTRIBUTING TO THE REGULATION OF THE SOLUTE PATH IN DEVELOPING FRUIT

The darkening and the deposition of apparently structureless electron dense material within the cells of the seed coat accompanied normal fruit maturation, but was especially evident in injured, stressed and phenotypically small fruit. The deposition of this material within the seed coat is thought to play a role in limiting solute transport, both apoplastically and symplastically.

It was thought that this material might be phytomelan, a chemically inert (Dafert and Miklauz 1912) cell wall polysaccharide that has been observed to be deposited in the seed coats of *Gasteria verrucosa* (Wittich and Graven 1998). Phytomelan is composed of spherical 15 nm units and is believed to be formed from Glu monomers and small polymers resulting from callose breakdown (Wittich and Graven 1998). Phytomelan is reported not to stain for light microscopy (Wittich and Graven 1995). In an attempt to localise phytomelan in the seed, seed coat and mesocarp tissue, an extensive staining protocol with periodic-Schiff's reagent, Congo red, Sudan IV, Ruthenium red, Amido black, Resorcinol blue, Aniline blue (aqueous and in lactophenol), Safranin, and fast green was undertaken (data not presented). Observations made during this study suggested that the electron dense material in the seed coat was not phytomelan.

However, staining with safranin suggested the seed coat was lined on both sides by cells encrusted with lignin (Figure 3.11a). Observation using UV light showed that these areas fluoresced and suggested the presence of phenolic (benzenoid) compounds (Figure 3.11b). It was thus thought that the material deposited in the seed coat was of phenolic origin and, therefore, soluble and insoluble phenols, anthocyanins and tannins were quantified in fruit tissue. Since all of these compounds are intrinsically linked via biosynthesis (acetate-malonate pathway, flavonoid biosynthetic pathway or the shikimic acid pathway) and are often associated with development, maturation and defence in the plant, it was thought that they may provide some indication about the dynamics of, and the inter-relationships of these compounds during, fruit growth.

Due to the osmiophilic nature of tannins, these polyphenols appear electron-opaque (Bussotti *et al.* 1998; and the references therein). The deposition of electron-opaque phenolic-like material in the seed coat, and the similarities in deposition patterns and appearance of these substances observed in this research and by Bussotti *et al.* (1998), suggested that the phenolic compounds deposited in the seed coat were tannins.

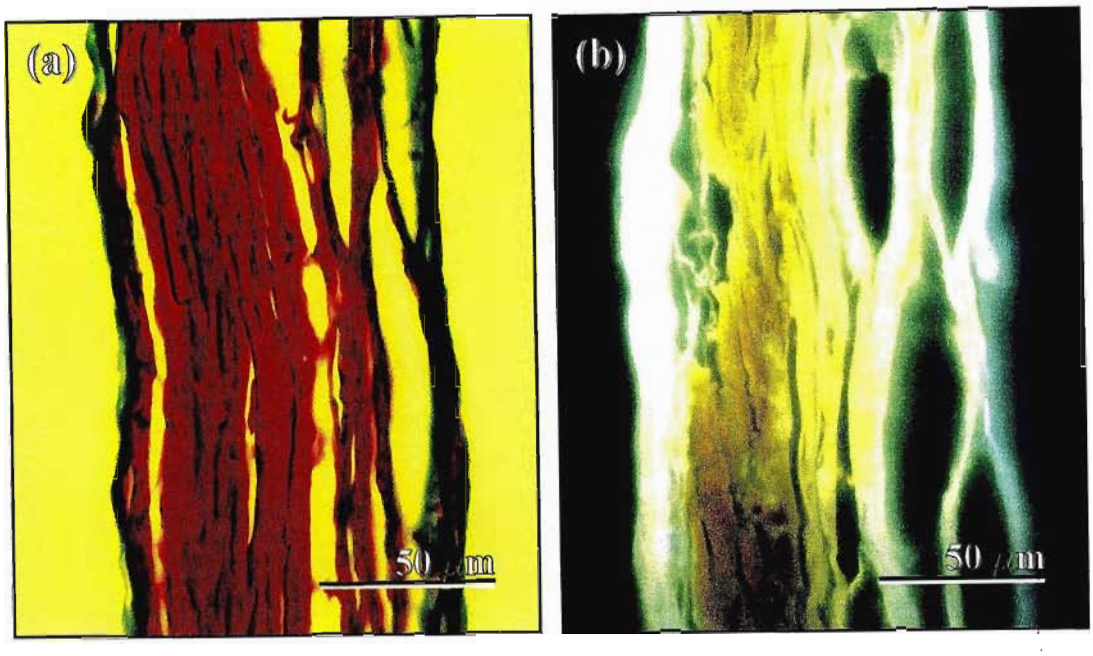


Figure 3.11 Light micrograph of a cross section of a degenerate seed coat showing (**a**) lignification on the inner region of the seed coat (lignin stains red with safranin), and (**b**) fluorescence of polyphenols in the outer layers of the seed coat under UV light. Images representative of a typical small seed coat observed in 12 fruit viewed under the light microscope.

Tannin deposition was initially observed as small droplets on the Pm, tonoplast and CW. These droplets gradually increased in size, became more numerous and ultimately merged, thereby occluding the entire cell (Figure 3.12). This type of deposition pattern was very prevalent in cells within the inner (seed facing) layer of the pachychalaza (Figure 3.13), forming an apparent barrier between the seed and seed coat. Such a formation can be expected to impact negatively on symplastic transport and together with lignification of the cell walls in the outer seed coat layer, on apoplastic transport as well.

High tannin concentrations were observed in the seed and seed coat tissues of 'Hass', constituting up to 11% DM in 120 DAFB seed coats (Figure 3.14). Tannin content increased in all tissues observed during development, and was most pronounced in seed and seed coat tissues (Figure 3.14).

Although the content of soluble phenols was high in the seed and the seed coat, it was very low in the mesocarp (Figure 3.15). A slight increase was observed in the soluble phenols of the seed over time, whereas a progressive decrease was seen in the endosperm and seed coat tissue.

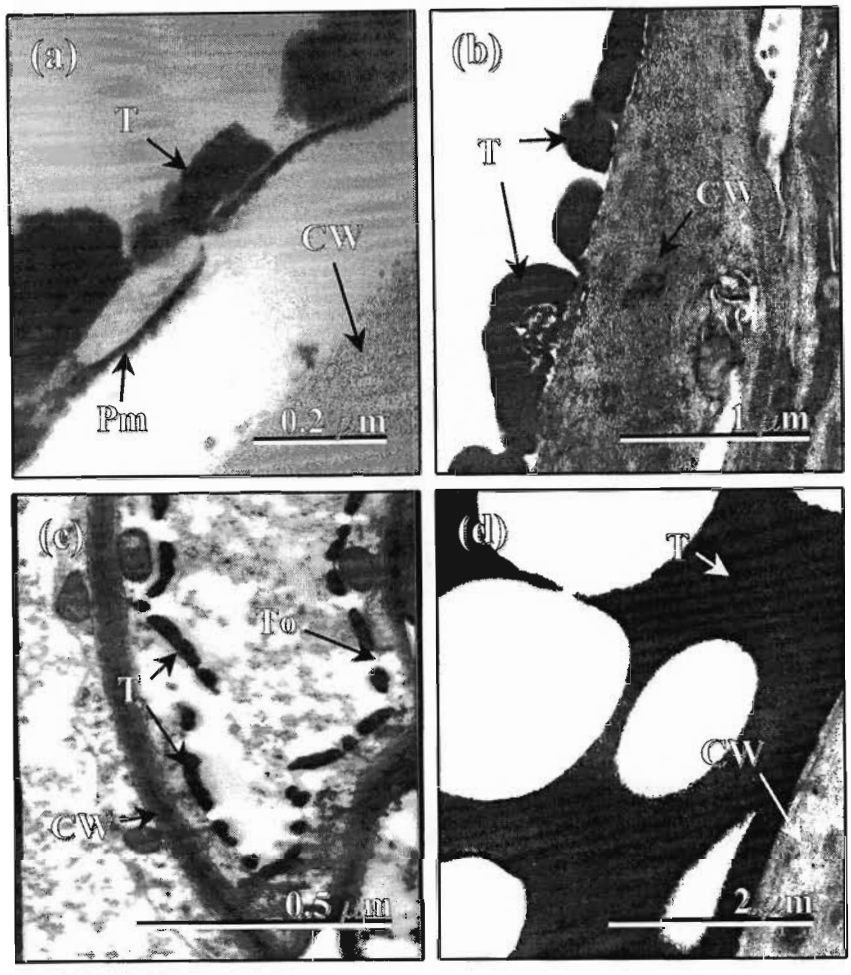


Figure 3.12 TEM micrographs showing patterns of tannin (T) deposition in the seed coat: (**a**) early tannin droplet accumulation on the Pm (90 DAFB), (**b**) on the cell wall (CW), (**c**) on inner-side of the tonoplast (To)(120 DAFB), and (**d**) ultimately occluding the whole cytoplasm (200 DAFB). Images are representative of observations made on 80 fruit over 3 seasons.

The insoluble phenol content was very low (in comparison to soluble phenols) in the seed, endosperm and seed coat tissues (Figure 3.16). However, insoluble phenol content increased significantly ($P \leq 0.01$) in endosperm and seed coat tissue, concomitant with the visible darkening of the seed coat and dehydration of the endosperm.

Anthocyanins were not detected in the mesocarp of 'Hass' avocado fruit (Figure 3.17), although the pink-coloured seed and darkly-coloured seed coat showed high levels of anthocyanins. In the seed the anthocyanin content did not change significantly with development. However, the endosperm and seed coat anthocyanin levels decreased substantially with fruit development.

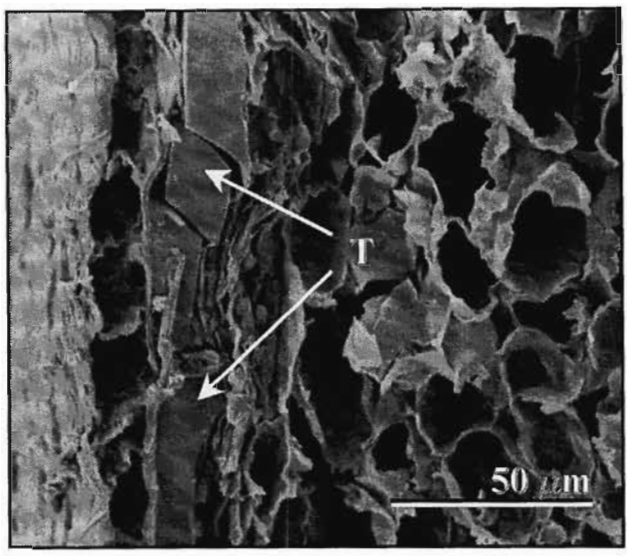


Figure 3.13 SEM micrograph of mature seed coat tissue with modified cells (endoblasts) thought to be totally filled with tannins (T) and thereby apparently forming an impermeable barrier in the inner (seed side) layer of the seed coat. The SEM image is representative of seed coat tissue observed from 12 fruit over 2 seasons.

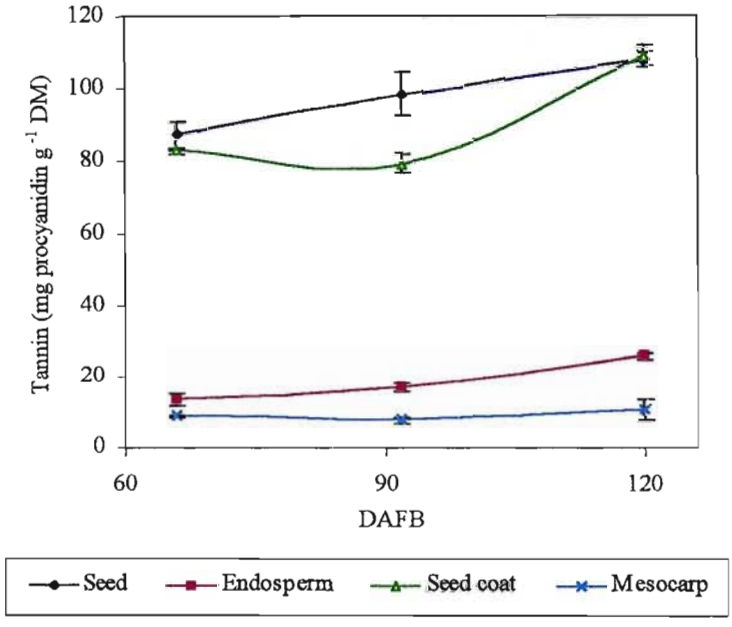


Figure 3.14 Tannin content in the seed, endosperm, seed coat and mesocarp tissue from developing 'Hass' fruit. n = 5. Error bars represent SD. $LSD_{0.05}$: Seed = 7.92; Endosperm = 4.54; Seed coat = 11.64; Mesocarp = 1.57.

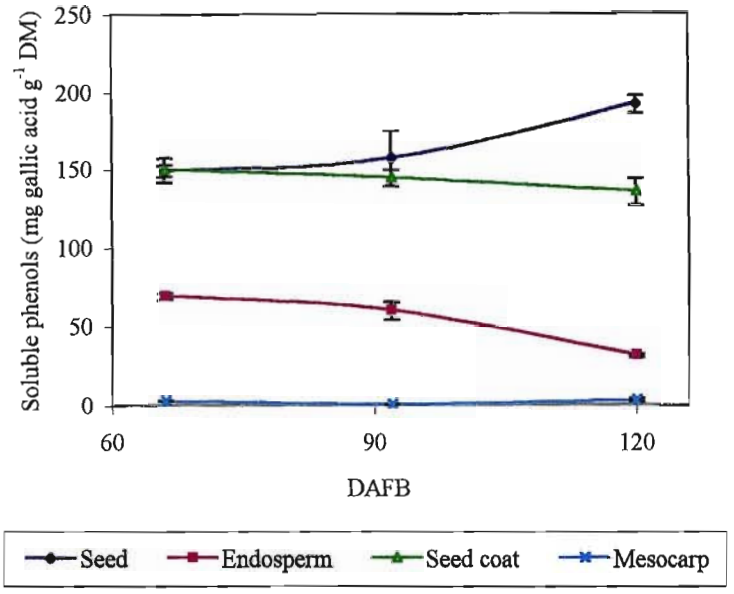


Figure 3.15 Soluble phenol content in the seed, endosperm, seed coat and mesocarp tissue from developing 'Hass' fruit. n = 5. Error bars represent SD. $LSD_{0.05}$: Seed = 20.86; Endosperm = 7.88; Seed coat = 6.74; Mesocarp = 1.37.

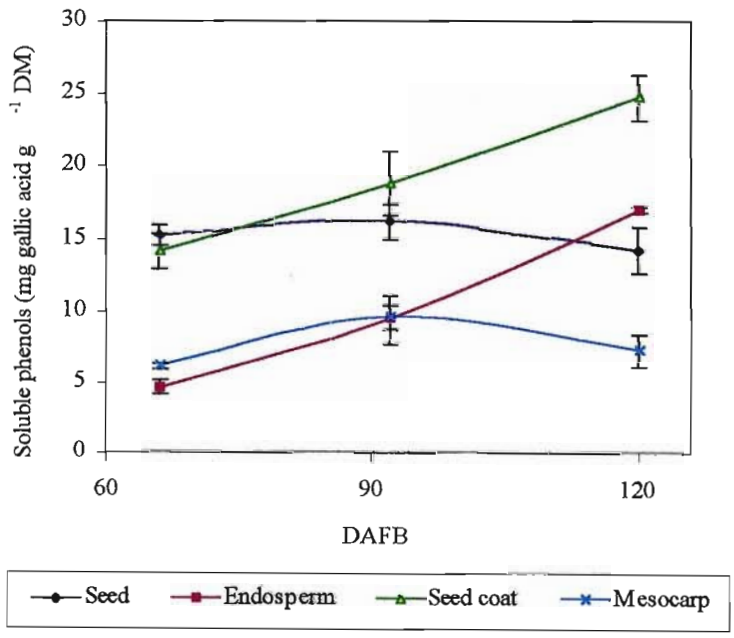


Figure 3.16 Insoluble phenol content in the seed, endosperm, seed coat and mesocarp tissue from developing 'Hass' fruit. n = 5. Error bars represent SD. $LSD_{0.05}$: Seed = 0.63; Endosperm = 2.46; Seed coat = 2.26; Mesocarp = 1.05.

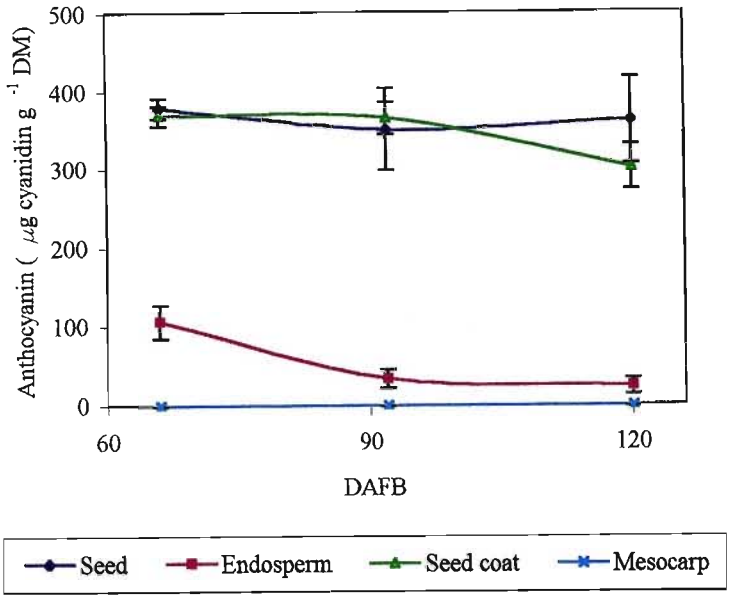


Figure 3.17 Anthocyanin content in seed, endosperm and seed coat tissue during early 'Hass' fruit development. Anthocyanins were not detected in mesocarp tissue. n = 5. $LSD_{0.05}$: Seed = 43.94; Endosperm = 65.15; Seed coat = 54.17; Mesocarp = ND.

3.3 SUMMARY

1. [^{14}C]-distribution studies suggested that solutes moved along the continuum: pedicel vasculature → mesocarp vasculature → mesocarp; or pedicel vasculature → mesocarp vasculature → seed coat → seed/mesocarp. The pachychalazal portion of the seed coat thus appears to assume primary importance in the supply of assimilates, via the endosperm in young fruit, to the seed.
2. The absence of aniline blue staining of callose in the phloem suggested that the phloem in 'Hass' fruit does not have callose associated with the Pd (or has few Pd). However, the high level of staining in surrounding tissues suggested high symplastic continuity in the tissues surrounding fruit vasculature and the possibility that the phloem is unloaded apoplastically along its entire length in the mesocarp tissue. Callose appeared to be localised to the cell wall matrix immediately surrounding the Pd sleeve, and did not appear to be localised within the Pd channel. This association suggested that callose deposition within the Pd itself does not play a role in the occlusion of Pd but, rather, that callose may function to stabilize the Pd or decrease the SEL by a sphincter-like mechanism.

3. Seed coat tissue from normal fruit consisted of two closely associated layers. The inner (seed side) layer has numerous Pd that were gathered in large pit fields, showed much branching, were associated with MC, and were often associated with a highly convoluted Pm. All of these can be considered as modifications that are believed to be associated with secondary Pd and an increased ability to traffic information molecules and/or permit enhanced symplastic fluxes between cells. Such modification can be expected to increase solute and molecule transport between cells and play an important role in the co-ordination of many physiological processes, including fruit growth.
4. The inner seed coat layer also appears to be the site of primary tannin and insoluble phenol deposition. Deposition of these polymers in the seed coat, together with lignification and the degeneration and dehydration of both the endosperm and seed coat, which accompany fruit growth, may form a possible apoplastic and symplastic barrier to solute flux from the seed coat into the seed. This phenomenon may be translated into a reduction in overall sink strength, solute flux into the fruit and, ultimately, fruit growth.

CHAPTER 4

CARBOHYDRATE METABOLISM IN DEVELOPING 'HASS' AVOCADO FRUIT

4.1 INTRODUCTION

Evergreen tree crops, such as citrus and avocado, frequently show a distinct pattern of carbohydrate cycling, in which carbohydrates are accumulated and stored in woody tissues during the quiescent period prior to flowering (Whiley *et al.* 1996; Goldschmidt 1999) and are subsequently mobilized to 'fuel' flowering, fruit set and early fruit growth and maintenance (Gonzalez-Ferrer *et al.* 1984; Scholefield *et al.* 1985). In these crops, a high yield season is often followed by low fruit yield, a phenomenon described as alternate-bearing, and attributed to low carbohydrate reserves in the tree (Goldschmidt and Golomb 1982; Monselise and Goldschmidt 1982; Van der Walt *et al.* 1993; Whiley *et al.* 1996). In 'on-years' there are a large number of smaller fruit, whilst in 'off-years' the fruit are larger but fewer (Paz-Vega 1997). Bearing-load, fruit size and abscission are considered to be strongly affected by the supply, and availability, of carbohydrates (Gonzalez-Carranza *et al.* 1998). This suggestion is supported by the positive effects of girdling and fruit thinning on fruit size (Cohen 1984; Guardiola 1988). Fruit size is determined by the accumulation of dry matter and water, the continued import of which is determined by both sink capacity, and supply of assimilates by the photosynthetic (source) tissues of the plant. In citrus, it is believed that sink strength, rather than assimilate supply, is the major determinant of fruitlet growth (Guardiola and García-Luis 2000). Thus the establishment and maintenance of sink strength is necessary for normal fruit development.

Early stages of fruit development are characterised by high metabolic activity (Gillaspy *et al.* 1993). During these initial stages, it is thought that sink strength is established. Sugar transport to and within the sink is highly regulated, and specific signalling pathways are involved in its control (Chiou and Bush 1998). The fate of imported photoassimilates partitioned into the fruit, and hence the maintenance of both sink strength and growth, is controlled by carbohydrate metabolism in the fruit itself (Schaffer *et al.* 1999). As the fruit grows, changes in enzymatic activity profiles parallel both developmental patterns and carbon storage, be it in the form of hexoses, Suc, starch or lipids. Extensive studies, performed largely in tomato, suggest a developmental transition from predominantly symplastic unloading to apoplastic unloading into the fruit (Ho 1988; Ruan and

Patrick 1995; Patrick 1997). The early symplastic stage is associated with starch accumulation, and the apoplastic period, which accompanies maturation, is associated with hexose accumulation. This transition from symplastic to apoplastic sugar transport is accompanied by a transition in Suc metabolism (Figure 4.1)(Robinson *et al.* 1988; Wang *et al.* 1993; Schaffer and Petreikov 1997a). The switch from symplastic to apoplastic transport of assimilates is associated with increased extracellular AI and accompanied by the accumulation of imported carbohydrate. In tomato fruit, AI activity is thought to control both sugar content and composition. Losses in AI have been associated with Suc accumulation and diminution of fruit size (Klann *et al.* 1996; Schaffer *et al.* 1999), suggesting a crucial role of Suc metabolism in the control of fruit growth.

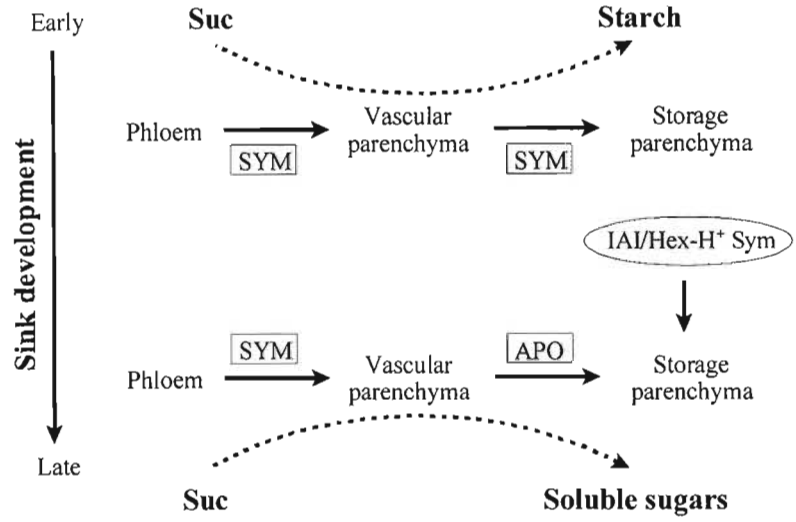


Figure 4.1 Scheme illustrating the path of Suc movement into developing tomato fruit, which accumulate starch during early fruit growth and soluble sugars at maturation. Abbreviations: APO = apoplastic; Hex-H^+ Sym = membrane-localised hexose/H^+ symporter; SYM = symplastic. Redrawn from Campbell *et al.* (2000).

With the exception of the research performed in our laboratory (Richings *et al.* 2000) and recent publications by Liu *et al.* (1999a; 1999b), very little literature is available on either sugar metabolism or sugar content and composition in young developing avocado fruit. Five major soluble sugars have been identified in 'Hass' fruit viz. the disaccharide Suc, the hexoses Glu and Fru, the 7C reducing sugar *manno*-heptulose, and its reduced polyol form, perseitol. These five sugars together constitute 98% the total soluble sugars (TSS) in 'Hass' fruit (Liu *et al.* 1999b). Traces of higher molecular weight oligosaccharides have also been found, especially in younger fruit (Liu *et al.* 1999b). Liu and co-workers (1999a; 1999b) found Suc levels to be continuously low and 7C sugars were the major non-structural carbohydrates present in avocado throughout the study. Thus these authors assumed that the 7C sugars play an essential role in fruit growth.

Liu and co-workers (1999b) proposed that avocado fruit growth follows two physiological carbon accumulation and utilisation phases. During the first phase, soluble sugars provide carbon for the increase in fruit biomass and this is the form in which photoassimilates are stored. The second phase accompanies slowing of fruit growth and coincides with the cessation of TSS accumulation, a decrease in soluble sugars, and the accumulation of oil. During post-harvest storage Suc, Glu, Fru and *manno*-heptulose decrease, suggesting they contribute to the respiratory process. If this is the case, an enzymatic mechanism exists for the metabolism of 7C sugars, which may thus act as a form of carbon storage or as a metabolic substrate (Liu *et al.* 1999b). The avocado fruit exocarp and mesocarp can fix CO_2, but their net assimilate rate is negative due to high respiration rates throughout growth (Whiley *et al.* 1992; Blanke and Whiley 1995). Dry matter accumulation, therefore, depends largely on carbon from the parent plant (Liu *et al.* 1999b). In most plants, Suc is used to support growth of heterotrophic tissues (Barker *et al.* 2000). Suc is relatively inactive as its catabolism depends on a few enzymes (Pontis 1978). For these reasons the relative activities of Suc metabolising enzymes are being investigated with reference to fruit growth. However, it is important to note that, although the nature of the transported sugars in avocado is unknown, Liu *et al.* (1999b) proposed that this role might be fulfilled by the sugar alcohol perseitol.

Previous work illustrated activity of IAI, SPS and SSy in both the cleavage (Clv) and synthesis (Syn) directions in the seed and mesocarp of 'Hass' fruit (Richings *et al.* 2000). In 206 DAFB fruit, i.e. fruit in the latter stages of phase II of fruit development, IAI activity was relatively high compared to that of the other enzymes. In avocado, fruit cell division continues whilst it is attached to the tree (Schroeder 1952; Valmayor 1967) and it is during this late phase that TSS decrease (Liu *et al.* 1999b). This suggests that sugar content and composition may parallel changes in enzyme activity and fruit growth in avocado. Recent work suggests that there are multiple sugar-signalling transduction pathways, involving the induction/repression of sugar sensitive genes via a sugar-sensing mechanism, such as HXK or SnRK1 (reviewed by Campbell *et al.* 2000). These sugar-sensing mechanisms cross-talk with plant hormone signalling pathways (Moore and Sheen 1999) and allow for the adjustment of resource allocation and metabolism (Koch 1996), so determining carbohydrate content and composition in developing sinks. In so doing, sugar content, composition and metabolism may control seed and fruit growth and play a central role in determining final fruit size (Cowan *et al.* 1997a; Higashi *et al.* 1999).

The metabolism, compartmentation and partitioning of solutes in sink tissue play an important role in phloem unloading, cellular transport and sink strength, cellular osmotic pressure and turgor,

gene expression, hormone physiology, cellular homeostasis and plant-environment interactions, carbon storage, plant defence mechanisms, as well as fruit growth. Phloem-transported Suc enters metabolism by cleavage into Glu and Fru, a step that is vital to both the maintenance of continued solute import, post-phloem transport and the subsequent partitioning of photoassimilate to the various metabolic pools in the fruit. Hence the relative distribution and activity of invertase, SSy and SPS will play an important role in Suc import/transport and solute allocation, dictating fruit sugar composition and augmenting changes in carbohydrate storage, gene expression and fruit growth.

Little is known about the enzymatic cleavage of Suc and/or sugar metabolism in avocado fruit. In the present study, changes in the activities of Suc metabolising enzymes, perseitol catabolism, α-amylase activity and carbohydrate accumulation in developing avocado fruit were assessed and attempts made to correlate these with growth in an attempt to establish developmental trends in Suc metabolism in growing 'Hass' fruit.

4.2 RESULTS

4.2.1 CARBOHYDRATE CONTENT AND COMPOSITION OF DEVELOPING 'HASS' AVOCADO FRUIT

4.2.1.1 Vascular sap composition

In an attempt to investigate whether Suc was transported in the vascular tissue, sap was collected from the tree and the leaf. Sap was extracted from the trunk by drilling a fine hole into the trunk before it brached out and drawing out the sap with a surgical syringe. Sap was collected from the petiole by making a small incision below the leaf blade and collecting the sap on Whatman No. 1 filter paper. Sugars were analysed as soluble sugars using HPLC.

The major constituents (Figure 4.2) of sap from 'Hass' leaf petioles appeared to be *manno*-heptulose (27%), perseitol (15%) and an unidentified sugar with a retention time of 8.84 min (40%). In sap samples from the trunk the same sugars predominated, with *manno*-heptulose, perseitol and the unknown sugar (retention time of 8.84 min) representing 17%, 35% and 21% respectively of the total TSS analysed (data not shown). In both cases, Suc and Fru each constituted about 5 % of the translocated sugars.

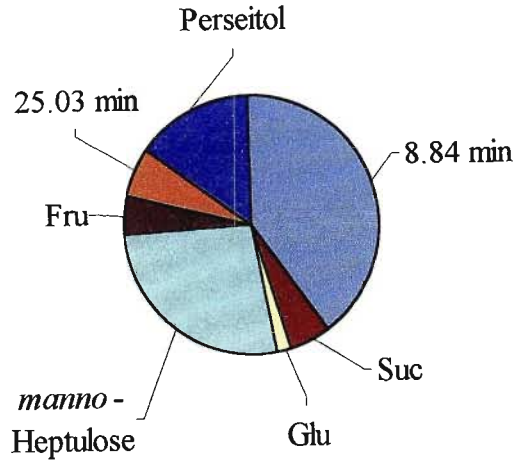


Figure 4.2 Average sugar composition of sap collected from the leaf petiole of 'Hass' avocado trees. Retention times represent unidentified sugars. $n = 3$.

4.2.1.2 Soluble sugar content and composition in developing 'Hass' fruit

TSS was assayed colorimetrically and soluble sugar composition determined by HPLC. Initially TSS was relatively high in all tissues assayed and, with the exception of the seed coat, declined during fruit development (Figure 4.3). There was little change (≈20% of the DM) in seed coat TSS throughout.

Ten soluble sugars were detected in the avocado fruit. In the absence of a GC-linked mass spectrophotometer, or quantities of sufficiently pure samples for nuclear magnetic resonance (NMR), HPLC was used to tentatively identify the sugars. On the basis of co-chromatography and retention time of authentic standards, five of the sugars were identified as Suc, Glu, Fru, perseitol and *manno*-heptulose. There were five unidentified sugars with the retention times 8.84, 11.85, 22.30, 32.65 and 35.97 min. Attempts were made to extract sufficient quantities of the compounds (assumed to be sugars, as unidentified peaks were also detected by GC analysis of sugars (Figure 2.8)) with retention times at 7.53 (a product from the saponification of avocado fruit sugars), 8.84 and 35.97 min using HPLC, for further NMR analysis. However, the sugars with retention times 7.53 and 8.84 min could not be separated to purity. Furthermore, attempts to clean pigmented matter from the extracts with C_{18} columns resulted in the loss of up to 80 % of these sugars (data not shown). The latter observation suggested that the sugars with retention times 7.53, 8.84 and 35.97 min may be lipopolysaccharides.

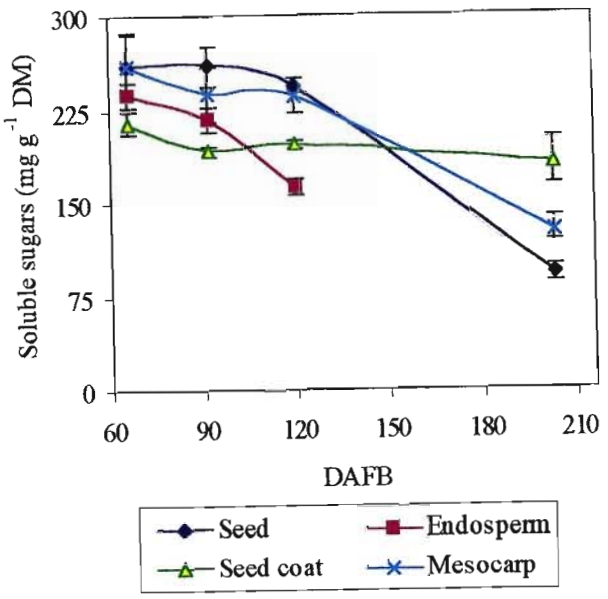


Figure 4.3 Total soluble sugars in avocado fruit tissue during development. n = 3. Error bars represent SD. $LSD_{0.05}$: Seed = 15.90; Endosperm = 30.07; Seed coat = 12.21; Mesocarp = 38.82.

The peak shape and long retention time of the sugar eluted after 35.97 min suggests this compound might be a sugar alcohol. Alkaline hydrolysis (saponification) of sugar extracts with 0.5M KOH in the dark at room temperature for 12 h, resulted in the 8.84 min peak being completely converted to a sugar with a retention time of 7.53 min. The sugar with the retention time of 35.97 min was converted to sugars with retention times 7.53, 13.36 (Suc) and 35.97 min in the ratio 16:4:1 respectively (data not presented). Alkaline hydrolysis of sugars extracted from endosperm tissue, which had high amounts of the sugar with a retention time 35.97, showed a large decrease in the sugars with retention times 17.03 (*manno*-heptulose), 26.82 (perseitol) and 35.97, and correspondingly, very large increases in Suc and Glu. These observations suggest that the peaks identified as perseitol and *manno*-heptulose may contain other co-elutive substances. These may also consist of the same sugars bound to fatty acids or additional, different, lipopolysaccharides.

Together, the sugars identified by co-chromatography constituted approximately 62%, 29%, 61% and 90% of the seed, endosperm, seed coat and mesocarp TSS respectively. Suc contents were consistently low in the seed, seed coat and mesocarp tissues, representing 5%, 3% and 4% of the TSS respectively (Figure 4.4). In young fruit (66 DAFB) Fru occurred in high concentrations in the seed and mesocarp tissues, and declined rapidly from 22% to 8% and 35% to 4% of the TSS respectively during fruit growth. This trend was also paralleled by Glu, with decreases from 14%

to 3% and 19% to 1% of the TSS in the seed and mesocarp, respectively, during the period under observation (Figures 4.4a; 4.4d). Perseitol levels remained relatively constant and accounted for 16-21% of the TSS (≈4-5% DM) in the seed and mesocarp during the study period. The 7C sugar *manno*-heptulose increased markedly (15% to 53% TSS) in the mesocarp tissue of the developing fruit, but remained relatively constant in the endosperm and seed coat tissues.

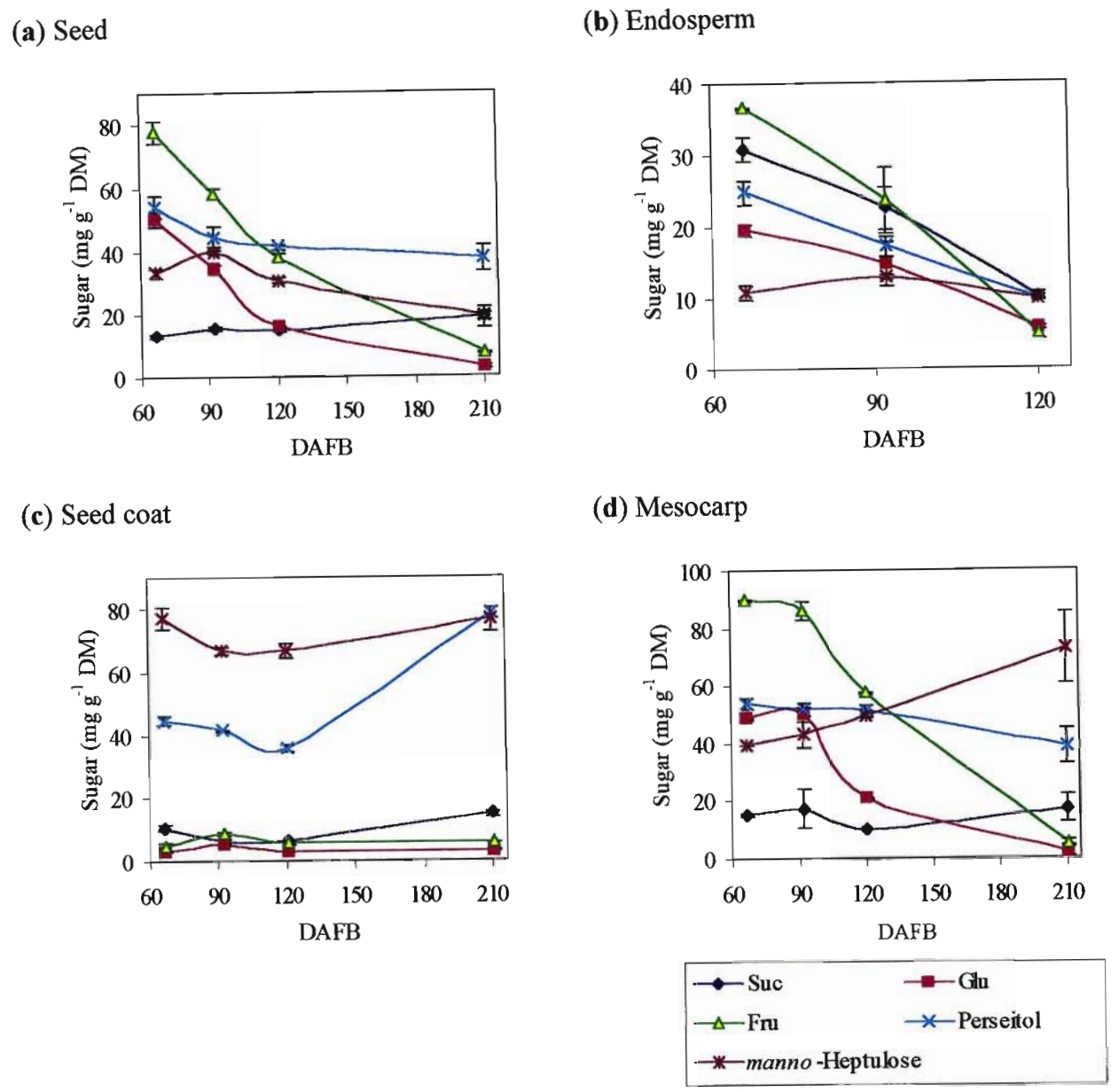


Figure 4.4 Changes in soluble sugar contents in the (**a**) seed; (**b**) endosperm; (**c**) seed coat; and (**d**) mesocarp tissue of developing 'Hass' fruit. $n = 3$. Error bars represent SD. $LSD_{0.05}$: Seed ~ Suc = 0.88; Glu = 12.29; Fru = 14.36; *manno*-Heptulose = 3.49; Perseitol = 5.11: Endosperm ~ Suc = 7.57; Glu = 5.14; Fru = 11.57; *manno*-Heptulose = 1.19; Perseitol = 5.40: Seed coat ~ Suc = 1.85; Glu = 1.00; Fru = 1.50; *manno*-Heptulose = 4.47; Perseitol = 3.29: Mesocarp ~ Suc = 3.63; Glu = 20.20; Fru = 24.97; *manno*-Heptulose = 6.48; Perseitol = 10.89.

In general, the identified soluble sugars decreased in content as the fruit developed, with the exceptions of perseitol in the seed coat and *manno*-heptulose in the mesocarp (Figures 4.4c; 4.4d). This trend was most pronounced in the endosperm tissue, which showed a rapid decline in all identified sugars (Figure 4.4b).

There was little variation in the unidentified sugars in the seed, seed coat and mesocarp (Table 4.1) during development. The unidentified sugar with a retention time of 8.84 min appeared to contribute significantly to the soluble sugar pool in the seed, endosperm and seed coat (Table 4.1). This sugar increased significantly (20% to 59% TSS) in the endosperm during fruit growth, and concurrently with endosperm degeneration. Relative to the other tissues assayed, the endosperm also had high levels of the sugars with retention times 11.85, 32.65 and 35.97 min (Table 4.1). These sugars were not detected or were detected in only very small amounts in the sap (Figure 4.2), showed little/no incorporation of label after feeding of fruit with [^{14}C]-Suc (Table 4.4), and their role in endosperm function and fruit growth remains to be elucidated. The endosperm tissue also showed a peak with the retention time of 22.30 min prior to endosperm degeneration.

4.2.1.3 Starch content in developing 'Hass' fruit

The seed starch concentration was initially low and with development rapidly increased from 18% to 72% of the DM (Figure 4.5). The starch content appeared to plateau during phase II of fruit growth. The endosperm tissue also showed a progressive increase in starch concentration prior to endosperm degeneration. Seed coat starch levels remained low (≈17% DM). In the mesocarp tissue the initially low levels of starch decreased with fruit development, from 14% to 7% of the DM (Figure 4.5). There was no apparent correlation of starch content with α-amylase activity (Section 4.2.3.2).

Table 4.1 Composition and content of the unidentified HPLC sugar peaks in developing 'Hass' seed, endosperm, seed coat and mesocarp tissue. $n = 3$. (mean±SD(% TSS)).

Rt (min)	Sugar content (mg^{-1} g DM) at different DAFB			$LSD_{0.05}$
	66	92	120	
Seed				
8.84	85.43±4.63 (24)	84.74±2.27 (27)	67.84±3.62 (29)	7.61
11.85	4.67±1.19 (1)	ND	3.49±0.893 (2)	1.93
22.30	16.58±0.23 (5)	16.47±0.53 (5)	10.03±0.21 (4)	2.69
32.65	ND	ND	ND	-
35.97	15.13±0.82 (4)	19.03±2.70 (6)	14.15±0.27 (6)	2.09
Endosperm				
8.84	60.21±3.92 (20)	59.83±8.63 (22)	126.01±7.62 (59)	30.75
11.85	46.97±5.75 (15)	46.63±5.29 (17)	1.79±0.32 (1)	27.36
22.30	7.92±1.42 (2)	8.53±6.25 (3)	19.26±1.10 (9)	5.13
32.65	2.92±0.40 (1)	4.40±0.46 (2)	5.29±0.65 (3)	0.90
35.97	71.78±1.31 (23)	58.46±8.84 (22)	22.34±1.76 (10)	18.59
Seed coat				
8.84	37.28±3.39 (17)	49.64±5.41 (23)	45.66±3.58 (24)	5.12
11.85	4.23±0.84 (2)	1.64±0.30 (1)	1.39±0.34 (1)	1.24
22.30	38.04±7.12 (17)	30.45±7.74 (14)	21.46±6.58 (11)	10.49
32.65	ND	ND	0.71±0.06 (<0.5)	-
35.97	8.65±0.70 (2)	9.89±0.83 (2)	6.63±0.80 (2)	1.29
Mesocarp				
8.84	12.60±0.02 (5)	12.86±1.74 (4)	15.58±0.26 (7)	1.78
11.85	ND	ND	ND	-
22.30	0.07±0.10 (<0.5)	0.51±0.36 (<0.5)	0.71±0.23 (<0.5)	0.39
32.65	ND	ND	ND	-
35.97	0.31±0.09 (<0.5)	ND	ND	-

Rt = Retention time
ND = Not detected

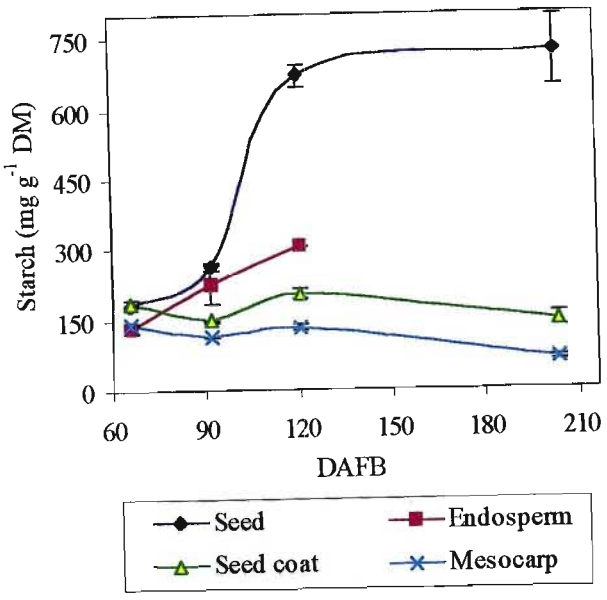


Figure 4.5 Starch content in seed, endosperm, seed coat and mesocarp tissue from avocado fruit during development. n = 3. Error bars represent SD. $LSD_{0.05}$: Seed = 189.25; Endosperm = 64.84; Seed coat = 22.45; Mesocarp = 10.78.

4.2.2 FATTY ACID CONTENT AND COMPOSITION OF SEED AND MESOCARP TISSUE

Total lipids were consistently low in the seed, representing approximately 3% of the DM (Figure 4.6). The mesocarp lipid content was much greater (Figure 4.6), remaining unchanged during the initial stages of phase II of fruit development (14% DM). As mesocarp growth progressed, lipids accumulated rapidly, accounting for 25% of the fruit DM 206 DAFB. Early seed development was characterised by high levels of linoleic, γ- and α-linolenic acid (25%, 18% and 46% of total fatty acids respectively). With fruit development linoleic acid accounted for >50% of the fatty acid composition, whilst γ- and α-linolenic acid decreased significantly. 120 DAFB, the fatty acids in the seed constituted primarily of palmitic (20%), petroselinic (35%) and linoleic (55%) acid (Table 4.2; 4.3). In the mesocarp, the fatty acids oleic, linoleic and α-linolenic acid occurred in high concentrations in the younger fruit, representing 19%, 22% and 25% of the total fatty acids respectively. As fruit growth progressed, linoleic acid levels remained relatively constant, whilst oleic and α-linolenic acids decreased. 120 DAFB, the fatty acids palmitic (19%), petroselinic (39%) and linoleic (18%) represented the dominant fatty acids of the lipid reserves (Table 4.3).

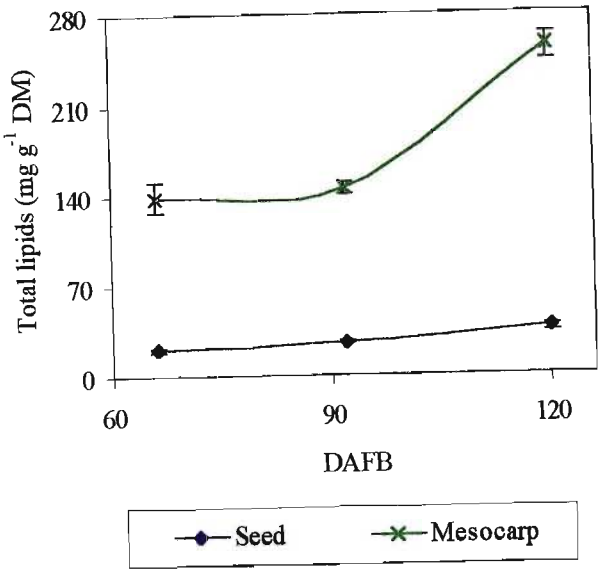


Figure 4.6 Total lipid content in avocado seed and mesocarp tissue during the early stages of phase II of fruit development. $n = 3$. Error bars represent SD. $LSD_{0.05}$: Seed = 5.90; Mesocarp = 46.40.

Table 4.2 Nomenclature and fatty acids identified in developing 'Hass' fruit seed and mesocarp tissue. Fatty acids were identified based on retention times with authentic standards, documented fatty acid relative retention times (compared to stearic acid) and equivalent chain lengths.

Symbol	Trivial name	Systematic name	Rt	$r_{18:0}$
14:0	Myristic	Tetradecenoic	8.761	0.73
16:0	Palmitic	Hexadecenoic	10.561	0.85
16:1(*n*-7)	Palmitoleic	*cis*-9-Hexadecenoic	11.165	0.90
18:0	Stearic	Octadecenoic	12.400	1.00
18:1(*n*-12)	Petroselinic	*cis*-6-Octadecenoic	12.916	1.04
18:1(*n*-9)	Oleic	*cis*-9-Octadecenoic	13.369	1.08
18:2(*n*-6)	Linoleic	9,12-Octadecadienoic	13.736	1.11
18:3(*n*-6)	γ-Linolenic	6,9,12-Octadecatrienoic	14.728	1.19
18:3(*n*-3)	α-Linolenic	9,12,15-Octadecatrienoic	15.184	1.22
18:4(*n*-3)		3,9,12,15-Octadecatetraenoic	15.940	1.29
20:4(*n*-6)	Arachidonic	Eicosanoic	16.690	1.39

Rt = Retention time (min)
$r_{18:0}$ = Relative retention time compared to that of stearic acid (18:0)

Table 4.3 Percentage fatty acid composition in the seed and mesocarp of developing 'Hass' fruit. $n = 3$. (mean±SD).

Rt	Fatty acid	% Fatty acid composition at different DAFB			$LSD_{0.05}$
		66	92	120	
Seed					
10.561	16:0	11.13±0.77	21.70±0.38	20.32±0.31	4.13
12.916	18:1(*n*-12)	10.86±1.23	28.12±0.39	35.16±0.47	8.96
13.736	18:2(*n*-6)	25.01±2.06	60.06±0.93	54.96±1.66	13.59
14.728	18:3(*n*-6)	17.84±1.48	11.47±0.57	9.69±1.14	3.15
15.184	18:3(*n*-3)	46.08±4.79	ND	ND	-
16.690	20:4(*n*-8)	0.24±0.01	0.48±0.03	0.31±0.09	0.09
Mesocarp					
8.761	14:0	0.33±0.05	0.17±0.05	0.06±0.08	0.14
10.561	16:0	8.73±0.32	9.92±0.38	18.96±0.30	4.01
11.165	16:1(*n*-7)	2.00±0.03	2.24±0.03	4.48±0.09	0.98
12.076	Unknown 1	0.68±0.06	0.73±1.03	ND	0.58
12.400	18:0	ND	ND	1.11±0.13	-
12.916	18:1(*n*-12)	9.15±.055	9.11±0.56	39.17±0.41	12.41
13.369	18:1(*n*-9)	18.95±0.96	19.15±0.28	6.10±0.02	5.37
13.621	Unknown 2	3.54±0.42	1.80±0.55	ND	1.57
13.736	18:2(*n*-6)	22.49±1.34	26.46±2.71	17.95±0.29	3.24
14.463	Unknown 3	0.56±0.10	ND	ND	-
14.728	18:3(*n*-6)	7.74±0.32	6.25±0.03	2.66±0.04	1.88
15.184	18:3(*n*-3)	25.41±3.08	23.59±0.32	2.37±0.21	9.23
15.940	18:4(*n*-3)	ND	ND	6.73±0.48	-
16.690	20:4(*n*-8)	0.43±0.20	0.59±0.20	0.44±0.02	0.12

Rt = Retention time (min)
ND = Not detected

4.2.3 CARBOHYDRATE METABOLISM IN DEVELOPING 'HASS' AVOCADO FRUIT

4.2.3.1 Incorporation of [^{14}C]-label sucrose into soluble sugars in 'Hass' avocado fruit

To ascertain the pattern of Suc metabolism in avocado, fruit (92 DAFB) were fed [^{14}C]-Suc via the pedicel (as described in Chapter 2.7). After incubation for 12 h at room temperature, the soluble sugars were separated by HPLC and the amount of label in each sugar fraction determined.

The distribution of [^{14}C]-label in the sugar fractions suggested that Suc was cleaved to Glu and Fru in the fruit (Table 4.4). Furthermore [^{14}C]-label, derived from Suc was incorporated into *manno*-heptulose and an unidentified sugar with a retention time of 8.84 min in significant amounts. On a whole fruit basis, 21% of the label apparently remained unchanged as Suc, whilst 25% and 22% was in the hexoses Glu and Fru, respectively.

Table 4.4 [^{14}C]-label percentage distribution in the soluble sugar pool from 'Hass' fruit tissue (92 DAFB) 12 h after pulsing with [^{14}C]-Suc. (mean±SD).

Rt (min)	Sugar	% [^{14}C]-label distribution					
		Pedicel	Seed	Endosperm	Seed coat	Mesocarp	Exocarp
8.84	Unknown	0.15±0.06	47.11±9.37	18.17±6.56	8.88±4.56	1.98±0.80	3.41±1.82
13.36	Suc	34.23±5.92	16.44±4.28	31.09±6.64	0.01±0.01	9.82±3.81	33.31±9.36
15.44	Glu	28.07±4.16	7.33±3.36	50.42±5.36	8.76±3.26	33.25±3.71	20.41±5.61
17.03	*m*-Hep	9.23±1.68	20.89±4.89	ND	21.80±4.67	11.79±2.40	2.53±1.58
18.63	Fru	19.65±5.19	8.23±2.58	ND	39.95±9.75	40.81±2.29	25.75±5.94
22.30	Unknown	ND	ND	0.32±0.05	4.54±2.21	1.46±0.06	13.31±5.28
26.82	Perseitol	8.68±4.27	ND	ND	ND	ND	1.28±1.00
35.97	Unknown	ND	ND	ND	16.06±1.76	0.90±0.27	ND
$LSD_{0.05}$		7.08	8.33	9.89	7.67	7.59	7.64

Rt = Retention time
m-Hep = *manno*-Heptulose
ND = Not detected

4.2.3.2 Enzymatic cleavage, resynthesis and partitioning of solutes in developing 'Hass' fruit

The pH optima and basic enzyme characterisation of AI, NI, SSy, SPS, perseitol catabolising enzymes and α-amylase from avocado fruit tissue was determined by assaying enzyme activity in the pH range 4.0-8.0 (Table 4.5). Enzyme kinetics were determined by assaying activity with increasing substrate concentrations until saturation and by performing Lineweaver-Burk plots. Crude SAI enzyme extract had a significantly larger Michaelis constant (K_m), suggesting a reduced affinity for Suc, and possible competitive inhibition for the active sites by 'background' sugars, which were largely removed by passing the extract through Sephadex micro-columns (Section 2.11).

NI, SSy, SPS and α-amylase activity in the insoluble phase represented <5% of that in the soluble phase (data not presented), and was ignored. Suc cleavage by invertases was confirmed by HPLC. HPLC analysis also suggested that the major product of perseitol catabolism was the reducing sugar *manno*-heptulose. This observation suggested that perseitol catabolism may involve an oxido-reductase enzyme and hence the enzyme responsible for perseitol catabolism was termed perseitol dehydrogenase (PDH). However, it must be emphasised that PDH activity was assayed in the absence of additional coenzymes (method described below). Furthermore, an extremely small increase in Glu was also observed (data not presented) during PDH assays, which may suggest the presence of an additional route for perseitol catabolism.

PDH was assayed in the same manner as invertase, using a Tris-HCl buffer (pH 6.10) containing 1 mM EDTA, 2 mM DTT, 2 mM $CaCl_2$ and 50 mM perseitol. In the absence of sufficient pure *manno*-heptulose, and no positive identification of the sugars with elution times of 8.84, 11.85, 22.30, 32.65 and 35.97 min, the metabolism of these sugars was not investigated further. The specificity of PDH was checked by using mannitol and sorbitol as alternate substrates. Enzyme activity with perseitol, sorbitol and mannitol was (mean±SD) 58.72±5.59, 18.40±3.19 and 52.99±6.58 nmol Glu mg^{-1} protein min^{-1}, respectively, in 120 DAFB fruit mesocarp tissue. These observations suggested PDH was not specific to perseitol, but also showed significant activity with mannitol and, to a lesser extent, sorbitol.

Table 4.5 Enzyme kinetics and pH optima of crude and partially purified 'Hass' avocado fruit tissue extracts assayed for the invertases, SSy, SPS, PDH and α-amylase. n = 3. Mean ± SD. Data presented is representative of both seed and mesocarp tissue, unless presented together or otherwise stated. Characterization of enzyme activities was not performed on endosperm and seed coat tissues.

Enzyme	Tissue	Purification	pH Optima	V_{max} (μmol substrate mg^{-1} protein min^{-1})	K_m (mM)
IAI	Mesocarp	Crude	4.50	0.27±0.01	23.03±5.58
SAI	Mesocarp	Crude	4.55	0.22±0.01	156.28±7.57
	Mesocarp	Sephadex	4.55	0.23±0.03	43.65±4.47
	Seed	Sephadex	4.55	0.49±0.01	37.91±4.78
NI	Mesocarp	Sephadex	6.80	1.04±0.03	65.69±4.58
	Seed	Sephadex	6.80	3.94±0.21	237.86±10.90
SSy (Clv)	Mesocarp	Sephadex	7.20	6.59±0.46	113.07±7.28
SSy (Syn)	Seed coat	Sephadex	7.20	0.29±0.01	66.94±7.28
SPS	Seed	Sephadex	7.20	1.55±0.06	128.30±5.36
PDH	Mesocarp	Sephadex	6.10	1.49±0.02	107.46±8.10
α-Amylase	Mesocarp	Sephadex	5.50	0.12±0.01	89.02±6.14

Clv = Suc cleavage direction
Syn = Suc synthesis direction

Generally, however, enzyme activity was initially high (corresponding to the end of growth phase I/beginning of growth phase II) and decreased as growth phase II progressed (Figures 4.7; 4.8; 4.9; 4.10).

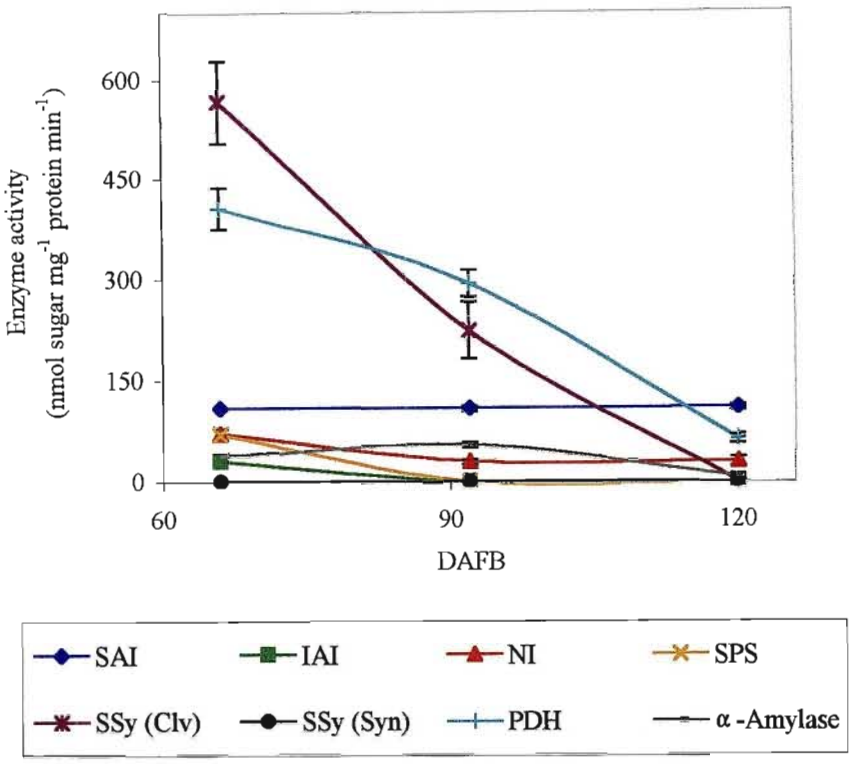


Figure 4.7 Activity of the enzymes SAI, IAI, NI, SPS, SSy, PDH and α-amylase in seed tissue from developing 'Hass' avocado fruit. n = 3, using extracts collected from at least 5 different fruit. Error bars represent SD. $LSD_{0.05}$: SAI = 1.90; IAI = 10.36; NI = 14.33; SPS = 23.07; SSy (Clv) = 184.12; SSy (Syn) = 3.94; PDH = 99.55; α-Amylase = 13.53.

In the seed tissue SAI activity was seen to be constant (≈110.00 nmol Glu mg^{-1} protein min^{-1}) from 66-120 DAFB (Figure 4.7). In the seed coat and mesocarp tissues, SAI was initially high and declined by 120 DAFB to <50.00 nmol Glu mg^{-1} protein min^{-1} by 120 DAFB (Figures 4.9: 4.10), remaining at these basal levels until 225 DAFB (data not presented).

IAI activity was consistently low in the seed and endosperm tissues (Figures 4.7; 4.8). In the seed coat tissue, IAI was initially low and increased by 1200% to 239.83 nmol Glu mg^{-1} protein min^{-1} by 120 DAFB (Figure 4.9). However, by 302 DAFB, IAI decreased to basal levels of <3.00 nmol Glu mg^{-1} protein min^{-1}. The high IAI activity of young mesocarp tissue declined rapidly to basal levels of <10.00 nmol Glu mg^{-1} protein min^{-1} (Figure 4.10) and remained so until 225 DAFB (data not presented).

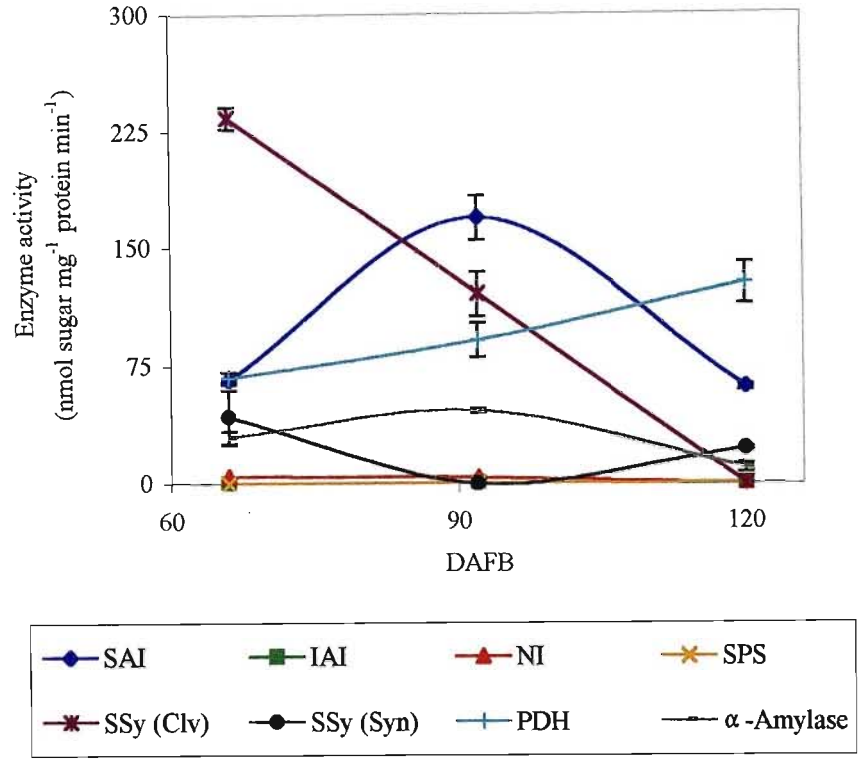


Figure 4.8 Activity of the enzymes SAI, IAI, NI, SPS, SSy, PDH and α-amylase in endosperm tissue from developing 'Hass' avocado fruit. n = 3, using extracts collected from at least 5 different fruit. Error bars represent SD. $LSD_{0.05}$: SAI = 34.57; IAI = ND; NI = 1.23; SPS = 0.26; SSy (Clv) = 70.90; SSy (Syn) = 13.11; PDH = 17.89; α-Amylase = 10.34.

NI activity was initially relatively high in the seed issue and declined rapidly to a consistent basal level of ±30.00 nmol Glu mg^{-1} protein min^{-1} (Figure 4.7). A similar, but much reduced trend was observed in seed coat tissue (Figure 4.9). In the endosperm and mesocarp tissues NI activity was consistently low throughout the period observed at <5 and ≈40 nmol Glu mg^{-1} protein min^{-1} respectively (Figures 4.8; 4.10).

In the endosperm, seed coat and mesocarp tissues, SPS activity was very low or not detected (<1 nmol Suc mg^{-1} protein min^{-1}) throughout the study period (Figures 4.8; 4.9; 4.10). However, high SPS activity (70.38 nmol Suc mg^{-1} protein min^{-1}) was detected in seed tissue, but this declined to undetectable levels by 120 DAFB. Interestingly, by 225 DAFB there was a significant increase in seed SPS activity (123.08 nmol Suc mg^{-1} protein min^{-1})(data not presented).

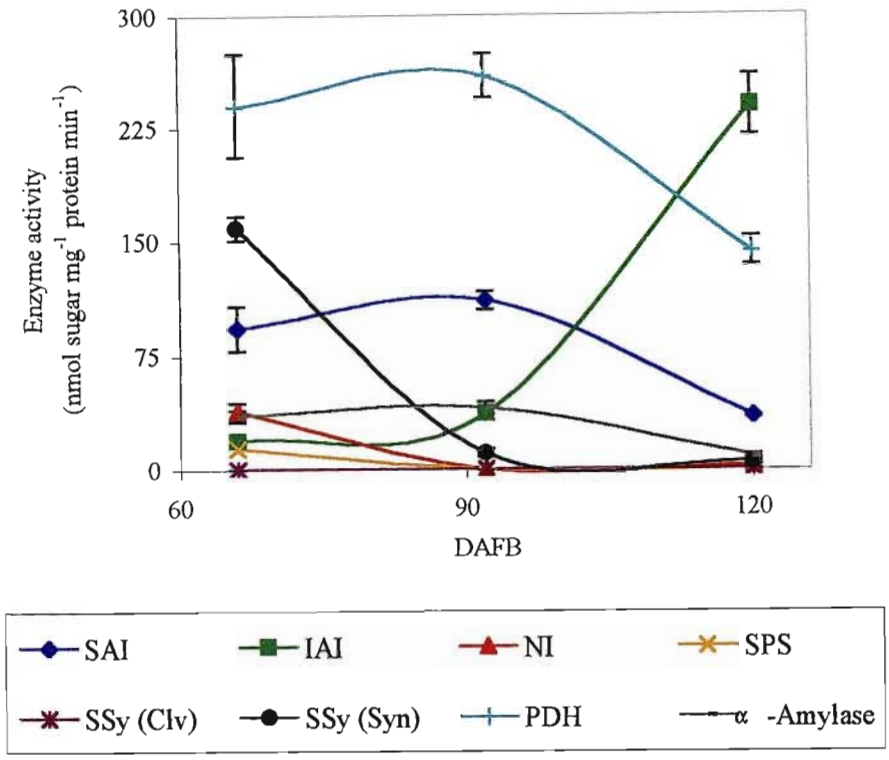


Figure 4.9 Activity of the enzymes SAI, IAI, NI, SPS, SSy, PDH and α-amylase in seed coat tissue from developing 'Hass' avocado fruit. n = 3, using extracts collected from at least 5 different fruit. Error bars represent SD. $LSD_{0.05}$: SAI = 23.09; IAI = 70.69; NI = 12.47; SPS = 4.55; SSy (Clv) = ND; SSy (Syn) = 49.32; PDH = 37.48; α-Amylase = 10.00.

Relative to all the other enzymes assayed SSy activity in the cleavage direction was very high. In the seed, endosperm and mesocarp tissues SSy (Clv) was initially very high, and as fruit growth progressed, SSy (Clv) activity decreased (Figures 4.7; 4.8; 4.10). In the mesocarp tissue the high SSy (Clv) activity continued to 92 DAFB. In the seed coat tissue SSy (Clv) activity was almost undetectable throughout the period under observation (Figure 4.9). On the other hand, SSy activity in the synthesis direction was very low in the seed, endosperm and mesocarp tissues (Figures 4.7; 4.8; 4.10). Seed coat SSy (Syn) activity was initially high and rapidly decreased to very low levels (<15 nmol Suc mg^{-1} protein min^{-1}), similar to those expressed in the other tissues (Figure 4.9).

PDH activity in the seed tissue was very high (405.37 nmol Glu mg^{-1} protein min^{-1}) early in fruit development, but declined rapidly with fruit development to 62.79 nmol Glu mg^{-1} protein min^{-1} (Figure 4.7). In contrast, in endosperm tissue (Figure 4.8), PDH activity increased gradually with fruit development. In the mesocarp tissue PDH activity followed a very similar pattern of activity to SSy (Clv) (Figure 4.10), peaking at 92 DAFB and declining gradually.

In all tissues α-amylase activity was both low and showed a gradual decline from ±40 nmol Glu mg^{-1} protein min^{-1} to ±10 nmol Glu mg^{-1} protein min^{-1} (Figures 4.7; 4.8; 4.9; 4.10).

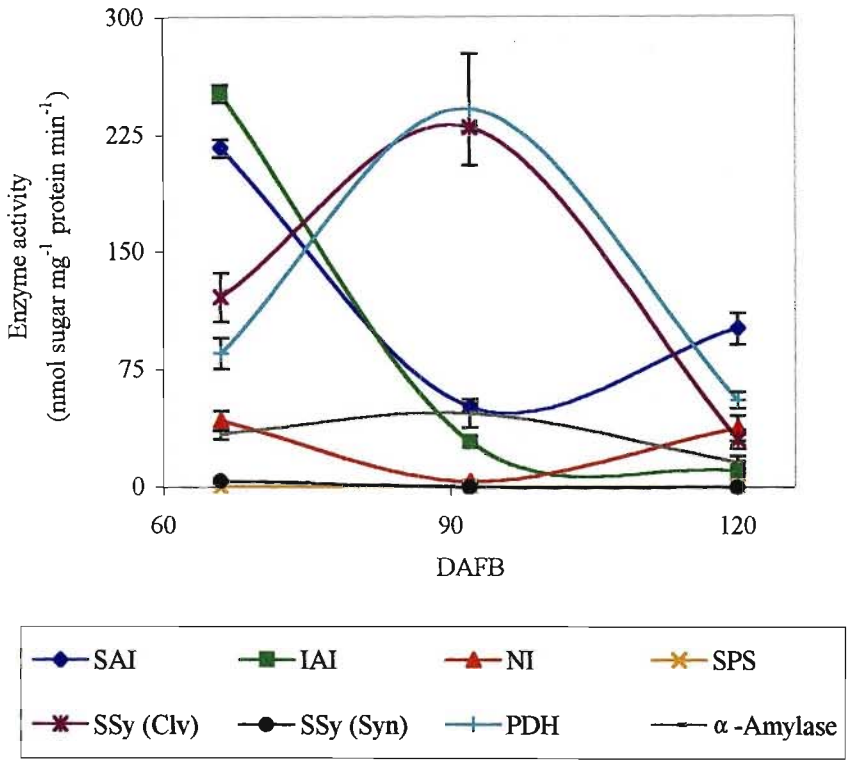


Figure 4.10 Activity of the enzymes SAI, IAI, NI, SPS, SSy, PDH and α-amylase in mesocarp tissue from developing 'Hass' avocado fruit. n = 3, using extracts collected from at least 5 different fruit. Error bars represent SD. $LSD_{0.05}$: SAI = 48.18; IAI = 75.93; NI = 12.43; SPS = 1.25; SSy (Clv) = 57.42; SSy (Syn) = 1.27; PDH = 57.70; α-Amylase = 9.49.

Thus, an overall pattern of changes in the enzyme activity may be characterised as:

1. No significant detectable activity, i.e. <5 nmol sugar mg^{-1} protein min^{-1}, e.g. the activity of IAI in the endosperm.
2. Low basal activity throughout development, e.g. SSy (Syn) activity in the mesocarp tissue.
3. Progressive reduction, either gradual (e.g. SSy (Clv) in the endosperm tissue) or rapid (e.g. IAI in the mesocarp tissue), to low, basal levels.
4. A gradual increase or late exponential increase in enzyme activity, as exemplified by PDH in endosperm tissue and α-amylase activity in seed coat tissue, respectively.

5. Bell shaped, either pronounced (low-high-low activity, e.g. SAI activity in the endosperm tissue) or gradual (medium-high-low activity, e.g. PDH activity in the seed coat tissue).

4.2.3.3 Metabolic control of the enzymatic cleavage and synthesis of sucrose in 'Hass' fruit seed coat tissue

An *in vitro* investigation was undertaken to determine possible reponses of some of the enzymes of Suc metabolism to changing levels of the 'key' substrates Glu, Fru and Suc, as it was thought endogenous changes in, and allosteric responses to, these substrates may have a profound effect on fruit growth. After incubation with Glu, Fru and/or Suc (method described in section 2.10.2), seed coat tissue from 80 DAFB fruit were homogenised in buffer and the activity of SAI, IAI, SPS and SSy determined (Figures 4.11; 4.12; 4.13; 4.14; and 4.15).

Figure 4.11 suggests that both Glu and Suc caused a rapid transient increase in SAI activity in the first 4 h, whilst Fru inhibited SAI activity. In contrast to SAI, IAI showed no striking differences in response to sugar treatment (Figure 4.12); however, Glu treatment appeared to inhibit IAI activity. The activity of SPS appeared to be 'short-lived' under the experimental conditions, and was essentially undetectable after 8 h (Figure 4.13). There was a short 'burst' of SPS activity in the control, and in the presence of Glu and the sugar-mix (a mixture of Glu, Fru and Suc in the ratio 2:3:2). Interestingly, although this 'burst' of activity was promoted by the sugar mixture, Glu, Fru and Suc alone were unable to elicit such a response. SSy (Clv) activity was markedly increased by Suc over the period 4-6 h, and Suc was the only sugar to give a pronounced response (Figure 4.14). Like SSy (Clv), SSy (Syn) underwent a 4-6 h induction period, peaking after 8 h (Figure 4.15). Of particular interest was the general inhibition or reduction in SAI, IAI, SPS, SSy (Clv) and SSy (Syn) activity in seed coat tissue incubated with Fru.

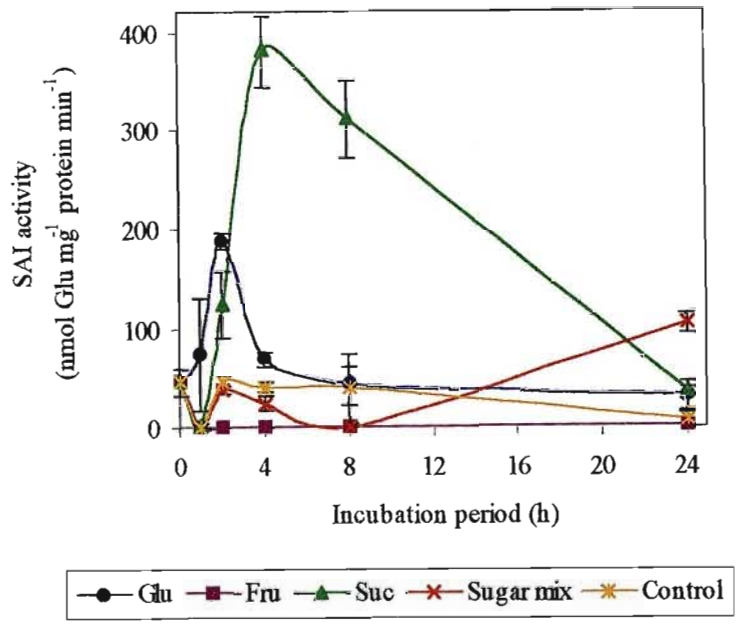


Figure 4.11 Activity of SAI from seed coat tissue incubated with Glu, Fru, Suc and a mixture of these three sugars in the ratio 2:3:2. $n = 5$. Error bars represent SD. $LSD_{0.05}$: Glu = 23.29; Fru = ND; Suc = 55.41; Sugar mix = 14.07; Control = 9.16.

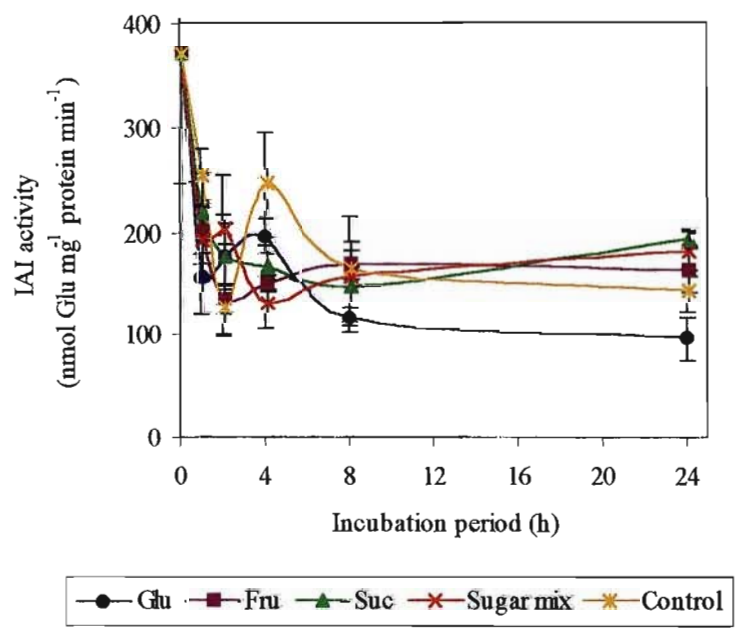


Figure 4.12 Activity of IAI from seed coat tissue incubated with Glu, Fru, Suc and a mixture of these three sugars in the ratio 2:3:2. $n = 5$. Error bars represent SD. $LSD_{0.05}$: Glu = 18.90; Fru = 17.83; Suc = 13.46; Sugar mix = 12.00; Control = 22.82.

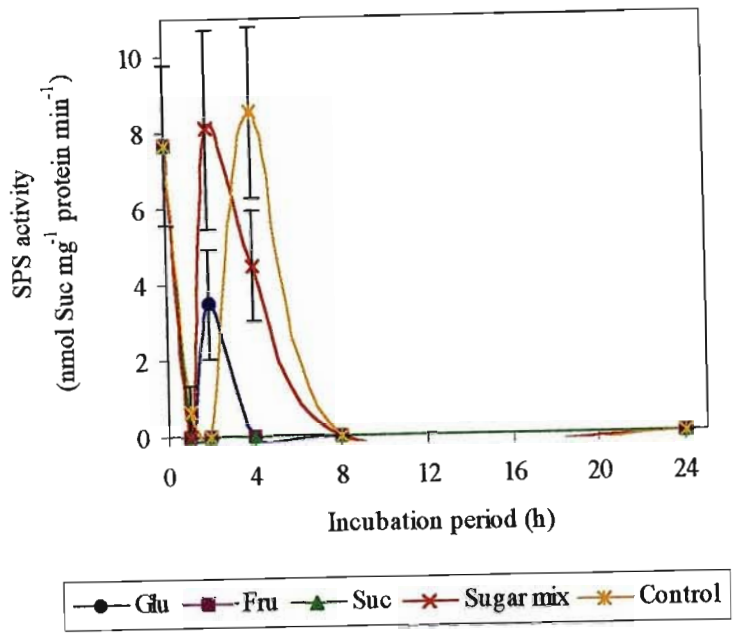


Figure 4.13 Activity of SPS from seed coat tissue incubated with Glu, Fru, Suc and a mixture of these three sugars in the ratio 2:3:2. n = 5. Error bars represent SD. $LSD_{0.05}$: Glu = 0.95; Fru = ND; Suc = ND; Sugar mix = 5.30; Control = 2.32.

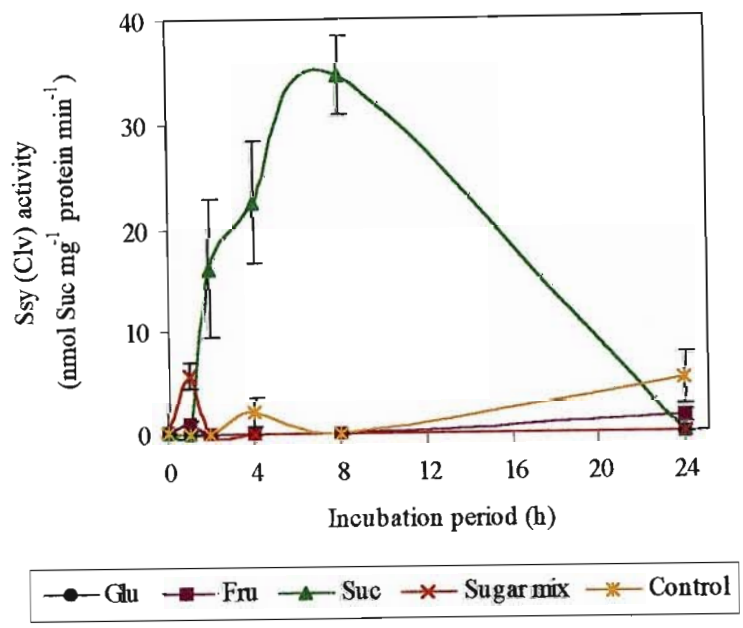


Figure 4.14 Activity of SSy (Clv) from seed coat tissue incubated with Glu, Fru, Suc and a mixture of these three sugars in the ratio 2:3:2. n = 5. Error bars represent SD. $LSD_{0.05}$: Glu = ND; Fru = 0.51; Suc = 10.20; Sugar mix = 1.62; Control = 1.55.

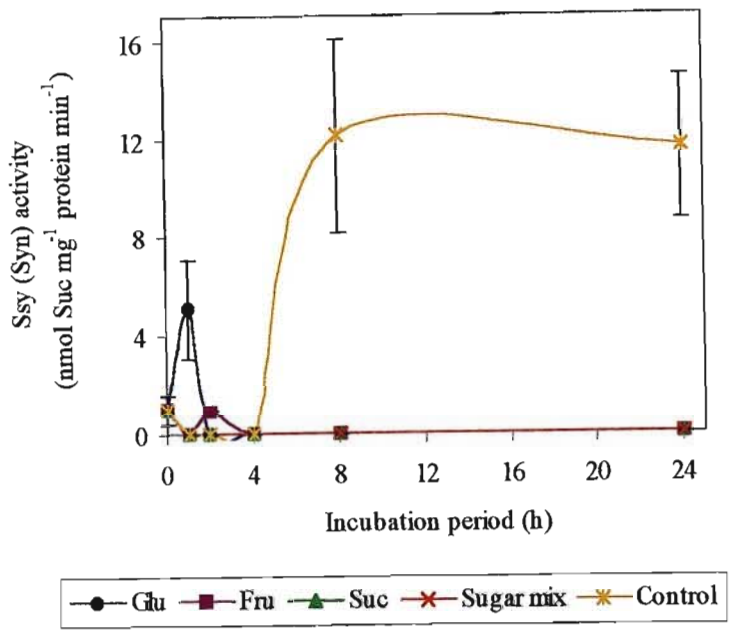


Figure 4.15 Activity of SSy (Syn) from seed coat tissue incubated with Glu, Fru, Suc and a mixture of these three sugars in the ratio 2:3:2. n = 5. Error bars represent SD. $LSD_{0.05}$: Glu = 1.28; Fru = 0.26; Suc = ND; Sugar mix = ND; Control = 2.52.

4.3 SUMMARY

1. The major constituents of vascular sap were found to be *manno*-heptulose, perseitol and an unidentified sugar with a retention time of 8.84 min. Although these sugars predominate, Suc was present in the sap, representing about 5% of the soluble sugars. The incorporation of [^{14}C]-labelled Suc into Glu and Fru suggested that there was active cleavage of Suc occurring in developing 'Hass' fruit. The enzymes SAI, IAI, NI, SPS, SSy, α-amylase and a perseitol catabolising enzyme (designated PDH) were shown to be active in developing 'Hass' fruit. Enzyme activity, in general, appeared to be high during early fruit development and decreased between 92-120 DAFB. Concurrently, the high levels of Glu and Fru levels seen in early fruit development declined as growth progressed. Activity of SSy (Clv) and PDH appeared to be substantially greater during early seed growth, whilst IAI, SAI and SSy (Clv) activity seemed to be more prominent in early mesocarp development, suggesting unique patterns of transport, metabolism and carbon accumulation. This observation was supported by the fact that the seed accumulated starch, whilst the mesocarp accumulated lipids with development. The enzyme tentatively

identified as PDH did not appear to be substrate-specific and its activity was highest in young seed tissue. Interestingly, perseitol content was very high in young seed tissue.

2. The concurrent decrease in general enzyme activity and TSS suggests a close relationship between enzyme activity and soluble sugar content in developing 'Hass' fruit. SAI, IAI, SPS and SSy are inhibited by increased Fru levels. Incubation with Suc is associated with an increase in SAI, IAI and SSy (Clv), and a decrease in SPS and SSy (Syn) activity. Clearly, there exists mechanisms for both substrate and product feed-back in the control of Suc metabolism in avocado fruit.

CHAPTER 5

INVESTIGATION OF A POSSIBLE RELATIONSHIP BETWEEN ABA, SOLUTE MOVEMENT AND THE 'HASS' SMALL FRUIT PHENOTYPE

5.1 INTRODUCTION

'Hass' avocado trees have a tendency to produce two phenotypically distinct populations of fruit (Zilkah and Klein 1987, Moore-Gordon *et al.* 1998), viz. small and normal fruit (Figure 5.1). The small fruit phenotype is usually less than 200 g FW and is characterized by a seed coat that appears to have undergone, what can be loosely termed, premature senescence. There are several characteristics frequently associated with the small fruit. These include pedicel 'ring neck', a narrow basal region, degeneration of the seed, a dead embryo, aberrant Pd structure and function (Moore-Gordon *et al.* 1998) and altered SSy and AI activity (Richings *et al.* 2000). In fact, it has been proposed that the initiation of expression of the small fruit phenotype may occur at any time during the development of the fruit and that the physiological processes that accompany this transition typically characterize avocado maturation (Cowan *et al.* 1997a).

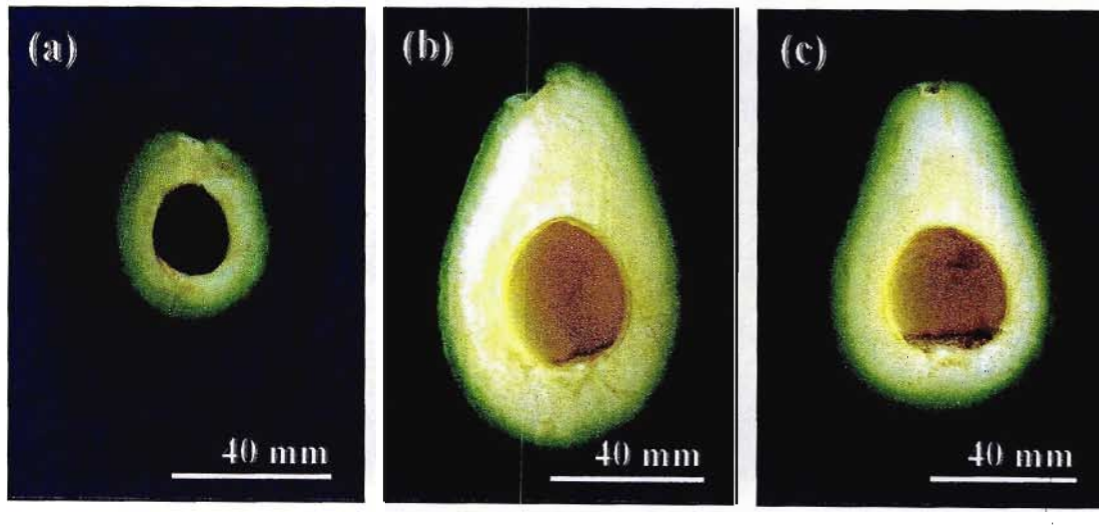


Figure 5.1 Longitudinal sections of (**a**) small; (**b**) normal; and (**c**) ABA-treated 'Hass' fruit harvested 200 DAFB, showing distinct difference in fruit size, shape and seed coat appearance. Images are representative of trends seen in excess of 100 fruit.

The occurrence of the small fruit phenotype is believed to follow the developmental continuum: abortion of the embryo→seed coat senescence→cessation of mesocarp cell division→reduction in growth→small fruit (Cowan *et al.* 2001). Although exacerbated by tree aging (Cutting 1993), large fruit loads (Lahav and Kalmer 1977), warmer and/or drier climates (Hilton-Barber 1992; Whiley and Schaffer 1994), and plant stress (Moore-Gordon *et al.* 1996; Whiley *et al.* 1996; Cowan *et al.* 1997a), the small fruit phenomenon is not restricted to diseased or unhealthy trees, with robust trees producing a significant proportion (20-40%) of small fruit (Kremer-Köhne and Köhne 1995). This population of small fruit in 'Hass' thus clearly arises from differences in fruit physiology. The occurrence of these two phenotypically different fruit on the same tree thus presents a system that allows one to study the metabolic control of fruit growth.

Cowan and co-workers (1997a) were able to show that activity of the enzyme HMGR and the end products of isoprenoid biosynthesis, especially ABA, CK and sterols, had a marked effect on avocado fruit growth. In the same study, the authors were able to show that the small fruit phenotype had elevated ABA levels in the mesocarp. Furthermore, the ABA content was found to be negatively correlated to fruit size in 'Hass' avocado (Moore-Gordon *et al.* 1998). It has been shown that CK inhibits ABA biosynthesis in avocado (Cowan and Railton 1987) or promotes its catabolism (Cowan *et al.* 1999), and the negative effects of ABA treatment on fruit growth can be reversed by simultaneous treatment with the CK, iP (Cowan *et al.* 1997a). Furthermore, in untreated (i.e. normal) fruit the iP:ABA ratio showed a linear relationship to increasing fruit size (Moore-Gordon *et al.* 1998). Final fruit size, therefore, appears to be strongly correlated with the endogenous CK:ABA ratio.

Interestingly, ABA has the ability to induce some of the phenomena associated with the small fruit phenotype at all stages of fruit growth, including the apparent premature senescence of the seed coat (Moore-Gordon *et al.* 1998). The effects of ABA on seed coat senescence were shown to be negated by co-treatment with molar equivalents of iP. Furthermore, ABA-treated fruit displayed Pd that were occluded by an electron dense material, suggestive of reduced seed coat function and reduced symplastic transport. A consequence of this might be reduction in carbohydrate supply to the fruit tissue and a state of sugar 'starvation' accompanied by reduced growth. The effects of ABA and CK on sink solute transport activities, Suc metabolism and the expression of sucrolysis-related mRNA are well documented (Tietz *et al.* 1981; Morris and Arthur 1984b; Schüssler *et al.* 1984; Ackerson 1985; Brenner and Cheikh 1995; Chraibi *et al.* 1995; Roitsch *et al.* 1995; Morris 1996; Ehness and Roitsch 1997; Godt and Roitsch 1997; Goupil *et al.* 1998). Similarly, sugars, like Glu, Fru and Suc (Koch 1996; Zhou *et al.* 1998) together with plant hormones (Mason *et al.*

1992; De Wald *et al.* 1994; Dijkwel *et al.* 1997; Mita *et al.* 1997; Perata *et al.* 1997) have the ability to modulate gene expression and hence control tissue physiology, growth and development. The involvement of HMGR, sterols, CK and ABA and differential accumulation, via altered solute transport and metabolism, of sugars within the fruit tissues can thus be expected to have a marked effect on fruit physiology and final fruit size.

In an attempt to gain an insight into the metabolic control of fruit growth and the occurrence of this aberrant fruit phenotype, the ultrastructure of, and the metabolism of Suc in, the small fruit phenotype was investigated.

5.2 RESULTS

5.2.1 GROWTH OF SMALL, ABA- AND CK-TREATED 'HASS' FRUIT

To determine the effect of ABA and iP on fruit size, length and/or dry mass of the small, untreated and hormone-treated normal fruit was investigated during phase II of fruit growth. The results in Figures 5.2 and 5.3 illustrate that both the small fruit phenotype and ABA-treated fruit exhibited a decrease in fruit length and dry mass. There were no significant differences ($P>0.05$) between fruit treated with only iP, a combination of ABA and iP, and control fruit length. Similarly, there was no significant difference in the percentage increase in fruit length between the untreated normal fruit phenotype and iP-treated fruit (data not presented). Small and ABA- treated fruit both exhibited a reduced (<50% of the control) seed and seed coat dry mass (Figure 5.3).

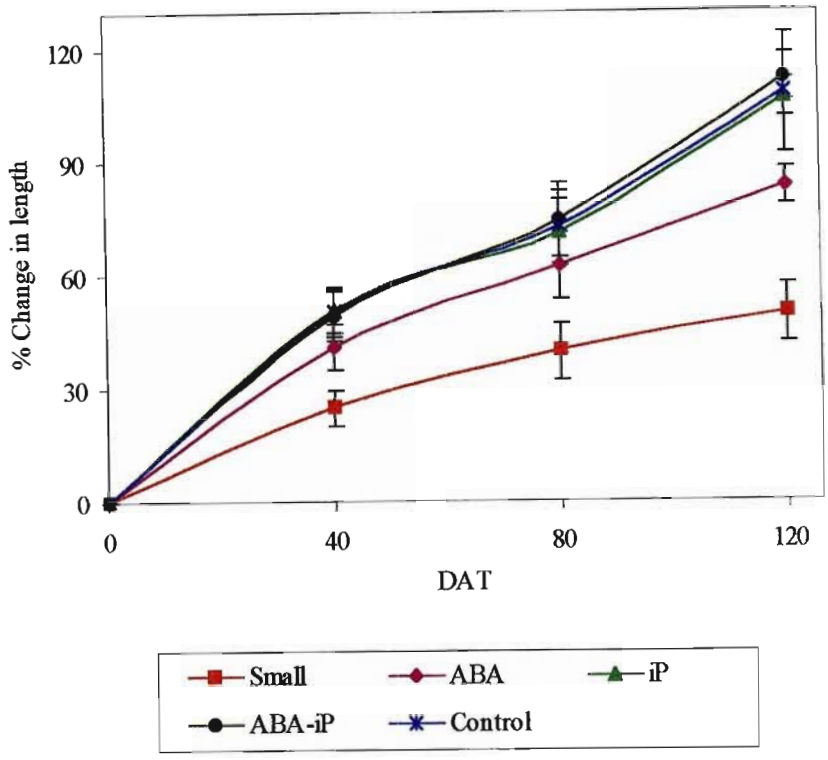


Figure 5.2 Increase in fruit length of small, ABA- and iP-treated 'Hass' fruit, treated 60 DAFB and measured for 120 DAT. n = 20. Error bars represent the standard deviation (SD). $LSD_{0.05}$: 40 DAT = 3.82; 80 DAT = 6.62; 120 DAT = 11.91.

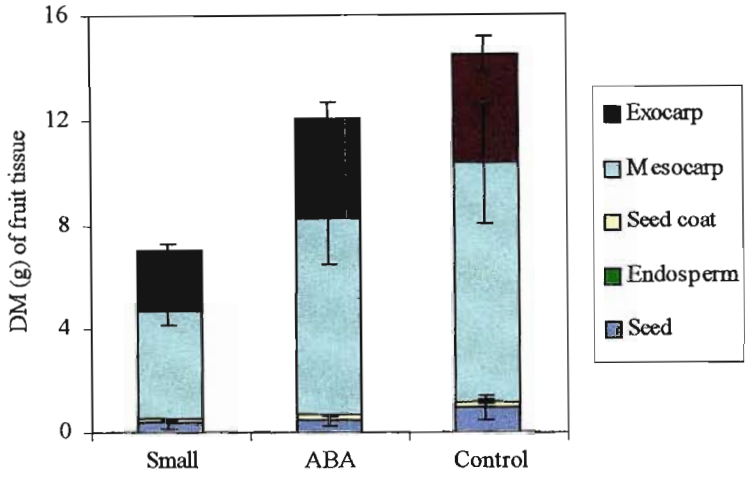


Figure 5.3 DM distribution in small and ABA-treated 'Hass' fruit 106 DAFB (40 DAT). n = 10. Error bars represent the standard deviation (SD). $LSD_{0.05}$: Seed = 0.27; Endosperm = 0.01; Seed coat = 0.04; Mesocarp = 1.24; Exocarp = 0.44.

5.2.2 MOVEMENT OF [^{14}C]-LABELLED SUCROSE IN SMALL AND TREATED- 'HASS' FRUIT

Feeding of small fruit (harvested 200 DAFB) with [^{14}C]-Suc via the pedicel indicated a poor movement of label into the seed (Figure 5.4). The exceptionally low level of labelling of the seed coat in the autoradiograph plate suggested very low movement of [^{14}C]-label into this tissue, and hence reduced solute unloading and transport by the seed coat of the small fruit phenotype. Quantitative values for the distribution of radioactivity derived from either [^{14}C]-Suc or [^{14}C]-Glu applied to intact small, ABA- and iP-treated fruit, is shown in Figures 5.5 and 5.6. In small fruit (Figure 5.5a) movement of label into the seed complex (i.e. the seed, endosperm and seed coat) and mesocarp was reduced, whilst relatively large amounts of label (32%) remained in the pedicel. Similarly, ABA-treated fruit mirrored this trend after 24 h (Figure 5.5b). Movement out of the pedicel and into the mesocarp and seed complex was reduced in small fruit treated 120 DAFB compared to fruit treated 92 DAFB (36% remained in the pedicel after 24 h in 120 DAFB fruit, whilst only 8% remained in the pedicel 92 DAFB; data not presented). A more detailed analysis of solute movement confirmed that after 24 h, ABA-treatment reduced the movement of label into the seed coat and seed (Figure 5.6a). Both iP- and ABA-iP co-treatment increased the amount of label in the seed. During extended periods of incubation the relative amount of label decreased in the mesocarp of ABA-treated fruit. However, only low levels of label were recovered from small and ABA-treated fruit, suggesting either reduced [^{14}C] uptake or elevated respiration rates.

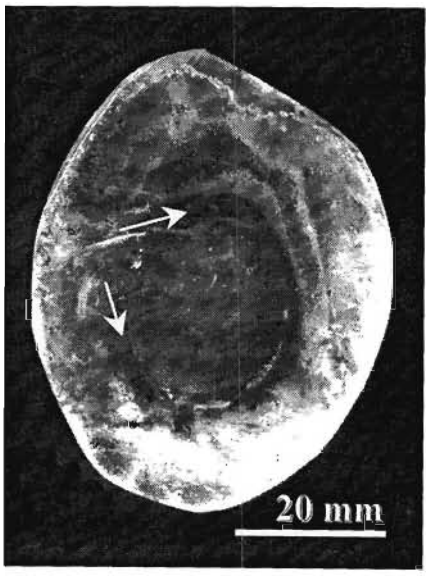
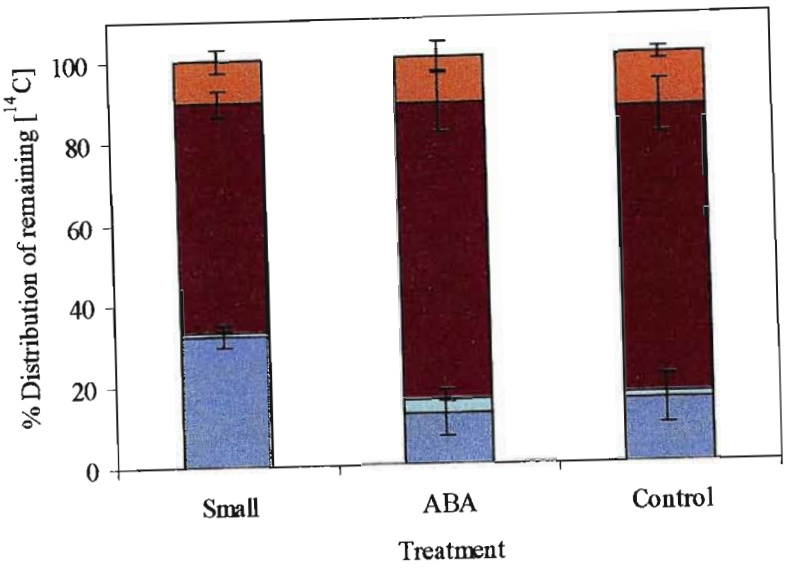


Figure 5.4 Autoradiograph showing the distribution of [^{14}C]-label, derived from exogenous Suc, in the small fruit phenotype 200 DAFB after 24 h. Light areas represent regions of incorporation of radioactivity. The dark seed coat (arrows) shows low [^{14}C] incorporation. Image is representative of 8 fruit from two seasons.

(**a**) 12 h after treatment



(**b**) 24 h after treatment

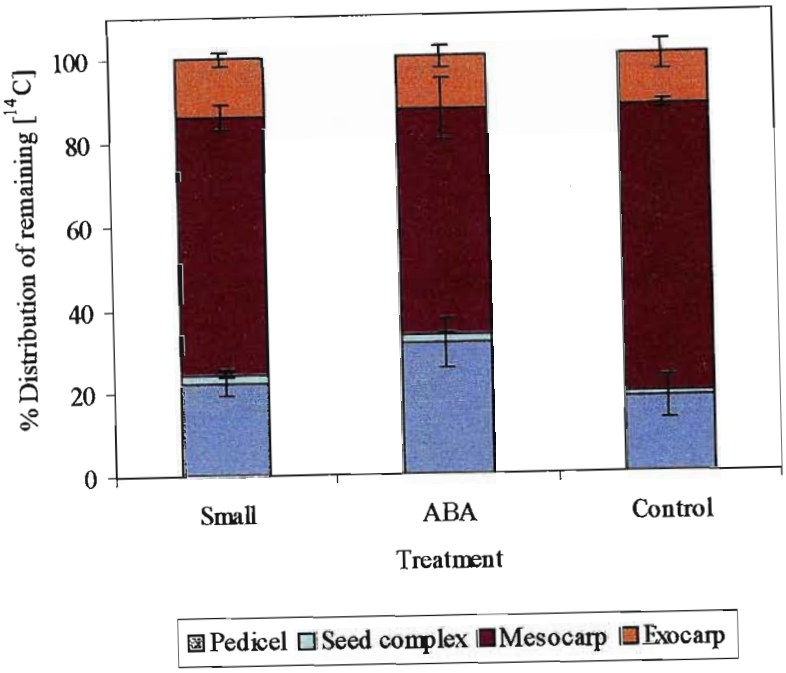


Figure 5.5 Distribution of [^{14}C]-label in developing 'Hass' small and ABA-treated (33 DAT) fruit after feeding with labelled Suc via the pedicel for, (**a**) 12 h; and (**b**) 24 h. The seed complex in the legend refers to the seed, endosperm and seed coat tissue combined. n = 3. Error bars represent the standard deviation (SD). $LSD_{0.05}$: 12 h ~ Pedicel = 7.50; Seed complex = 0.60; Mesocarp = 7.99; Exocarp = 1.29: 24 h ~ Pedicel = 5.11; Seed complex = 0.80; Mesocarp = 4.67; Exocarp = 1.81.

Less label from Glu, compared to Suc, moved into the seed coat. Label derived from Glu appeared to remain predominantly in the mesocarp of iP-treated and ABA-iP co-treated fruit (Figures 5.6c; 5.6d). After 48 h of incubation, ABA-treated fruit showed an increase in the amount of label derived from Glu remaining in the seed and seed coat. The differences in observed [^{14}C]-label distribution derived from Suc and Glu may be a consequence of unique routes of metabolism, and suggests that these pathways are differentially affected by ABA- and iP-treatment.

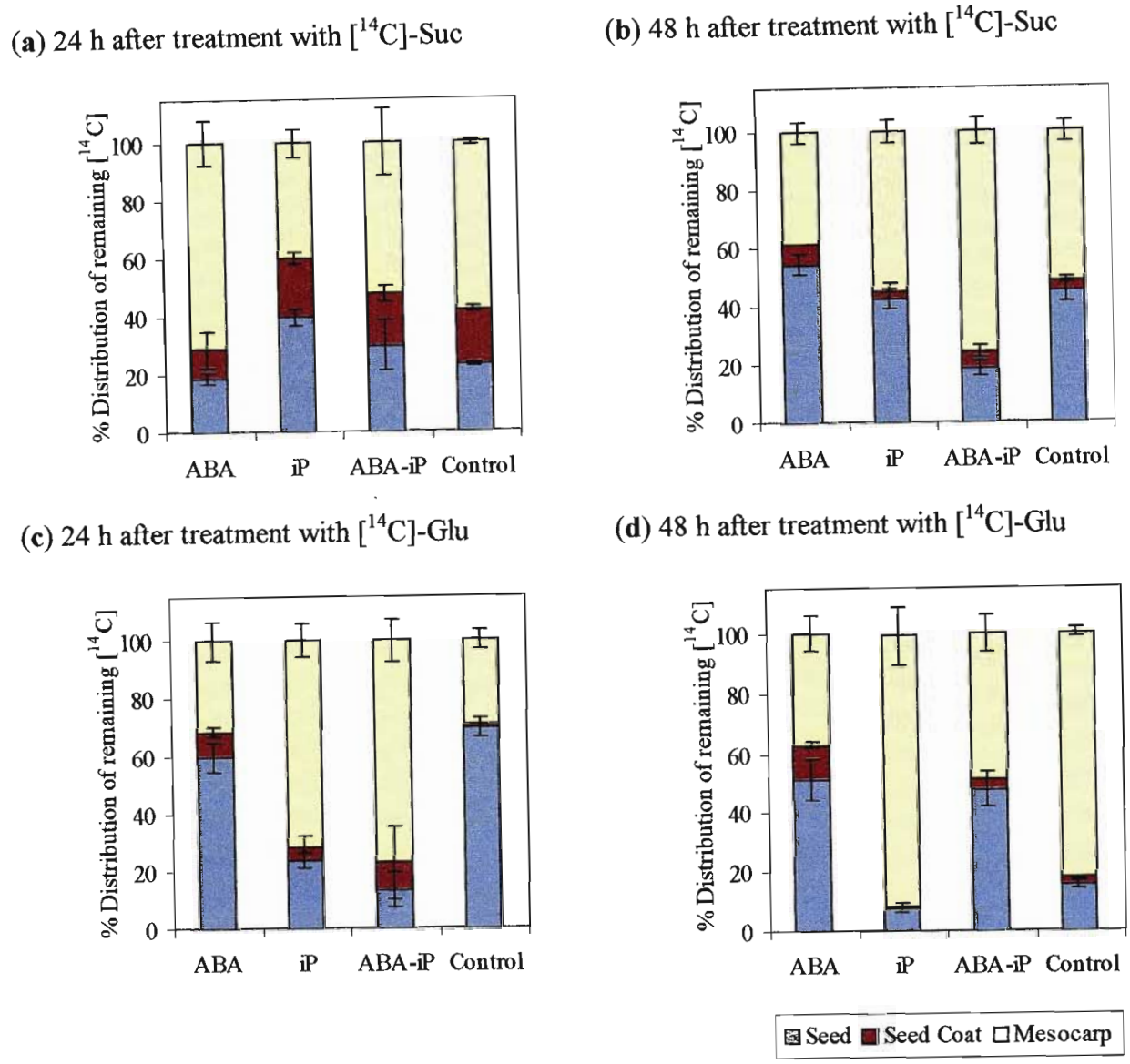


Figure 5.6 Distribution of [^{14}C]-label in untreated, and ABA- and iP-treated (120 DAT) developing 'Hass' fruit after feeding [^{14}C]-Suc via the pedicel (**a**) after 24 h; and (**b**) after 48 h: and [^{14}C]-Glu (**c**) after 24 h; and (**d**) after 48 h. n = 3. Error bars represent the standard deviation (SD). $LSD_{0.05}$: Suc 24 h ~ Seed = 5.25; Seed coat = 2.82; Mesocarp = 7.52: Suc 48 h ~ Seed = 11.93; Seed coat = 1.24; Mesocarp = 12.46: Glu 24 h ~ Seed = 15.07; Seed coat = 3.73; Mesocarp = 14.72: Glu 48 h ~ Seed = 13.10; Seed coat = 2.48; Mesocarp = 15.06.

5.2.3 SEED COAT ULTRASTRUCTURE IN SMALL AND ABA-TREATED 'HASS' FRUIT

The seed coat from the small fruit is highly reduced, consisting of degenerate cells that have either collapsed or been occluded by the deposition of phenolic-like polymers (Figure 5.8). The deposition of phenolic-like polymers is especially evident in the peripheral regions of the seed coat, and in the layer of cells on the inner surface. The degenerate nature of the seed coat, and phenolics and tannins within it, are similar to that observed in the seed coat of mature normal fruit.

The seed coat from ABA-treated fruit showed a similar deposition of phenolic-like substances in the outer cell layers (Figure 5.7). The deposition pattern appeared indistinguishable to that previously described for normal fruit development (Chapter 3.2.4).

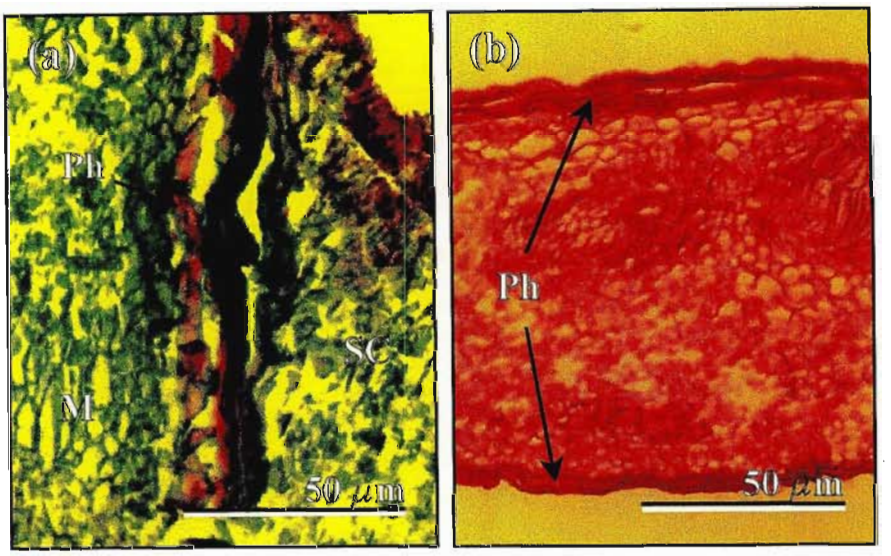


Figure 5.7 Light micrographs of the seed coat from ABA-treated fruit stained with (**a**) fast green and safranin and (**b**) Ruthenium red. Degenerate appearance of inner layers is due to poor wax infiltration, possibly enhanced by deposition of phenolics in the outer seed coat layers. Images are representative of seed coat tissue collected from 3 ABA-treated fruit. Abbreviations: M = mesocarp; Ph = phenolics; SC = seed coat.

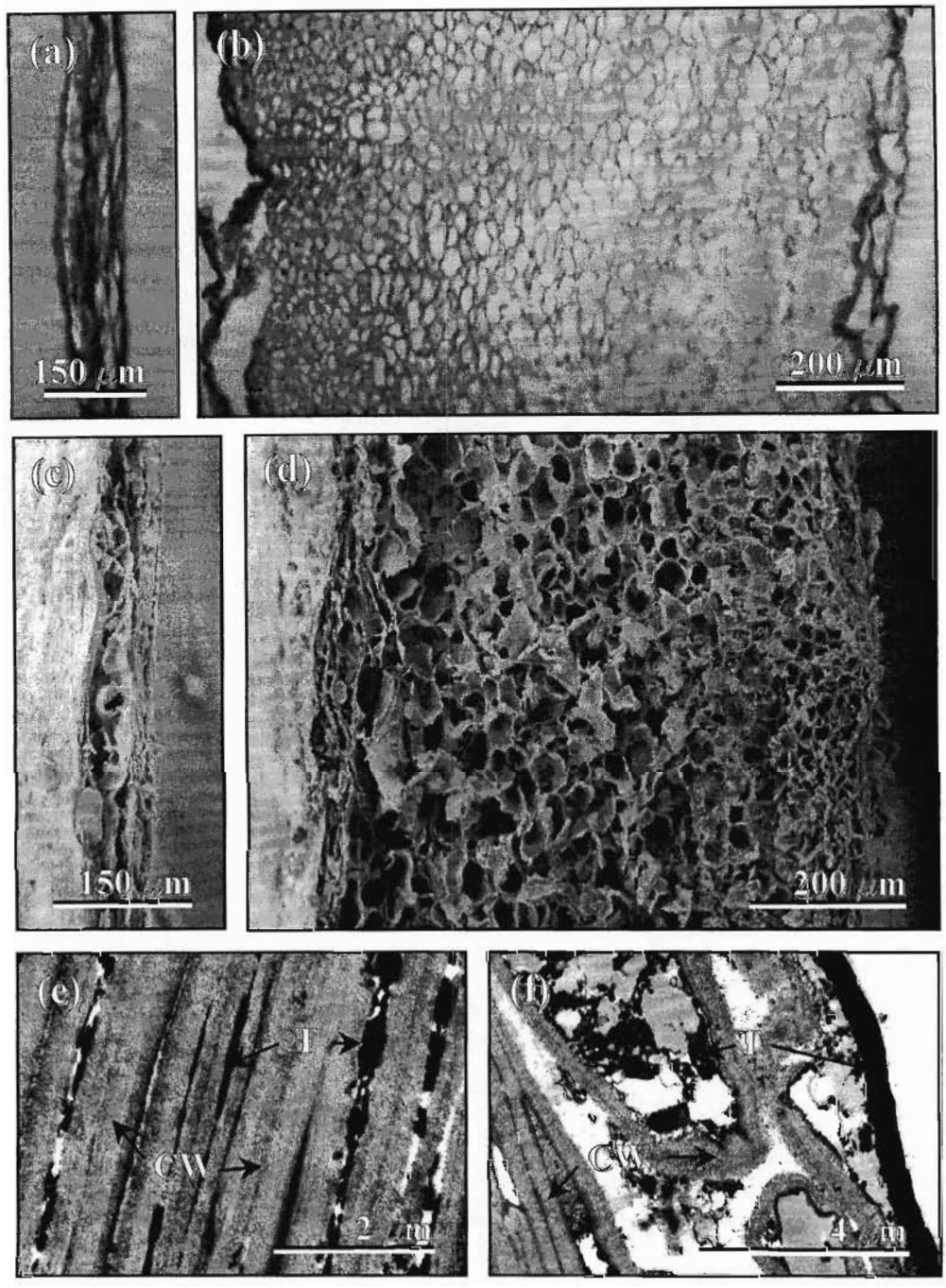


Figure 5.8 Light and SEM micrographs of the seed coat from (**a** and **c**) small and (**b** and **d**) normal 'Hass' fruit 200-210 DAFB. TEM micrographs of transverse sections of small fruit seed coat showing the (**e**) highly flattened nature of the cells; and (**f**) the deposition of phenolic-like compounds on the edge of the seed coat. Light microscopy images stained with aniline blue in lactophenol. Images are representative of trends seen in excess of 100 fruit.

5.2.3.1 Phenolic content in small and hormone-treated 'Hass' fruit seed coat tissue

The degeneration of the seed coat in small fruit is associated with both a dark colour and the apparent deposition of phenolic-like compounds (Figures 5.1; 5.7). Similarly, ABA-treated fruit displayed a similar darkening of the seed coat (Figure 5.1). The premature senescence of, and the deposition of phenolics within, the seed coat may impair the movement of solutes into the developing seed. To investigate the nature of, and potential role in solute partitioning/transport by, these phenolic-like compounds, tannin, phenol and anthocyanin content was determined.

The tannin content of the seed, endosperm, seed coat and mesocarp was higher in the small fruit phenotype (Figure 5.9); however, this was only significant ($P<0.05$) in seed coat tissue. The elevated tannin levels in the seed coat remained largely unchanged at approximately 100 mg procyanidin g^{-1} DM throughout the period of observation (120 DAFB: data not presented). Interestingly, the seed tissue of the small fruit showed an increase in tannin levels during early development, from 68 mg procyanidin g^{-1} DM at 66 DAFB to 105 mg procyanidin g^{-1} DM at 92 DAFB, after which the tannin content remained unchanged, indicating a possible role of tannins in seed maturation.

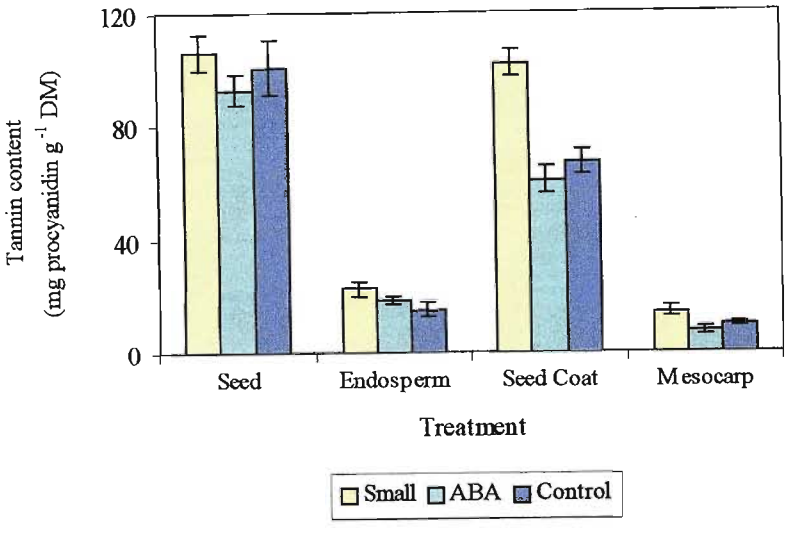


Figure 5.9 Tannin content of small and ABA-treated 'Hass' fruit tissue harvested 40 DAT. $n = 5$. Error bars represent SD. $LSD_{0.05}$: Seed = 7.95; Endosperm = 3.55; Seed coat = 17.28; Mesocarp = 2.60.

Soluble phenol content of fruit tissues followed a similar pattern to that seen with tannin content (Figure 5.11a). The only significant difference was in the phenol content of seed coat tissue, in which the soluble phenol content was significantly ($P<0.01$) higher in the small fruit variant. ABA-treatment increased soluble phenol levels slightly, but not significantly. In the seed and seed coat tissue, soluble phenols and tannins represent 9-18 % and 6-18 % of the tissue dry mass, respectively, and may reflect a 'shunting' of carbon into metabolic pools other than those associated with an increase in biomass. In younger tissue (106 DAFB, 40 DAT), there were no significant differences in insoluble phenol content between treatments (Figure 5.11b). In both the endosperm and seed coat tissue the insoluble phenol content increased with fruit development over the period 66-120 DAFB (data not presented). In seed coat tissue from fruit assayed 220 DAFB, the insoluble phenol content of the small fruit is almost twice that of the normal fruit phenotype (Figure 5.10).

In small and ABA-treated fruit, seed coat anthocyanin levels were high in the seed coat tissue (Figures 5.12; 5.13). Notably, iP-treatment alone reduced the anthocyanin content in seed coat tissue.

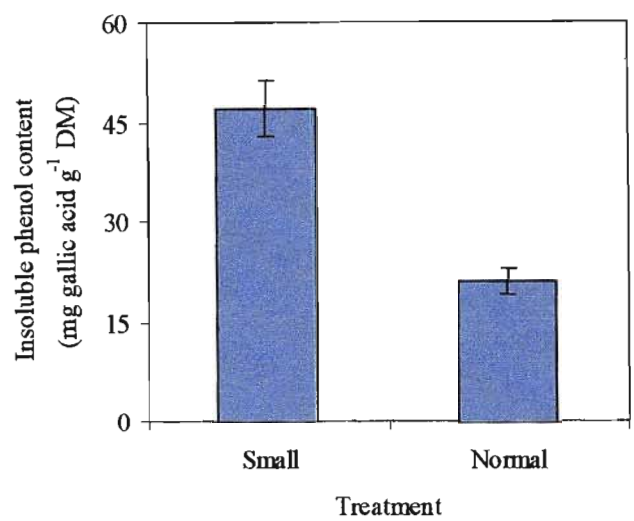
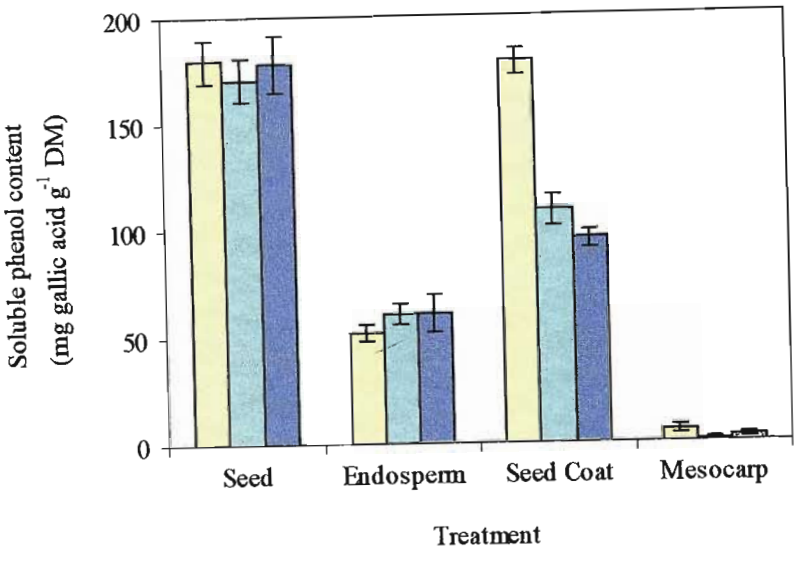


Figure 5.10 Insoluble phenol content of the seed coat from small and normal fruit harvested 220 DAFB. $n = 3$. Error bars represent SD. $LSD_{0.05} = 18.88$.

(a) Soluble phenol content



(b) Insoluble phenol content

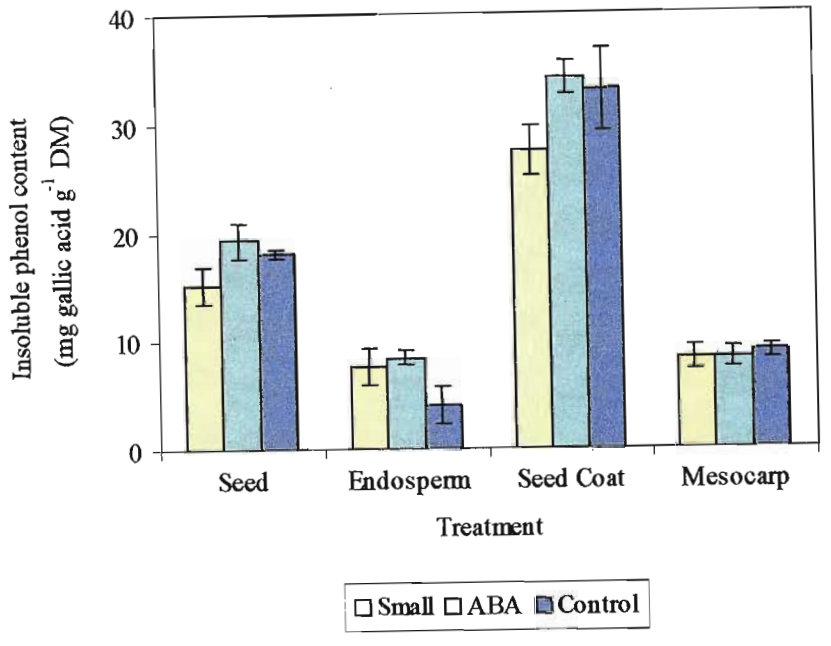


Figure 5.11 (**a**) Soluble and (**b**) insoluble phenol content of small and ABA-treated fruit tissue harvested 106 DAFB (40 DAT). n = 5. Error bars represent SD. $LSD_{0.05}$: Soluble phenols ~ Seed = 38.62; Endosperm = 5.00; Seed coat = 31.23; Mesocarp = 3.65: Insoluble phenols ~ Seed = 1.56; Endosperm = 3.72; Seed coat = 9.48; Mesocarp = 1.23.

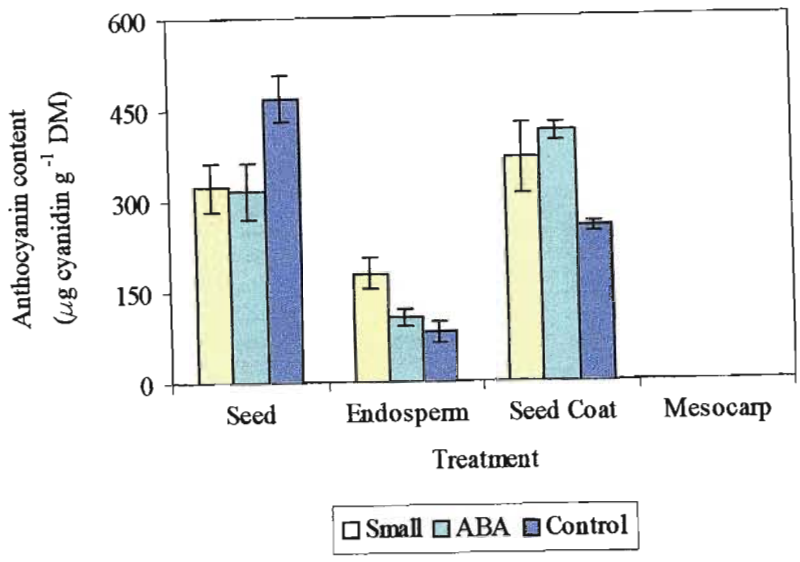


Figure 5.12 Anthocyanin content of small and ABA-treated 'Hass' fruit tissues harvested 40 DAT (106 DAFB). n = 5. Error bars represent SD. $LSD_{0.05}$: Seed = 90.93; Endosperm = 130.07; Seed coat = 59.21. No anthocyanins were detected in the mesocarp.

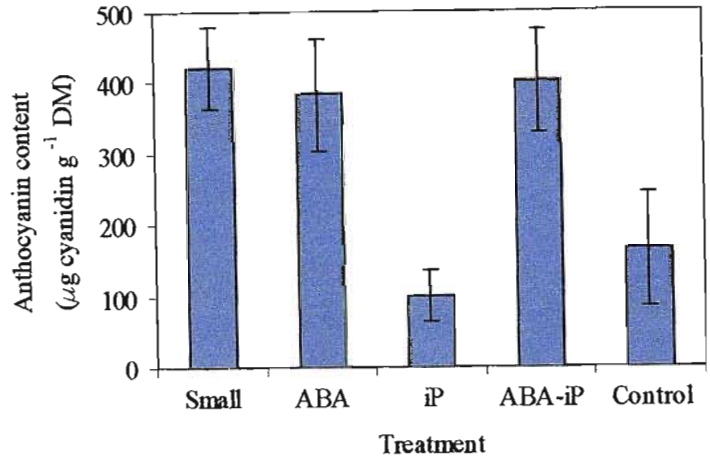


Figure 5.13 Anthocyanin content of seed coat tissue from small and treated 'Hass' fruit harvested 120 DAT (210 DAFB). n = 3. Error bars represent SD. $LSD_{0.05}$ = 93.60.

5.2.4 PLASMODESMATA STRUCTURE AND SYMPLASTIC CONTINUITY IN SMALL AND ABA- TREATED FRUIT

Symplastic solute flux can often be attributed to Pd frequency and function. Moore-Gordon *et al.* (1998) reported a loss of cell-to-cell communication in small and ABA-treated avocado fruit. Ultrastructural studies revealed that the small fruit has low Pd frequencies in the seed, seed coat

and mesocarp (Table 5.1). The Pd in the mesocarp (Figure 5.14) also appear to be simpler in structure and more primary in nature than those observed in the seed coat and mesocarp tissue in the normal fruit phenotype (Figure 3.7). Furthermore, in the degenerate seed coat it was very difficult to distinguish between the different layers in the seed coat at the TEM level, and to locate or identify Pd, suggesting that during the senescence of the seed coat there is a loss of Pd structure and function and hence symplastic continuity.

Table 5.1 Plasmodesmatal, pit field and mitochondrial frequency in seed, seed coat (pachychalazal portion) and mesocarp of small fruit. The values represent the averages of counted structures in a 60-80 nm thick section of 30 complete cells randomly selected from several sections taken from at least 2 different fruit. Single Pd were not regarded as coming from a pit field. (mean±SD).

Tissue	Layer orientation	Cellular Structure		
		Plasmodesmata	Pit field	Mitochondria
Seed		1±1	1±1	2±1
Seed coat	Indistinguishable	1±1	1±1	1±1
Mesocarp		3±2	3±1	7±4

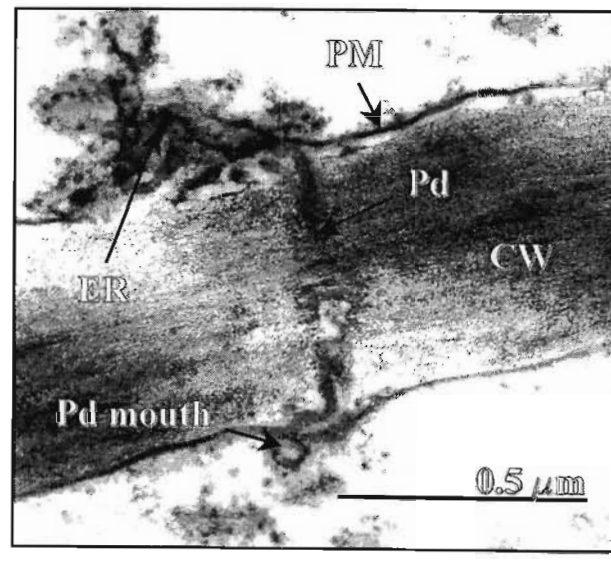


Figure 5.14 View of a single, simple Pd frequently found in mesocarp tissue from the small fruit phenotype.

There was no apparent difference in Pd number or structure between ABA- and iP-treated fruit (data not presented). Neither did ABA- and iP-treated fruit Pd, pit field and mitochondria frequencies differ significantly from those of the normal fruit phenotype (Table 3.1). These results suggest symplastic continuity in ABA-treated avocado fruit is, in the short term, affected by Pd function and not Pd frequency.

To determine whether Pm-bound proteins may be responsible for the occlusion of Pd, proteins were separated by SDS-PAGE from membranes prepared from fresh tissue and protoplasts isolated from the mesocarp tissue of small and normal 'Hass' fruit. No observable differences were seen in membrane-bound proteins from the two fruit variants (data not presented). A distinct protein band corresponding to 26.6 kD was observed, and although the identification of this band was not confirmed by immunology, it may be homologous with the connexin family of gap junction proteins.

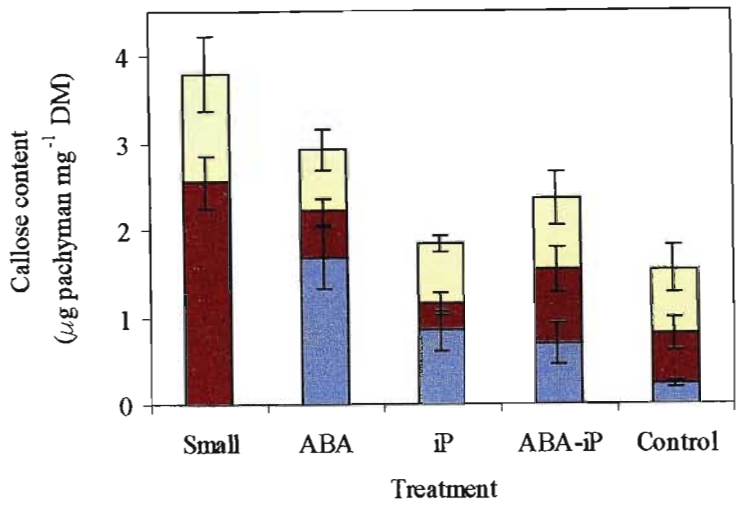
The association of callose with the Pd, and a reported loss of Pd function in small fruit seed coat and mesocarp tissue (Moore-Gordon *et al.* 1998) begs the question: 'Does callose affect symplastic transport in small fruit?'. Since plugging of the Pd by callose is likely to exert a profound role in avocado fruit morphogenesis and physiology through altered symplastic transport, a histochemical study was undertaken to investigate the potential role of callose in the expression of the small fruit phenotype. With the techniques available, differences in callose association/deposition at the Pd could not be observed between small, normal and hormone-treated fruit. In an attempt to address this question the endogenous amounts of callose and the rates of callose synthesis were thus assayed.

5.2.4.1 Quantification and *in vivo* synthesis of callose in small and treated 'Hass' fruit

The callose content (Figure 5.15a) in the seed coat and mesocarp tissue of small fruit was high, relative to that of the control-fruit. In the small fruit both callose levels and rates of synthesis were very low in the seed (Figure 5.15). Treatment with ABA, or ABA and iP simultaneously, was found to increase seed callose levels over the control (5.15a), but this was not evident from enzyme levels (5.15b).

The rate of callose synthesis in the small fruit phenotype and ABA-treated fruit is low (Figure 5.15b), whilst fruit treated with iP show slightly higher rates of callose synthesis.

(a) Callose content



(b) Rate of callose synthesis

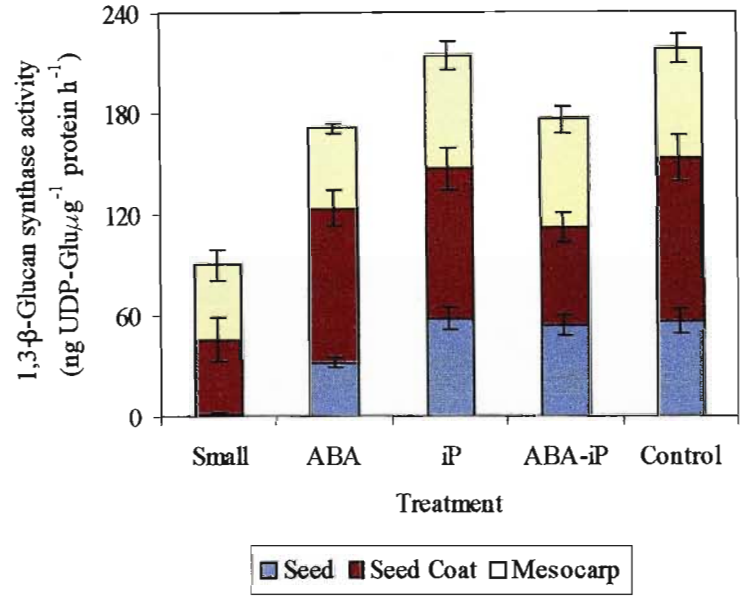
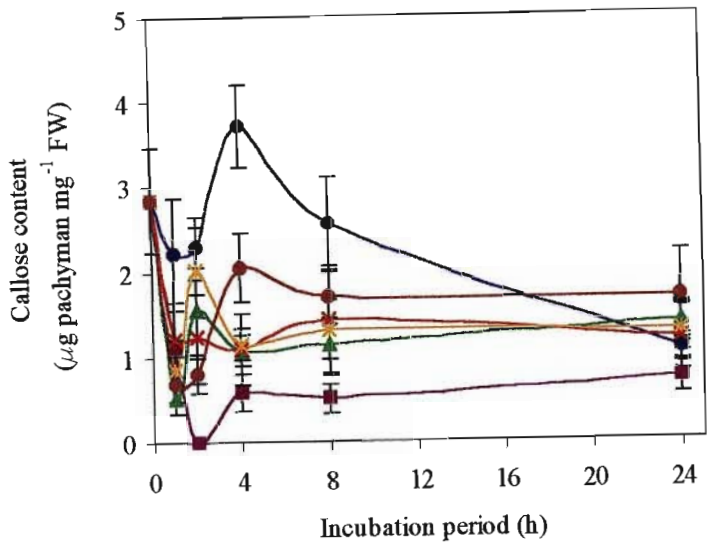


Figure 5.15 Callose (**a**) content, and (**b**) rates of synthesis in the seed, seed coat and mesocarp from small, ABA- and iP-treated 'Hass' fruit harvested 203 DAFB (115 DAT). n = 8. Error bars represent SD. $LSD_{0.05}$: Callose content ~ Seed = 0.34; Seed coat = 0.29; Mesocarp = 0.26: Rate of callose synthesis ~ Seed = 9.61; Seed coat = 17.86; Mesocarp = 11.93.

5.2.4.2 Effect of ABA and sugar on the *in vitro* synthesis of callose in seed coat tissue

The apparent increase in callose content in small and ABA-treated fruit, with a corresponding decrease in 1,3-β-glucan synthase activity, suggested that callose content may have been influenced by ABA. Furthermore, the close association of sugar transport, metabolism, hormones

(**a**) Callose content



(**b**) Rate of callose synthesis

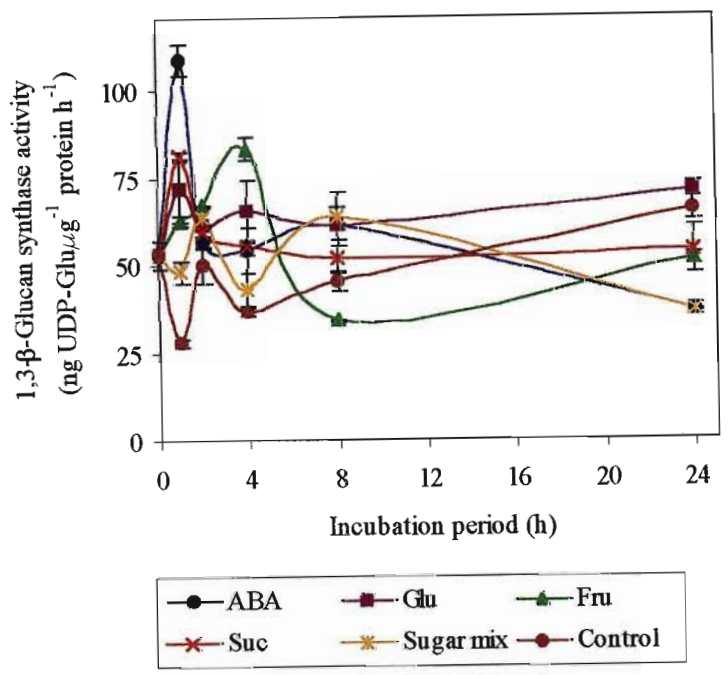


Figure 5.16 Effect of ABA, Glu, Fru, Suc, and a mix of these three sugars (2:3:2, w/w/w) on (**a**) callose content and (**b**) the *in vitro* rate of callose synthesis in seed coat tissue. $n = 5$. Error bars represent SD. $LSD_{0.05}$: Callose content ~ 1 h = 0.31; 2 h = 0.27; 4 h = 0.33; 8 h = 0.27; 24 h = 0.16: Rate of callose synthesis ~ 1 h = 9.27; 2 h = 10.58; 4 h = 52.50; 8 h = 3.93; 24 h = 4.77.

and fruit growth necessitated a comparison of the effect of ABA and different sugars on callose content and synthesis. The results in Figure 5.16 indicate that ABA treatment caused a transient increase in the callose content of seed coat tissue, whilst having only a small, relatively rapid (after ±1 h) transient effect on callose synthesis. Similarly, Suc, Glu and Fru increased the rates of callose synthesis after 1 h (Figure 5.16b). In addition, Glu, Fru, Suc and a mixture of these sugars in the ratio 2:3:2 increased the callose content in the short term (after 2 h), but subsequently reduced the callose content in the seed coat tissue (Figure 5.16a).

5.3 SUMMARY

1. Small and ABA-treated fruit exhibited a reduced growth rate and show poor uptake of [^{14}C]-label, with reduced allocation of label to the seed and seed coat.
2. Both small and ABA-treated fruit appear to have prematurely senesced seed coats, rich in phenolic-like compounds deposited in the cells peripheral layers. The tannin, soluble and insoluble phenols and anthocyanin contents were higher in the small fruit.
3. The small fruit variant was characterised by low Pd frequency and the presence of simple, single Pd. ABA- and iP-treatment did not appear to have a significant influence on the frequency and 'differentiation' of Pd. Thus the differences in Pd conductivity described by Moore-Gordon *et al.* (1998) must be attributed to differences in Pd function.

CHAPTER 6

THE RELATIONSHIP BETWEEN ABA TREATMENT, CARBOHYDRATE METABOLISM AND THE APPEARANCE OF THE 'HASS' SMALL FRUIT PHENOTYPE

6.1 INTRODUCTION

Recent models of phloem unloading in fruit suggest a transition with development from symplastic to apoplastic transport (Figure 4.1). Whilst phloem unloading, sugar metabolism and sink strength are affected by the fruits normal developmental programme, changes in hormone homeostasis can be expected to have far-reaching effects on fruit physiology and growth. One such effect would be an alteration in the metabolism of sugars and subsequent alterations in solute content and composition, sink strength and continued photoassimilate unloading.

Symplastic unloading may be modulated by plant hormones such as ABA via inositol triphosphate/diacyl glycerol signal transduction (Morris 1996). Although ABA has been shown to inhibit Suc transport in wheat kernels (Borkovec and Procházka 1992), it is generally believed to promote photosynthate unloading (Tietz *et al.* 1981, Schüssler *et al.* 1984; Clifford *et al.* 1990) and has been associated with increased TSS and Suc, Glu, Fru and sorbitol contents (Hartung *et al.* 1980; Ackerson 1985; Kojima *et al.* 1995; Kobashi *et al.* 2000). Similarly, ABA has been found to be high in both young developing tomatoes (Fraser *et al.* 1995) and avocado (Cowan *et al.* 1997a) when the establishment of sink strength is paramount, and declines during the course of development. In part, these changes in sugar content and phloem unloading can be attributed to changes in Suc cleavage. The activity of the enzymes AI, NI, SSy, SPS and α-amylase have all been shown to be modified by ABA and/or CK, with a resultant increase in Suc hydrolysis and a reduction in starch formation (Hartung *et al.* 1980; Ackerson 1985; Zieslin and Khayat 1990; Antognozzi *et al.* 1996; Pagano *et al.* 1997; Goupil *et al.* 1998; Kashem *et al.* 1998). Furthermore, the metabolism of the sugar alcohol sorbitol in peach fruit is similarly increased by ABA treatment (Kobashi *et al.* 1999).

Moore-Gordon and co-workers (1996; 1997a) suggested that the occurrence of the small fruit phenotype is aggravated by stress, particularly water stress. Stress has been reported to induce a bulk increase in endogenous ABA in plant tissue (Cowan *et al.* 1997b), and a strong relationship

has been found between water-stress and increasing ABA levels (Lopez-Carbonell *et al.* 1994). Thus ABA acts, in part, as a relay between the environment and the plant (Chandler and Robertson 1994). This interaction between the stimulus and cell response by ABA occurs through the induction of a cascade of events, including the induction of numerous polypeptides (Lin and Ho 1986; Bush *et al.* 1993; Marttila *et al.* 1996; Bonetta and McCourt 1998). Furthermore, CK usually decreases with water stress (Pospíšilová *et al.* 2000). CK appear to antagonize many physiological processes mediated by ABA (Schmülling *et al.* 1997; Cowan *et al.* 1999; Pospíšilová *et al.* 2000) and a decrease in endogenous CK may thus 'amplify' the effect of increasing ABA. It is, therefore, not surprising that the occurrence of the small fruit phenotype has been attributed to alterations in the CK:ABA balance in the fruit (Moore-Gordon *et al.* 1998). ABA and sugar content in plants are intrinsically linked and have the ability to modulate gene expression and hence control solute transport and metabolism, sink strength, cell cycle activity and plant growth and development (Mason *et al.* 1992; De Wald *et al.* 1994; Koch 1996; Dijkwel *et al.* 1997; Mita *et al.* 1997; Perata *et al.* 1997; Zhou *et al.* 1998).

Water stress in maize has been associated with a decrease in ovary growth and altered sugar metabolism (Zinselmeier *et al.* 1995). In *Arabidopsis*, genes encoding for SSy are increased by exposure to stress (drought, cold and O_2 deficiency), a phenomenon mimicked by the feeding of metabolizable sugars such as Suc and Glu (Déjardin *et al.* 1999). The comparison of small and normal fruit phenotypes showed a four-fold increase in total Suc hydrolysis in the small fruit (Cowan *et al.* 1998), high seed IAI activity, low mesocarp IAI and reduced seed TSS, but high Glu levels as a percentage of TSS (Cripps *et al.* 1999; Richings *et al.* 2000). Furthermore, the small fruit phenotype was found to have high respiration rates and low levels of starch accumulation (Cripps and Cowan 2000). [^{14}C]-Suc movement indicated that in small and ABA- treated fruit accumulation of label was reduced in the seed and seed coat (Cowan *et al.* 1998). Many of these differences between small and normal fruit, and the similarities between small and ABA-treated fruit, suggest differences in Suc metabolism that are similar to those induced by ABA and/or drought stress. Furthermore, Richings and co-workers (2000) showed that the small fruit phenotype of avocado had elevated Glu, decreased HMGR activity and increased ABA metabolism. They also showed that treatment with mevastatin, an inhibitor of HMGR, resulted in increased Glu as a percentage of TSS with a concurrent decrease in HMGR activity. Similarly, incubation with Glu resulted in decreased HMGR activity in the seed, and increased ABA biosynthesis/turnover. HMGR appears to be a member of the SnRK1-type protein subfamily (Barker *et al.* 1996). Thus it is plausible that HMGR activity is affected by the endogenous Glu concentrations, and that the sugar content/composition and relative levels of ABA, arising

independently of the mevalonate pathway, may act together to modulate the expression and/or activity of HMGR and so affect seed solute transport, cell division and fruit growth. The differential accumulation and subsequent breakdown of imported sugars in small, normal and ABA-treated fruit can thus be expected to have far-reaching effects on fruit physiology and size.

This chapter describes experiments that were carried out to establish the effect of an altered sugar content, composition and metabolism on the control of final fruit size in 'Hass' avocado. Attempts were made to simulate stress conditions similar to those thought to cause appearance of the small fruit, by treating fruit with ABA, and the relationship between carbohydrate metabolism and fruit growth was investigated.

6.2 RESULTS

6.2.1 CARBOHYDRATE CONTENT AND COMPOSITION IN SMALL AND TREATED 'HASS' FRUIT

The effects of ABA treatment on TSS, and sugar content and composition, are shown and contrasted with small fruit in Figures 6.1-6.3 and Table 6.1. ABA treatment resulted in a significant increase ($P \leq 0.05$) in seed (120 DAT) TSS, an observation that mirrored that in seed tissue of the small fruit phenotype (Figures 6.1; 6.2). Treatment with iP alone did not cause any significant changes in TSS compared to the control. ABA treatment also caused a slight increase in TSS in the seed coat tissue 40 DAT; however, this increase appears transient as TSS levels at 120 DAT were the same in control fruit as well as in ABA- and iP-treated fruit. In small fruit, TSS levels in the seed coat were very low.

ABA-treatment resulted in increased levels of Suc, Glu, Fru, perseitol and, with the exception of the endosperm and mesocarp, *manno*-heptulose (Figure 6.3). Except for the mesocarp, sugar levels for control tissue and small fruit were generally similar, whereas in ABA-treated fruit the levels were often markedly increased. Hence, the high TSS in small and ABA-treated fruit must be attributed to high levels of the unidentified sugar with an elution time of 8.84 min (Table 6.1). On a percentage composition basis, the only significant differences between small, ABA- and control-fruit sugar levels (Figure 6.3) was that of Glu and Fru in the endosperm, which was almost twice that of the control in small and ABA-treated fruit. In fruit 120 DAT, ABA increased the percentage of Fru (+40%) in the mesocarp, and Glu (+25%), Fru (+77%) and *manno*-heptulose (+56%), whilst reducing Suc (-22%) and perseitol (-25%) in the seed (data not presented). These

changes in sugar composition were reflected in the small fruit. The content of Suc, Glu, Fru, perseitol and manno-heptulose was also increased in both small and ABA-treated fruit 120 DAT (data not presented).

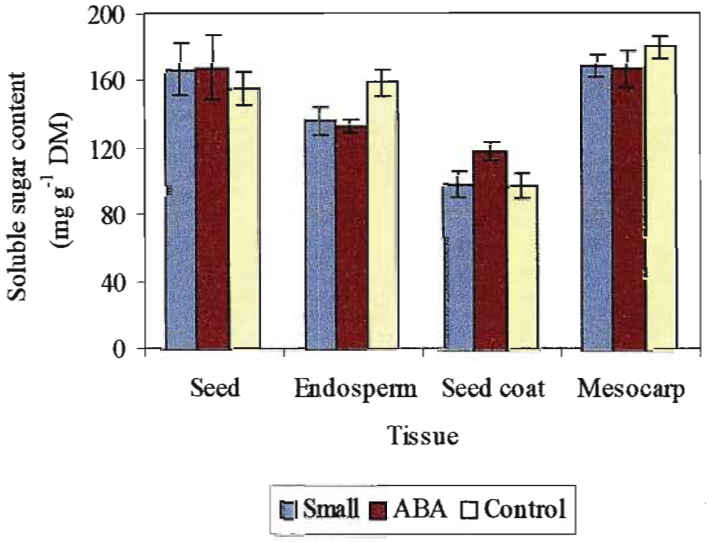


Figure 6.1 TSS in seed, endosperm, seed coat and mesocarp tissue from small and ABA-treated 'Hass' fruit, harvested 40 DAT. $n = 3$. $LSD_{0.05}$: Seed = 14.17; Endosperm = 15.26; Seed coat = 12.57; Mesocarp = 19.78.

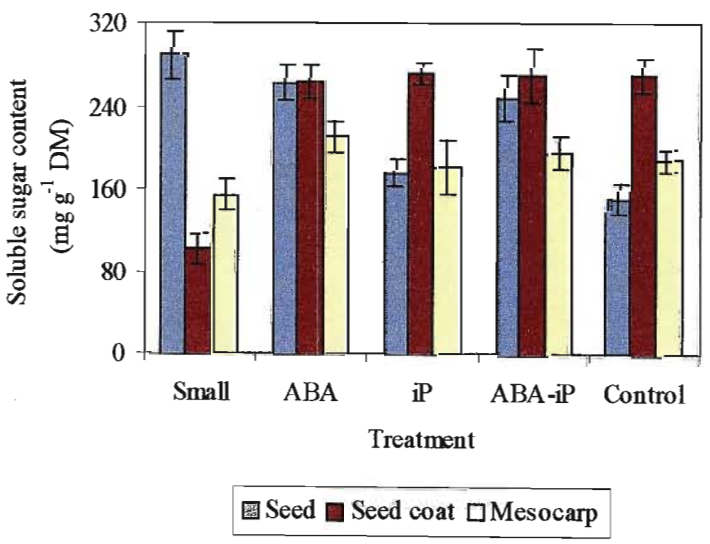


Figure 6.2 TSS in seed, seed coat and mesocarp tissue from small, ABA-, and iP-treated 'Hass' fruit, harvested 120 DAT (210 DAFB). $n = 3$. $LSD_{0.05}$: Seed = 18.63; Seed coat = 23.21; Mesocarp = 8.15.

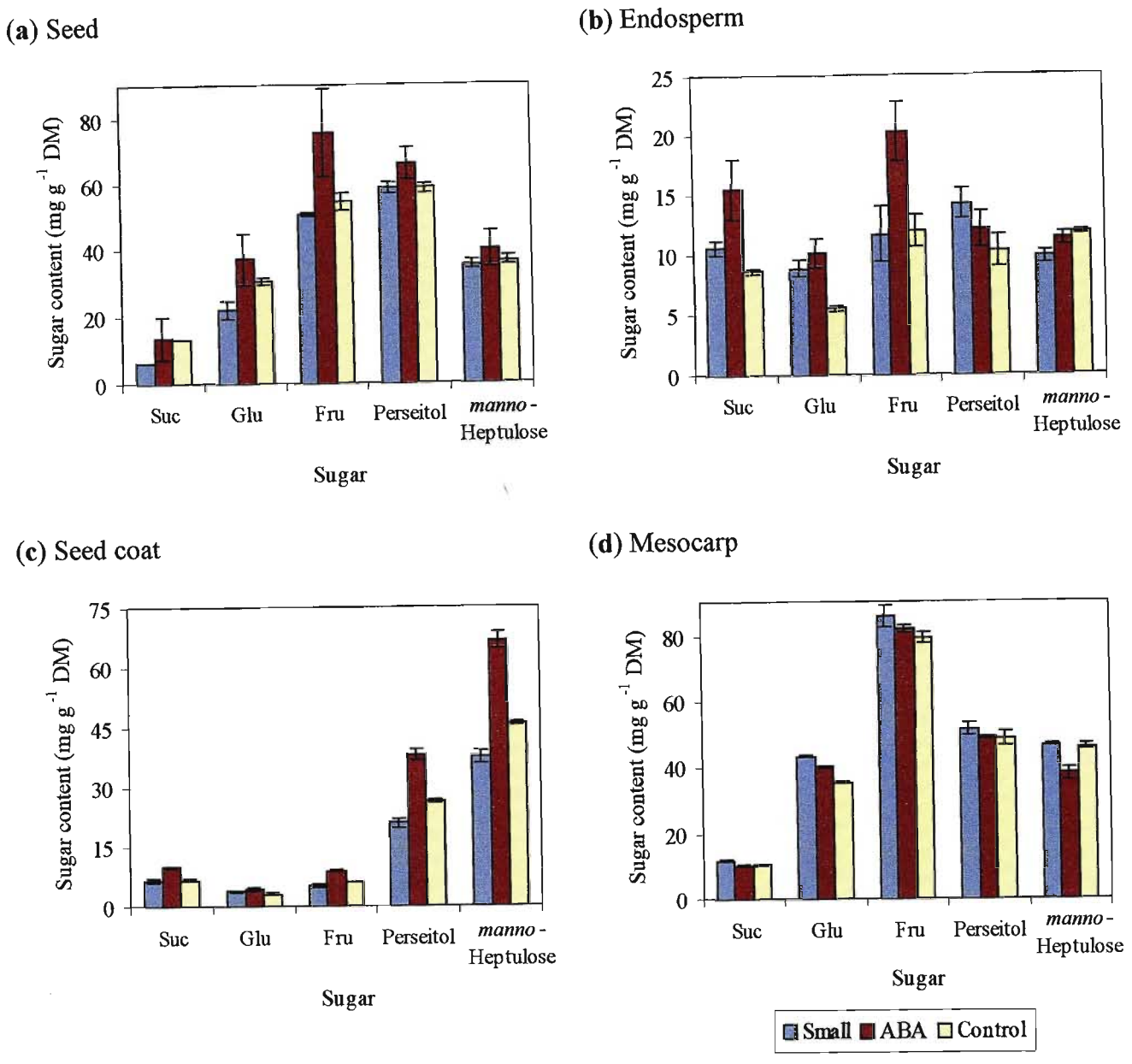


Figure 6.3 Soluble sugar content in (a) seed; (b) endosperm; (c) seed coat and (d) mesocarp tissue from small and ABA-treated 'Hass' fruit harvested 40 DAT (106 DAFB). $n = 3$. $LSD_{0.05}$: Seed ~ Suc = 3.68; Glu = 5.87; Fru = 14.27; Perseitol = 5.12; *manno*-Heptulose = 3.53: Endosperm ~ Suc = 4.07; Glu = 2.70; Fru = 5.02; Perseitol = 1.51; *manno*-Heptulose = 0.42: Seed coat ~ Suc = 1.84; Glu = 0.69; Fru = 1.66; Perseitol = 6.77; *manno*-Heptulose = 11.83: Mesocarp ~ Suc = 0.26; Glu = 2.89; Fru = 1.80; Perseitol = 1.23; *manno*-Heptulose = 4.63.

Of the unidentified sugars found in 'Hass', the only significant differences were an increase in the sugars eluted after 8.84 min and 22.30 min in the seed and endosperm respectively, and a large decrease in the sugar with the elution time of 22.30 min in the mesocarp of small and ABA-treated fruit (Table 6.1).

Table 6.1 Retention times, composition and content of the unidentified sugars separated by HPLC from small and ABA-treated 'Hass' seed, endosperm, seed coat, and mesocarp tissue harvested 40 DAT (106 DAFB). $n = 3$. (mean±SD(% total soluble sugars)).

Rt (min)	Sugar content (mg^{-1} g DM)			$LSD_{0.05}$
	Small	ABA	Control	
Seed				
8.84	91.94±10.82 (31)	103.27±8.44 (28)	78.13±3.47 (25)	15.13
11.85	8.38±1.01 (3)	3.32±0.48 (1)	9.70±0.42 (3)	3.62
22.30	16.58±0.23 (4)	17.28±0.34 (5)	13.01±0.93 (4)	2.48
32.65	ND	ND	ND	-
35.97	18.64±1.19 (6)	19.33±0.92 (5)	17.74±0.39 (6)	1.11
Endosperm				
8.84	130.98±1.89 (52)	127.35±16.18 (45)	142.93±20.55 (51)	20.11
11.85	4.29±0.53 (2)	2.77±0.12 (1)	6.83±0.47 (2)	2.32
22.30	3.60±1.70 (2)	8.17±11.27 (3)	0.62±0.08 (<0.5)	7.69
32.65	5.22±0.13 (2)	5.50±1.02 (2)	5.04±1.02 (2)	0.86
35.97	60.16±2.77 (22)	67.47±8.80 (24)	76.28±6.77 (27)	8.02
Seed coat				
8.84	44.69±0.60 (29)	46.02±1.26 (23)	39.24±1.45 (25)	3.99
11.85	4.96±0.31 (3)	1.74±0.11 (1)	4.62±0.29 (3)	1.63
22.30	29.88±31.94 (16)	15.64±9.03 (8)	23.38±0.08 (15)	7.69
32.65	ND	ND	ND	-
35.97	5.38±0.52 (4)	5.02±0.14 (3)	3.37±2.76 (2)	8.02
Mesocarp				
8.84	11.55±1.67 (4)	12.23±0.32 (4)	17.80±5.65 (5)	4.87
11.85	1.10±0.07 (>0.5)	1.55±0.49 (1)	2.25±0.20 (1)	0.49
22.30	111.29±15.74 (21)	81.37±9.57 (22)	103.67±17.33 (30)	17.71
32.65	ND	ND	ND	-
35.97	ND	ND	ND	-

Rt = Retention time
ND = Not detected

Small and ABA-treated fruit exhibited a decrease in the amount of starch in the seed tissue 40 DAT and 120 DAT (Figures 6.4; 6.5). The small fruit also showed slightly higher levels of starch in the seed coat and mesocarp tissues, but these were not significant.

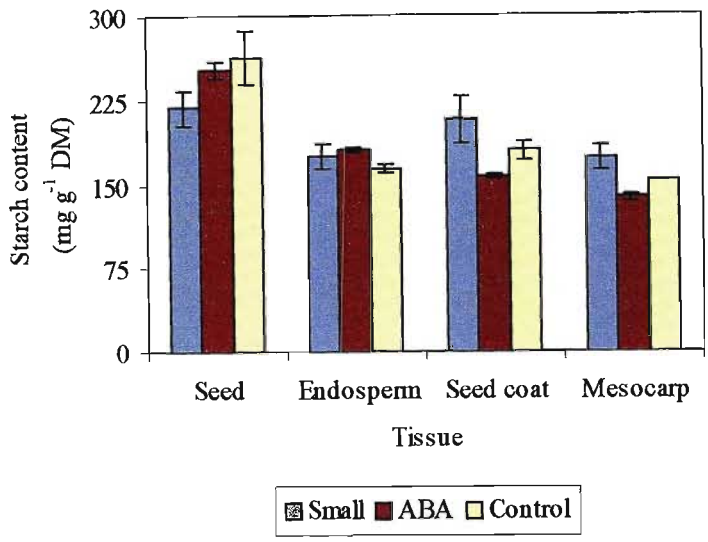


Figure 6.4 Starch content in seed, endosperm, seed coat and mesocarp tissue from small and ABA-treated 'Hass' fruit harvested 40 DAT (106 DAFB). n = 3. $LSD_{0.05}$: Seed = 23.08; Endosperm = 12.95; Seed coat = 14.37; Mesocarp = 13.10.

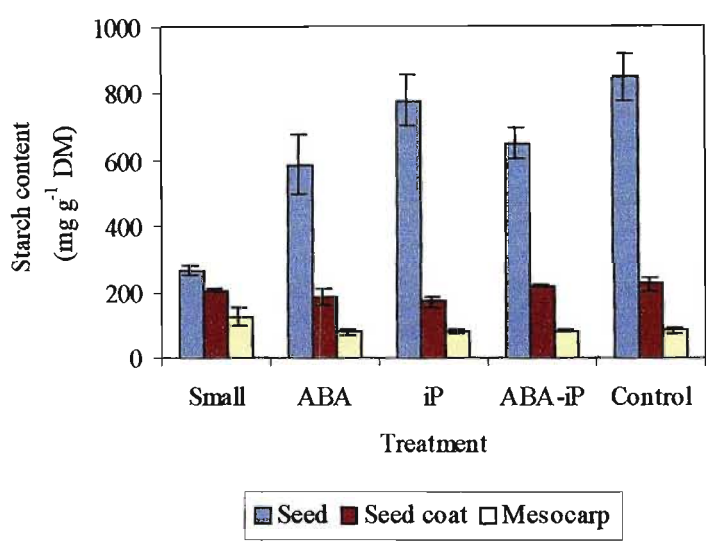


Figure 6.5 Starch content in seed, seed coat and mesocarp tissue from small ABA- and iP-treated 'Hass' fruit harvested 120 DAT (210 DAFB). n = 3. $LSD_{0.05}$: Seed = 71.01; Seed coat = 8.52; Mesocarp = 7.32.

6.2.2 TOTAL LIPIDS AND FATTY ACID CONTENT IN SMALL AND TREATED 'HASS' FRUIT

It was found that total lipids 40 DAT were slightly higher, but not significantly so, in the mesocarp of the small fruit (Figure 6.6). Furthermore, only slight changes in fatty acid composition were observed (Table 6.2). The seed tissue of small and ABA-treated fruit showed a slight and similar increase in the percentages of palmitic and petroselinic acids and decrease in linoleic acid. In mesocarp tissue, both small and ABA-treated fruits showed a small increase in oleic acid and in the fatty acid with a retention time of 13.621 min, which may be an unidentified 18C fatty acid.

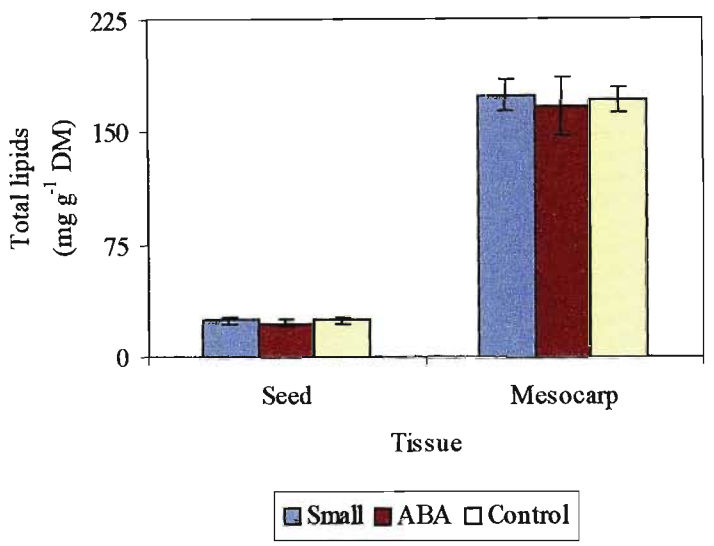


Figure 6.6 Total lipid content in seed and mesocarp tissue from small and ABA-treated 'Hass' fruit 40 DAT (106 DAFB). n = 3. Error bars represent SD. $LSD_{0.05}$: Seed = 1.90; Mesocarp = 2.33.

Table 6.2 Percentage fatty acid composition of lipids extracted from the seed and mesocarp tissue from control, small and ABA-treated 'Hass' fruit harvested 40 DAT (106 DAFB). n = 3. (mean±SD).

Rt	Fatty acid	% Fatty acid composition			$LSD_{0.05}$
		Small	ABA	Control	
Seed					
10.561	16:0	23.42±0.29	22.75±0.12	25.74±1.14	1.94
12.916	18:1(*n*-12)	24.36±0.64	23.58±0.18	31.29±4.97	5.19
13.736	18:2(*n*-6)	43.38±0.58	44.72±0.02	47.86±8.15	4.94
14.728	18:3(*n*-6)	8.84±0.69	8.96±0.33	10.12±2.02	1.33
15.184	18:3(*n*-3)	ND	ND	ND	-
16.690	20:4(*n*-8)	ND	ND	ND	-
Mesocarp					
8.761	14:0	0.13±0.10	0.16±0.23	0.15±0.21	0.18
10.561	16:0	11.92±0.23	11.71±0.46	13.53±0.69	1.13
11.165	16:1(*n*-7)	2.81±0.17	2.80±0.07	3.39±0.21	0.35
12.076	Unknown 1	0.57±0.08	1.29±0.10	1.05±0.15	0.17
12.400	18:0	0.39±0.06	ND	ND	-
12.916	18:1(*n*-12)	19.31±0.44	16.10±0.65	23.72±1.06	4.37
13.369	18:1(*n*-9)	22.54±3.48	19.24±0.88	15.75±0.50	2.05
13.621	Unknown 2	3.27±1.16	2.03±0.87	ND	1.99
13.736	18:2(*n*-6)	21.58±1.58	25.48±3.86	22.63±0.24	2.72
14.463	Unknown 3	ND	ND	ND	-
14.728	18:3(*n*-6)	7.58±1.65	9.34±0.99	8.15±2.09	1.48
15.184	18:3(*n*-3)	8.49±3.72	11.62±2.39	11.40±0.93	1.46
15.940	18:4(*n*-3)	1.11±1.00	ND	ND	-
16.690	20:4(*n*-8)	0.30±0.04	0.23±0.01	0.24±0.03	0.02

Rt = Retention time (min)
ND = Not detected

6.2.3 CARBOHYDRATE METABOLISM IN SMALL AND TREATED 'HASS' FRUIT DURING PHASE II OF FRUIT DEVELOPMENT

Several metabolic pools exist in developing sink tissues, some of which support growth and maintenance (e.g. amino acids, organic acids and lipids), defense (e.g. polyphenolic compounds), and transport and storage (e.g. sugars and starch) (Kleiner *et al.* 1999). However, before photosynthate can be utilized within these pools, it must be imported into sink tissue and converted into metabolically active compounds. The enzymatic regulation of the cleavage of assimilates may be related to the route of sugar transport into sink cells, the provision of metabolic substrates and hence growth (Ho 1996). A study was undertaken of the enzymes AI, NI, SSy, SPS, PDH and α-amylase in an attempt to understand the occurrence of the small fruit phenomenon and the possible role of ABA in the inhibition of fruit growth. Respiration rates were also measured as these might provide an indicator of total catabolic activity in tissues.

6.2.3.1 Respiration rates in developing small, normal and ABA-treated 'Hass' fruit

Fruit respiration has been related to the maintenance of a Suc gradient into sink tissue, dry matter accumulation and fruit growth (Walker and Thornley 1977; Hole and Barnes 1980; De Jong and Goudriaan 1989; Nakano *et al.* 1998). The small fruit phenotype was associated with high respiration rates relative to normal fruit (Figure 6.7). In both small and normal fruit respiration rates were initially very high and declined with fruit growth.

In an experiment to assess the influence of ABA on respiratory rate, fruits were treated with ABA *in vivo* and assayed 40 DAT (Section 2.6). Treatment with ABA was found to increase respiration by 115% (Figure 6.8). However, this value was substantially lower than that of small fruit, which showed more than a 150% increase in respiration relative to the control.

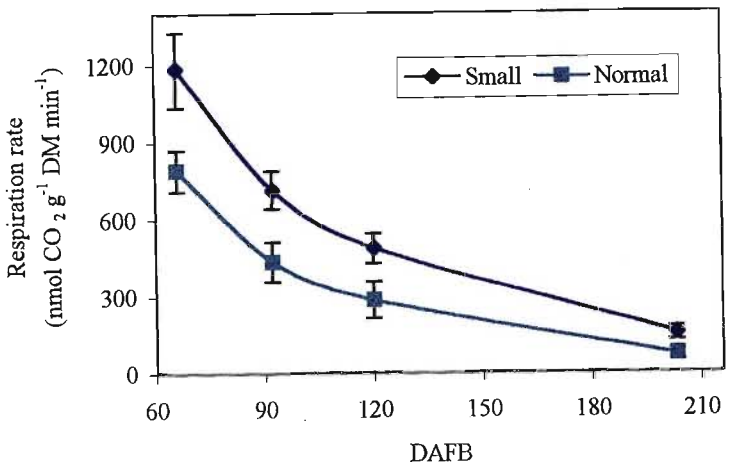


Figure 6.7 Respiration rates of small and normal fruit during phase II of fruit growth. n = 5. Error bars represent SD. $LSD_{0.05}$: Within treatment ~ Small = 201.45; Normal = 116.92: Between treatments ~ 66 DAFB = 248.70; 92 DAFB = 118.19; 120 DAFB = 86.91; 200 DAFB = 45.30.

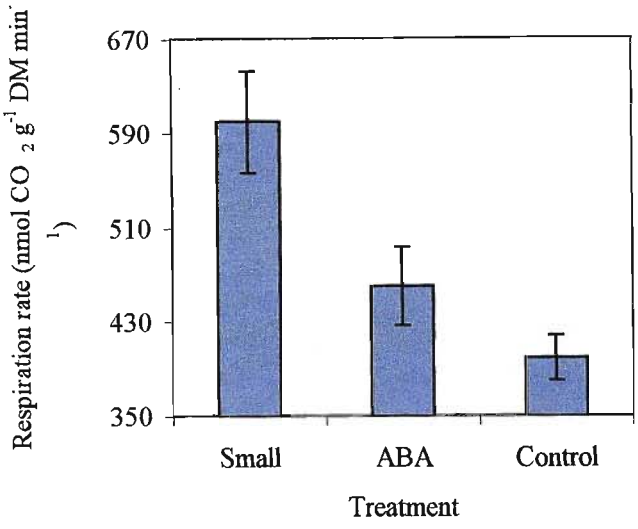


Figure 6.8 Respiration rates of control, small and ABA-treated fruit harvested 40 DAT (106 DAFB). n = 5. Error bars represent SD. $LSD_{0.05}$ = 45.29.

6.2.3.2 Sucrose, perseitol and starch metabolism in small and treated 'Hass' fruit

The activity of carbohydrate metabolizing enzymes in small and ABA-treated fruit is shown in Figures 6.9-6.16. In seed coat and mesocarp tissue from small and ABA-treated fruit the activity of IAI was significantly (P<0.01) greater than the control (Figure 6.9a). In older fruit (120 DAT), a slight increase in seed, seed coat and mesocarp IAI activity was observed in small and ABA-

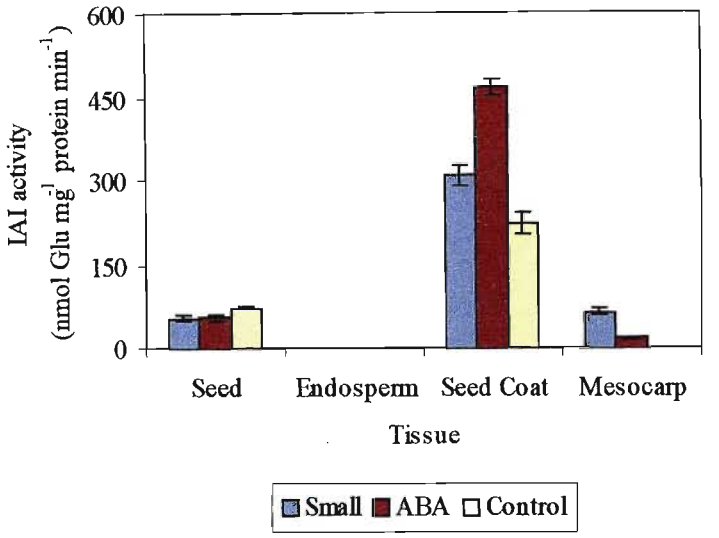
treated fruit (Figure 6.9b). However, of these results, only that of the seed was significant. The *in vitro* incubation of seed coat tissue with ABA revealed that IAI activity was increased by exogenously applied ABA (Figure 6.17a). Compared to the results obtained with normal fruit (Figure 4.12), the small fruit phenotype showed an earlier increase in IAI activity in the seed coat, peaking at a much higher level (542.39 nmol Glu mg^{-1} protein min^{-1}) at 90 DAFB (data not presented).

40 DAT (106 DAFB), SAI activity was significantly higher, by an average of 415% and 382%, in all tissues from small and ABA-treated fruit, respectively (Figure 6.10a). Older (120 DAT, 210 DAFB) small and ABA-treated fruit showed a slight, but non-significant decrease in SAI activity, whilst fruits treated with ABA or simultaneously with ABA and iP showed significantly ($P<0.05$) increased SAI activity in the mesocarp tissue (Figure 6.10b). In seed coat tissue incubated with ABA *in vitro*, SAI activity was increased slightly after 4 h and remained slightly higher than that of the control (Figure 6.17b). Relative to the normal fruit, SAI activity was similar in the seed and seed coat tissue during development, but almost 3 times that of the mesocarp (data not presented).

NI activity was 463% higher in the seed tissue of ABA-treated fruit than the control (Figure 6.11). The seed coat and mesocarp tissue from the small fruit had substantially higher NI activity than that of the control, a trend that was also seen in ABA treatments.

The small fruit phenotype also displayed high levels of SSy activity in the cleavage direction relative to ABA-treated fruit and the control (Figure 6.12a). Whilst this trend was not seen in the seed and mesocarp tissue, ABA treatment did increase the activity of SSy (Clv) in the endosperm and seed coat tissues. Treatment with iP appeared to completely reduce SSy (Clv) activity (Figure 6.12b). Furthermore, co-treatment with ABA and iP negated the inhibitory effect of ABA and iP treatment on SSy (Clv) activity. In seed coat tissue (280 DAFB) incubated with ABA *in vitro*, SSy (Clv) activity was totally abolished (Figure 6.17c).

(a) 40 DAT



(b) 120 DAT

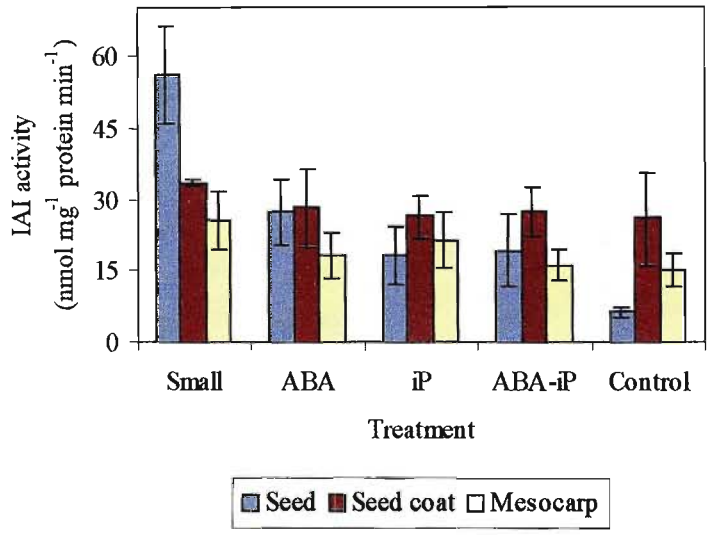


Figure 6.9 IAI activity in control, small and treated 'Hass' fruit harvested (**a**) 40 DAT (106 DAFB) and (**b**) 120 DAT (210 DAFB). n = 3. Error bars represent SD. $LSD_{0.05}$: 40 DAT ~ Seed = 9.74; Endosperm = ND; Seed coat = 110.01; Mesocarp = 7.75: 120 DAT ~ Seed = 3.52; Seed coat = 2.53; Mesocarp = 1.76.

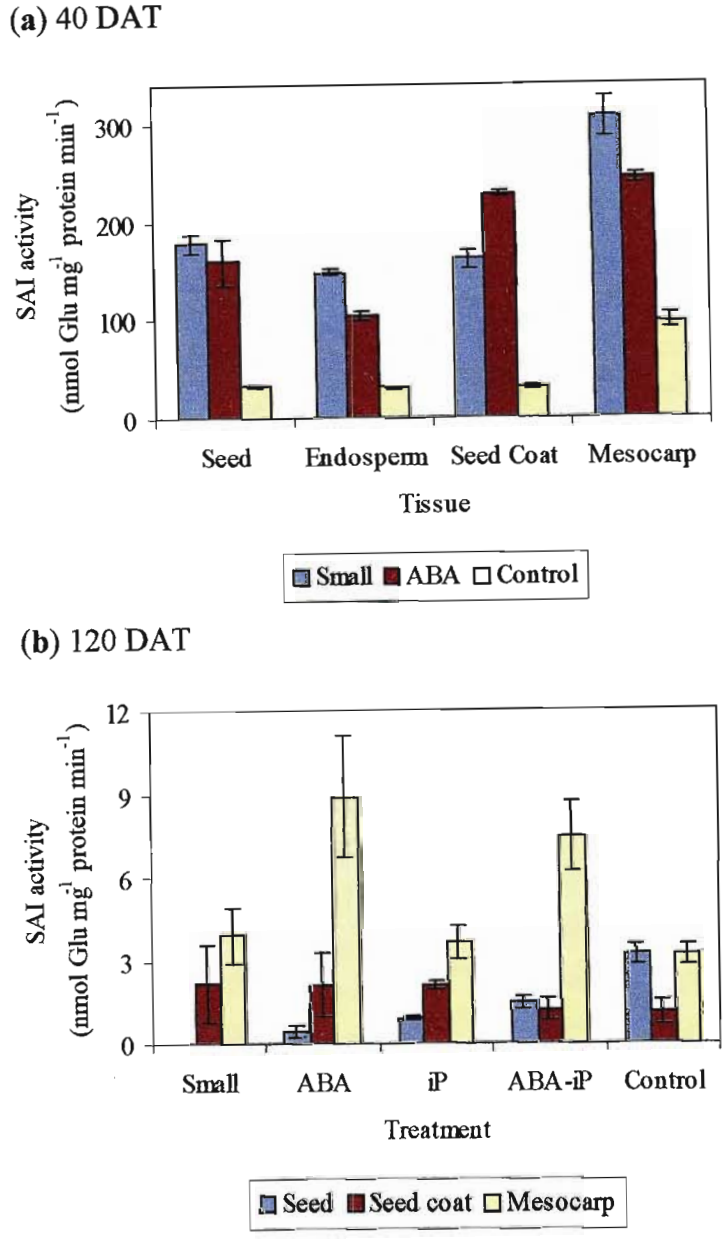


Figure 6.10 SAI activity in control, small and treated 'Hass' fruit harvested (**a**) 40 DAT and (**b**) 120 DAT. n = 3. Error bars represent SD. $LSD_{0.05}$: 40 DAT ~ Seed = 56.46; Endosperm = 31.84; Seed coat = 86.03; Mesocarp = 64.29: 120 DAT ~ Seed = 0.40; Seed coat = 0.29; Mesocarp = 1.03.

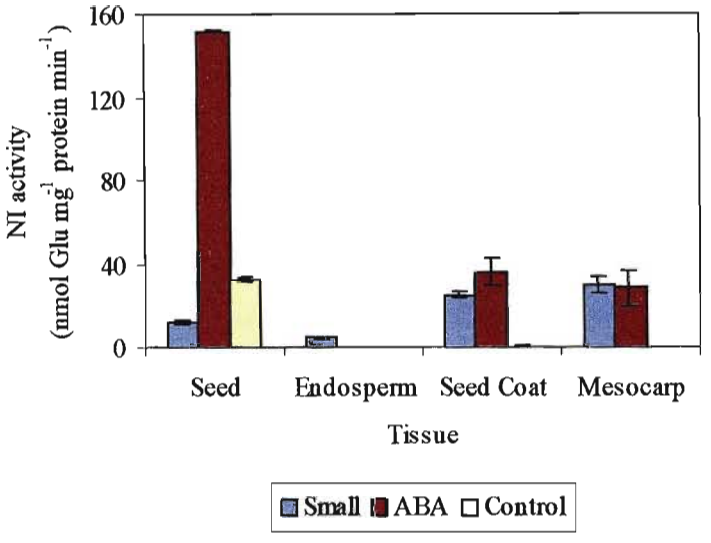


Figure 6.11 NI activity in control, small and ABA-treated 'Hass' fruit harvested 40 DAT. n = 3. Error bars represent SD. $LSD_{0.05}$: Seed = 52.09; Endosperm = ND; Seed coat = 15.93; Mesocarp = 13.15.

The activity of SSy in the synthesis direction was high in the seed of ABA-treated fruit at 40 DAT (Figure 6.13a). Furthermore, both small and ABA-treated fruit had elevated SSy (Syn) activity in the seed, seed coat and mesocarp tissue. However, in more mature fruit (210 DAFB), no SSy (Syn) activity was detected in the small fruit (Figure 6.13b). In mesocarp tissue, ABA and ABA-iP co-treatment appeared to increase SSy (Syn) activity slightly over that of the control. *In vitro* incubation of seed coat tissue in exogenous ABA suggested that SSy (Syn) was induced by ABA after 4-8 h, and ABA treatment increased SSy (Syn) activity in seed coat tissue after this period (Figure 6.17d). These results were corroborated by the fruit treatment studies where ABA resulted in an increase in SPS activity in all tissues, except the endosperm (Figure 6.14a). The small fruit phenotype showed some elevation of SPS activity in the endosperm, seed coat and mesocarp tissues 40 DAT. There was no detectable SPS activity in the seed and seed coat tissue of fruit harvested 210 DAFB (Figure 6.14b). Again, ABA-treatment appeared to increase SPS activity in the seed and seed coat. Interestingly, iP-treatments increased mesocarp SPS activity markedly. Incubation of seed coat tissue with ABA revealed that ABA-treatment caused a transient increase in SPS activity after 1 h (Figure 6.17e).

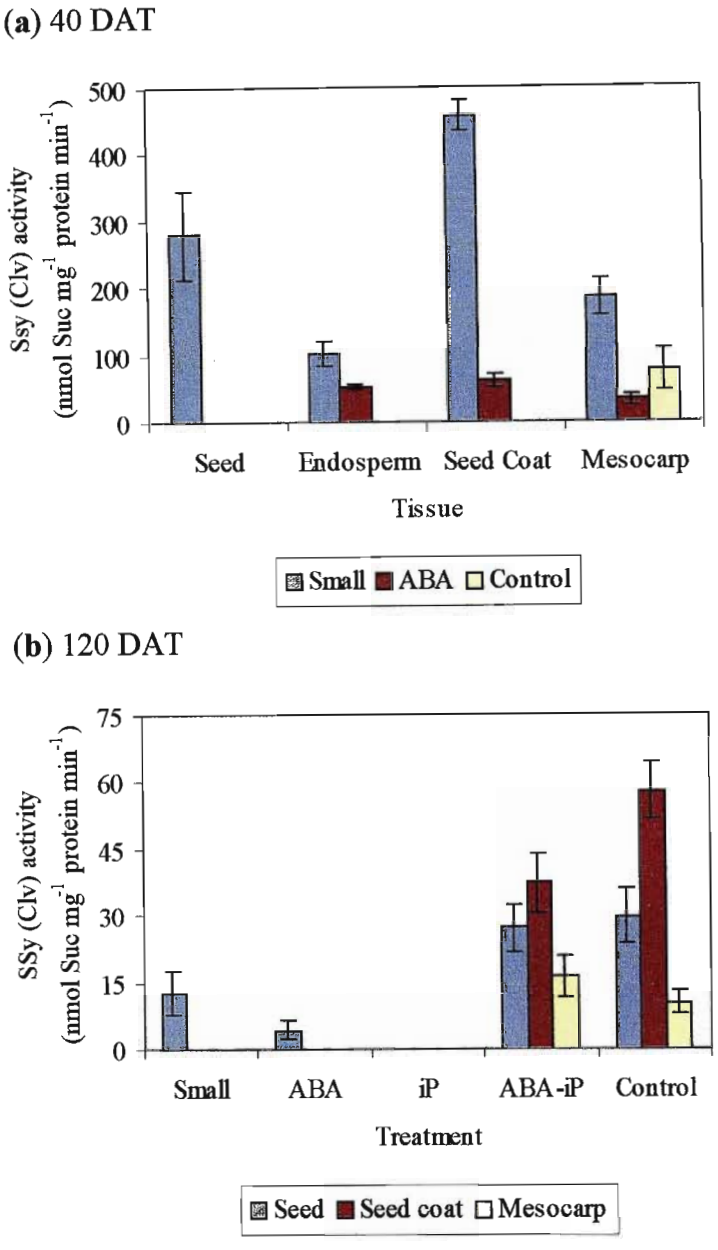


Figure 6.12 SSy (Clv) activity in control, small and treated 'Hass' fruit harvested (**a**) 40 DAT and (**b**) 120 DAT. $n = 3$. Error bars represent SD. $LSD_{0.05}$: 40 DAT ~ Seed = 200.10; Endosperm = 25.60; Seed coat = 27.32; Mesocarp = 25.56: 120 DAT ~ Seed = 6.69; Seed coat = 9.88; Mesocarp = 3.01.

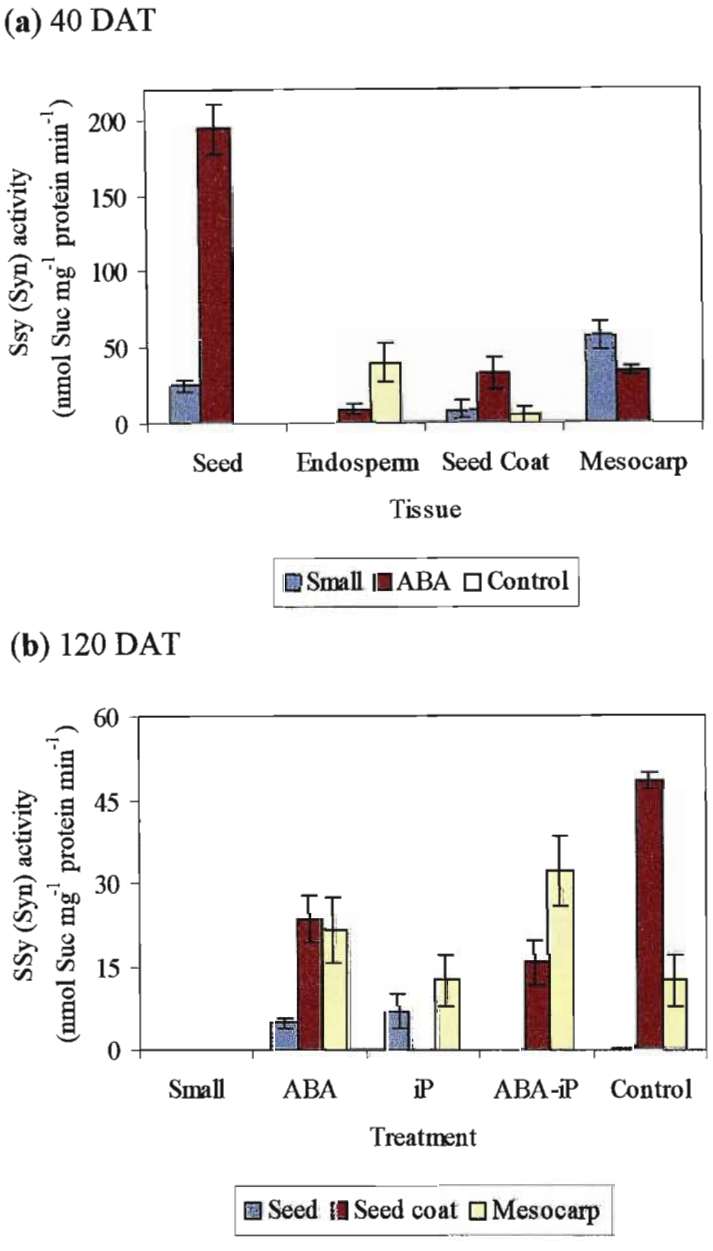
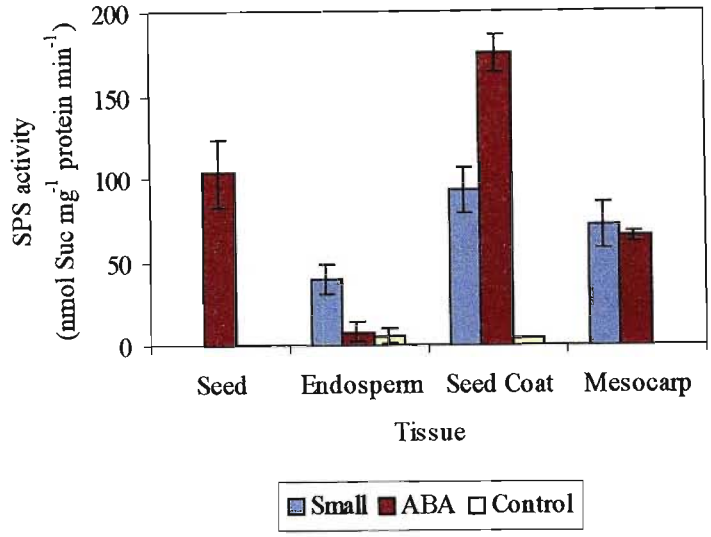


Figure 6.13 SSy (Syn) activity in small and treated 'Hass' fruit harvested (**a**) 40 DAT and (**b**) 120 DAT. n = 3. Error bars represent SD. $LSD_{0.05}$: 40 DAT ~ Seed = 85.31; Endosperm = 15.32; Seed coat = 15.17; Mesocarp = 14.89: 120 DAT ~ Seed = 1.21; Seed coat = 5.97; Mesocarp = 3.61.

(a) 40 DAT



(b) 120 DAT

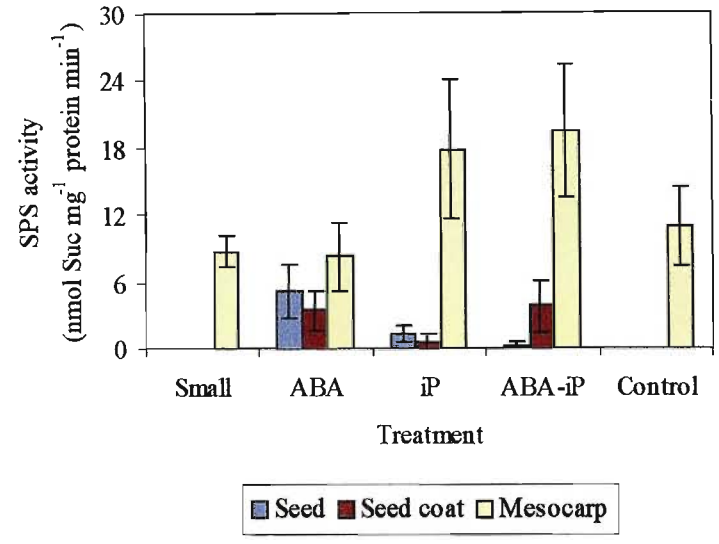


Figure 6.14 SPS activity in small and treated 'Hass' fruit harvested (**a**) 40 DAT and (**b**) 120 DAT. $n = 3$. Error bars represent SD. $LSD_{0.05}$: 40 DAT ~ Seed = 46.51; Endosperm = 3.74; Seed coat = 75.40; Mesocarp = 28.52: 120 DAT ~ Seed = 0.89; Seed coat = 0.82; Mesocarp = 2.45.

In the small fruit, PDH activity was elevated over the control value in the seed, seed coat and mesocarp (Figure 6.15). This trend was also paralleled by ABA treatment. The activity of α-amylase was consistently high in all tissues in the small fruit phenotype (Figure 6.16), and much lower levels were seen in the tissues of all ABA-treated fruit.

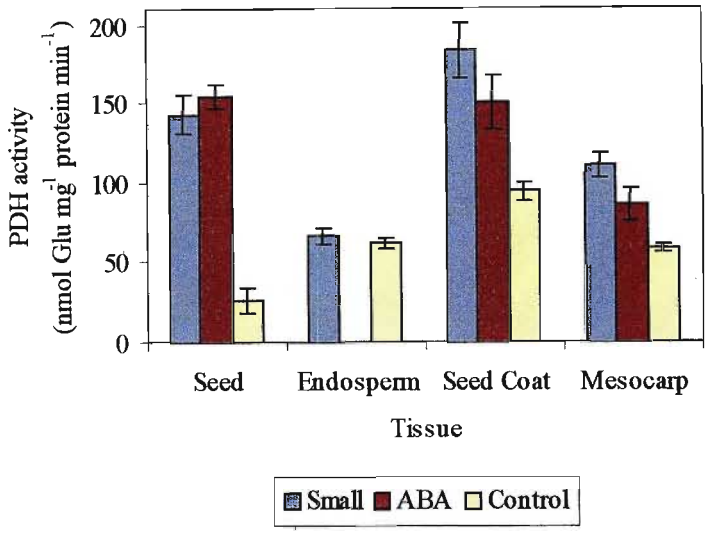


Figure 6.15 PDH activity in small and ABA-treated 'Hass' fruit harvested 40 DAT. n = 3. Error bars represent SD. $LSD_{0.05}$: Seed = 56.64; Endosperm = 27.22; Seed coat = 26.29; Mesocarp = 13.21.

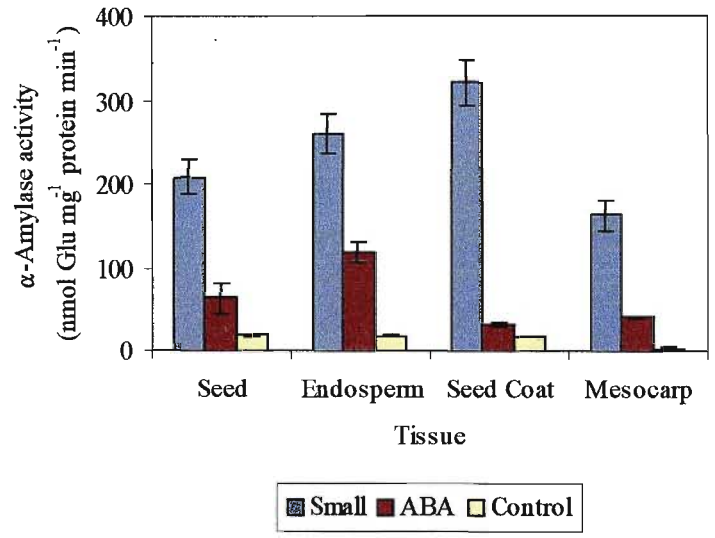


Figure 6.16 α-Amylase activity in control, small and ABA-treated 'Hass' fruit harvested 40 DAT. n = 3. Error bars represent SD. $LSD_{0.05}$: Seed = 21.58; Endosperm = 44.08; Seed coat = 6.30; Mesocarp = 17.10.

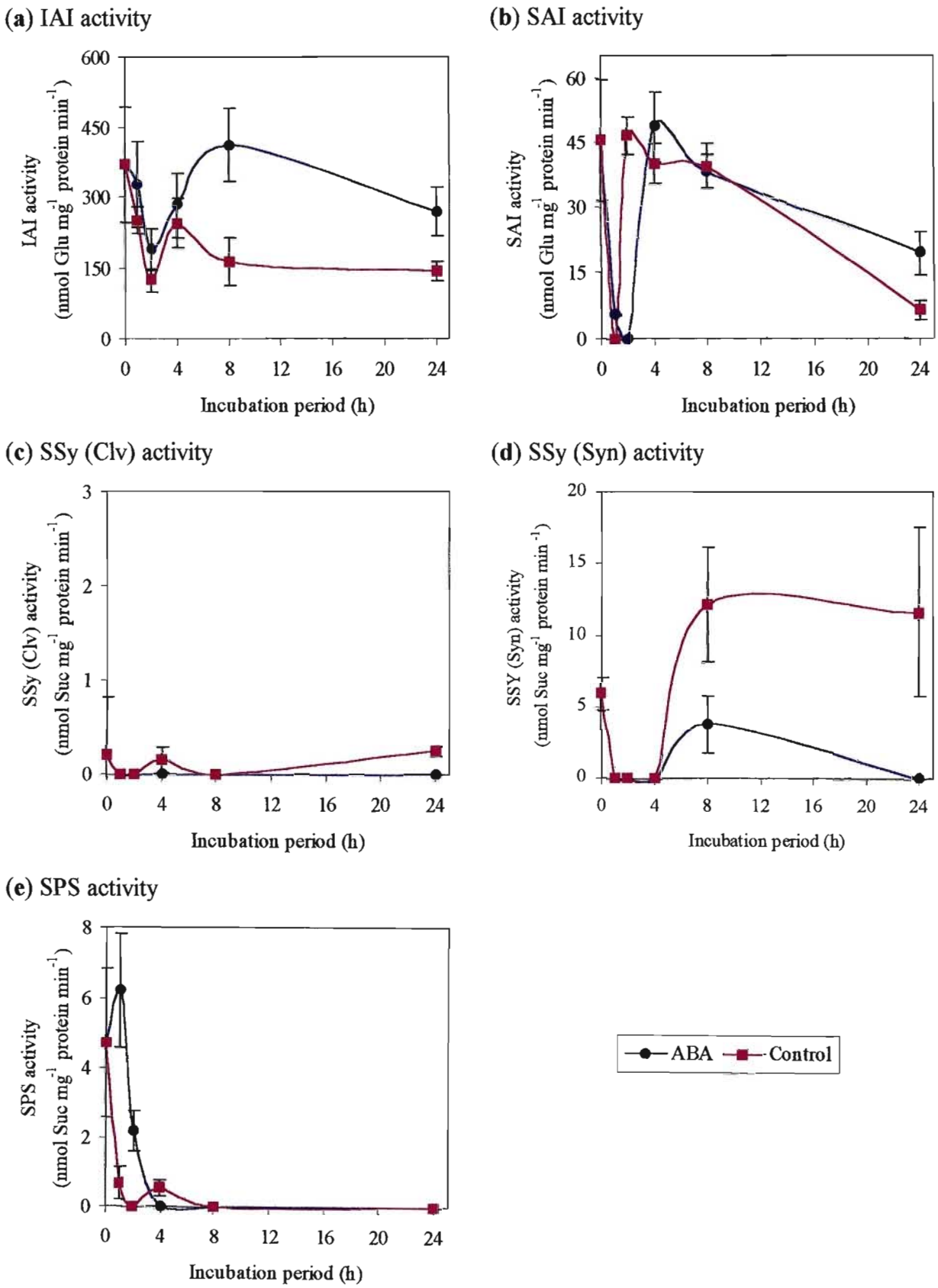


Figure 6.17 Effect of *in vitro* ABA-treatment on (**a**) IAI; (**b**) SAI; (**c**) SSy (Clv); (**d**) SSy (Syn) and (**e**) SPS activity in seed coat tissue from untreated 'Hass' fruit harvested 280 DAFB. $n = 5$. Error bars represent SD. $LSD_{0.05}$: Overall ~ IAI = 25.04; SAI = 5.78; SSy (Clv) = 0.10; SSy (Syn) = 6.78; SPS = 1.44.

6.3 SUMMARY

1. The small fruit phenotype was characterised by increased TSS, Suc, Glu, Fru and perseitol contents during the early stage of phase II of fruit growth. The changes in sugar content and composition were mirrored by ABA-treatment. Several novel and still unidentified sugars were observed in the fruit tissue and these increased in the seed and endosperm from small and ABA-treated fruit. The seed tissue from small and ABA-treated fruit also had a reduced starch content.
2. Although total lipid content was unaffected by ABA treatment at early stages of fruit growth, a distinct pattern emerged with respect to fatty acid composition in small and ABA-treated fruit. Both small and ABA-treated fruit had increased palmitic and petroselinic acids in the seed, and oleic acid in the mesocarp. Conversely, linoleic and γ-linolenic acids decreased in the seed.
3. Normal avocado fruit have high rates of respiration, and in the small fruit phenotype respiration rates are further elevated. ABA-treatment increased respiration rates in 'Hass' fruit. Similarly, both small and ABA-treated fruit show an elevation of enzyme activity associated with carbohydrate metabolism in most tissues. However, SSy (Clv) activity was inhibited by ABA- and iP- treatment, although co-treatment with ABA-iP negated this inhibitory affect. In view of these results, it is evident that the small fruit is characterised by high rates of carbohydrate metabolism and that this phenomenon can be induced by ABA-treatment.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 INTRODUCTION

The initiation and occurrence of the small fruit phenotype in 'Hass' is a long-standing problem in the South African avocado industry, and is believed to cause losses of up to 20% of the export crop[2]. Recently, substantial advances have been made in understanding some of the metabolic processes associated with avocado fruit growth and the occurrence of the small fruit phenotype (reviewed by Cowan *et al.* 2001). However, many of the metabolic processes associated with fruit development, and indeed the specific stimulus that results in the initiation of the small fruit phenotype, remain to be elucidated. One facet of this intricate phenomenon is the metabolism and transport of carbohydrates in avocado fruit. Recourse of the literature, with exception of the work done by our study group and recent work by Liu *et al.* (1999a; 1999b), revealed surprisingly little information on the transport and metabolism of sugars in avocado. In an attempt to lay the foundations of an understanding in carbohydrate metabolism in 'Hass', with the long-term intention of addressing the small fruit problem, the present study was undertaken. The research presented characterises the transport and metabolism of sugars, primarily Suc, in developing 'Hass' fruit and demonstrates the potential role of ABA in the occurrence of the small fruit phenotype.

7.1.1 CHARACTERIZATION OF THE SOLUTE PATHWAY AND METABOLISM OF CARBOHYDRATE IN 'HASS' FRUIT

7.1.1.1 Movement of solutes into developing 'Hass' fruit

In the current trial, autoradiography and [^{14}C] movement suggested that solute flow into developing avocado fruit may occur by two routes: firstly, solutes may be unloaded along the axial path of the phloem in the mesocarp; secondly, solutes may enter the seed coat where they are unloaded and subsequently transported to the seed and/or mesocarp.

[2]D. Donkin (2001) South African Avocado Growers Association. Tzaneen, South Africa.

The avocado mesocarp is a large, relatively homogenous tissue with high metabolic activity and it is unlikely that the photosynthate requirement for mesocarp growth comes via the seed coat. In avocado fruit, the vascular tissue ramifies extensively throughout and, in some cases, ends blindly within the mesocarp. Moore-Gordon (1997) was able to show an absence of lateral diffusion of eosin from vascular traces, suggesting restricted symplastic unloading of the phloem in the mesocarp and seed coat of avocado. However, the present study has shown that high amounts of [^{14}C]-label moved into the mesocarp tissue and there is an apparent ring of cells with numerous Pd surrounding the vasculature in the mesocarp (as shown by staining with aniline blue). These observations suggest radial unloading of the phloem throughout the mesocarp, and indicate that unloading of the phloem must occur via an apoplastic step. Subsequent distribution from parenchyma cells in the immediate vicinity of the phloem may follow a symplastic route. The high Pd frequency within the mesocarp and the rapid cell-to-cell transfer of Lucifer yellow (Moore-Gordon *et al.* 1998) provided some indication of a probable symplastic route for radial photosynthate transport in the mesocarp. In fact, throughout the mesocarp tissue, there appear numerous well-developed pit fields containing highly branched secondary Pd, often with large median cavities. This is clearly characteristic of Pd that have differentiated to allow for rapid, efficient transport. The axial unloading of the phloem and radial distribution of assimilate can be expected to facilitate the large metabolic demands of the developing avocado mesocarp.

The avocado embryo may be likened to that of a pea. Both consist of an embryonic axis (constituting the plumule and radicle) and two large cotyledons (where starch accumulation takes place). The embryo develops within the seed coat, which is a maternal organ and ensures the symplastic isolation of the embryo from the mother plant. In the pea, it has been established that phloem unloading occurs in the seed coat and assimilate subsequently migrates toward the embryo (Déjardin *et al.* 1997). The distribution of [^{14}C] in the developing avocado suggested that pedicel-fed solutes do enter the seed coat. Furthermore, the relative amount of label in the seed coat decreased over the 12 h incubation period, whilst that in the seed showed a concurrent and significant ($P \leq 0.01$) increase, suggesting that the label was being unloaded from the seed coat into the seed tissue. The pea seed coat is not a passive layer and active starch-Suc interconversion takes place, allowing the seed coat to act as a transient storage zone and buffer the supply of nutrients to the embryo (Rochat and Boutin 1992). The placento-chalazal region in maize is thought to play a similar role, facilitating the absorption and transport of photoassimilate and nutrients to the developing albuminous seed (Schel *et al.* 1984). In wheat, after Suc is unloaded from the SE/CC complex, it enters an extra-phloem Suc pool from which it is released across the Pm of the nucellar cells into the endosperm cavity (Fisher and Wang 1993; Wang and Fisher 1994). Thus, it is highly

likely that the seed coat, especially the pachychalazal region, may be the route for early assimilate distribution to the seed. During early fruit development in avocado, the pachychalazal region of the seed coat is separated from the seed by a thin layer of endosperm tissue, which is proposed to act as a conduit between the seed and seed coat. The numerous Mi and Pd, grouped in large pit fields in the inner layer of the seed coat (the layer adjacent to the endosperm) suggested high metabolic activity associated with the active unloading and transport of sugar, and a potentially high degree of symplastic continuity within this zone. During fruit development the endosperm degenerated and was crushed by the developing seed to form a layer of flattened cells on the inner side of the seed coat. In miniature maize mutants, the loss of the chalazal bridge leads to the near arrest of starch accumulation and embryo development (Lowe and Nelson 1946). The loss of structural integrity of the endosperm tissue in avocado can thus be expected to reduce or inhibit transport into the seed, and may play a crucial role in the transition of the fruit into growth phase III, once seed development has progressed sufficiently to permit maturation. The pachychalaza also does not adhere as tightly to the seed as to the mesocarp, suggesting a more integrated function with the mesocarp. The testal region appears to have a number of veins ending blindly in a region localised to the base of the seed in an area that is common to both cotyledons and the young developing embryo. It is plausible that the supply of assimilates to these tissues may, during later development, be localised to the testal/basal region of the seed. Thus, the seed coat may act as a temporary unloading zone for the phloem, from where solutes are allocated to the seed and the mesocarp. In addition, messenger molecules, e.g. hormones, synthesised in the seed may be transported to the mesocarp via the seed coat, thereby allowing the seed to exhibit a degree of control over mesocarp development. Assimilate import is a multi-step process, involving distinct types of transport in unique tissues. These transport steps are anatomically separate and are to some extent controlled independently by the transport processes that occur within the individual tissues. The termination of the vasculature in the seed coat also provides a means of insuring symplastic isolation of the filial tissue, permitting the existence of two distinct metabolic zones, one geared towards the accumulation of starch and the other towards the accumulation of lipids. Furthermore, the lack of Pd connections between seed, endosperm and seed coat will exclude the symplastic entry of mobile elements such as viruses into the seed (Felker and Shannon 1980; Thorne 1985).

These findings suggest that the pathway of solute flow into the developing 'Hass' fruit occurs along three possible continua: pedicel vasculature → mesocarp vasculature → mesocarp; or pedicel vasculature → mesocarp vasculature → seed coat → endosperm → seed; or pedicel vasculature → mesocarp vasculature → seed coat → mesocarp. The results presented here also

emphasise the importance of endosperm and seed coat structural and functional integrity, in addition to sink activity and size, photosynthesis and respiration (Henton *et al.* 1999), in the supply of carbon to the developing avocado fruit.

7.1.1.2 Metabolism of carbohydrates during growth of 'Hass' fruit

In plants, reproduction involves intense biosynthetic activity and large amounts of photosynthate are allocated to developing reproductive structures (Zinselmeier *et al.* 1999). Suc hydrolysis is considered to be key mediator between carbohydrate unloading and metabolism in developing fruit and contributes to the establishment and maintenance of sink strength (Ho *et al.* 1987; Ho 1988; Wang *et al.* 1993). Sap composition from 'Hass' suggested that Suc is transported to the fruit via the phloem, and some of the enzymes involved in the metabolism of this disaccharide were shown to exist in avocado in the present study. [^{14}C]-labelled Suc was incorporated into other fractions within the fruit and the activity of the enzyme IAI, SAI, NI, SSy (in both the cleavage and synthesis directions) and SPS were detected in 'Hass' fruit tissue. Generally, the activity of these enzymes in developing 'Hass' fruit, with the exception of IAI in the seed coat tissue, was initially high and subsequently declined during phase II of fruit growth.

SSy activity is strongly correlated with sink strength, the control of Suc import capacity, turnover and content, and rate of growth in developing sink tissues (Sung *et al.* 1989; Wang *et al.* 1993; D'Aoust *et al.* 1999; Nguyen-Quoc *et al.* 1999; Usuda *et al.* 1999; Chopra *et al.* 2000). Hence, SSy is believed to play a crucial role as a determinant for fruit set and development. In developing 'Hass' fruit, it was observed that SSy (Clv) was the major contributor to Suc cleavage and sugar metabolism in the seed and endosperm during early fruit development. Furthermore, mesocarp SSy (Clv) appeared to consistently contribute significantly to Suc cleavage. These results suggest that SSy plays an important role in the provision of carbon during seed development and the linear phase of avocado mesocarp growth. SSy has UDP-transferase activity and thus forms UDP-Glu. This has two advantages. Firstly, the product of Suc cleavage does not require ATP-dependent phosphorylation, and hence SSy catalysed cleavage of Suc has an energetic advantage over that of invertase. Secondly, the sugar-nucleotide products can be used directly for the synthesis of structural and storage polysaccharides during tissue development (Ruan and Chourey 1998; Hauch and Magel 1998). Indeed, distribution of SSy mRNA revealed by *in situ* hybridisation in young tomato fruit closely paralleled starch distribution, and SSy activity has been correlated to transient levels of starch (Wang *et al.* 1993; Wang *et al.* 1994). Furthermore, during early stages of fruit development Suc is unloaded symplastically directly into the cytosol (Damon *et al.* 1988; Dali *et*

al. 1992). SSy, whilst localised to the cytosol, has also been reported to be part of a membrane-bound biosynthetic enzyme complex (Ruan *et al.* 1997), and is thus ideally positioned to contribute to both symplastic solute transport and cell wall development.

In developing 'Hass' fruit SAI activity in the seed and endosperm tissue remained relatively high. Apoplastically transported Suc may enter the symplast by diffusion and be stored in the vacuole. SAI will thus contribute to the maintenance of a Suc gradient into the seed and play a role in the provision of carbon substrates primarily for metabolism subsequent to the decrease in SSy (Clv) activity in the seed. Furthermore, it is possible that the vacuolar cleavage of Suc may contribute to the generation of Glu-1-P for starch biosynthesis (Hendrix 1990), and thus SAI may also play a minor role in starch accumulation in the seed, especially once SSy activity decreases and polyphenol deposition within the seed coat restricts further carbon movement.

In the seed coat SAI activity was initially high and decreased, whilst IAI activity was initially very low and increased during fruit development. Conversely, SSy (Syn) activity was initially high in the seed coat and declined during fruit development. These results suggest that seemingly 'futile cycling' of Suc may occur in the seed coat during early fruit development, and this may contribute to the establishment of a concentration gradient into this tissue and to the maintenance of internal osmotic pressure by countering the dilution of vacuolar contents caused by water uptake. The seed coat is assumed to be the conduit between the phloem and both the seed and mesocarp, and it is believed that the seed derives its carbon apoplastically. Initially Pm bound hexose symporters may transport symplastically cleaved hexoses into the apoplast. The initial high levels of PDH activity may also contribute to the supply of carbon to the apoplast and developing seed. As the fruit matured, IAI activity in the seed coat increased. This elevation in IAI occurred concurrently with the deposition of polyphenols in the seed coat and the associated expected loss of transport between the symplast and the apoplast. Roitsch and Tanner (1996) proposed a model in which Suc is released from the sieve elements into the apoplast, and is subsequently split by invertase. The hexoses are then imported into the sink cell via a hexose transporter. IAI is uniquely positioned to fulfil this role, and links the transport of Suc to hexose transporters (Roitsch *et al.* 2000). Thus it appears that, as seed coat integrity is lost, so is cytosolic and vacuolar Suc cleavage, and that this apparent loss is compensated for by an increase in apoplastic Suc cleavage. A similar increase in invertase has been observed in potato in response to the down-regulation of SSy (Zrenner *et al.* 1995), suggesting losses in activity of one enzyme can be physiologically compensated by increases in others.

In mesocarp tissue, both IAI and SAI were initially high and decreased with development. Invertase-catalysed cleavage of sugars can affect turgor pressure. Over-expression of a yeast-derived invertase in the vacuole of transgenic tobacco plants resulted in an increase of turgor pressure in the cells (Hoffmann-Benning *et al.* 1997). Hence uptake of hexoses into developing cells may allow for both the turgor-driven expansion of the cells and play a crucial role in the initial establishment of sink strength and rapid cell expansion of the mesocarp during phase II of fruit growth. Although cell division occurs throughout the ontogeny of the avocado, cell division slows as the fruit matures, and mesocarp physiology can be expected to change with the onset of lipid accumulation. Thus it can be expected that invertase activity will decrease, and the initial high levels of both SAI and IAI, observed in the current study, appear to play a role in the establishment of sink strength. Once sink strength is established, SSy (Clv) and PDH activity appear elevated and are assumed to contribute to the supply of carbon to the developing mesocarp tissue.

Concurrent with the decrease in enzyme activity in the tissues examined, there was a general decrease in identifiable sugars and TSS. Most obvious was the decrease in Fru and/or Glu in the seed, endosperm and mesocarp tissues. With the exception of the endosperm, Suc levels were consistently low in all fruit tissues. In all tissues, by 210 DAFB perseitol and *manno*-heptulose represented the bulk of the identified sugars. This suggested a fundamental change in sugar metabolism during fruit growth, in which early fruit growth is characterised by high cleavage of sugars to Fru and Glu and this decreases as the fruit develops. Presumably these hexoses play an important role in the supply of carbon for growth and respiration, and their utilization is vital for the establishment of fruit sink strength. The high levels of hexoses during early fruit development may also contribute to the control of fruit growth and the suppression of fruit maturation via sugar sensing mechanisms. If this were the case a decrease in the enzymatic production of Fru and Glu may play a crucial role in the transition of fruit from growth phase I to growth phase II and may accompany the reduction of rapid cell division experienced during early fruit development. Indeed, Bean (1958) observed that the level of sugars in avocado mesocarp tissue did not change during the initial stages of growth, but declined rapidly as the fruit approached maturity.

The metabolism of Suc in the mesocarp and seed was interesting as it suggested a significant amount of [^{14}C]-labelled Suc entering the fruit is converted to *manno*-heptulose. Although the mechanism of this conversion is not well understood, the potential pathways of 7C sugar metabolism are discussed in Chapter 7.1.1.3. What is also particularly intriguing is the incorporation of large amounts of label into an unidentified sugar with a retention time of 8.84

min. Several sugars have previously been identified in avocado in very small quantities, including the octuloses D-*glycero*-D-*manno*-octulose and D-*glycero*-L-*galacto*-octulose; the nonuloses D-*erythro*-L-*gluco*-nonulose and D-*erythro*-L-*galacto*-nonulose; and the octitol D-*erythro*-D-*galacto*-octitol (Charlson and Richtmyer 1960; Sephton and Richtmyer 1963a; Sephton and Richtmyer 1963b; Sephton and Richtmyer 1966). The large amounts of unidentified sugars detected in young 'Hass' fruit, especially in the seed and endosperm tissue, do not appear to be those observed by the above authors, who only recorded extremely small amounts of sugars from large amounts of fruit. However, it must also be noted that these researchers used mature whole fruit. The results presented in the current study suggest that these sugars decrease in the seed as maturation progresses. Additionally, the endosperm represents a very small portion of the fruit and degenerates during fruit development, and thus it is expected that these sugars will occur in smaller amounts in mature fruit. Since the unidentified sugars with retention times of 8.84 and 35.97 min could be hydrolysed under alkaline conditions, and the fact that they were retained on a C_{18} cartridge, strongly suggests that these sugars may be associated with fatty acids. In bacteria, the presence of lipopolysaccharides of 7C and larger sugars is well documented, and they are usually associated with membrane systems, especially that of the cell wall (Sonesson *et al.* 1994). It is, therefore, possible that these sugars exist as lipopolysaccharides and play a role in storage and/or membrane function, transport or stability. The partitioning of carbon into these unidentified (lipopolysaccharide) sugar pools and also into perseitol may assist in maintaining a solute gradient into the developing seed, thereby contributing to the massive accumulation of starch in the cotyledons. This is of particular interest as sugars enter starch biosynthesis via Glu-6-phosphate. Fru-6-phosphate, a potential product of perseitol catabolism, is converted to Glu-6-phosphate by Glu-6-phosphate isomerase, providing a possible entry point for 7C sugars into starch biosynthesis, and hence suggesting a important role for perseitol in seed development. The activity of the enzyme tentatively identified as PDH and the perseitol content were very high in young seed tissue. This suggests that perseitol accumulation/import in young seed tissue may play an important role in the provision of carbon for starch accumulation.

Three important physiological features characterise sink activity: phloem unloading and post-phloem transport, assimilate utilization, and carbohydrate storage (Herbers and Sonnewald 1998). Efficiency of phloem unloading is therefore determined by the sink's capacity to remove the imported sugar from the same sugar pool and so create a concentration gradient into the sink tissue. This can be achieved by compartmentation within the vacuole, apoplast and symplast, or by partitioning into other carbon forms, including high molecular weight compounds such as starch and oil.

In the current investigation, α-amylase activity was low in developing 'Hass' avocado fruit throughout the period assayed and followed a similar trend in all tissues, being initially relatively high and then decreasing with fruit development. Starch levels, however, increased dramatically in the seed whilst that of the seed coat and mesocarp remained consistently low. The starch content in the seed appeared to plateau during phase II of fruit growth, suggesting seed maturity may be reached before mesocarp maturity, and this may be linked to seed coat (and possibly even endosperm) degradation. These results suggest that α-amylase activity may contribute to the provision of Glu during early fruit development and counteract starch accumulation. During the growth and maturation of non-starch storing fruits, starch accumulated in early fruit development is broken down to provide Suc. Starch may thus serve as a transient carbohydrate reservoir during growth (N'tchobo *et al.* 1999). Starch can be degraded by the enzymes α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2), γ-amylase (EC 3.2.1.3) and starch phosphorylase (EC 2.4.1.1). α-Amylase, β-amylase and starch phosphorylase have all been identified in fruit (Seymour and Tucker 1993). α-Amylase (an endoamylase) hydrolyses the internal α-(1,4)-linkages of amylose at random to produce a mixture of Glu and maltose, with the reducing groups in an α-configuration. Maltose is then hydrolysed to Glu by glucosidase (EC 3.2.1.20). Endoamylases are thought to play a key role in the coarse control of starch degradation (Abe *et al.* 1996; Kashem *et al.* 1998), although the entire reaction sequence of starch degradation and the contribution of each enzyme is poorly defined. The cleavage of starch by α-amylase has been suggested to be essential under some circumstances, such as anoxia, for the continued supply of carbon (Arpagaus and Braendle 2000). However in avocado, the consistently low starch and α-amylase activity suggested that starch cycling may play only a minor, insignificant role in ensuring a permanent supply of free sugars for tissue growth. α-Amylase is thought to be the primary enzyme in the hydrolytic break-up of amyloplasts (Perata *et al.* 1998; Witt and Sauter 1995). However, starch accumulation and amyloplast formation occurs during seed development and appears to be unaffected by the basal levels of α-amylase activity. Thus it is apparent that this enzyme plays only a minor role in the contribution of sugars to the soluble sugar pool, and subsequently the solute gradient into the seed of developing fruit.

Total lipid content increased in the mesocarp of developing fruit but remained consistently low in the seed. Traditionally oil content has been used as an indication of fruit maturity (Eaks and Sinclair 1978; Young and Lee 1978), with mesocarp lipid content increasing with fruit age (Kaiser 1993), and lipid production levelling off once maturity is reached. Oleic acid has been shown to occur in high levels in avocado mesocarp tissue (Davenport and Ellis 1959; Gaydou *et al.* 1987), especially during early fruit development. In the cultivar 'Fuerte' palmitic, linoleic and linolenic

acids increase in the mesocarp during development (Kikuta and Erickson 1968). The most abundant fatty acids found by Kaiser (1993) in the mature mesocarp tissue from 'Hass' were palmitic and oleic acid. In the current study, the increase in fatty acids could be largely attributed to increases in palmitic and petroselinic acids. The discrepancies observed between these two investigations may be attributed to differences in the age of the fruit, and the fact that Kaiser (1993) used a 2 m × 3 mm ID glass column packed with 10% Silar 5CP on 100/120 mesh Supelcuport 100G. This column is expected not to give as good a separation of fatty acids as the column used in this trial, and thus not discriminate between the octadecenoic acids oleic and petroselinic. Lipids, such as the glycolipids containing linolenic acid, are essential for chloroplast functioning (playing a role in thylakoid orientation) and are associated with the Mi. Thus the transition from higher octadecatrienoic acids composition to high octadecadienoic acid levels may be associated with the reduction of chloroplast functioning and the accumulation of triacylglycerols which act as a compact, easily-metabolised and non-hydrated energy store during mesocarp maturation.

Studies on lipogenesis in avocado suggest that up to 30% of Glu metabolised by catabolic pathways in the fruit was directly oxidised to provide about 50% of the total reducing power for fatty acid synthesis (Kikuta and Erickson 1969). Furthermore, lipid accumulation in the mesocarp is accompanied by a decrease in alcohol-soluble and -insoluble sugars (Davenport and Ellis 1959). These observations suggest a high rate of resource allocation to lipid biosynthesis as the fruit approaches maturity. Kaiser (1993) found that a larger seed mass resulted in lower monounsaturated fatty acid levels in the mesocarp during fruit growth and delayed lipid accumulation, and suggested that the seed acts as a sink for assimilates at the expense of fatty acid accumulation. It is thus apparent that seed maturation must precede lipid accumulation in the mesocarp. If this is the case, seed maturation and seed coat function can be expected to play a key role in the onset of lipid accumulation in the mesocarp, and can thus be envisaged to influence aspects of mesocarp maturation.

There may be two distinct fatty acid synthesis systems in the avocado mesocarp plastids: a particulate system forming palmitate and oleate from acetate or acetyl-CoA, and a soluble system forming stearate from malonyl-CoA (Wearie and Kekwick 1975). The contribution of 7C sugars to these systems remains to be elucidated. However, it is plausible that 7C sugar metabolism (discussed in 7.1.1.3) may be a source of glyceraldehyde-3-phosphate (G3P), a precursor in the synthesis of glycerol, and hence in triacylglycerols (and for isopentenyl pyrophosphate, an intermediate in isoprenoid biosynthesis). The accumulation of triacylglycerols appears to occur

concurrently with the decrease in 7C sugars in avocado during fruit development and thus it is possible that *manno*-heptulose and perseitol may contribute to lipid accumulation during fruit maturation.

In conclusion, it appears that sink strength in avocado is established during early fruit growth by high SAI and IAI activity, especially during the period of rapid cell division, and maintained by SSy and PDH activity, during the phase of cell expansion. Seed maturation and starch accumulation accompany seed coat degeneration and precede mesocarp maturation and lipid accumulation. Maturation of the seed and mesocarp tissue is also preceded by a decrease in both the enzymes of sugar metabolism and soluble sugar content.

7.1.1.3 Seven carbon sugars in 'Hass' avocado

The presence of the 7C sugar *manno*-heptulose (D-*manno*-2-ketoheptose; Figure 7.1a) and the related alcohol perseitol (D-*glycero*-D-*galacto*-heptitol; Figure 7.1b) as the sugars in developing avocado fruit is of much interest. Although *manno*-heptulose was first isolated by La Forge in 1917 and has subsequently been isolated from a variety of plants including gymnosperms from the family Taxodiaceae, 22 dicotyledonous families (including commercially important families such as Lauraceae, Leguminosae, Moraceae, Rosaceae, Salicaceae) and the monocotyledonous family Iridaceae, very little is known about the function and synthesis of this ketoheptose in plants (La Forge 1917; Nordal and Benson 1954; Charlson and Richtmyer 1960; Bevenue *et al.* 1961; White and Secor 1961; Begbie and Richtmyer 1966; Ogata *et al.* 1972; Okuda and Mori 1974; Shaw *et al.* 1980).

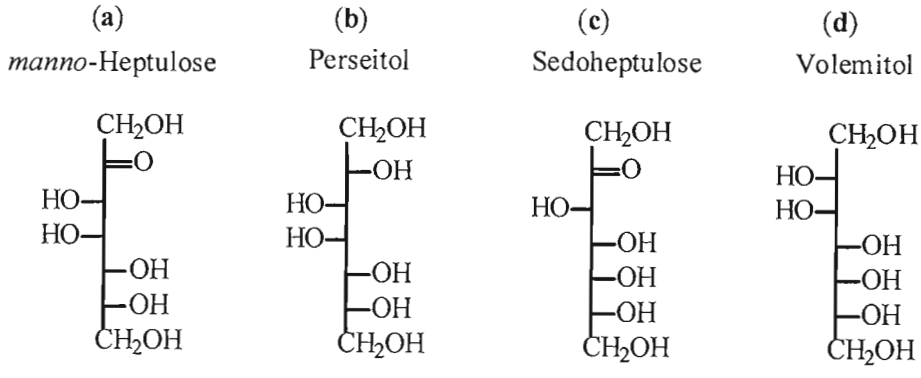


Figure 7.1 Fischer projection formulae of the 7C sugars and alditols (**a**) *manno*-heptulose; (**b**) perseitol; (**c**) sedoheptulose; and (**d**) volemitol.

Similarly, the heptahydroxy alcohol perseitol was first described by Avequin in 1831 and, to date, very little progress has been made with regard to the synthesis, metabolism and function of this non-structural carbohydrate in plants.

In this study *manno*-heptulose was found in relatively high levels in the seed, seed coat and mesocarp tissue representing at least 15 % of TSS and constituting 27% of the sugars identified in the phloem sap. These results are significant for several reasons.

Firstly, *manno*-heptulose is transported to the fruit via the phloem, an observation supported by the fact that *manno*-heptulose and its monophosphate have been isolated from avocado leaves (Nordal and Benson 1954). Little is known about the synthesis of *manno*-heptulose in plants. However, the synthesis of the 7C sugar sedoheptulose (D-*altro*-2-heptulose; Figure 7.1c) is understood (Figure 7.2). Sedoheptulose is an intermediate in the Calvin cycle, playing an important role in the fixation of CO_2, and occurs universally in the plant kingdom. Free sedoheptulose has rarely been described in plants and normally only exists in its mono- and bis-phosphate ester forms in the pentose phosphate cycles (Häfliger *et al.* 1999). Sedoheptulose is also speculated to play a role in carbon storage, cryo-protection, and as a metabolic precursor (Kull 1967; Häfliger *et al.* 1999). These roles may also be fulfilled by the structurally similar *manno*-heptulose. Removal of P_i from the phosphorylated forms of sedoheptulose by a phosphatase and simultaneous/subsequent rotation of the hydroxyl group at C-4 by an epimerase may be a possible route for *manno*-heptulose synthesis. It is interesting to note that the aldolase catalysed condensation of an aldehyde and dihydroxyacetone-P (DHAP) yields a stereospecific D-*threo* configuration of the hydroxyl groups at C-3 and C-4. If *manno*-heptulose is to be synthesised by a similar pathway, it would be necessary for epimerase activity on C-4, or, alternatively, it suggests the presence of another enzyme(s) in the synthesis of *manno*-heptulose and higher carbon sugars in plants (Jones and Sephton 1960; Sephton and Richtmyer 1963).

Secondly, differences in phloem and tissue *manno*-heptulose contents suggest that this 7C monosaccharide is undergoing active metabolism in the fruit. Again, no information could be found regarding the metabolism of *manno*-heptulose and, hence, that of sedoheptulose is presented as a guideline. Häfliger and co-workers (1999) found that crude enzyme extract from hybrid polyanthus (*Primula* × *polyantha*) leaves catalysed the reduction of sedoheptulose by NADPH to volemitol and proposed the existence of a novel NADPH-dependent ketose reductase called sedoheptulose reductase. It has previously been shown that *manno*-heptulose can be reduced (by sodium amalgam) to perseitol (La Forge 1917). Volemitol (D-*glycero*-D-*manno*-heptitol; Figure

7.1d), like perseitol in avocado, appears to be a major non-structural, phloem-mobile carbohydrate in polyanthus. Sedoheptulose reductase did not show any activity with other 7C sugars, including *manno*-heptulose, and could not be isolated from avocado leaves (Häfliger *et al.* 1999). Thus, although this enzyme is specific to volemitol-containing tissue, it is conceivable that a similar mechanism exists for the metabolism of *manno*-heptulose in perseitol-containing tissues, in which *manno*-heptulose is reduced to perseitol by the activity of a *manno*-heptulose reductase-like enzyme.

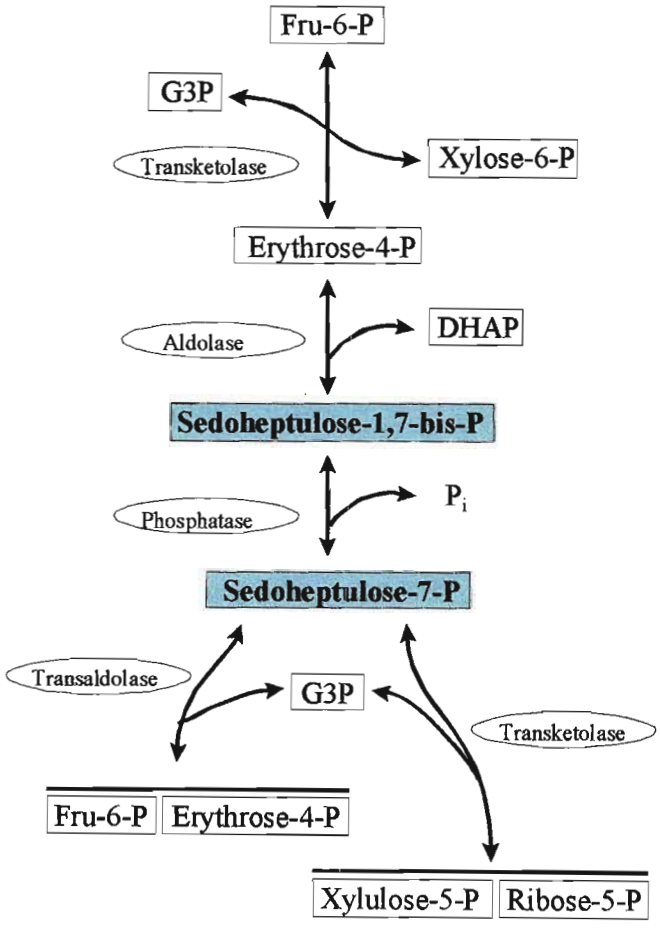


Figure 7.2 Schematic illustration of the synthesis of sedoheptulose in plants. Adapted from Mathews and van Holde 1990. DHAP = dihydroxyacetone-P; G3P = glyceraldehyde-3-P; P = phosphate; P_i = phosphate ion.

Thirdly, during growth phase II *manno*-heptulose levels increase in the mesocarp, suggesting a role in the provision of carbon for fruit growth and respiration during the stage of rapid cell expansion. This role supports the proposal that *manno*-heptulose is an active substrate for respiration and plays an important part in the metabolic processes associated with fruit growth in avocado (Davenport and Ellis 1959; Liu *et al.* 1999b).

Finally, avocado fruit do not normally ripen whilst attached to the tree and remain in a mature, unripe state until harvested. It has been proposed that ripening is thus delayed by a 'factor' translocated from the tree or pedicel (Biale and Young 1971; Adato and Gazit 1974; Tingwa and Young 1975a). Plant hormones, namely IAA and ethylene, polyamines and calcium levels/activity have been suggested to contribute to this phenomenon (Tingwa and Young 1975b; Adato and Gazit 1976; Wills and Tirmazi 1982; Winer and Apelbaum 1986); however the identity and nature of this 'ripening factor' remains unknown. The sugar *manno*-heptulose is a potent inhibitor of respiration, preventing the entry of Glu into the glycolytic cycle (Simon and Kracier 1966; Board *et al.* 1995), and acts as a specific and competitive inhibitor of HXK, potentially altering the expression of sugar regulated genes (Salas *et al.* 1965; Prata *et al.* 1997; Chiou and Bush 1998; Smeekens 1998; Pego *et al.* 1999). Many vital processes, such as stress response, fruit growth and senescence are influenced by sugar signalling and/or metabolism (reviewed by: Jang and Sheen 1997; Sheen *et al.* 1999). Substantial decreases in 7C sugars have been observed during ripening of avocado fruit (Davenport and Ellis 1959; Ogata *et al.* 1972; Shaw *et al.* 1980; Liu *et al.* 1999b) and it is plausible that this phloem-derived 7C sugar acts to inhibit ripening whilst the fruit is still attached to the tree. This may occur by the inhibition of HXK-mediated ripening processes within the avocado fruit, such as the respiration climacteric. It is assumed that endosperm degeneration and seed maturation precede mesocarp ripening, and hence the decrease in endosperm and seed *manno*-heptulose levels observed in this trial may permit or accompany the early stages of fruit maturation. During this stage, the mesocarp is still within the linear phase of fruit growth and the high *manno*-heptulose levels within the fruit may prevent premature ripening.

Sugar alcohols are compounds in which the aldehyde or keto group has been reduced to a hydroxyl group, and are also known as acylic polyols, alditols, polyalcohols and polyhydric alcohols. Examples of sugar alcohols include mannitol, sorbitol, ribitol, and the 7C alditols volemitol and perseitol. The physiological functions of alditols include photosynthetic assimilation, translocation and storage of carbon, the storage of reduced carbon and reducing power, osmoregulation, serve as a compatible solute, protection against different types of stress and free radical scavenging, and regulation of co-enzymes (reviewed by: Bieleski 1982; Loescher 1987; Pharr *et al.* 1995; Steinitz 1999). Like *manno*-heptulose, perseitol was shown to occur in high concentrations in developing 'Hass' fruit. This could be expected as perseitol represents the reduced form of *manno*-heptulose.

Perseitol was also found to constitute 15% of TSS in the phloem sap. Alditols, like volemitol, sorbitol and mannitol function as phloem-translocated carbohydrates in some higher plants (Zimmermann and Zeigler 1975; Bieleski 1982; Häfliger *et al.* 1999; Lo Bianco *et al.* 2000). Thus

although perseitol is supplied to the fruit, its high composition in fruit relative to the phloem suggests that perseitol is further synthesised in the fruit.

The conversion of perseitol to *manno*-heptulose observed in this trial suggests the presence of some enzyme that catalyses the oxidation of perseitol. Sorbitol dehydrogenase (EC 1.1.1.14; SDH) and mannitol dehydrogenase (EC 1.1.1.138; MDH) are believed to play an important role in the metabolism of sorbitol and mannitol respectively and the eventual use of alditol-derived carbon in growth and metabolism in sink tissues (Loescher 1987; Pharr *et al.* 1995; Lo Bianco *et al.* 1998; Archibold 1999). Both SDH and MDH catalyse the NAD-dependent oxidation of an alditol to Fru-6-P. Oxidases are capable of catalysing a similar reaction; however, these enzymes only participate in a small proportion of reactions as they use molecular oxygen as an electron acceptor and usually form hydrogen peroxide as a by-product. NAD is readily available in the cell medium, and in all likelihood the dehydrogenase is responsible for the oxidation of *manno*-heptulose to perseitol in avocado fruit tissue (Figure 7.3). Furthermore, dehydrogenase activity would result in the synthesis of NADH (or NADPH) required for the synthesis of various carbon reserves (Chopra *et al.* 2000). The relatively high levels of PDH observed in this study suggested that perseitol may play an important role in the provision of substrates for respiration, cell division and cell wall synthesis, especially during early fruit growth when PDH activity is most pronounced.

Various environmental stresses, such as drought or chilling, promote the production of free oxygen radicals, such as superoxide, hydrogen peroxide and the hydroxyl radical, in plant cells (Moran *et al.* 1994; Prasad 1996; Murphy *et al.* 1998; Pfeiffer and Höftberger 2001; Tausz *et al.* 2001). These reactive oxygen species (ROS) are normally a consequence of the side reactions of metabolism (in cells involved in oxidation-reduction processes), disruption of the mitochondrial electron transport chain or electron leakage, and mediate the degradation of membrane components (lipid peroxidation), the oxidation of protein sulphydryl groups, the formation of gel phase domains and the loss of membrane function (Fridovich 1984; Elstner and Oßwald 1994; Quartacci *et al.* 1995; Sgherri *et al.* 1996; Minibayeva *et al.* 1998; Lam *et al.* 1999; Navari-Izzo and Rascio 1999). ROS levels within the cell are under rigid control by a multilevel antioxidant system, composed of hydrophilic (ascorbic acid, glutathione) and lipophyllic (tocopherols) antioxidants, the action of detoxifying enzymes (such as superoxide dismutase (EC 1.15.1.1; SOD) and ascorbate peroxidase (EC 1.11.1.11)), and enzyme repair and regeneration processes (Foyer 1997; Sgherri *et al.* 2000). Interestingly, alditols have been shown to accumulate in response to environmental stress (Wyn Jones 1984; Smirnoff and Cumbes 1989; Pharr *et al.* 1995; Naidu 1998; Steinitz 1999; Lo Bianco *et al.* 2000).

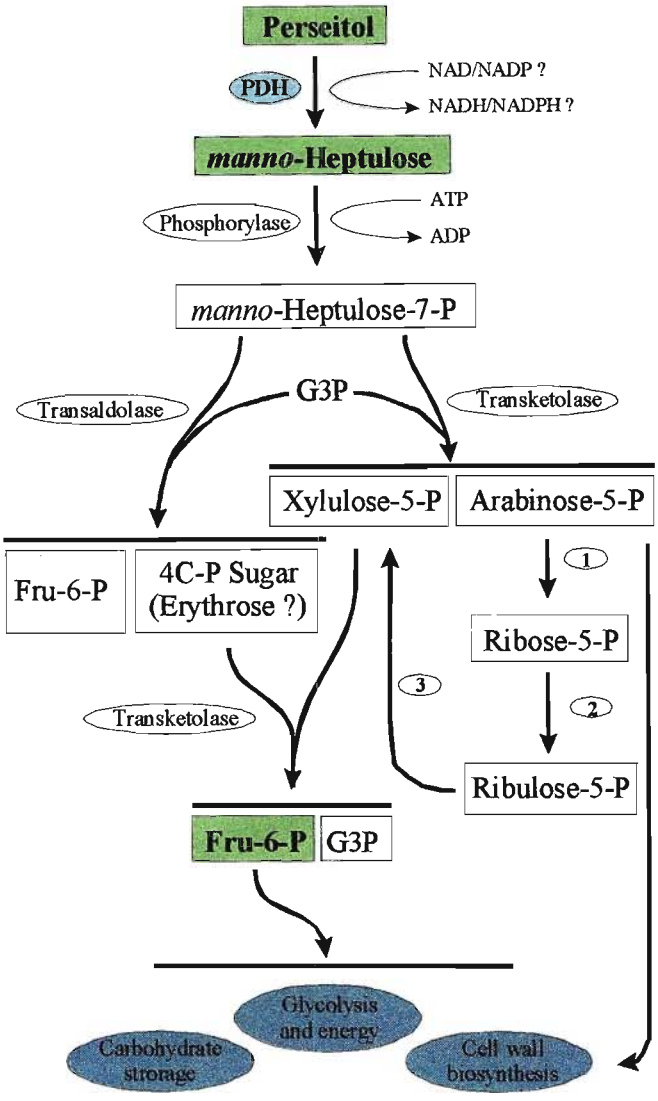


Figure 7.3 Simplified scheme illustrating the possible pathway of perseitol catabolism in avocado fruit. Enzymes: 1 = Epimerase on C-2; 2 = Epimerase on C-3; 3 = Phosphopentose epimerase. The activities of the enzymes 1–3 may be substituted by a phosphopentose isomerase.

Alditols are highly hydroxylated molecules and are thought to mimic the structure of water and create an artificial sphere of hydration around macromolecules so protecting them from oxidation, play an important role in the neutralization of ROS, act as compatible solutes and osmoprotectants/-regulators, and stimulate the biosynthesis of secondary plant metabolites (Lewis 1984; Loescher 1987; Tholakalabavi *et al.* 1997; Steinitz 1999). Richings and Cowan (2000) theorised that perseitol may act to protect enzymes, such as HMGR, from oxidative damage or ROS-induced inactivation. These authors proposed a model (Figure 7.4) that suggests elevated ABA and subsequently ROS levels reduce HMGR activity and so alter isoprenoid metabolism, and impact of fruit growth. The inter-conversion of *manno*-heptulose to perseitol may serve to scavenge ROS and so ensure the maintenance of normal fruit growth. It may be noteworthy that if the proposed oxidation of perseitol by PDH is inhibited, and an oxidase-catalysed mechanism

promoted, ROS production can be expected to increase. Thus, situations of 'abnormal' 7C sugar metabolism may also negatively affect fruit growth by increasing ROS and so alter isoprenoid metabolism. This scheme is of particular interest as environmental stress, such as drought and low temperatures, have been associated with increases in endogenous ABA (Chen *et al.* 1983; Macháčková *et al.* 1989; Walker-Simmons *et al.* 1989; Dörffling *et al.* 1990; Taylor *et al.* 1990; Lång *et al.* 1994; Grossi *et al.* 1995; Pruvot *et al.* 1996; Veisz *et al.* 1996; Bravo *et al.* 1998; Kadlecová *et al.* 2000). Treatment of avocado fruit with ABA resulted in a general elevation in tissue perseitol content, suggesting that perseitol may be accumulated in response to stress. A similar observation was made by Lo Bianco and co-workers (2000) who observed an elevation of sorbitol levels in drought-stressed peaches. Ironically, ABA treatment also resulted in an elevation of PDH activity; however, this may be associated with increased perseitol/*manno*-heptulose inter-conversion.

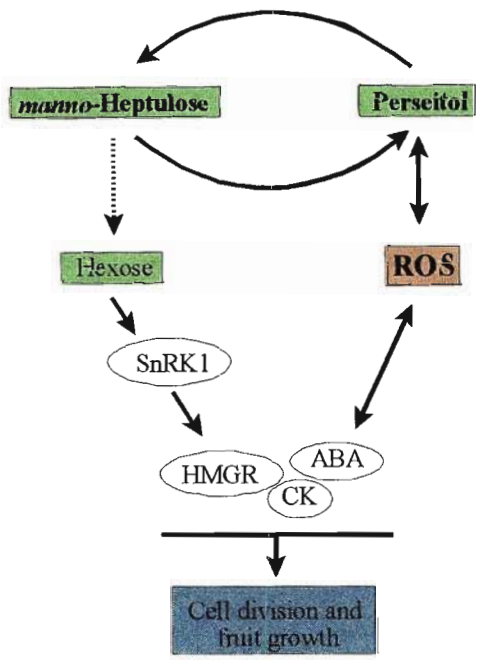


Figure 7.4 Scheme to illustrate the possible interaction between 6C and 7C sugar metabolism, isoprenoid metabolism and ROS in the control of fruit size. Redrawn from Richings and Cowan (2000).

In grape (*Vitis vinifera* L.) a *Vitis vinifera* hexose transporter 1 (*Vvht1*) gene has been identified which appears to be potentially regulated by ethylene-, ABA-, and sugar-responsive *cis*-element sequences (Fillion *et al.* 1999). Interestingly, this *Vvht1* promoter was shown to possess very similar properties to an alcohol dehydrogenase promoter expressed at the same time during ripening. In addition, MDH activity repression appears to be mediated by HXK (Prata *et al.* 1997).

These observations suggest co-regulation of gene expression by endogenous sugars, alditols and hormones, and that a putative alditol-responsive transduction sequence similar, to that described for hexoses, may exist. In the current study, with the exception of the seed coat, perseitol levels decreased slightly throughout the period observed. Furthermore, as with *manno*-heptulose, perseitol levels decrease with ripening in avocado fruit (Shaw *et al.* 1980; Liu *et al.* 1999b). It is thus conceivable that the expression of an alcohol dehydrogenase (such as PDH) or oxidase gene, via a HXK-in/dependent signalling pathway during ripening may contribute to the depletion of perseitol in maturing fruit. This obviously would occur concurrently with the catabolism of *manno*-heptulose, perhaps via phosphorylation and subsequent cleavage. The depletion of 7C sugars, especially *manno*-heptulose, may allow for the expression of HXK-dependant genes associated with ripening, and thus permit this phenomenon to proceed.

These results suggest that *manno*-heptulose and perseitol may play an important role in the transport and provision of carbon, protection of the fruit/plant to cold and osmotic stress and subsequent ROS, and the regulation of fruit growth and ripening.

7.1.2 THE METABOLIC CONTROL OF 'HASS' AVOCADO FRUIT GROWTH

Fruit set is resource-limited (Stephenson 1981). The ability of the fruit to grow once set is also resource limited. This limitation may not only be due to a limited source, but may arise from the inability to establish and maintain sufficient sink strength. Sink strength, in part, is determined by the ability of the fruit to metabolise, partition and compartment incoming photosynthate. These processes are determined by the enzymology of the sink. In turn, enzyme activity is differentially modulated by hormones, nitrogen supply, sugar status, intracellular pH, anoxia, water stress and the expression of levels of enzyme protein (Mackintosh 1998). The relative rates of solute metabolism need to be regulated in response to sugar import to allow for carbohydrate storage and the diversion of photosynthate into pathways for amino acid, membrane and polyphenol synthesis.

7.1.2.1 Constraints to the apoplastic and symplastic transport of solutes in the seed coat

In developing cukes (underdeveloped fruits that usually do not have a seed during late development), isolation of the embryo sac from nutritive tissue and chalazal flow of nutrients causes starvation and subsequent cessation of embryo development (Steyn *et al.* 1993). In normally developing avocado fruit, wounding of the seed coat at early stages of development can induce fruit drop, and rapid embryo growth normally diminishes concurrently with the drying of

the seed coat, suggesting severance from its food source (Blumenfeld 1970). Not only does the seed coat act as a conduit for solutes and developmental/physiological signals from the tree, but the seed has been proposed to be a major source of growth regulators (such as IAA and GA)(Lee 1987) that direct sink activities and growth. GA and CK have been shown to be high in the endosperm and seed coat (Blumenfeld and Gazit 1971) of avocado. Blumenfeld (1970) and Blumenfeld and Gazit (1971) were able to conclude that promotive growth substances in avocado fruit were chiefly produced in the seed, in particular the embryo, and transported via the seed coat to the mesocarp where they sustained and/or promoted cell division. These 'messengers' supplied by the seed may act to keep the mesocarp tissue in a 'juvenile' state during seed development, ensuring that the seed reaches full maturation before the mesocarp, and that the mesocarp maintains fruit sink strength and so ensures a continued supply of assimilates to the fruit (and seed) until the seed and embryo are suitably developed. In the current study, it was also shown that treatment with iP negated the effects of ABA on fruit growth and phenolic composition, and increased the amount of [^{14}C]-label entering the seed. This suggests that in ABA-treated fruit, a reduction in fruit growth can be correlated with increased polyphenolics in the seed coat and reduced solute import into the seed. Interestingly, levels of inhibitory plant growth regulators increase in the mesocarp as the fruit matures and the levels of these correlate negatively with the rate of cell division (Cowan *et al.* 1997a; Moore-Gordon *et al.* 1998). Cowan *et al.* (1997a) found that the ABA concentration declines during the course of normal fruit development, whilst the ABA content of small fruit mesocarp was substantially higher at all stages of fruit growth. Thus the role of the seed coat in communication is bidirectional. These observations, along with the apparent degradation of the seed coat and the accumulation of polyphenolics during normal fruit development and in small and ABA-treated fruit, suggested that the functional and structural integrity of the seed coat plays a crucial role in the import of solutes and the control of fruit development. The polyphenol deposition within the seed coat is believed to act as both a symplastic and apoplastic barrier, reducing the transport of assimilates to the seed and, likewise, reducing the transport of growth regulators and chemical messengers from the seed to the mesocarp.

It must be stressed at this point that, although the seed coat appears to be senesced, it is still metabolically active, indicating that while its function may be largely reduced, it is by no means redundant and may still take part in communication and transport between the mesocarp and seed, albeit at reduced rates.

The concept of the interaction of plant stress, ABA, ROS, alditols and the manifestation of the small fruit is very interesting due to the apparent role of polyphenols in seed coat senescence. Polyphenols play an important role in normal plant developmental processes and response to both biotic and abiotic stress. The mechanism of phenolic action appears to be attributed to interference with plant hormones, especially IAA (Vickery and Vickery 1981). Phenolic synthesis is regulated by a variety of enzymes, whose formation can be induced by diverse environmental conditions, including light, pathogen related elicitors, nutrient deficiency and drought stress (Bussotti *et al.* 1998; Noh and Spalding 1998; and the references therein). A variety of compounds can be categorised as polyphenols, including flavonoids (e.g. anthocyanins and phytoalexins), tannins and lignin. Avocados are rich in a complex mixture of polyphenolic acids, ranging from the simple (+)-catechin and (-)-epicatechin to highly polymeric substances, such as tannins (Geissman and Dittmar 1965). Numerous phenolic compounds have been identified in avocado, most abundant being *p*-coumaric and ferulic acid, but also present are the phenolic acids: *p*-hydroxybenzoic, pyrocatechuic, γ-resorcylic, α-resorcylic, protocatechuic, gallic, isovanillic, vanillic, syringic, *o*-coumaric, *m*-coumaric, caffeic, *p*-coumaroylquinic, caffeolquinic acids; the flavan-3-ols: catechin and epicatechin; and the miscellaneous phenols: dopamine and serotonin (Macheix *et al.* 1990).

During normal fruit maturation, the seed coat is seen to darken in colour and then senesce. It is thought that this occurs once the seed, particularly the embryo, has reached 'physiological maturity' and is believed to occur concurrently or initiate changes in physiology that are associated with mesocarp maturation and lipid accumulation. In this investigation, it was shown that the darkening of the seed coat was associated with the deposition of tannins and insoluble phenols, especially within the inner layer of the seed coat. Seed coat degeneration was also associated with the lignification of the outer layers of the seed coat. Furthermore, it is currently accepted that premature seed coat abortion contributes to smaller fruit size (Blumenfeld and Gazit 1974; Steyn *et al.* 1993). It would, therefore, appear that one characteristic of the small fruit is premature seed coat senescence, a phenomenon that could be induced artificially in this study by ABA treatment. The small fruit was shown to have substantially higher levels of tannin in the seed coat than that of the control. Similarly, both the small and ABA-treated fruit had elevated soluble and insoluble phenol, as well as increased anthocyanins in the seed coat. This suggests that elevated ABA levels may contribute to the initiation/occurrence of seed coat senescence.

The term senescence refers to the controlled and programmed process of cell death. Programmed cell death (PCD) refers to the genetically-determined death of a cell through changes in nuclear morphology, activation of proteases and nucleases, and internucleosomal fragmentation of nuclear

DNA (Young and Gallie 2000; and the references therein). The execution of cell death involves the influx of Ca^{2+} into the cell and is manifested by rapid collapse of the large hydrolytic vacuole and cessation of cytoplasmic streaming (Groover and Jones 1999), a process that is likely to result in the instant cessation of symplastic transport. In addition, the involvement of Ca^{2+} in PCD is interesting as it is believed that Ca^{2+} also plays a role in callose and cellulose deposition (Delmer *et al.* 1985; Köhle *et al.* 1985; Kauss and Jeblick 1986; Hayashi *et al.* 1987; Kauss 1987; Waldmann *et al.* 1988; Dearnaley *et al.* 1997; Holdaway-Clarke *et al.* 2000), and will thus further contribute to a loss of symplastic transport.

PCD plays an important role in embryonic, juvenile and adult phases of plant development (Kawasaki *et al.* 1999; Jones 2001), allowing for the differentiation of specialised cells within the plant and the isolation of tissues during normal development. An example of such a situation could be the PCD or senescence of the seed coat during normal avocado fruit maturation. Another example that can almost be considered functionally analogous to the role of the seed coat in developing avocado would be the death of the suspensor subsequent to embryo maturation. Many developmental programmes culminating in cell death have been shown to be regulated by hormones, such as ethylene, GA, CK and IAA (Dalessandro and Roberts 1971; Fukuda 1994; He *et al.* 1996; Wang *et al.* 1996). The ordered suspensor PCD in *Arabidopsis* mutants suggests that both inductive and inhibitory signals from the embryo control the onset of PCD (Jones and Dangl 1996). Thus, it is highly likely that similar signals from the seed may control the senescence of the seed coat in avocado. During maize endosperm development it is believed that the balance between ABA and ethylene establishes the onset and progression of PCD (Young and Gallie 2000). Davenport and Manners (1982) found that young avocado fruit undergoing nucellus and seed coat senescence experienced a large, transient increase in the rate of ethylene synthesis by these tissues. It is thus possible that elevated ABA production by the seed and seed coat stimulates ethylene synthesis in the endosperm and seed coat and triggers the onset of PCD in these tissues.

Rapid PCD at a site of pathogen entry or as a consequence of interactions with the environment is a common feature of plant resistance, and is termed the hypersensitive response (HR) (Jones and Dangl 1996; Pennell and Lamb 1997). HR is an active process in which accumulation of ROS leads to elevated cytosolic Ca^{2+}, and triggers a protein-mediated PCD response (Pennell and Lamb 1997; and the references therein). The ROS burst can occur within minutes, and sometimes seconds, after the stress is applied. A second burst may occur 2-3 hours after the inducing stimulus. These elevated ROS levels induce the synthesis of enzymes that may relate to defence against the stress (e.g. enzymes associated with phytoalexin synthesis) or for protection from the

oxidative burst (e.g. ROS scavengers) (Murphy *et al.* 1998; and the references therein). Interestingly, ABA is commonly referred to as 'the stress hormone', and similar to ROS, ABA is associated with many abiotic and biotic stress factors, including water stress, cold stress and even pathogenic invasion/infection (Westgate *et al.* 1986). The seed coat from small and ABA-treated fruit show many characteristics associated with ROS induced HR. Firstly, in cells that have undergone PCD due to HR, the cell corpse is often crushed by the surrounding tissue (Groover and Jones 1999). In small and ABA-treated fruit, the cells from the seed coat are typically flattened and compressed without any distinct cytoplasm or cellular organelles. Secondly, elevated ROS and the associated HR leads to the peroxidation of membrane lipids and cross linking of cell-wall material, including polyphenols, so generating an impervious wall (Murphy *et al.* 1998; and the references therein). In addition, Cowan *et al.* (1997a) theorised that ABA accumulation may cause the loss of membrane integrity. In small and ABA-treated fruit the seed coat showed extensive lignification and an apparent absence of the Pm, or other membrane bound organelles. Thirdly, environmental stress, elevated ROS and the HR have been associated with increased production of phenolic compounds such as flavonoids and phenylpropanoids in plants (Dixon and Paiva 1995; Grace *et al.* 1998; Murphy *et al.* 1998). Indeed, many phenolic compounds have a hydrogen-donating anti-oxidant capacity and exhibit a strong relationship with the oxygen radical absorbance capacity of a tissue (Cao *et al.* 1997; Grace *et al.* 1998; Phippen and Simon 1998; Prior *et al.* 1998). Again, small and ABA-treated fruit had elevated soluble and insoluble phenols, and anthocyanin levels which may represent a response to limit or quench free radical damage.

In stressed beech (*Fagus sylvatica*) and pine leaves, it has been suggested phenols, especially tannins, are produced within the cytoplasm and cluster together forming droplets in the vacuoles. These droplets fuse and ultimately completely fill the vacuole. Once this occurs the cytoplasm degenerates, the organelles disappear and, finally, there is a release of the vacuolar contents leading to the death of the cell (Zobel and Nighswander 1990; Bussotti *et al.* 1998). Furthermore, tannins observed in the vacuoles of beech leaves and released into the cytoplasm appear to be translocated to the cell wall, where they form an electron opaque layer (Bussotti *et al.* 1998). Plant tannins are a unique group of phenolic compounds of relatively high molecular weight, that have the ability to form strong complex compounds with carbohydrates and proteins (Porter 1989; Ayres *et al.* 1997), potentially forming an impermeable barrier within the cell wall. The plant extracellular matrix comprises of a complex array of polysaccharides, glycoproteins, phenolics and other macromolecules (reviewed by: McNeil *et al.* 1984; Carpita and Gibeaut 1993). This macromolecular complex is a dynamic structure that plays an important role in the interaction of the plant with biotic and abiotic factors, cellular transport processes and growth/development. The

cell wall components will thus also play an important role in cell-to-cell communication and the control of morphogenesis. The pattern of tannin deposition in beech and pine is very similar to that observed in 'Hass' seed coat tissue, and clearly illustrates the role of polyphenolics in the isolation of the symplast, PCD and the control of fruit growth. Phenolic compounds provide the monomeric precursors, derived from hydroxycinnamic acids, required for the synthesis of lignin (Grisebach 1981). Lignins are polyphenolic polymers of three cinnamyl alcohol derivatives, namely *p*-coumaryl, coniferyl and sinapyl alcohols (Vickery and Vickery 1981). Lignins are believed to limit cell wall extensibility and represent a major carbon/phenylpropanoid sink in plant tissues (Boudet 1998). Thus a controlled change in lignin content, as a consequence of elevated ROS and/or ABA, may suppress cell wall extensibility as well as alter the profile of soluble phenolics within the fruit. These observations suggest that some of the characteristics associated with the prematurely senesced seed coat in the small fruit phenotype may be a consequence of elevated ROS levels and are mediated via ABA.

Not only do phenolics limit cell wall extensibility, cellular transport and play a role in PCD, but they also have growth regulatory effects (Vickery and Vickery 1981). The *o*-diphenols are believed to inhibit IAA-oxidase activity, whilst the monophenols, such as *p*-courmaric acid, are believed to be activators of IAA-oxidase activity (Lee *et al.* 1982). Thus it is possible that the accumulation of monophenolics will result in the reduction of free IAA and so reduce both the polar transport of IAA, and impact directly on cell wall plasticity, reducing fruit growth. IAA is involved in the *de novo* synthesis of ethylene and endogenous avocado phenolic compounds have been shown to inhibit the *in vitro* oxidation of ACC to ethylene (Vioque *et al.* 1981; Sitrit *et al.* 1988), it is thus plausible that the phenolic content and/or the ratio of diphenols to monophenols during fruit growth may impact on many physiological processes influenced by ethylene, such as fruit ripening and the formation of abscission zones. Furthermore, several naturally occurring flavonoids have been shown to block Glu uptake in animal myelocytic cells (Park 1999). It may, therefore, also be possible that elevated polyphenols may further affect fruit growth not only by altering hormone metabolism, but also by interfering with sugar uptake, and hence all processes affected by sugar content and composition.

Phytoalexins are low molecular weight flavonoid compounds produced by plants in response to infection or stress. In cotton, sesquiterpenoids that include the terpenoid phytoalexin 'gossypol' and its derivatives are synthesised through the isoprenoid pathway and hence subject to control by HMGR activity (Joost *et al.* 1995; Loguercio *et al.* 1999). Furthermore, parasitization of tobacco plants by the parasitic angiosperm *Orobanche* (broomrape) has been shown to induce the

expression of *hmg2*, a gene that encodes HMGR and is associated with the production of sesquiterpene phytoalexins (Westwood *et al.* 1998; and the references therein). Thus, it is apparent that phytoalexin production can be induced by stress. These observations suggest an additional link between ROS and isoprenoid biosynthesis and an important role for HMGR in the interaction between stress, ROS and ABA.

During early fruit development, especially at the highly vulnerable stage of early fruit set, it is possible that a short period of water stress, an unseasonal drop in daily temperature, or any pathogen- or insect-induced damage may induce a burst in ABA and/or ROS levels in the young fruit and initiate the premature senescence of the seed coat (Figure 7.5). The period of this stress does not need to be long. ROS bursts have been documented to occur within minutes and sometimes seconds after a stress is applied (Murphy *et al.* 1998; and the references therein), and may thus only affect fruit that are at a crucial stage of development. As the period of anthesis in avocado is extended and pollination occurs throughout this period not all fruit set are of exactly the same age. Neither will they all be set under the same conditions or be at the same stage of development and will thus differ in sensitivity and response to stress. During early fruit set the implications of this change in ABA and/or ROS may not be apparent and may only be visually manifested during the linear phase of fruit growth (phase II). This would explain the apparent anomaly of small and normal fruit occurring side-by-side on the same branch.

The huge input of resources into secondary plant metabolites, such as tannins, phenols and anthocyanins, represents a significant energy investment in developing avocado fruit and may impact on the partitioning of assimilates in the developing fruit. Although no research was undertaken on the subsequent metabolism of polyphenols in the avocado, it is known that phenol-degrading enzymes such as polyphenol oxidase (EC 1.10.3.1) occur in these fruit (Knapp 1965; Golan *et al.* 1977; Van Rensburg and Englbrecht 1986). However, the partitioning of sizable proportions of sugars into this metabolic fraction may contribute to the maintenance of a solute gradient and sink strength, between the parent plant and the fruit, and the various tissues within the fruit.

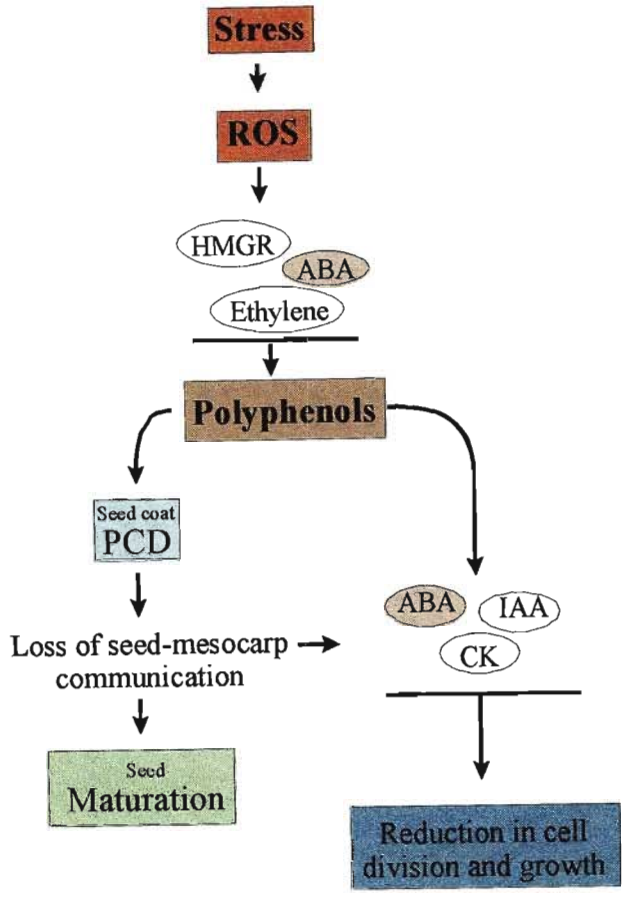


Figure 7.5 Scheme to illustrate the possible interaction between stress, polyphenol accumulation and seed coat senescence. Young fruit subjected to stress, such as water stress, exhibit a temporary burst in ROS resulting in altered HMGR activity and increased ABA content and ethylene synthesis. The resultant change in hormone balance stimulates polyphenol accumulation/deposition, which has a two-pronged effect. Firstly, PCD occurs within the seed coat and endosperm resulting in reduced symplastic and/or apoplastic transport and cell wall extensibility. Secondly, the polyphenols interact with plant hormones, especially IAA, and impact directly on cell division and carbon utilisation. Consequently there is a reduction in the supply of growth promotive factors to the mesocarp from the seed, a reduction in solute import via the seed coat and an overall reduction in cell division activity, manifested as a 'small fruit'.

7.1.2.2 Control of intercellular solute flux

Symplastic transport is the movement of small molecules (that, with the exception of viruses and some proteins, are usually less than 1 kD) and water from cell-to-cell via the Pd (Goodwin 1983; Terry and Robards 1987; Robards and Lucas 1990; Balachandran *et al.* 1997; Imlau *et al.* 1999). Many of these molecules, such as simple sugars, small proteins and some nucleic acids, play important roles as signalling molecules, substrates or intermediates involved in gene expression,

metabolism and the cell cycle, growth and development (Fisher *et al.* 1992; Lucas *et al.* 1995; Goshroy *et al.* 1997; Ding 1998). Under vegetative and developmental circumstances, symplastic transport is considered to be the primary route for the movement of these molecules. Thus any factor that affects symplastic transport can be expected to impact on overall tissue growth and development. One such factor would be the conductivity of the Pd. Pd are connections that link the cytoplasm of adjacent protoplasts, forming a symplastic continuum and are functionally analogous to gap junctions in animal cells. These dynamic pores traverse the plant cell wall and allow for high rates of cytoplasmic transport between contiguous cells (reviewed by: Gunning and Robards 1976; Robards and Lucas 1990; Beebe and Turgeon 1991; Lucas and Wolf 1993; Lucas *et al.* 1993; Oparka 1993; Mezitt and Lucas 1996; Overall and Blackman 1996; Goshroy *et al.* 1997; McLean *et al.* 1997; Crawford and Zambryski 1999; Oparka and Roberts 2001), with Pd frequencies having been used (though not conclusively) to characterise symplastic pathways (Overall and Gunning 1982; McLean *et al.* 1997).

Seed, seed coat and mesocarp tissue from normal fruit had numerous well-developed branched Pd grouped into large pit fields, that were often joined by a MC within the cell wall. Small fruit showed very few Pd in all tissue types examined, whilst no significant differences were observed in Pd number or ultra-structure between ABA-, iP-treated and control fruit. However, both small and ABA-treated fruit showed poor [^{14}C] uptake and distribution. In the small fruit this may be attributed to a reduction in Pd number, whilst in ABA-treated fruit this must suggest that there is a reduction in Pd function and not number. Pd function may therefore be inhibited by ABA treatment.

Primary Pd are simple channels formed during cell plate formation during cytokinesis (Ghoshroy *et al.* 1997), and appear simple and unbranched. Such Pd were commonly seen in tissue taken from small fruit. In phenotypically normal fruit the Pd appeared to be more branched, occurred in large pit fields and were often associated with rough ER, i.e. characteristics commonly associated with secondary Pd. Similar observations were made in phenotypically normal avocado fruit by Botha *et al.* (1996). This suggests that the normal fruit (and ABA-/iP-treated fruit) have secondary Pd. Secondary Pd are formed *de novo* in pre-existing cell walls, by the deposition of cell wall material over branched ER strands, and allow for greater cytoplasmic integration of cells (Lucas *et al.* 1993). The formation and the nature of Pd may be regulated during the course of morphogenesis (Kwaitkowska and Maszewski 1985). Hence, the small fruit phenotype appears to have limited Pd development. Contrary to the findings in this study, Moore-Gordon *et al.* (1998) found ABA application resulted in reduced Pd branching in avocado mesocarp tissue. Thus

elevated ABA may contribute to the inhibition of secondary Pd in the small fruit phenotype and, subsequently, to reduced symplastic continuity and cell-to-cell transport.

Application of ABA also resulted in the deposition of an electron dense, particulate material in the neck region of the Pd in the mesocarp and seed coat tissue (Moore-Gordon *et al.* 1998). This was not visible in iP-treated fruit or observed in the current trial, although an osmiophilic substance was observed in the Pd channel. However, micro-iontophoretic techniques confirmed retarded dissipation of Lucifer yellow, and hence reduced cell-to-cell transport, in ABA-treated seed coat and mesocarp tissues (Moore-Gordon *et al.* 1998), suggesting the deposition of some material as a consequence of ABA treatment within the Pd that reduced Pd conductivity. Pd are lined by the Pm and traversed by the ER, which is tightly appressed to form the desmotubule (Gunning and Overall 1983; Thompson and Platt-Aloia 1985; Overall and Blackman 1996). This axial component is surrounded by proteinaceous electron lucent particles that are thought to be associated with actin and/or myosin (White *et al.* 1994; Ding *et al.* 1996; Blackman and Overall 1998; Radford and White 1998). The Pd mouth is also frequently associated with a distinct raised collar (Willison 1976), below which lies an extracellular ring of opaque structures. Transport through the plasmodesmata occurs via two possible routes:

1. Through the 'central rod' of the desmotubule, which has been suggested to form a proteinaceous channel and serve as a pathway for molecular transport when the desmotubule is dilated (Waigmann *et al.* 1997);
2. Through the cytoplasmic sleeve between the two cytosolic components.

Control of transport through these two routes is, in part, determined by the hydrodynamic radius of molecules (Terry and Robards 1987) and the size of the Pd, i.e. the SEL, which is normally in the range of 0.8-1 kD (diameter 3 nm). This is, in part, a consequence of the tortuous route created by the cytoplasmic sleeve subunits and the presence of proteins embedded in the inner and outer leaflets of the endoplasmic reticulum and the plasma membrane, which form a supramolecular complex that divides the annulus into 8-10 micro channels. The SEL has also been suggested to be controlled (often rapidly) by the action of the external molecules acting as sphincters or valves (Olesen 1979; Robards 1982) as regulated by transcellular turgor pressure gradients (Oparka and Prior 1992), protein kinases (Yahalom *et al.* 1998), cytosolic ATP levels and chemical signals, such as Ca^{2+} (Tucker 1988; Tucker 1993; Cleland *et al.* 1994; Salisbury 1995; Tucker and Boss 1996; Trewavas and Malhó 1997; Holdaway-Clarke *et al.* 2000). Alterations in the size (even complete closure) (Robards and Lucas 1990), structure (van Bel and Kempers 1990) and

macromolecular organization (Thompson and Platt-Aloia 1985) of the Pd allows the SEL to be regulated throughout development in a temporal and position-dependant manner (Crawford *et al.* 1999). This permits the Pd to have different functional states and hence play an important role in controlling (Lucas and Wolf 1993) and synchronising (Ehlers and Kollmann 2000) developmental processes, including the demarcation of symplastic domains that is believed to be prerequisite for differential morphogenesis (Ehlers *et al.* 1999) in plant tissues.

The deposition within the Pd microchannel and in the Pd mouth (forming a cap-like structure which occluded the Pd) in avocado (Moore-Gordon *et al.* 1998), and the similar occlusion of Pd by the deposition of an osmiophilic, dense material located in the microchannels of the Pd cytoplasmic sleeves in protoplast cultures of *Solanum nigrum* (Ehlers *et al.* 1999), can thus be expected to play a crucial role in intercellular transport and growth/development. In avocado, Botha and Cross (2000) observed the rapid deposition, within 10-15 min, of an electron opaque material, interpreted as a mixture of proteinaceous and carbohydrate material, in the collar region of Pd following wounding. These authors defined this material as callose and suggested that the deposition of this material may play a role in conformational sub-structural changes in Pd and hence in Pd gating. Such closure of Pd is expected to result in loss of functionality and the termination or down-regulation of transport. It must be noted, however, that under normal circumstances callose is electron lucent (Kauss 1996). Callose is a structural polysaccharide consisting of spirally arranged glucose residues linked by β- (1→3) glycosidic bonds, which is frequently deposited in response to wounding or stress in plants. Callose has been localised to the Pd and/or the micro-domain that surrounds the Pd (Northcote *et al.* 1989; Turner *et al.* 1994; Kauss 1996; Enkerli *et al.* 1997; Roy *et al.* 1997), and has been suggested to play a role in the transient blocking/gating of Pd (Olesen and Robards 1990; Lucas *et al.* 1993). In the present investigation, callose was localised to the Pd by light microscopy with aniline blue. Furthermore, contrast staining at the electron microscope level suggests that callose may form a ring-like structure surrounding the outer Pd. Frequently the Pd also appeared to be associated with an osmiophilic substance that was localised within the Pd channel. These results suggest that callose may play a role in controlling the conductivity of the plasmodesmata by either occluding or constricting the Pd and so reducing cell-to-cell transport. It is also is possible that the rings merely provide structural support to the Pd and loss of these callose rings may similarly be expected to result in the collapse of the Pd and hence loss of transport function.

Interestingly, callose synthesis can be rapidly induced by elicitors such as polycations or amphipathic compounds, which are known to cause changes in membrane permeability, as

determined by the leakage of cellular elecrolytes into the medium (Waldmann *et al.* 1988; and the references therein). Indeed, callose formation normally occurs after membrane perturbation (Kauss 1996), one of the consequences of elevated ROS levels. Like ROS, callose deposition is a well-characterised response to wounding and stress (Pooviah 1974; Van de Venter and Currier 1977; Jaffe and Goren 1988). From the results of this study, small fruit had slightly elevated callose contents, a phenomenon that could be induced by either ABA or simultaneous ABA and iP treatment *in vivo*. However, small and ABA-treated fruit showed a reduction in the rates of callose synthesis. Furthermore, ABA treatment increased callose levels *in vitro* and transiently increased 1,3-β-glucan synthase activity. These observations suggest that ABA treatment, although transiently increasing the rate of callose synthesis, plays a more important role in the inhibition of callose turnover. Callose has been shown to undergo degradation and turnover by enzymes present in wall preparations, such as exo-1,3-β-glucanase (EC 3.2.1.84) and β-glucosidase (EC 3.2.1.21) (Bucheli *et al.* 1985; 1987; Francey *et al.* 1989). Thus it is possible that the small and ABA-treated fruit have high callose levels as a consequence of reduced callose turnover, not increased callose deposition, and the ABA-induced reduction of callose catabolism may be a means in which the conductivity of Pd can be reduced or controlled. Conversely, the sugars Glu, Fru and Suc increased the rate of callose synthesis, but were associated with reduced callose contents, suggesting these sugars contribute to elevated rates of callose turnover. Callose deposition also occurs, normally transiently, in a developmentally regulated manner at specific sites within the plant, such as sieve plates, pollen tubes and around the Pd in primary walls (Northcote *et al.* 1989; Turner *et al.* 1998). Taking into account the localisation of callose to the Pd, callose may thus act as a short-term method for the crude control of Pd conductivity. These results, therefore, suggest that avocado symplastic continuity is controlled developmentally and/or in response to stress.

Finer control of Pd function may occur via changes in conformation of Pd-localised proteins. Actin, myosin and centrin have been localised to Pd in higher plants and show Ca^{2+}-dependant interactions or contractions. These proteins may thus play a role in the constriction of the cytoplasmic annulus and the restriction of cell-to-cell movement of ions and small molecules (White *et al.* 1994; Blackman and Overall 1998; Radford and White 1998; Blackman *et al.* 1999; Holdaway-Clarke *et al.* 2000). Similarly, Epel and co-workers (1996) found a 41 kD protein in the soluble fraction associated with the Pd, and suggested it may be located at or within the Pd pore and/or the desmotubule. Botha and Cross (2000) have suggested that a mixture of protein and callose forms a plug that prevents transport via the Pd and that this plugging can be stimulated by ABA treatment. There is also evidence that plugging of the Pd by osmiophilic electron-dense material precedes desynchronization of mitotic cycles (Kwiatkowska and Maszewski 1976). As

the Pd is lined by the Pm it is speculated that the production or deposition of this material must occur via a Pm-localised enzyme. 1,3-β-glucan synthase has been localised to the Pm (Kauss *et al.* 1983; Delmer *et al.* 1993; Turner *et al.* 1998). However, in the present investigation, little difference was observed in the membrane-bound proteins between small and normal fruit. A 26.6 kD protein was isolated from the Pm fraction, and although not identified, it may relate to the plant homologue of a protein with the same mass from the connexin family of gap junction proteins (Meiners *et al.* 1991; Yahalom *et al.* 1991). The presence of such a polypeptide within the Pd would allow for organisational control of the transit channel between plant cells, and changes in the macromolecular organization of the Pd would be indicative of different functional states during development and maturation.

Both callose and protein deposition or conformational changes in the Pd offer very promising means of altering the SEL of Pd. Callose levels, via reduced callose turnover, have been shown to be increased by ABA treatment. ABA-treated and the small fruit phenotype both display elevated callose levels. Symplastic transport is believed to be more efficient and permit the greater flux of solutes from cell-to-cell transport, and the stress or ABA induced loss of this can be expected to negatively impact on phloem unloading, sink strength and fruit growth.

7.1.2.3 Effects of ABA on carbohydrate metabolism and fruit growth

Results presented in the current investigation provide good evidence to support an interaction between elevated ABA levels and the appearance of the small fruit phenotype. In addition, it is noteable that both small and ABA-treated fruit showed similar responses with respect to sugar content. The small fruit phenotype was characterised by high TSS, Suc, Glu, Fru and perseitol during the early stages of phase II of fruit growth. ABA-treatment resulted in an increase in Suc, Glu, Fru, perseitol in all tissues, and *manno*-heptulose in the seed and seed coat tissue. Of particular interest was the increase (almost double) of Glu and Fru in the endosperm tissue of small and ABA-treated fruit relative to control and iP-treated fruit. Both small and ABA-treated fruit were also associated with an increase in the content of the sugars with a retention time of 8.84 and 22.3 min in the seed and endosperm tissues respectively, as well as a significant decrease in starch content. Prior to endosperm degeneration, the sugars with the retention times 8.84 and 22.30 min were seen to increase in the endosperm tissue from the normal fruit phenotype. This observation may suggest a relationship between the presence of these sugars and the onset of endosperm and seed degeneration. These results are not surprising as ABA and stress have been documented to influence carbohydrate partitioning, increase the levels of free sugars, decrease

starch levels and increase sugar uptake (Sowokinos *et al.* 1985; Schüssler *et al.* 1991; Roitsch *et al.* 2000). A similar pattern of altered sugar metabolism was observed in small and ABA-treated 'Hass' fruit. In addition, the changes in sugar profile, especially increased Glu and Fru levels can be expected to have far-reaching effects on fruit growth through the control of gene expression. This is a result of the fact that both substrates and products of sugar metabolism can act as signal molecules. Like hormones, and in combination with hormones and other stimuli, these sugars can regulate many aspects of plant development from gene expression to long-distance nutrient allocation (Roitsch *et al.* 2000).

Sugar sensing in plants is thought to occur by two mechanisms, HXK and SnRK1. HXK is the first enzyme in hexose metabolism and believed to be one of the glucose sensors in plants and to mediate sugar regulation of gene expression. HXK activity has been detected in extracts from mature avocado mesocarp (Copeland and Tanner 1988). However, as HXK is competitively inhibited by *manno*-heptulose (Pego *et al.* 1999), and this sugar occurs in very high concentrations in the avocado mesocarp (Richtmyer 1970, Ogata *et al.* 1972, Shaw *et al.* 1980), its role in the control of avocado fruit growth remains questionable. Sucrose nonfermenting 1 protein kinase (SNF1) is a protein-serine/threonine kinase that has been shown to play a key role in the main Glu repression/derepression pathway in yeast, regulating fundamental metabolic pathways in response to nutritional and environmental stresses that deplete ATP or induce alterations in AMP/ATP ratios (Hardie *et al.* 1998; Gibson and Graham 1999; and the references therein). This calcium/calmodulin-dependent protein kinase (Stone and Walker 1995) is believed to be a central component in a highly conserved protein kinase cascade that appears in most eukaryotic cells (Hardie *et al.* 1998). SnRK1 is the higher plant homologue to yeast SNF1, and is thought to protect the cell from nutritional and/or environmental stresses by regulating both metabolism and gene expression (Halford and Hardie 1998). Interestingly the plant HMGR kinase has been found to be related to SnRK1 (Barker *et al.* 1996). SnRK1 rapidly phosphorylates and inactivates HMGR, nitrate reductase and SPS *in vitro* (Sugden *et al.* 1999), and its activity has been detected in avocado mesocarp tissue (MacKintosh *et al.* 1992). SnRK1 thus may potentially regulate Suc synthesis, nitrogen assimilation and isoprenoid biosynthesis in avocado fruit, and hence play a role in the control of sink strength, carbon partitioning, the interaction between carbon and nitrogen metabolism, and the cell division cycle (Purcell *et al.* 1998; Dickinson *et al.* 1999). The sugars Glu, Fru and Suc have also been shown to induce the autophosphorylation of several Ca-dependant protein kinases associated with the regulation of sugar transport across the Pm (Ohto and Nakamura 1995). In addition, transnodal transport via the Pd in *Nitella flexilis* is partly 'active' and requires the input of metabolic energy (Trębacz *et al.* 1988). Though this might not be

the case in avocado, the interaction of sugars with solute transport suggests changes in HMGR activity, ABA levels, sugar content and metabolism, and symplastic continuity are intrinsically linked.

With the exception of SSy (Clv), all enzymes assayed showed a general increase in relative rates of activity in small and ABA-treated fruit. Similarly, ABA-treatment of seed coat discs *in vitro* resulted in the elevation of IAI, SAI, SSy (Syn) and SPS activity. This increased activity was delayed by 4-8 h, suggesting that ABA may be responsible for inducing changes in gene expression and the *de novo* synthesis of enzymes such as IAI, SAI and SSy (Syn). On the other hand, SSy (Clv) activity was decreased by incubation with ABA *in vitro*. *In vivo*, SSy (Clv) activity was inhibited by ABA- and iP- treatment alone; however, when simultaneously treated with ABA and iP, this inhibitory affect was negated. SSy activity in the cleavage direction was inhibited by treatment with Glu, which was elevated in both small and ABA-treated fruit. Results from the current investigation suggest that SSy (Clv) plays a major role in the provision of carbon during seed development and the linear phase of mesocarp growth. Thus a loss of SSy (Clv) activity in small fruit and ABA-treated tissue, is expected to significantly reduce carbon metabolism and hence fruit growth. Purcell *et al.* (1998) observed that SSy gene expression was decreased by the expression of antisense SnRK1 in potato tubers. Furthermore, SSy activity is modulated by tissue carbohydrate status (Koch *et al.* 1992). The accumulation of Glu mediated by ABA, and the elevated levels of ABA in the small fruit may, therefore, via SnRK1 activity result in altered Suc channeling into certain metabolic pathways by SSy and possibly reduced sugar uptake.

Interestingly, SSy (Clv) is thought to be the source of UDP-Glu needed for callose synthesis (Carpita and Delmer 1981; Nolte *et al.* 1995). Exogenous application of UDP-Glu has been shown to increase the rate of callose deposition (Clark and Villemez 1972; Jaffe and Goren 1988). Thus the reduced rates of callose synthesis in small and ABA-treated fruit may be linked to a reduction in SSy (Clv) activity as a consequence of either elevated ABA or Glu levels.

Exogenous ABA treatment has been previously documented to increase SAI, NI and Glu activity/levels (Morris and Arthur 1984b; Ackerson 1985; Goupil *et al.* 1998). Similarly, stress-related stimuli and Glu have been reported to stimulate AI or elevate mRNA levels associated with invertase (Matsushita and Uritani 1974; Sturm and Chrispeels 1990; Ohyama *et al.* 1995; Roitsch *et al.* 1995; Ehness and Roitsch 1997; Godt and Roitsch 1997). In the current investigation, both small and ABA-treated fruit had elevated IAI activity. This high extracellular invertase activity

appeared concurrently with an apparent loss of symplastic continuity. Furthermore, the genes for invertase are expressed maximally when supplies of metabolizable sugar are limited (Xu *et al.* 1995). Evidently there appears to be a limited supply of sugars to the seed, which is compensated for by increased IAI activity. And, indeed, hydrolysis of Suc by IAI is believed to contribute to the establishment of sink strength (Weber *et al.* 1995; Roitsch 1999). Overexpression of AI has, however, been associated with morphological changes (Tymowska-Lalanne and Kreis 1998), stunted growth (Dickinson *et al.* 1991) and the arrested development of secondary Pd (Ding *et al.* 1993). All of these being traits observed in both small and ABA-treated fruit.

Miller and Chourey (1992) presented genetic evidence to show that the developmental stability of maternal cells in the pedicel at the base of maize seeds is determined by the genotype of the developing endosperm. These authors suggested that the pollen donor may influence the development of the maize kernel. Interestingly, in the same research they were able to show that miniature seed mutants had very low levels of SAI and IAI, and suggested that the invertase-mediated maintenance of a physiological gradient of photosynthate between the pedicel and endosperm constitutes the rate-limiting step in the structural stability of the maternal cells as well as in normal development of the endosperm and seed. Miller and Chourey (1992) also proposed that the cellular changes associated with the cellular destruction of placento-chalazal region and the formation of a gap between the pedicel and endosperm in miniature maize seed mutants may arise as a consequence of a transient osmotic imbalance due to the impaired movement of photosynthate in the endosperm. This reduced photosynthate movement arises from a change in invertase activity in the basal endosperm region, which drives the continuous flow of photosynthate from the maternal tissues to the cells into the filial generation. Anatomically the pedicel and endosperms tissues of miniature and normal maize seeds were indistinguishable, but histochemical staining for invertase showed a marked reduction of this enzyme in the miniature variant. Furthermore, this phenomenon (refered to as xenia where only the seed is affected and metaxenia where the morphology of the whole fruit is affected) has been observed in some one-seeded fruits, such as pecan nuts, pistachio nuts, chestnuts and dates, in which fruit size is influenced by pollen donor (Sedgley and Griffin 1989). This research by Miller and Chourey (1992) offers many exciting new facets to the occurrence of the small fruit phenotype. Firstly, it brings into play the question of pollen donor viability. The potential role of pollination in the occurrence of the small fruit phenotype is a theory of long standing. The avocado exhibits dianthesis and synchronous dichogamy when flowering (Davenport 1986). Thus it appears to outcross, and self-pollination is normally only permitted when the weather is cool enough to allow an overlap in flower opening. It is known that cross-pollination increases the yield in some

cultivars, including 'Hass' (Bergh 1975b; Davenport 1986), reduces the rate of fruit abscission (Degani *et al.* 1989) and increases embryo size and sink strength. There is, however, substantial evidence to suggest pollination is not the limiting factor in fruit set and size in avocado (Clark 1923; Cintron 1947; Degani and Gazit 1984). However, if pollen compatibility/viability was to cause structural instability in the maternal cells of the pachychalazal region and, subsequently, an osmotic imbalance (i.e. a stress) and further structural instability within the endosperm and hence between the filial and maternal tissue, it is plausible that these tissues might experience a rise in ROS and ABA levels. Such a response would result in poor sink establishment, an altered enzyme profile and, subsequently, reduced fruit growth and even fruitlet abortion. This response would be exacerbated by environmental stress, such as drought stress, cold stress or excessive heat during pollination and fruit set. This proposal also emphasises the intimate link between plant stress, ABA, sugar metabolism and the developmental interactions between the endosperm and the maternal cells. It also provides a very plausible explanation for the occurrence and random distribution of the small fruit phenotype. Furthermore, the metaxenial influence is also proposed to occur via plant growth regulators produced by the embryo and endosperm (Sedgley and Griffon 1989; and the references therein). The loss of communication between the embryo and the mesocarp at this early stage of fruit development can be expected to have a profound effect on overall mesocarp physiology and morphogenesis.

Plant growth regulators such as GA and IAA, synthesised by the embryo, also play an important role in the control of the activity and *de novo* synthesis of sugar-metabolising enzymes (Broughton and McComb 1971; Gordon and Flood 1980; Schaffer *et al.* 1987b; Estruch and Beltrán 1991; Wu *et al.* 1993b; Miyamoto *et al.* 2000). Bangerth (1989) presented a hypothesis termed 'primigenic dominance', which proposes that the polar export of IAA from earlier developed sinks inhibits the IAA export of later developed sinks and so leads to the inhibition of subordinate sink/fruit growth. Taylor and Cowan (2000) postulated the ratio of CK and IAA to ABA plays a key role in the control of fruit size, and that reduced IAA oxidase and elevated ABA aldehyde oxidase activity contributes to decreased IAA and increased ABA levels in the small fruit. The effect of this would be two-fold; firstly, an environment unfavourable for cell division would be created; secondly, the polar export of IAA may be reduced. Polar IAA transport has been proposed to play a role in the differentiation of vascular tissue, direction of assimilate transport, inhibition of organ abscission and the regulation of calcium status (Bangerth 1989; and the references therein). Normally nutrient status and cell size must be allowed to reach certain critical values before the transition from G1 phase to S phase in the cell division cycle can occur (Hirt 1996). The direction of assimilate import and metabolism by hormones, such as IAA, ABA and CK, will affect sugar composition and

availability in the fruit, and so impact on cell differentiation and cell cycle activity (Webster and Henry 1987). The availability of some of these hormones is also determined by the condition of the seed coat, which is affected by endogenous ABA levels. ABA has also been shown to cause the strong transcriptional down-regulation of cdc2 gene expression (John *et al.* 1993). The cdc2 gene is normally expressed prior to cell division (Hemerly *et al.* 1993) and plays a crucial role in the cell division cycle. Artlip *et al.* (1995) observed that water deficit reduced the rate of cell division in developing maize endosperm and concluded cell division is highly responsive to water stress. Conversely, CK levels were positively correlated with the mesocarp growth rate in avocado (Blumenfeld 1970). These observations suggest that stress and elevated ABA levels, or rather, an altered ABA to CK and IAA ratio, impact directly on the cell division cycle by reducing entry into the phase of DNA replication and histone synthesis. Furthermore, seed senescence will alter communication between the seed and mesocarp, and between the seed and parent tree. This will consequently impact on sugar supply, metabolism, content and composition, and subsequently cell division, hormone and sugar homeostasis, and ultimately fruit growth.

Avocado fruit has very high rates of respiration. When comparing avocado cultivars of different size, Blanke and Whiley (1995) proposed that 'Hass' was smaller than 'Fuerte' due to the higher respiration rates and hence greater energy demands during fruit growth and development. The current investigation showed that the small fruit variant had much higher respiratory rates, and that ABA treatment resulted in increased respiration rates. During the climacteric of avocado, the respiration rates, as well as the ability to produce ABA, increase (Adato and Gazit 1976). Fruit respiration is closely related to fruit size in persimmon and is important for maintaining sink strength (Nakano *et al.* 1998). Many processes controlling assimilate import, such as phloem unloading, sugar uptake by sink cells, and compartmentation of sugars within cells require respiratory energy, and a close relationship between dry mass accumulation and respiration rates has been observed in several fruit (Walker and Thornely 1977; Hole and Barnes 1980; De Jong and Goudriaan 1989). The high respiration rate in the small fruit may contribute to its reduced size, or be a result of assimilates being allocated to respiration instead of processes associated with growth and development. In addition, the high respiration rates in the small fruit may be indicative of earlier fruit maturation/climacteric, a phenomenon, not unlike the premature senescence of the seed coat, that can be induced by ABA application.

Also associated with avocado maturation is the accumulation of starch and oils in the seed and mesocarp, respectively. Normal and iP-treated fruit accumulated more starch, predominantly in the seed. On the other hand, small and ABA-treated fruit had lower starch levels in the seed. Small

and ABA-treated fruit were also characterised by elevated α-amylase activity. It has been reported that in maize, feeding of substrate quantities of Suc to the stems of excised parent plants, resulted in the persistence of starch and a reduction in the arrest of embryo development (Zinselmeier *et al.* 1999). This suggests that interruption of carbohydrate supply during development may induce starch degradation to ensure the continued supply of carbohydrates to sustain metabolism. In the event of insufficient sugar supply to sustain embryo growth, early embryo abortion may occur. It is thus proposed that in the small fruit senescence of the seed coat together with elevated respiration rates results in reduced assimilate supply to the seed and necessitates the hydrolysis of starch by α-amylase to sustain embryonic development/respiration. The same suggestion may be applicable to ABA-treated fruit. Ironically, the inhibition of ABA biosynthesis by fluoridone has been shown to increase α-amylase activity (Pagano *et al.* 1997). Similarly, treatment with (+)-trifluoro-ABA, an ABA analog, has been shown to inhibit the formation of α-amylase (Kashem *et al.* 1998). However, these observations were made in the presence of adequate carbohydrate substrates and it is assumed that the demand for carbon needed for respiration overrides the inhibition of α-amylase by ABA in small and ABA-treated fruit.

In avocado, although total lipid content shows little difference in the early stages of fruit growth, a distinct pattern emerges with respect to fatty acid composition in small and ABA-treated fruit. Both small and ABA-treated fruit showed an increase/decrease in the same fatty acids, namely, an increase in palmitic and petroselinic acid in the seed and oleic acid in the mesocarp, while linoleic and γ-linolenic acids decreased in the seed. The changes in seed fatty acid content associated with small and ABA-treated fruit mimic the changes seen in the fatty acid profile of normal fruit during development. These observations suggest that small and ABA-treated fruit may be undergoing premature maturation and, although they are chronologically the same age as the control fruit, they may be developmentally more mature.

The metabolism of carbohydrates, sugar sensing and hormone homeostasis within the fruit are thus intrinsically linked. The small fruit phenotype displays distinct characteristics with respect to sugar metabolism and carbon content and storage. These characteristics appear to be inducible by treatment with ABA and thus it is suggested that ABA plays a crucial role in the metabolic control of developing 'Hass' fruit.

7.1.2.4 An integrated model for the metabolic control of 'Hass' fruit growth

What can be considered as the 'normal' pattern (associated with the normal fruit phenotype) of carbon metabolism, seed coat senescence and fruit growth has been established by the current research. The small fruit phenotype is believed to arise as a consequence of some form of stress and displays a distinct pattern of early seed coat senescence, altered carbohydrate metabolism and transport, and apparently premature maturation. These characteristics can be induced by ABA treatment, both *in vivo* and *in vitro*. ABA is considered a 'stress-hormone' and many of the characteristics observed in both the small and ABA-treated fruit have been associated with various plant stress responses. Presented in Figure 7.6 is a model integrating the stimulus and metabolic responses believed to be associated with the induction and manifestation of the 'Hass' small fruit phenotype.

In this model, fruit size is determined by cell number and hence by the extent and duration of cell division. The small fruit is believed to undergo premature maturation and, as a consequence, the period of cell division is very much reduced. The precise stimulus for the induction of the small fruit phenotype still needs to be elucidated, but it is proposed to be tissue stress, as a consequence of reduced placento-chalazal stability associated with pollen donor and/or environmental stress, especially water, cold or heat stress, during the period of pollination and early fruit set when the tissue is most sensitive. Such a stress is proposed to induce the synthesis of ROS and ABA. Elevated ROS and ABA may result in HR, PCD, the synthesis, accumulation, and deposition of polyphenolics, and a reduction in 7C sugars. Polyphenolic accumulation and PCD within the seed coat results in the reduction of communication between the seed and the mesocarp. In addition, membrane perturbation and changes in Ca^{2+} associated with PCD will result in increased association of callose with the Pd and a reduction of symplastic continuity. Accompanying this will be a reduction in the movement of promotive growth factors (such as IAA and CK) synthesised in the seed, into the mesocarp, and consequently a reduction in cell division and growth. Associated with the alteration in 7C sugar levels and profile may be the loss of the so-called 'juvenility factor' and hence the induction of premature maturation. Premature maturation can be expected to be associated with the reduction of secondary Pd formation and hence a further reduction in symplastic continuity. Isolation of the seed and seed coat from phloem-derived carbon as a consequence of PCD (i.e. premature senescence) of the seed coat, and callose deposition within the Pd, will result in the establishment of a pattern of carbon metabolism similar to that of tissues subjected to stress and carbon starvation. Consequently, hexose levels would increase and the genes regulated by sugar levels will be differentially expressed. Through the action of HXK

and SnRK1 the activity of key enzymes such as HMGR, AO, XDH, SSy and AI could in turn be altered and thus sugar-regulated gene expression would further impact on sugar metabolism, hormone homeostasis, PCD, solute movement, cell division and fruit maturation. Of particular importance in this model would be a loss of SSy (Clv) activity and increased apoplastic Suc cleavage in the seed coat. The consequence of this entangled, spiralling chain of events will be a reduction in fruit growth, premature seed coat senescence and fruit maturation as manifested by the small fruit phenotype.

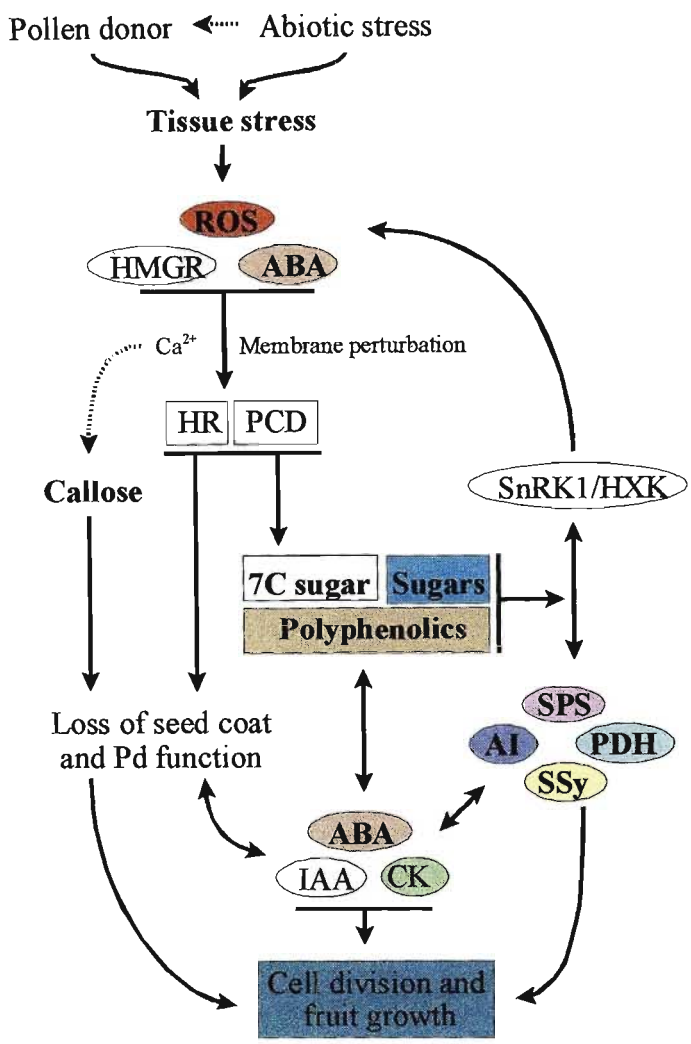


Figure 7.6 Hypothetical scheme illustrating the relationship between ABA, sugar transport, metabolism and sensing, and the appearance of the small fruit phenotype.

7.1.3 PRACTICAL IMPLICATIONS OF THIS RESEARCH

The small fruit problem is physiological and occurs without pathogen involvement (Blanke and Bower 1991). It is, however, aggravated by poor cultural techniques and becomes more pronounced with tree age (Cutting 1993). This problem is also more prevalent in orchards situated in drier/warmer climates (Hilton-Barber 1992; Whiley and Schaffer 1994). Studies by Moore-Gordon and Wolstenholme (1996) revealed that mulching reduced the incidence of premature seed coat senescence and pedicel ring-neck, both characteristics associated with plant water stress and the small fruit phenotype. These observations, the results from the present investigation, and the scheme illustrated in Figure 7.6, suggest plant stress plays a key role in the occurrence of the small fruit phenotype, and confirm the proposal by Moore-Gordon (1997) that the small fruit phenotype is associated with plant stress. Whilst logistics and current technology limit the practical applications of the current research, the work presented here facilitates the understanding of the small fruit problem and lays a foundation for further research that can be more practically applied. However, it does emphasise the importance of good orchard management practices, such as careful irrigation, and the use of pine bark (Moore-Gordon and Wolstenholme 1996; Moore-Gordon *et al.* 1996; 1997) and sugarcane filter-press mulches (van Niekerk *et al.* 1999) to alleviate/minimize water stress during crucial periods of fruit set and growth.

Robbertse and co-workers (1996) have investigated 'Hass' yield and fruit size as influenced by the pollen donor. Their study suggests 'Ettinger' pollen outperformed 'Hass' pollen in terms of germination and pollen tube growth. Pollination with 'Ettinger' pollen resulted in increased 'Hass' fruit and seed size. It is therefore possible that the interplanting of 'Hass' orchards with high quality pollen donors such as 'Ettinger' may alleviate the small fruit problem. The question of pollen source/compatibility/viability also needs to be addressed to ascertain the significance of the contribution of pollen donor to the occurrence of the small fruit phenotype.

Ultimately fruit size is fundamentally determined by genome. Thus the most logical long term approach to address the 'Hass' small fruit problem is to breed or select new high yielding, larger fruit cultivars that are less susceptible to both abiotic and biotic stress, especially water or cold stress. Progress is being made in this field with a breeding programme being undertaken by the Institute for Tropical and Subtropical fruits (Bijzet *et al.* 1996). In addition, testing of 'Hass'-like cultivars, such as 'Lamb Hass', 'Iriet' and 'Gil' has revealed some promising results (Kremer-Köhne 1999; 2000). In the interim, it may be beneficial to find new/improved ways to manage

orchards, especially during the crucial period of fruit set when the small fruit is believed to be induced.

7.2 CONCLUSIONS AND FUTURE PROSPECTS

The limited literature available on solute transport and metabolism in avocado has necessitated a lot of speculation, based both on the findings of the current investigation and published research in other plant species. However, this study has, in part, elucidated the metabolism of Suc in developing 'Hass' fruit. In so doing, the role of stress, ABA and potentially ROS has been linked to the induction and expression of the small fruit phenotype. Furthermore, efforts have been made to address the potential role of 7C sugars in the supply of carbon for metabolism, growth and storage in developing avocado fruit, and the contribution of these sugars to fruit maturation and the expression of the small fruit phenotype.

Of equal importance is the fact that this research has highlighted the need for further investigations into the role of ROS in the induction of the small fruit phenotype during early fruit set, the mechanism of Pd gating and in particular the role of callose as a 'gate-keeper' in these dynamic intercellular channels, and finally the pathways of synthesis and catabolism of *manno*-heptulose and perseitol and the role of these abundant 7C sugars in avocado physiology. In addition, this study has emphasised the need to find a practical method to reduce plant stress, or the synthesis of ROS/ABA during fruit set, and thereby to minimize the occurrence of the small fruit phenotype.

REFERENCES

Abe T, Kudo M, Oka Y, Yamaguchi J, Sasahara T (1996) Changes in α-amylase activity during plant regeneration from rice calli. J Plant Physiol 149: 592-598

Ackerson RC (1985) Invertase activity and abscisic acid in relation to carbohydrate status in developing soybean reproductive structures. Crop Sci 25: 615-618

Adato I, Gazit S (1974) Postharvest responses of avocado fruits of different maturity to delayed ethylene treatments. Plant Physiol 53: 899-902

Adato I, Gazit S (1976) Response of harvested avocado fruits to supply of indole-3-acetic acid, gibberellic acid and abscisic acid. J Agric Food Chem 24: 1165-1167

Ahmed EM, Barmore CR (1980) Avocado. *In*: Tropical and subtropical fruits: composition, properties and uses (Eds. Nagy S, Shaw PE). AVI Publishing, Westport. Pp 121-156. ISBN 0-87055-350-X

Aloni B, Wyse R, Griffith S (1986) Sucrose transport in stems of *Vicia faba*. Possible involvement of a sucrose carrier and osmotic regulation. Plant Physiol 81: 482-487

Amor Y, Haigler CH, Johnson S, Waiscott M, Delmer DP (1995) A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. Proc Natl Acad Sci USA 92: 9353-9357

Anon. (2001) Abstract of agricultural statistics. National Department of Agriculture, Pretoria. P 46. ISBN 1-86871-091-2

Antognozzi E, Battistelli A, Famiani F, Moscatello S, Stanica F, Tombesi A (1996) Influence of CPPU on carbohydrate accumulation and metabolism in fruits of *Actinidia deliciosa* (A. Chev.). Scientia Horticulturae 65: 37-47

ap Rees T (1974) Pathways of carbohydrate breakdown in higher plants. Int Rev Sci Plant Biochem 11: 89-127

ap Rees T (1987) Compartmentation of plant metabolism. *In*: The biochemistry of plants - A comprehensive treatise. Vol 12. Physiology of metabolism (Ed. Davies DD). Academic Press, San Diego. Pp 87-115. ISBN 0-12-675412-X

ap Rees T (1988) Hexose phosphate metabolism by nonphotosynthetic tissues of higher plants. *In*: The biochemistry of plants - A comprehensive treatise. Vol 14. Carbohydrates (Ed. Preiss J). Pp 1-30. ISBN 0-12-675414-4

ap Rees T, Hill SA (1994) Metabolic control analysis of plant metabolism. Plant Cell Environ 17: 587-599

Archbold DD (1999) Carbohydrate availability modifies sorbitol dehydrogenase activity of apple fruit. Physiologia Plantarum 105: 391-395

Arpagaus S, Braendle R (2000) The significance of α-amylase under anoxia stress in tolerant rhizomes (*Acorus calamus* L.) and non-tolerant tubers (*Solanum tuberosum* L., var. Désirée). J Exp Bot 51: 1475-1477

Artlip TS, Madison JT, Setter TL (1995) Water deficit in developing endosperm of maize: Cell division and nuclear DNA endoreduplication. Plant Cell Environ 18: 1034-1040

Avequin JB (1831) J Chim Méd Pharm Toxicol 7: 464-467. *In*: La Forge (1917); Charlson and Richtmyer (1960)

Avigad G (1982) Sucrose and other disaccharides. *In*: Plant carbohydrates. I. Intracellular carbohydrates. Vol 13A (Eds. Loewus FA, Tanner W). Springer-Verlag, Berlin. Pp 216-234. ISBN 3-540-11007-0

Ayres MP, Clausen TP, MacLean SF Jr., Redman AM, Reichardt PB (1997) Diversity of structure and antiherbivore activity in condensed tannins. Ecology 78: 1696-1712

Balachandran S, Xiang Y, Schobert C, Thompson GA, Lucas WJ (1997) Phloem sap proteins from *Cucurbita maxima* and *Ricinus communis* have the capacity to traffic cell-to-cell through plasmodesmata. Proc Natl Acad Sci USA 94: 14150-14155

Balibrea ME, Parra M, Bolarín MC, Pérez-Alfocea F (1999) Cytoplasmic sucrolytic activity controls tomato fruit growth under salinity. Aust J Plant Physiol 26: 561-568

Bandu VH (1991) The production and use of tungsten-coated knives for semi-thin and ultra-thin sectioning. Electron Microscopy Soc SA 21: 13-14

Bangerth F (1989) Dominance among fruits/sinks and the search for a correlative signal. Physiologia Plantarum 76: 608-614

Barker JHA, Slocombe SP, Ball KL, Hardie DG, Shewry PR, Halford NG (1996) Evidence that barley 3-hydroxy-3-methylglutaryl-coenzyme A reductase kinase is a member of the sucrose nonfermenting-1-related protein kinase family. Plant Physiol 112: 1141-1149

Barker L, Kühn C, Weise A, Schulz A, Gebhardt C, Hirner B, Hellmann H, Schulze W, Ward JM, Frommer WB (2000) SUT2, a putative sucrose sensor in sieve elements. Plant Cell 12: 1153-1164

Barklay GF, Fensom DS (1984) Physiological evidence for the existence of pressure-sensitive valves in plasmodesmata between internodes of *Nitella*. *In*: Membrane transport in plants (Eds. Cram WJ, Janacek K, Rybova R, Sigler K). Whiley, New York. Pp 316-317. ISBN 0-471-90467-8

Bean RC (1958) Changes in sugars during growth and storage of avocados. CA Avo Soc Yrbk 42: 90-93

Beebe DW, Turgeon R (1991) Current perspectives on plasmodesmata: structure and function. Physiologia Plantarum 83: 194-199

Begbie R, Richtmyer NK (1966) The isolation of some heptoses, heptuloses, octuloses, and nonuloses from *Primula officinalis* Jacq. Carbohyd Res 2: 272-288

Bergh BO (1975a) Avocados. *In*: Advances in plant breeding (Eds. Janick J, Moore JM). Purdue University Press, West Lafayette. Pp 541-567. ISBN 0-911198-36-9

Bergh BO (1975b) Avocado research in Israel. CA Avo Soc Yrbk 58: 103-126

Bergh B, Ellstrand N (1986) Taxonomy of avocado. CA Avo Soc Yrbk 70:127-133

Bevenue A, White LM, Secor GE, Williams KT (1961) The occurrence of two heptuloses in the fig plant. J Assoc Offic Agr Chem 44: 265-266

Biale JB, Young RE (1971) The avocado pear. *In*: The biochemistry of fruits and their products (Ed. Hulme AC). Academic Press, London. Pp 1-63. ISBN 12-361202-0

Bieleski RL (1982) Sugar alcohols. *In*: Encyclopedia of plant physiology - New series. Vol. 13A (Eds. Loewus F, Tanner W). Springer-Verlag, Berlin. Pp 158-192. ISBN 3-540-11060-7

Bieleski RL (2000) The bigger picture – phloem seen through horticultural eyes. Aust J Plant Physiol 27: 615-624

Bijzet Z, Cilliers B, Sippel AD, Snijder B (1996) Avocado cultivar breeding – results and prospects. SA Avo Growers' Assoc Yrbk 19: 11-12

Blackman LM, Overall RL (1998) Immunolocalization of the cytoskeleton to plasmodesmata of *Chara corallina*. Plant J 14: 733-741

Blackman LM, Harper JDI, Overall RL (1999) Localisation of a centrin-like protein to higher plant plasmodesmata. Eur J Cell Biol 78: 297-304

Blanke MM, Bower JP (1991) Small fruit problem in citrus trees. Trees 5: 239-243

Blanke MM, Whiley AW (1995) Bioenergetics, respiration costs and water relations in developing avocado fruit. J Plant Physiol 145: 87-92

Blumenfeld A (1970) Hormonal systems in avocado fruits. PhD. Thesis, The Hebrew University of Jerusalem, Israel.

Blumenfeld A, Gazit S (1971) The role of the seed coat in avocado fruit development. CA Avo Soc Yrbk 54: 100-104

Blumenfeld A, Gazit S (1974) Development of seeded and seedless avocado fruits. J Amer Soc HortSci 99: 442-448

Board MA, Colquhoun A, Newsholme EA (1995) High K_m glucose-phosphorylating (glucokinase) activities in a range of tumor cell lines and inhibition rates of tumor growth by the specific enzyme inhibitor mannoheptulose. Cancer Res 55: 3278-3285

Bohner J, Bangerth F (1988) Cell numbers, cell size and hormone levels in semi-isogenic mutants of *Lycopersicon pimpinellifolium* differing in fruit size. Physiologia Plantarum 72: 316-320

Bonetta D, McCourt P (1998) Genetic analysis of ABA signal transduction pathways. Trends in Plant Sci 3: 231-235

Borkovec V, Procházka S (1992) Pre-anthesis interaction of cytokinins and ABA in the transport of ^{14}C-sucrose to the ear of winter wheat (*Triticum aestivum* L.). J Agronomy Crop Sci 169:13-19

Botha CEJ, Cross RHM (2000) Towards reconciliation of structure with function in plasmodesmata – who is the gatekeeper? Micron 31: 713-721

Botha CEJ, Cowan AK, Cross RHM, Moore-Gordon C, Bertling I (1996) Plasmodesmatal structure and cell-to-cell communication in avocado fruit. Micro Soc SA – Proceedings 26: 53

Boudet A-M (1998) A new view of lignification. Trends in Plant Sci 3: 67-71

Bradford MM (1976) A rapid and sensitive method for the quantitative determination of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

Brander U, Wattendorff J (1987) Staining callose for electron microscopy. Experimental conditions. La Cellule 74: 43-55

Bravo LA, Zuñiga GE, Alberdi M, Corcuera LJ (1998) The role of ABA in freezing tolerance and cold acclimation in barley. Physiologia Plantarum 103: 17-23

Brenner ML, Cheikh N (1995) The role of hormones in photosynthate partitioning and seed filling. *In*: Plant hormones: Physiology, biochemistry and molecular biology (Ed. Davies PJ). Kluwer Academic Publishers, Dordrecht. Pp 649-670. ISBN 0-7923-2984-8

Broughton WJ, McComb AJ (1971) Changes in the pattern of enzyme development in gibberellin-treated pea internodes. Ann Bot 35: 213-228

Brundrett MC, Enstone DE, Peterson CA (1988) A berberine-aniline blue fluorescent staining procedure for suberin, lignin, and callose in plant tissue. Protoplasma 146: 133-142

Bucheli P, Dürr M, Buchala AJ, Meier H (1985) β-glucanases in developing cotton (*Gossypium hirsutum* L.) fibres. Planta 166: 530-536

Bucheli P, Buchala AJ, Meier H (1987) Autolysis *in vitro* of cotton (*Gossypium hirsutum* L.) fibre cell walls. Physiologia Plantarum 70: 633-638

Bucke C, Oliver IR (1975) Location of enzymes metabolizing sucrose and starch in the grasses *Pennisetum purpureum* and *Muhlenbergia montana*. Planta 122: 45-52

Bush DS, Biswas AK, Jones RL (1993) Hormonal regulation of Ca^{2+} transport in the endomembrane system of the barley aleurone. Planta 189: 507-515

Bussotti F, Gravano E, Grossoni P, Tani C (1998) Occurrence of tannins in leaves of beech trees (*Fagus sylvatica*) along an ecological gradient, detected by histochemical and ultrastructural analyses. New Phytol 138: 469-479

Buysse J, Merckx R (1993) An improved colorimetric method to quantify sugar content of plant tissue. J Exp Bot 44: 1627-1629

Campbell T, Richings EW, Cripps RF, Taylor NJ, Cowan AK (2000) The plant SnRK1 complex: Sugar sensing and cell metabolism in avocado fruit growth. S Afr J Bot 66: 104-111

Cano-Medrano R, Darnell RL (1997) Sucrose metabolism and fruit growth in parthenocarpic vs. seeded blueberry (*Vaccinium ashei*) fruits. Physiologia Plantarum 99: 439-446

Cao G, Sofic E, Prior LR (1997) Antioxidant and pro-oxidant behaviour of flavonoids: Structure-activity relationships. Free Radicals Biol Med 22: 749-760

Carlson SJ, Chourey PS (1996) Evidence for plasma-membrane associated forms of sucrose synthase in maize. Mol Gen Genet 252: 303-310

Carpita N, Delmer D (1981) Concentration and metabolic turnover of UDP-glucose in developing cotton fibres. J Biol Chem 256: 308-315

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: Consistency of molecular structure with the physical properties of the walls during growth. Plant J 3: 1-30

Chandler WH (1958) Evergreen Orchards – 2nd Ed. Henry Kimpton, London. Pp 205-228. ISBN none

Chandler PM, Robertson M (1994) Gene expression regulated by abscisic acid and its relation to stress tolerance. Annu Rev Plant Physiol Plant Mol Biol 45: 113-141

Charlson AJ, Richtmyer NK (1960) The isolation of an octulose and an octitol from natural sources: D-*glycero*-D-*manno*-octulose and D-*erythro*-D-*galacto*-octitol from the avocado and D-*glycero*-D-*manno*-octulose from *Sedum* species. J Am Chem Soc 82: 3428-3434

Chen YC, Chourey PS (1989) Spatial and temporal expression of the two SuSy genes in maize: Immunohistological evidence. Theor Appl Genet 78: 553-559

Chen JQ, Black CC (1992) Biochemical and immunological properties of alkaline invertase from sprouting soybean hypocotyls. Arch Biochem Biophys 295: 61-69

Chen HH, Li PH, Brenner ML (1983) Involvement of abscisic acid in potato cold acclimation. Plant Physiol 71-75

Cheng GW, Breen PJ (1991) Activity of phenylalanine ammonia-lyase (PAL) and concentrations of anthocyanins and phenolics in developing strawberry fruit. J Amer Soc Hort Sci 116: 865-869

Cheng W-H, Taliercio EW, Chourey PS (1996) The *Miniature1* seed locus of maize encodes a cell wall invertase required for normal development of endosperm and maternal cells in the pedicel. Plant Cell 8: 971-983

Cheng W-H, Taliercio EW, Chourey PS (1999) Sugars modulate an unusual mode of control of the cell-wall invertase gene (*Incw*1) through its 3′ untranslated region in a cell suspension culture of maize. Proc Natl Acad Sci 96: 10512-10517

Chengappa S, Loader N, Shields R (1998) Cloning, expression and mapping of a second tomato (*Lycopersicon esculentum*) sucrose synthase gene, sus3 (accession nos AJ011319, AJ011543)(PGR 98-190). Plant Physiol 118: 1533

Chengappa S, Guilleroux M, Phillips W, Shields R (1999) Transgenic tomato plants with decreased sucrose synthase are unaltered in starch and sugar accumulation in the fruit. Plant Mol Biol 40: 213-221

Chiou T-J, Bush DR (1998) Sucrose is a signal molecule in assimilate partitioning. Plant Biol 95: 4784-4788

Chin CK, Weston GD (1973) Distribution in excised *Lycopersicon esculentum* roots of the principle enzymes involved in sucrose metabolism. Phytochemistry 12: 1229-1235

Choi Y-H, Kobayashi M, Sakurai A (1996) Endogenous gibberellin A_1 level and α-amylase activity in germinating rice seeds. J Plant Growth Regul 15: 147-151

Chopra J, Kaur N, Gupta AK (2000) Ontogenic changes in enzymes of carbon metabolism in relation to carbohydrate status in developing mungbean reproductive structures. Phytochemistry 53: 539-548

Chourey PS, Nelson O (1976) The enzymatic deficiency conditioned by the shrunken 1 mutations in maize. Biochem Genet 14: 1041-1055

Chourey PS, Taliercio EW, Kane EJ (1991) Tissue specific expression and anaerobically induced post-transcriptional modulation of sucrose synthase genes in *Sorghum bicolor* M. Plant Physiol 96: 485-490

Chourey PS, Taliercio EW, Carlson SJ, Ruan YL (1998) Genetic evidence that the two isozymes of sucrose synthase present in developing maize endosperm are critical, one for cell wall integrity and the other for starch biosynthesis. Mol Gen Genet 259: 88-96

Chraibi A, Palms B, Druart N, Goupil P, Gojon A, Rambour S (1995) Influence of ABA on nitrogen partitioning, sucrose metabolism and nitrate reductase activity of chicory suspension cells. J Exp Bot 46: 1525-1533

Cintron RH (1947) Fruit setting and fruit development of avocados in Texas. CA Avo Soc Yrbk 31: 62-66

Clark OI (1923) Avocado pollination and bees. CA Avo Soc Yrbk 7: 57-62

Clark G (1981) Staining procedures - 4th Ed. Williams and Wilkens, Baltimore. Pp 189-190, 323-324. ISBN 0-683-01707-1

Clark AF, Villemez CL (1972) The formation of β-1,4-glucan from UDP-α-D-glucose catalysed by *Phaseolus aureus* enzyme. Plant Physiol 50: 371-374

Claussen W, Loveys, BR, Hawker JS (1985) Comparative investigations on the distribution of sucrose synthase activity within growing, maturing and old leaves of some C_3 and C_4 plant species. Physiologia Plantarum 65: 275-280

Cleland RE, Fujiwara T, Lucas WJ (1994) Plasmodesmal mediated cell to cell transport in wheat roots is modulated by anaerobic stress. Protoplasma 178: 81-85

Clifford PE, Ross GS, McWha JA (1990) Why are the effects of abscisic acid on photosynthate unloading so variable? A possible answer from a source-limited experimental system. J Plant Physiol 137: 151-154

Cohen A (1984) Citrus fruit enlargement by means of summer girdling. J Hort Sci 59: 119-125

Coombe BG (1976) The development of fleshy fruit. Annu Rev Plant Physiol 27: 507-528

Copeland L (1990) Enzymes of sucrose metabolism. Methods Plant Biochem 3: 73-85

Copeland L, Tanner GJ (1988) Hexose kinase of avocado. Physiologia Plantarum 74: 531-536

Cordenunsi BR, Lajolo FM (1995) Starch breakdown during banana ripening: Sucrose synthase and sucrose phosphate synthase. J Agric Food Chem 43: 347-351

Cowan AK (1997) Why are small Hass fruit small? SA Avo Growers' Assoc Yrbk 20: 52-54

Cowan AK, Railton ID (1987) Cytokinins and ancymidol inhibit abscisic acid biosynthesis in *Persea gratissma*. J Plant Physiol 130: 273-277

Cowan AK, Moore-Gordon CS, Bertling I, Wolstenholme BN (1997a) Metabolic control of avocado fruit growth: isoprenoid growth regulators and the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Physiol 114: 511-518

Cowan AK, Richardson GR, Maurel JCG (1997b) Stress-induced abscisic acid transients and stimulus-response-coupling. Physiologia Plantarum 100: 491-499

Cowan AK, Richings EW, Cripps RF, Cairns ALP (1998) Metabolic control of Hass avocado fruit growth. SA Avo Growers' Assoc Yrbk 21: 48-51

Cowan AK, Cairns ACP, Bartels-Rahm B (1999) Regulation of abscisic acid metabolism: Towards a metabolic basis for abscisic acid-cytokinin antagonism. J Exp Bot 50: 595-603

Cowan AK, Cripps RF, Richings EW, Taylor NJ (2001) Fruit size: towards an understanding of the metabolic control of fruit growth using avocado as a model system. Physiologia Plantarum 111: 127-136

Crawford KM, Zambryski PC (1999) Plasmodesmata signaling: Many roles, sophisticated statutes. Current Opinions Plant Biol 2: 382-387

Cripps RF, Cowan AK (2000) Sucrose movement and metabolism in small Hass avocado fruit. SA Avo Growers' Assoc Yrbk 23: 63-69

Cripps RF, Richings EW, Taylor NJ, Cowan AK (1999) The Hass small fruit syndrome: solving a 50 million rand per season problem. SA Avo Growers' Assoc Yrbk 22: 1-6

Cummings K, Schroeder CA (1942) Anatomy of the avocado fruit. CA Avo Soc Yrbk 67: 56-64

Cutting JGM (1993) The cytokinin complex related to small fruit in 'Hass' avocados. Acta Hort 329: 147-149

Dafert FW, Miklauz R (1912) Untersuchungen über die kohleähnliche Masse der Kompositen (Chemischer Teil). Deutsch Akad Wiss Wien Math Naturwiss KI 78: 143-152. [Title translation: Investigations into the 'carbohydrate-like' mass of Compositae (Chemical post)]. *In*: Wittich and Graven (1995)

Daie J (1985) Carbohydrate partitioning and metabolism in crops. HortReviews 7: 69-103

Dalessandro G, Roberts L (1971) Induction of xylogenesis in pith parenchyma explants of *Lactuca*. Am J Bot 58: 378-385

Dali N, Michaud D, Yelle S (1992) Evidence for the involvement of sucrose phosphate synthase in the pathway of sucrose accumulation in sucrose accumulating tomato fruits. Plant Physiol 99: 434-438

Damon S, Hewitt J, Nieder M, Bennett AB (1988) Sink metabolism in tomato fruit. II. Phloem unloading and sugar uptake. Plant Physiol 87: 731-736

Davenport TL (1986) Avocado flowering. Hort Reviews 8: 257-289

Davenport JB, Ellis SC (1959) Chemical changes during growth and storage of the avocado fruit. Aust J Biol Sci 12: 445-454

Davenport TL, Manners MM (1982) Nucellar senescence and ethylene production as they relate to avocado fruitlet abscission. J Exp Bot 33: 815-825

D'Aoust M-A, Yelle S, Nguyen-Quoc B (1999) Antisense inhibition of tomato fruit sucrose synthase decreases fruit setting and the sucrose unloading capacity of young fruit. Plant Cell 11: 2407-2418

Dearnaley JDW, Levina NN, Lew RR, Heath IB, Goring DR (1997) Interrelationships between cytoplasmic Ca^{2+} peaks, pollen hydration and plasma membrane conductances during compatible and incompatible pollinations of *Brassica napus* papillae. Plant Cell Physiol 38: 985-999

Degani C, Gazit S (1984) Selfed and crossed proportions of avocado progenies produced by caged pairs of complementary cultivars. HortSci 19: 258-260

Degani C, Goldring A, Gazit S (1989) Pollen parent effect on outcrossing rate in 'Hass' and 'Fuerte' avocado plots during fruit development. J Amer Soc Hort Sci 114: 106-111

Déjardin A, Rochat C, Maugenest S, Boutin J-P (1997) Purification, characterization and physiological role of sucrose synthase in the pea seed coat (*Pisum sativum* L.). Planta 201: 128-137

Déjardin A, Sokolov LN, Kleczkowski LA (1999) Sugar/osmoticum levels modulate differential abscisic acid-independent expression of two stress-responsive sucrose synthase genes in *Arabidopsis*. Biochem J 344: 503-509

De Jong TM, Goudriaan J (1989) Modeling peach fruit growth and carbohydrate requirements: Reevaluation of the double-sigmoid growth pattern. J Amer Soc Hort Sci 114: 800-804

De Jong A, Koerselman-Kooij JW, Schuurmans JAMJ, Borstlap AC (1996) Characterization of the uptake of sucrose and glucose by isolated seed coat halves of developing pea seeds. Evidence that a sugar facilitator with diffusional kinetics is involved in seed coat unloading. Planta 199: 486-492

Delmer DP, Cooper G, Alexander D, Cooper J, Hayashi T, Nitsche C, Thelen M (1985) New approaches to the study of cellulose biosynthesis. J Cell Sci 2: 33-50

Delmer DP, Volokita M, Solomon M, Fritz U, Delphendahl W, Herth W (1993) A monoclonal antibody recognises a 65 kDa higher plant membrane polypeptide which undergoes cation-dependent association with callose synthase in vitro and co-localizes with sites of high callose deposition in vivo. Protoplasma 176: 33-42

Demnitz-King A, Ho LC, Baker DA (1997) Activity of sucrose hydrolysing enzymes and sugar accumulation during tomato fruit development. Plant Growth Regul 22: 193-201
Dey PM (1990) Oligosaccharides. *In*: Methods in plant biochemistry. Vol 2. Carbohydrates (Eds. Dey PM, Harborne JB). Academic Press, London. Pp 195-218. ISBN 0-12-799214-6
De Wald DB, Sadka A, Mullet JE (1994) Sucrose modulation of soybean vsp gene expression is inhibited by auxin. Plant Physiol 104: 439-441
DeWitt ND, Sussman MR (1995) Immunocytological localization of an epitope-tagged plasma membrane proton pump (H^+-ATPase) in phloem companion cells. Plant Cell 7: 2053-2067
Dickinson CD, Altabella T, Chrispeels MJ (1991) Slow-growth phenotype of transgenic tomato expressing apoplastic invertase. Plant Physiol 95: 420-425
Dickinson JR, Cole D, Halford NG (1999) A cell cycle role for plant sucrose nonfermenting-1-related protein kinase (SnRK1) is indicated by expression in yeast. Plant Growth Regul 28: 169-174
Dijkwel PP, Huijser C, Weisbeek PJ, Chua H-N, Smeekens SCM (1997) Sucrose control of phytochrome A signalling in *Arabidopsis.* Plant Cell 9: 583-595
Ding B (1998) Intercellular protein trafficking through plasmodesmata. Plant Mol Biol 38: 279-310
Ding B, Haudenshield JS, Willmitzer L, Lucas WJ (1993) Correlation between arrested secondary plasmodesmata development and onset of accelerated leaf senescence in yeast acid invertase transgenic tobacco plants. Plant J 4: 179-189
Ding B, Kwon MO, Warnberg L (1996) Evidence that actin filaments are involved in controlling the permeability of plasmodesmata in tobacco mesophyll. Plant J 10: 157-164
Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7: 1085-1097
Doehlert DC, Felker FC (1987) Characterization and distribution of invertase activity in developing maize (*Zea mays*) kernals. Physiologia Plantarum 70: 51-57
do Nascimento JRO, Cordenunsi BR, Lajolo FM (2000) Sucrose synthase activity and expression during development and ripening in bananas. J Plant Physiol 156: 605-611
Dörffling K, Shulenburg S, Lesselich G, Dörffling H (1990) Abscisic acid and proline levels in cold hardened winter wheat leaves in relation to variety-specific differences in freezing resistance. J Agron Crop Sci 165: 230-239
Eaks I, Sinclair WB (1978) Pectin and related constituents in avocado fruit ontogeny. J Amer Soc Hort Sci 103: 846-849
Echeverria E (1995) Intracellular localization of sucrose-phosphate phosphatase in storage cells. Physiologia Plantarum 95: 559-562
Echeverria E, Gonzalez PC, Brune A (1997a) Characterization of proton and sugar transport at the tonoplast of sweet lime (*Citrus limmetioides*) juice cells. Physiologia Plantarum 101: 291-300
Echeverria E, Salvucci ME, Gonzalez P, Paris G, Salerno G (1997b) Physical and kinetic evidence for an association between sucrose-phosphate synthase and sucrose-phosphate phosphatase. Plant Physiol 115: 223-227
Edwards J, ap Rees T (1986) Sucrose partitioning in developing embryos of round and wrinkled varieties of *Pisum sativum*. Phytochemistry 25: 2027-2032
Ehlers K, Kollmann R (2000) Synchronization of mitotic activity in protoplast-derived *Solanum nigrum* L. microcalluses is correlated with plasmodesmal connectivity. Planta 210: 269-278
Ehlers K, Binding H, Kollmann R (1999) The formation of symplastic domains by plugging of plasmodesmata – a general event in plant morphogenesis. Protoplasma 209: 181-192
Ehness R, Roitsch T (1997) Co-ordinated induction of mRNAs for extracellular invertase and a glucose transporter in *Chenopodium rubrum* by cytokinins. Plant J 11: 539-548
Elliott E (1993) A study of proteinases of invasive cells using cryoultramicrotomy and immunogold labelling. PhD. Thesis, University of Natal, South Africa. Pp 50-51
Elstner EF, Oßwald W (1994) Mechanisms of oxygen activation during plant stress. Proc Royal Soc Edinburgh B Biol 102: 131-154

Enkerli K, Hahn MG, Mims CW (1997) Immunogold localisation of callose and other plant cell wall components in soybean roots infected with the oomycete *Phytophthora sojae*. Can J Bot 75: 1509-1517

Epel BL, van Lent JWM, Cohen L, Kotlizky G, Katz A, Yahalom A (1996) A 41 kDa protein isolated from maize mesocotyl cell walls immunolocalizes to plasmodesmata. Protoplasma 191: 70-78

Esau K (1977) The anatomy of seed plants – 2nd Ed. John Wiley and Sons, New York. Pp 157-171. ISBN 0-471-24520-8

Eschrich W (1980) Free space invertase, its possible role in phloem unloading. Ber Dtsch Bot Ges 93: 363-378

Estruch JJ, Beltrán JP (1991) Changes in invertase activity precede ovary growth induced by gibberellic acid in *Pisum sativum*. Physiologia Plantarum 81: 319-326

Evans NA, Hoyne PA, Stone BA (1984) Characteristics and specificity of the interaction of a fluorochrome from aniline blue (Sirofluor) with polysaccharides. Carb Polym 4: 215-230

Famiani F, Walker RP, Técsi L, Chen Z-H, Proietti P, Leegood RC (2000) An immunohistochemical study of the compartmentation of metabolism during the development of grape (*Vitis vinifera* L.) berries. J Exp Bot 51: 675-683

Feingold DS, Avigad G (1980) Sugar nucleotide transformations in plants. *In*: The biochemistry of plants – A comprehensive treatise. Vol. 3. Carbohydrates: structure and function (Ed. Preiss J). Academic Press, New York. Pp 101-170. ISBN 0-12-675403-1

Felker FC, Shannon JC (1980) Movement of 14C-labeled assimilates into kernels of *Zea mays* L. III. An anatomical examination and microautoradioraphic study of assimilate transfer. Plant Physiol 65: 864-870

Fillion L, Ageorges A, Picaud S, Coutos-Thévenot P, Lemoine R, Romieu C, Delrot S (1999) Cloning and expression of a hexose transporter gene expressed during the ripening of grape berry. Plant Physiol 120: 1083-1093

Finazzo SF, Davenport TL (1987) Source-sink relations during avocado development. Proc Plant Growth Regul Soc Amer 14: 233-235

Fisher DB, Oparka KJ (1996) Post-phloem transport: Principles and problems. J Exp Bot 47 (special ed.): 1141-1154

Fisher DB, Wang N (1993) A kinetic and microautoradiographic analysis of [^{14}C] sucrose import by developing wheat grains. Plant Physiol 101: 391-398

Fisher DB, Wang N (1995) Sucrose concentration gradients along the post-phloem transport pathway in the maternal tissues of developing wheat grains. Plant Physiol 109: 587-592

Fisher DB, Wu Y, Ku MSB (1992) Turnover of soluble proteins in the wheat sieve tube. Plant Physiol 100: 1433-1441

Flora LL, Madore MA (1993) Stachyose and mannitol transport in olive (*Olea europaea* L.). Planta 189: 484-490

Foyer C (1997) Oxygen metabolism and electron transport in photosynthesis. *In*: Oxidative stress and the molecular biology of antioxidant defence (Ed. Scandalios JG). Cold Spring Harbour Laboratory Press, New York. Pp 687-721. ISBN 0-87969-502-1

Francey Y, Jaquet JP, Cairoli S, Buchala AJ, Meier H (1989) The biosynthesis of β-glucans in cotton (*Gossypium hirsutum* L.) fibres of ovules cultured in vitro. J Plant Physiol 134: 485-491

Fraser PD, Hedden P, Cooke DT, Bird CR, Schuch W, Bramley PM (1995) The effect of reduced activity of phytoene synthase on isoprenoid levels in tomato pericarp during fruit development and ripening. Planta 196: 321-326

Frick J, Nielson SS, Mitchell CA (1994) Yield and seed oil content response of dwarf, rapid-cycling Brassica to nitrogen treatments, planting density, and carbon dioxide enrichment. J Amer Soc Hort Sci 119: 1137-1143

Fridovich I (1984) Overview: Biological sources of $O_2^{\cdot-}$. Methods Enzymol 105: 59-61

Frommer WB, Hirner B, Kühn C, Harms K, Martin T, Riesmeier JW, Schulz B (1996) Sugar transport in higher plants. *In*: Membranes: Specialized functions in plants (Eds. Smallwood M, Knox JP, Bowles DJ). Bios Scientific Publishers, Oxford. Pp 319-335. ISBN 1-85996-200-9

Fu H, Park WD (1995) Sink- and vascular-associated sucrose synthase functions are encoded by different gene classes in potato. Plant Cell 7: 1369-1385

Fukuda H (1994) Redifferentiation of single mesophyll cells into tracheary elements. Int J Plant Sci 155: 262-271

Gao Z, Petreikov M, Zamski E, Schaffer AA (1999) Carbohydrate metabolism during early fruit development of sweet melon (*Cucumis melo*). Physiologia Plantarum 106: 1-8

Garbus J, De Luca HF, Loomans ME, Strong FM (1963) The rapid incorporation of phosphate into mitochondrial lipids. J Biol Chem 238: 59-63

Gaydou EM, Lizana Y, Ratovohery J (1987) Triglyceride and fatty acid compositions in the mesocarp of *Persea americana* during fruit development. Phytochemistry 26: 1595-1597

Geigenberger P, Stitt M (1993) Sucrose synthase catalyses a readily reversible reaction *in vivo* in developing potato tubers and other plant tissues. Planta 189: 329-339

Geissman TA, Dittmar HFK (1965) A proanthocyanidin from avocado seed. Phytochemistry 4: 359-368

Getz HP (1991) Sucrose transport in tonoplast vesicles of red beet roots is linked to ATP hydrolysis. Planta 185: 261-268

Giaquinta R (1977) Sucrose hydrolysis in relation to phloem transport in Beta vulgaris. Plant Physiol 60: 339-343

Giaquinta RT (1983) Phloem loading of sucrose. Annu Rev Plant Physiol 34: 347-387

Gibson SI, Graham IA (1999) Another player joins the complex field of sugar-regulated gene expression in plants. Proc Natl Acad Sci USA 96: 4746-4748

Gifford RM, Evans LT (1981) Photosynthesis, carbon partitioning, and yield. Annu Rev Plant Physiol 32: 485-509

Gillaspy G, Ben-David H, Gruissem W (1993) Fruits: A developmental perspective. Plant Cell 5: 1439-1451

Glasziou KT, Gayler KR (1972) Storage of sugars in stalks of sugar cane. Bot Rev 38: 471-490

Godt DE, Roitsch T (1997) Regulation and tissue-specific distribution of mRNAs for three extracellular invertase isoenzymes of tomato suggest an important function in establishing and maintaining sink metabolism. Plant Physiol 115: 273-282

Golan A, Kahn V, Sadouski AY (1977) Relationship between polyphenols and browning in avocado mesocarp. Comparison between the Fuerte and Lerman cultivars. J Agric Food Chem 25: 1253-1260

Goldschmidt EE (1999) Carbohydrate supply as a critical factor for citrus fruit development and productivity. HortSci 34: 1020-1024

Goldschmidt EE, Golomb A (1982) The carbohydrate balance of alternate-bearing citrus trees and the significance of reserves for flowering and fruiting. J Amer Soc Hort Sci 107: 206-208

Goldschmidt EE, Huber SC (1992) Regulation of photosynthesis by end-product accumulation in leaves of plants storing starch, sucrose, and hexose sugars. Plant Physiol 99: 1443-1448

Gonzalez-Carranza ZH, Lozoya-Gloria E, Roberts JA (1998) Recent developments in abscission: Shedding light on the process. Trends in Plant Sci 3: 10-14

Gonzalez-Ferrer J, Agusti M, Guardiola JL (1984) Fruiting pattern and retranslocation of reserves in the Navelate and Washington navel oranges. Proc Intl Soc Citricult 1: 194-200

Goodwin PB (1983) Molecular size limit for movement in the symplast of the *Elodea* leaf. Planta 157: 124-130

Gordon AJ, Flood AE (1980) 2,4-Dichlorophenoxyacetic acid and the de novo synthesis of invertase in chicory root tissue. Phytochemistry 19: 505-508

Goshroy S, Lartey R, Sheng J, Citovsky V (1997) Transport of proteins and nucleic acids through plasmodesmata. Annu Rev Plant Physiol Plant Mol Biol 48: 27-50

Goupil P, Loncle D, Druart N, Bellettre A, Rambour S (1998) Influence of ABA on nitrate reductase activity and carbohydrate metabolism in chicory roots (*Cichorium intybus* L.). J Exp Bot 49: 1855-1862

Grace SC, Logan BA, Adams III WW (1998) Seasonal differences in foliar content of chlorogenic acid, a phenylpropanoid antioxidant, in *Mahonia repens*. Plant Cell Environ 21: 513-521

Greenland AJ, Lewis DH (1981) The acid invertases of the developing third leaf of oat. I. Changes in activity of invertase and concentrations of ethanol-soluble carbohydrates. New Phytol 88: 265-277

Greutert H, Martinoia E, Keller F (1998) Mannitol transport by vacuoles of storage parenchyma of celery petioles operates by facilitated diffusion. J Plant Physiol 153: 91-96

Grisebach H (1981) Lignins. *In*: The biochemistry of plants – A comprehensive treatise. Vol. 7. Secondary plant products (Ed. Conn EE). Academic Press, New York. Pp 457-478. ISBN 0-12-675407-1

Groover A, Jones AM (1999) Tracheary element differentiation uses a novel mechanism coordinating programmed cell death and secondary cell wall synthesis. Plant Physiol 119: 375-384

Grossi M, Gulli M, Stanca AM, Cattivelli L (1995) Characterization of two barley genes that respond rapidly to dehydration stress. Plant Sci 105: 71-80

Guardiola JL (1988) Factors limiting productivity in citrus: A physiological approach. Proc Intl Soc Citricult 1: 381-394

Guardiola JL, García-Luis A (2000) Increasing fruit size in citrus. Thinning and stimulation of fruit growth. Plant Growth Regul 31: 121-132

Gunning BES, Overall R (1983) Plasmodesmata and cell-to-cell transport in plants. Bioscience 33: 260-265

Gunning BES, Robards AW (1976) Plasmodesmata: Current knowledge and outstanding problems. *In*: Intercellular communication in plants: Studies on plasmodesmata (Eds. Gunning AW, Robards AW). Springer, Heidelberg. Pp 297-311. ISBN 3-540-07570-4

Gurr MI (1980) The biosynthesis of triacylglycerols. *In*: The biochemistry of plants – A comprehensive treatise. Vol 4. Lipids: structure and function (Ed. Stumpf PK). Academic Press, New York. Pp 205-248. ISBN 0-12-675404-7

Häfliger B, Kindhauser E, Keller F (1999) Metabolism of D-*glycero*-D-*manno*-heptitol, volemitol, in polyanthus. Discovery of a novel ketose reductase. Plant Physiol 119: 191-197

Halford NG, Hardie DG (1998) SNF1-related protein kinases: Global regulators of carbon metabolism in plants? Plant Mol Biol 37: 735-748

Hardie DG, Carling D, Carlson M (1998) The AMP-activated/SNF1 protein kinase subfamily: Metabolic sensors of the eukaryotic cell? Annu Rev Biochem 67: 821-855

Hartung W, Ullrich-Eberius CI, Luttge U, Bocher M, Novacky A (1980) Effect of abscisic acid on membrane potential and transport of glucose and glycine in *Lemma gibba* Gl. Planta 148: 256-261

Hatch MD, Glasziou KT (1963) Sugar accumulation cycle in sugar cane. II. Relationship of invertase activity to sugar content and growth rate in storage tissue plants grown in controlled environments. Plant Physiol 38: 344-348

Hauch S, Magel E (1998) Extractable activities and protein content of sucrose-phosphate synthase, sucrose synthase and neutral invertase in trunk tissue of *Robinia pseudoacacia* L. are related to cambial wood production and heartwood formation. Planta 207: 266-274

Hawker JS (1985) Sucrose. *In*: Biochemistry of storage carbohydrates in green plants (Eds. Dey PM, Dixon RA). Academic Press, London. Pp 1-51. ISBN 0-12-274680-8

Hayashi T, Read SM, Bussell J, Thelen M, Lin FC, Brown RMJ, Delmer DP (1987) UDPglucose:(1,3)-glucan synthases from mung bean and cotton: Differential effects of Ca^{2+} and Mg^{2+} on enzyme properties and on macromolecular structure of high glucan product. Plant Physiol 83: 1054-1062

Hayat MA (1989) Principles and techniques of electron microscopy. Biological applications - 3rd Ed. Macmillan Press, Hampshire. Pp 280-281, 291-292. ISBN 0-333-45294-1

He C, Morgan P, Drew M (1996) Transduction of an ethylene signal is required for cell death and lysis in the root cortex of maize during aerenchyma formation induced by hypoxia. Plant Physiol 112: 463-472

Hedley PE, Maddison AL, Davidson D, Machray GC (2000) Differential expression of invertae genes in internal and external phloem tissues of potato (*Solanum tuberosum* L.). J Exp Bot 51: 817-821

Heim U, Weber H, Baumlein H, Wobus U (1993) A sucrose synthase gene of *Vicia faba* L.: Expression pattern in developing seeds in relation to starch synthesis and metabolic regulation. Planta 191: 394-401

Heinlein M, Starlinger P (1989) Tissue- and cell-specific expression of two sucrose synthase isozymes in developing maize kernels. Mol Gen Genet 215: 441-446

Helmerhorst E, Stokes GB (1980) Microcentrifuge desalting: A rapid, quantitative method for desalting small amounts of protein. Anal Biochemistry 104: 103-135

Hendrix DL (1990) Carbohydrate and carbohydrate enzymes on developing cotton ovules. Physiologia Plantarum 78: 85-92

Henton SM, Piller GJ, Gander PW (1999) A fruit growth model dependent on both carbon supply and inherent fruit characteristics. Ann Bot 83: 509-514

Herbers K, Sonnewald U (1998) Molecular determinants of sink strength. Current Opinions Plant Biol 1: 207-216

Hermely AS, Ferreira P, de Almeida-Engler J, van Montagu M, Engler G, Inze D (1993) CDC2a expression in Arabidopsis linked with competence for cell division. Plant Cell 5: 1711-1723

Higashi K, Hosoya K, Ezura H (1999) Histological analysis of fruit development between two melon (*Cucumis melo* L. *reticulatus*) genotypes setting a different size of fruit. J Exp Bot 50: 1593-1597

Hilton-Barber D (1992) 'Hass' crisis turned to PR success. AVOKAD: J SA Avo Growers' Assoc 12: 1

Hirt H (1996) In and out the plant cell cycle. Plant Mol Biol 31: 459-464

Ho LC (1988) Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. Annu Rev Plant Physiol Plant Mol Biol 39: 355-378

Ho LC (1996) The mechanism of assimilate partitioning and carbohydrate compartmentation in fruit in relation to the quality and yield of tomato. J Exp Bot 47 (special ed.): 1239-1243

Ho LC, Grange RI, Picken AJ (1987) An analysis of tomato accumulation of water and dry matter in tomato fruit. Plant Cell Environ 10: 157-162

Hoffmann-Benning S, Willmitzer L, Fisahn J (1997) Analysis of growth, composition and thickness of the cell wall of transgenic tobacco plants expressing a yeast-derived invertase. Protoplasma 200: 146-153

Holdaway-Clarke TL, Walker NA, Helper PK, Overall RL (2000) Physiological elevations in cytoplasmic free calcium by cold or ion injection result in transient closure of higher plant plasmodesmata. Planta 210: 329-335

Hole CC, Barnes A (1980) Maintenance and growth components of carbon dioxide efflux from growing pea fruits. Ann Bot 45: 295-307

Hough T, Bernhardt P, Knox RB, Williams EG (1985) Applications of fluorochromes to pollen biology. II. The DNA probes ethidium bromide and Hoechst 33258 in conjunction with the callose-specific aniline blue fluorochrome. Stain Technology 60: 155-162

Hubbard NL, Huber SC, Pharr DM (1989) Sucrose phosphate synthase and acid invertase as determinants of sucrose concentration in developing muskmelon (*Cucumis melo* L.) fruits. Plant Physiol 91: 1527-1534

Hubbard NL, Pharr DM, Huber SC (1990) Role of sucrose phosphate synthase in sucrose biosynthesis in ripening bananas and its relationship to the respiratory climacteric. Plant Physiol 94: 201-208

Hubbard NL, Pharr DM, Huber SC (1991) Sucrose phosphate synthase and other sucrose metabolizing enzymes in fruits of various species. Physiologia Plantarum 82: 191-196

Huber SC (1989) Biochemical mechanism for regulation of sucrose accumulation in leaves during photosynthesis. Plant Physiol 91: 656-662

Huber SC, Huber JL (1996) Role and regulation of sucrose phosphate synthase in higher plants. Annu Rev Plant Physiol Plant Mol Biol 47: 431-444

Imlau A, Truernit E, Sauer N (1999) Cell-to-cell long-distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. Plant Cell 11: 309-322

Irving DE, Hurst PL, Ragg JS (1997) Changes in carbohydrates and carbohydrate metabolizing enzymes during development, maturation, and ripening of buttercup squash (*Cucurbita maxima* D. 'Delica'). J Amer Soc Hort Sci 122: 310-314

Isla MI, Vattuone MA, Sampietro AR (1991) Modulation of potato invertase activity by fructose. Phytochemistry 30: 423-426

Isla MI, Salerno G, Pontis H, Vattuone MA, Sampietro AR (1995) Purification and properties of the soluble acid invertase from *Oryza sativa*. Phytochemistry 38: 321-325

Isla MI, Vattuone MA, Ordòñez RM, Sampietro AR (1999) Invertase activity associated with the walls of *Solanum tuberosum* tubers. Phytochemistry 50: 525-534

Iwatsubo T, Nakagawa H, Ogura N, Hirabayashi T, Sato T (1992) Acid invertase of melon fruits: Immunochemical detection of acid invertases. Plant Cell Physiol 33: 1127-1133

Jaffe J, Goren R (1988) Deposition of callose in relation to abscission of citrus leaves. Physiologia Plantarum 72: 329-336

Jang J-C, Sheen J (1997) Sugar sensing in higher plants. Trends in Plant Sci 2: 208-214.

Jensen WA (1962) Botanical histochemistry. W. H. Freeman and company, San Francisco. Pp 199, 201-202. ISBN none

Johansen DA (1940) Plant microtechnique. McGraw-Hill Book Company, Inc., New York. Pp 20, 80-82. ISBN none

John PCL, Zhang K, Dong C, Diederich L, Wightman F (1993) P34-cdc2 related proteins in control of cell cycle progression, the switch between division and differentiation in tissue development, and stimulation of cell division by auxin and cytokinin. Austr J Plant Physiol 20: 503-520

Jones AM (2001) Programmed cell death in development and defense. Plant Physiol 125: 94-97

Jones JKN, Sephton HH (1960) Synthesis of sugars from smaller fragments. Part XII. Synthesis of D-glycero-D-altro-, L-glycero-L-galacto-, D-glycero-L-gluco- and D-glycero-L-galacto-octulose. Canadian J Chem 38: 753-760

Jones AM, Dangl JL (1996) Logjam at the Styx: Programmed cell death in plants. Trends in Plant Sci 1: 114-119

Joost O, Bianchini G, Bell AA, Benedict CR, Magill CW (1995) Differential induction of 3-hydroxy-3-methylglutaryl CoA reductase in two cotton species following inoculation with *Verticillium*. Mol Plant-Microbe Interactions 8: 880-885

Kaiser C (1993) Some physiological aspects of delayed harvest of 'Hass' avocado (*Persea Americana* Mill.) in the Natal midlands. MSc. Thesis, University of Natal (Pietermaritzburg), South Africa. Pp 1-13, 101-106

Kadlecová Z, Faltus M, Prášil I (2000) Relationship between abscisic acid content, dry weight and freezing tolerance in barley cv. Lunet. J Plant Physiol 157: 291-297

Kashem MA, Hori H, Itoh K, Hayakawa T, Todoroki Y, Hirai N, Ohigashi H, Mitsui T (1998) Effects of (+)-8',8',8'-trifluoroabscisic acid on α-amylase expression and sugar accumulation in rice cells. Planta 205: 319-326

Kato T, Kubota S (1978) Properties of invertases in sugar storage tissues of citrus fruit and changes in their activities during maturation. Physiologia Plantarum 42: 67-72

Kaur S, Gupta AK, Kaur N (1998) Gibberellin A_3 reverses the effect of salt stress in chickpea (*Cicer arietinum* L.) seedlings by enhancing amylase activity and mobilization of starch in cotyledons. Plant Growth Regul 26: 85-90

Kauss H (1987) Some aspects of calcium-dependent regulation in plant metabolism. Annu Rev Plant Physiol 38: 47-72

Kauss H (1996) Callose synthesis. *In*: Membranes: specialized functions in plants (Eds. Smallwood M, Knox JP, Bowles DJ). Bios Scientific Publishers Ltd, Oxford. Pp 77-92. ISBN 1-85996-200-9

Kauss H, Jeblick W (1986) Influence of free fatty acids, lysophosphatidylcholine, platelet-activating factor, acrlycarnitine, and echinocandin B on 1,3-β-D-glucan synthase and callose synthesis. Plant Physiol 80: 7-13

Kauss H, Köhle H, Jeblick W (1983) Proteolytic activation and stimulation by Ca^{2+} of glucan synthase from soybean cells. FEBS Lett 158: 84-88

Kawasaki T, Henmi K, Ono E, Hatakeyama S, Iwano M, Satoh H, Shimamoto K (1999) The small GTP-binding protein Rac is a regulator of cell death in plants. Proc Natl Acad Sci USA 96: 10922-10926

Keller F (1992) Transport of stachyose and sucrose by vacuoles of Japanese artichoke (*Stachys sieboldii*) tubers. Plant Physiol 98: 442-445

Kempers R, van Bel AJE (1997) Symplastic connection between sieve element and companion cell in the stem of phloem of *Vicia faba* have a molecular exclusion limit of at least 10 kDa. Planta 201: 195-201

Kikuta Y, Erickson LC (1968) Seasonal changes in avocado lipids during fruit development and storage. CA Avo Soc Yrbk 52: 102-108

Kikuta Y, Erickson LC (1969) Metabolism of glucose in relation to the lipid synthesis in the fruit of *Persea americana* Mill. Plant Cell Physiol 10: 563-574

Kingston-Smith AH, Pollock CJ (1996) Tissue level localization of acid invertase in leaves: An hypothesis for the regulation of carbon export. New Phytol 134: 423-432

Klann EK, Chetelat RT, Bennett AB (1993) Expression of acid invertase gene controls sugar composition in tomato (*Lycopersicon*) fruit. Plant Physiol 103: 863-870

Klann EM, Hall B, Bennett AB (1996) Antisense acid invertase (TIV1) gene alters soluble sugar composition and size in transgenic tomato fruit. Plant Physiol 112: 1321-1330

Kleiner KW, Raffa KF, Dickson RE (1999) Partitioning of 14C-labeled photosynthate to allelochemicals and primary metabolites in source and sink leaves of aspen: Evidence for secondary metabolite turnover. Oecologia 119: 408-418

Knapp FW (1965) Some characteristics of eggplant and avocado polyphenol oxidase. J Food Sci 30: 930-936

Knoblauch M, van Bel AJE (1998) Sieve tubes in action. Plant Cell 10: 35-50

Kobashi K, Gemma H, Iwahori S (1999) Sugar accumulation in peach fruit as affected by abscisic-acid (ABA) treatment in relation to some sugar metabolizing enzymes. J Japan Soc Hort Sci 68: 465-470

Kobashi K, Gemma H, Iwahori S (2000) Abscisic acid content and sugar metabolism of peaches grown under water stress. J Amer Soc Hort Sci 125: 425-428

Koch KE (1996) Carbohydrate-modulated gene expression in plants. Annu Rev Plant Physiol Plant Mol Biol 47: 509-540

Koch KE, Nolte KD, Duke ER, McCarty DR, Avigne WT (1992) Sugar levels modulate differential expression of maize sucrose synthase genes. Plant Cell 4: 59-69

Köhle H, Jeblick W, Poten F, Blaschek W, Kauss H (1985) Chitosan-elicited callose synthesis in soybean cells as a Ca^{2+}-dependent process. Plant Physiol 77: 544-551

Kojima K, Yamada Y, Yamamoto M (1995) Effects of abscisic acid injection on sugar and organic acid contents of citrus fruit. J Japan Soc Hort Sci 64: 17-21

Komor E, Thom M, Maretzki A (1981) The mechanism of sugar uptake by sugarcane suspension cells. Planta 153: 181-192

Kremer-Köhne S (1999) Evaluation of new Hass-like avocado cultivars at Merensky Technological Services. SA Avo Growers' Assoc Yrbk 22: 120-122

Kremer-Köhne S (2000) New Hass-like avocado cultivars at Merensky Technological Services – progress report. SA Avo Growers' Assoc Yrbk 23: 52-55

Kremer-Köhne S, Köhne JS (1995) Approaches to solving the small 'Hass' fruit problem: Progress report. SA Avo Growers' Assoc Yrbk 18:59-60

Kühn C, Franceschi V, Schulz A, Lemoine R, Frommer WB (1997) Macromolecular trafficking indicated by localization and turnover of sucrose transporters in enucleate sieve elements. Science 275: 1298-1300

Kühn C, Barker L, Bürkle L, Frommer WB (1999) Update on sucrose transport in higher plants. J Exp Bot 50 (special ed.): 935-953

Kull U (1967) Zum physiologischen Verhalten der Sedoheptulose im Rahmen des Kohlenhydrathaushaltes einiger Crassulaceen. Ber Dtsch Bot Ges 80: 187-198 [Title translation: The physiological role of sedoheptulose in carbohydrate metabolism in some Crassulaceae]

Kwaitkowska M, Maszewski J (1976) Plasmodesmata between synchronously and asynchronously developing cells of the antheridial filaments of *Chara vulgaris* L. Protoplasma 96: 59-74

Kwaitkowska M, Maszewski J (1985) Changes in ultrastructure of plasmodesmata during spermatogenesis in *Chara vulgaris* L. Planta 166: 46-50

La Forge FB (1917) D-mannoketoheptose, a new sugar from avocado. J Biol Chem 28: 511-522

Lahav E, Kalmer D (1977) Water requirements of avocado in Israel. II. Influence on yield, fruit growth and oil content. Aust J Agric Res 28: 869-877

Lalonde S, Boles E, Hellmann H, Barker L, Patrick JW, Frommer WB, Ward JM (1999) The dual function of sugar carriers: Transport and sugar sensing. Plant Cell 11: 707-726

Lam E, del Pozo O, Pontier D (1999) BAXing in the hypersensitive response. Trends in Plant Sci 44: 419-421

Lang A, Thorpe MR (1986) Water potential, translocation and assimilate partitioning. J Exp Bot 37: 495-503

Lång V, Mäntylä E, Welin B, Sundberg B, Palva ET (1994) Alteration in water status, endogenous abscisic acid, and expression of *rab18* gene during the development of freezing tolerance in *Arabidopsis thaliana*. Plant Physiol 104: 1341-1349

Lange H, Shropshire W, Mohr H (1971) An analysis of phytochrome-mediated anthocyanin synthesis. Plant Physiol 47: 649-655

Larsson C, Widell S, Kjellbom P (1987) Preparation of high purity plasma membranes. Methods in Enzymology 148: 558-568

Lee TD (1988) Patterns of fruit and seed production. *In*: Plant reproductive ecology - patterns and strategies (Eds. Doust JL, Doust LL). Oxford University Press, Oxford. Pp 179-202. ISBN 0-19-505175-0

Lee H-S, Sturm A (1996) Purification and characterization of neutral and alkaline invertase from carrot. Plant Physiol 112: 1513-1522

Lee TT, Starratt AN, Jevnikar JJ (1982) Regulation of enzymatic oxidation of indole-3 acetic acid by phenols: Structure-activity relationships. Phytochemistry 21: 517-523

Leigh RA, ap Rees T, Fuller WA, Banfield J (1979) The location of invertase activity and sucrose in the vacuoles of storage roots of beetroot (*Beta vulgaris*). Biochem J 178: 539-547

Lerchl J, Geigenberger P, Stitt M, Sonnewald U (1995) Impaired photoassimilate partitioning caused by phloem-specific removal of pyrophosphate can be complemented by phloem-specific cytosolic yeast-derived invertase in transgenic plants. Plant Cell 7: 259-270

Lewis DH (1984) Physiology and metabolism of alditols. *In*: Storage carbohydrates in vascular plants (Ed. Lewis DH). Cambridge University Press, Cambridge. Pp 157-179. ISBN 0-521-23698-3

Lin LS, Ho TH-D (1986) Mode of action of abscisic acid in barley aleurone layers. Induction of new proteins by abscisic acid. Plant Physiol 93: 289-297

Lingle SE, Dunlap JR (1987) Sucrose metabolism in netted muskmelon fruit during development. Plant Physiol 84: 386-389

Little G, Edelman J (1973) Solubility of plant invertases. Phytochemistry 12: 67-71

Liu XL, Robinson PW, Madore MA, Witney GW, Arpaia ML (1999a) 'Hass' avocado carbohydrate fluctuations. I. Growth and phenology. J Amer Soc Hort Sci 124: 671-675

Liu XL, Robinson PW, Madore MA, Witney GW, Arpaia ML (1999b) 'Hass' avocado carbohydrate fluctuations. I. Fruit growth and ripening. J Amer Soc Hort Sci 124: 676-681

Lo Bianco R, Rieger M, Sung SS (1998) A simple, rapid extraction and assay procedure for NAD^+-dependent sorbitol dehydrogenase (SDH) in peach. J Amer Soc Hort Sci 123: 1065-1068

Lo Bianco R, Rieger M, Sung S-JS (1999) Activities of sucrose and sorbitol metabolizing enzymes in vegetative sinks of peach and correlation with sink growth rate. J Amer Soc Hort Sci 124: 381-388

Lo Bianco R, Rieger M, Sung S-JS (2000) Effect of drought on sorbitol and sucrose metabolism in sinks and sources of peach. Physiologia Plantarum 108: 71-78

Locquin MV, Langeron M (1983) Handbook of microscopy. Butterworths, London. Pp 206. ISBN 0-408-10679-4

Loescher WH (1987) Physiology and metabolism of sugar alcohols in higher plants. Physiologia Plantarum 70: 553-557

Loguercio LL, Scott HC, Trolinder NL, Wilkins TA (1999) HMG-coA reductase gene family in cotton (*Gossypium hirsutum* L.): Unique structural features and differential expression of *hmg2* potentially associated with synthesis of specific isoprenoids in developing embryos. Plant Cell Physiol 40: 750-761

Lopez-Carbonell M, Alegre L, van Onckelen H (1994) Changes in cell ultrastructure and endogenous abscisic acid and indole-3-acetic acid concentrations in *Fatsia japonica* leaves under polyethylene glycol-induced water stress. Plant Growth Regul 15: 165-174

Lovall CA, Tomlinson PT, Koch KE (1989) Sucrose-metabolizing enzymes in transport tissues and adjacent sink structures in developing citrus fruit. Plant Physiol 90: 1394-1402

Lowe J, Nelson OE Jr (1946) Miniature seed – a study in the development of a defective caryopsis in maize. Genetics 31: 525-533

Lucas WJ, Madore MA (1988) Recent advances in sugar transport. *In*: The biochemistry of plants - A comprehensive treatise. Vol 14. Carbohydrates (Ed. Preiss J). Academic Press, San Diego. Pp 35-84. ISBN 0-12-675414-4

Lucas WJ, Wolf S (1993) Plasmodesmata: The intercellular organelles of green plants. Trends in Cell Biol 3: 308-315

Lucas WJ, Ding B, van der Schoot C (1993) Plasmodesmata and the supracellular nature of plants. New Phytol 125: 435-476

Lucas WJ, Bouche-Pillon S, Jackson DP, Nguyen L, Baker L, Ding B, Hake S (1995) Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. Sci Wash 270: 1980-1983

Lunn JE, Furbank RT (1999) Tansley review no. 105: Sucrose biosynthesis in C_4 plants. New Phytol 143: 221-237

Macháčková I, Hanišová A, Krekule J (1989) Levels of ethylene, ACC, MACC, ABA and proline as indicators of cold hardening and frost resistance in winter wheat. Physiol Plant 76: 603-607

Macheix J-J, Fleuriet A, Billot J (1990) Fruit phenolics. CRC Press, Boca Raton. Pp 110, 239-294. ISBN 0-8493-4968-0

MacKintosh C (1998) Regulation of cytosolic enzymes in primary metabolism by reversible protein phosphorylation. Current Opinions Plant Biol 1: 224-229

MacKintosh RW, Davies SP, Clarke PR, Weeks J, Gillespie JC, Gibb BJ, Hardie GD (1992) Evidence for a protein kinase cascade in higher plants. Eur J Biochem 209: 923-931

Maddison AL, Hedley PE, Meyer RC, Aziz N, Davidson D, Machray GC (1999) Expression of tandem invertase genes associated with sexual and vegetative growth cycles in potato. Plant Mol Biol 41: 741-751

Manning K, Maw GA (1969) Distribution of acid invertase in the tomato plant. Phytochemistry 14: 1965-1969

Mansfield SG, Briarty LG (1991) Early embryogenesis in *Arabidopsis thaliana*. II. The developing embryo. Can J Bot 69: 461-476

Martin T, Frommer WB, Salanoubat M, Willmitzer L (1993) Expression of an *Arabidopsis* sucrose synthase gene indicates a role in metabolization of sucrose both during phloem loading and in sink organs. Plant J 4: 367-377

Marttila S, Tenhola T, Mikkonen A (1996) A barley (*Hordeum vulgare* L.) LEA3 protein, HVA1, is abundant in protein storage vacuoles. Planta 199: 602-611

Mason HS, De Wald DB, Creelman RA, Mullet JE (1992) Coregulation of soybean vegetative storage protein gene expression by methyl jasmonate and soluble sugars. Plant Physiol 98: 859-867

Masuda H, Takahashi T, Sugawara S (1987) The occurrence and properties of alkaline invertase in mature roots of sugar beets. Agric Biol Chem 51: 2309-2314

Masuda H, Takahashi T, Sugawara S (1988) Acid and alkaline invertases in suspension cultures of sugar beet cells. Plant Physiol 86: 312-317

Mathews CK, van Holde KE (1990) Biochemistry. Benjamin/Cummings Publishing Company, Redwood City. Pp 494-500, 660-664. ISBN 0-8053-5015-2

Matsushita K, Uritani I (1974) Change in invertase activity of sweet potato in response to wounding and purification and properties of its invertases. Plant Physiol 54: 60-66

McLean BG, Hempel FD, Zambryski PC (1997) Plant intercellular communication via plasmodesmata. Plant Cell 9: 1043-1054

McNeil M, Darvill A, Fry SC, Albersheim P (1984) Structure and function of the primary cell walls of plants. Annu Rev Biochem 53: 625-663

Meiners S, Xu A, Schindler M (1991) Gap junction protein homologue from *Arabidopsis thaliana*: Evidence for connexins in plants. Proc Natl Acad Sci 88: 4119-4122

Mezitt LA, Lucas LA (1996) Plasmodesmal cell-to-cell transport of proteins and nucleic acids. Plant Mol Biol 32: 251-273

Miller ME, Chourey PS (1992) The maize invertase-deficient *miniature-1* seed mutation is associated with aberrant pedicel and endosperm development. Plant Cell 4: 297-305

Milner ID, Ho LC, Hall JL (1995) Properties of proton and sugar transport at the tonoplast of tomato (*Lycopersicon esculentum*) fruit. Physiologia Plantarum 94: 399-410

Minibayeva FV, Kolesnikov OP, Gordon LK (1998) Contribution of a plasma membrane redox system to the superoxide production by wheat root cells. Protoplasma 205: 101-106

Miron D, Schaffer AA (1991) Sucrose phosphate synthase, sucrose synthase, and invertase activities in developing fruit of *Lycopersicon esculentum* Mill. and the sucrose accumulating *Lycopersicon hirsutum* Humb. and Bonpl. Plant Physiol 95: 623-627

Mita S, Hirano H, Nakamuro K (1997) Negative regulation in the expression of a sugar-inducable gene in *Arabidopsis thaliana*. A recessive mutant causing enhanced expression of a gene for β-amylase. Plant Physiol 114: 575-582

Mitchell DI (2000) *In vitro* culture of avocado: A model system for studying the biochemistry of fruit growth. MSc. Thesis, University of Natal (Pietermaritzburg), South Africa. Pp 22-26, 29-30

Miyamoto K, Ito E, Yamamoto H, Ueda J, Kamisaka S (2000) Gibberellin-enhanced growth and sugar accumulation in growing subhooks of etiolated *Pisum sativum* seedlings: Effects of actinomycin D on invertase activity, soluble sugars and stem elongation. J Plant Physiol 156: 449-453

Mizukami Y, Fischer RL (2000) Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. PNAS 97: 942-947

Monselise S, Goldschmidt E (1982) Alternate bearing in fruit trees. Hort Rev 4: 128-173

Moore B, Sheen J (1999) Plant sugar sensing and signalling – a complex reality. Trends in Plant Sci 4: 248-249

Moore-Gordon CS (1997) Aspects of avocado fruit growth and development: Towards understanding the 'Hass' small fruit syndrome. PhD. Thesis, University of Natal, Pietermaritzburg, South Africa. Pp 1-113

Moore-Gordon CS, Wolstenholme BN (1996) The Hass small-fruit problem: role of physiological stress and its amelioration by mulching. SA Avo Grower's Assoc Yrbk19: 82-85

Moore-Gordon C, Wolstenholme BN, Levin J (1996) Effect of composted pinebark mulching on *Persea americana* Mill. cv. Hass fruit growth and yield in a cool subtropical environment. J S Afr Soc Hort Sci 6: 23-27

Moore-Gordon C, Cowan AK, Wolstenholme BN (1997) Mulching of avocado orchards to increase Hass yield and fruit size and boost financial rewards – a three season summary of research findings. SA Avo Growers' Assoc Yrbk 20: 46-49

Moore-Gordon CS, Cowan AK, Bertling I, Botha CEJ, Cross RHM (1998) Symplastic solute transport and avocado fruit development: A decline in cytokinin/ABA ratio is related to appearance of the Hass small fruit variant. Plant Cell Physiol 39: 1027-1038

Moran JF, Becana M, Iturbeormaetxe I, Frechilla S, Klucas RV, Apariciotejo P (1994) Drought induces oxidative stress in pea plants. Planta 194: 346-352

Morell M, Copeland L (1985) Sucrose synthase of soybean nodules. Plant Physiol 78: 140-154

Morris DA (1996) Hormonal regulation of source-sink relationships: An overview of potential control mechanisms. *In*: Photoassimilate distribution in plants and crops (Eds. Zamski E, Schaffer AA). Marcel Dekker, New York. Pp 441-465. ISBN 0-8247-9440-0

Morris DA, Arthur ED (1984a) An association between acid invertase activity and cell growth during leaf expansion in *Phaseolus vulgaris* L. J Exp Bot 35: 1369-1379

Morris DA, Arthur ED (1984b) Invertase and auxin-induced elongation in internodal segments of *Phaseolus vulgaris*. Phytochemistry 23: 2163-2167

Münch E (1930) Die Stoffbewegungen in der Pflanze. Jena, Germany: Fischer. [Title translation: Transport of metabolites in plants]

Murphy R (1986) Symplastic sieve tube unloading in seed coats of *Phaseolus vulgaris* is a necessary requirement for pressure-driven translocation. *In*: Phloem transport, Plant Biology. Vol 1. (Eds. Cronshaw J, Lucas WJ, Giaquinta RT). Alan R. Liss Inc., New York. Pp 259-262. ISBN 0-8451-1800-5

Murphy R (1989) Water flow across the sieve tube boundary: Estimating turgor and some implications for phloem loading and unloading. II. Root tips and seed coats. Ann of Bot 63: 571-579

Murphy TM, Asard H, Cross AR (1998) Possible sources of reactive oxygen during the oxidative burst in plants. *In*: Plasmamembrane redox systems and their role in biological stress and disease (Eds. Asard H, Bérczi A, Caubergs RJ). Kluwer Academic Publishers, Dordrecht. Pp 215-246. ISBN 0-7923-5467-2

Naidu BP (1998) Separation of sugars, polyols, proline analogues, and betaines in stressed plant extracts by high performance liquid chromatography and quantification by ultra violet detection. Aust J Plant Physiol 25: 793-800

Nakano R, Yonemori K, Sugiura A (1998) Fruit respiration for maintaining sink strength during final swell at growth stage III of persimmon fruit. J Hort Sci Biotech 73: 341-346

Narváez-Vásquez J, Pearce G, Orozco-Cardenas ML, Franceschi VR, Ryan CA (1995) Autoradiographic and biochemical evidence for the systemic translocation of systemin in tomato plants. Planta 195: 593-600

Navari-Izzo F, Rascio N (1999) Plant response to water-deficit conditions. *In*: Handbook of plant and crop stress (Ed. Pessarakli M). Marcel Dekker Inc., New York. Pp 231-270. ISBN 0-8247-8987-3

Nelson N (1944) A photometric adaption of the Somogyi method for the determination of glucose. J Biol Chem 153: 375-380

Newman GR, Hobot JA (1993) Resin microscopy and on-section immunocytochemistry. Springer-Verlag, Heidelberg. Pp 30-34, 88-90. ISBN 3-540-56429-2

Nguyen-Quoc B, Krivitzky M, Huber SC, Lecharny A (1990) Sucrose synthase in developing maize leaves. Regulation of activity by protein level during the import to export transition. Plant Physiol 516-523

Nguyen-Quoc B, N'tchobo H, Foyer CH, Yelle S (1999) Overexpression of sucrose phosphate synthase increases sucrose unloading in transformed tomato fruit. J Exp Bot 50: 785-791

Nielson TH, Skjaerbaek HC, Karlsen P (1991) Carbohydrate metabolism during fruit development in sweet pepper (*Capsicum annuum*) plants. Physiologia Plantarum 82: 311-319

Noh B, Spalding EP (1998) Anion channels and the stimulation of anthocyanin accumulation by blue light in Arabidopsis seedlings. Plant Physiol 116: 503-509

Nolte KD, Koch KE (1993) Companion-cell specific localization of sucrose synthase in zones of phloem loading and unloading. Plant Physiol 101: 899-905

Nolte KD, Hendrix DL, Radin JW, Koch KE (1995) Sucrose synthase localization during initiation of seed development and trichome differentiation in cotton ovules. Plant Physiol 109: 1285-1293

Nordal A, Benson AA (1954) Isolation of mannoheptulose and identification of its phosphate in avocado leaves. J Amer Chem Soc 76: 5054-5055

Northcote DH, Davey R, Lay J (1989) Use of antisera to localize callose, xylan and arabinogalactan in the cell-plate, primary and secondary walls of plant cells. Planta 179: 353-366

N'tchobo H, Dali N, Nguyen-Quoc B, Foyer CH, Yelle S (1999) Starch synthesis in tomato remains constant throughout fruit development and is dependent on sucrose supply and sucrose synthase activity. J Exp Bot 50: 1457-1463

Offler CE, Horder B (1992) The cellular pathway of short distance transfer of photosynthates in developing tomato fruit. Plant Physiol 99: 41

Ofosu-Anim J, Kanayama Y, Yamaki S (1996) Sugar uptake into strawberry fruit is stimulated by abscisic acid and indolacetic acid. Physiologia Plantarum 97: 169-174

Ogata JN, Kawano Y, Bevenue A, Casarett LJ (1972) The ketoheptulose content of some tropical fruits. J Agric Food Chem 20: 113-115

Ohto M, Nakamura K (1995) Sugar-induced increase of calcium-dependent protein kinases associated with the plasma membrane in leaf tissues of tobacco. Plant Physiol 109: 973-981

Ohyama A, Ito H, Sato T, Nishimura S, Imai T, Hirai M (1995) Suppression of acid invertase activity by antisense RNA modifies the sugar composition of tomato fruit. Plant Cell Physiol 36: 369-376

Okuda T, Mori K (1974) Distribution of *manno*-heptulose and sedoheptulose in plants. Phytochemistry 13: 961-964

Olesen P (1979) The neck constriction in plasmodesmata: Evidence for a peripheral sphincter-like structure revealed by fixation with tannic acid. Planta 144: 349-358

Olesen P, Robards AW (1990) The neck region of plasmodesmata: General architecture and some functional aspects. *In*: Parallels in cell-to-cell junctions in plants and animals (Eds. Robards AW, Lucas WJ, Pitts JD, Jonsma HJ, Spray DC). Springer, Heidelberg. Pp 145-170. ISBN 0-387-51768-5

Oparka KJ (1993) Signalling via the plasmodesmata – the neglected pathway. Semin Cell Biol 4: 131-138

Oparka KJ, Prior DAM (1987) ^{14}C-sucrose efflux from the perimedula of growing potato tubers. Plant Cell Environ 10: 667-675

Oparka KJ, Prior DAM (1988) Movement of lucifer yellow CH in potato tuber storage tissues: A comparison of symplastic and apoplastic transport. Planta 176: 533-540

Oparka KJ, Prior DAM (1992) Direct evidence for pressure-generated closure of plasmodesmata. Plant J 2: 741-750

Oparka KJ, Santa Cruz S (2000) The great escape: Phloem transport and unloading of macromolecules. Annu Rev Plant Physiol Plant Mol Biol 51: 323-347

Oparka KJ, Roberts AG (2001) Plasmodesmata. A not so open-and-shut case. Plant Physiol 125: 123-126

Osborne RE (1992) Factors affecting the rooting of *Pinus patula* Scheide *et* Deppe stem cuttings. MSc. Thesis, University of Natal (Pietermaritzburg), South Africa. Pp 51-54

Ovardi J (1991) Physiological significance of metabolic channeling. J Theor Biol 152: 1-22

Overall RL, Blackman LM (1996) A model of the macromolecular structure of plasmodesmata. Trends in Plant Sci 1: 307-311

Overall RL, Gunning BES (1982) Intercellular communication in *Azolla* roots: II. Electrical coupling. Protoplasma 111: 151-160

Pagano EA, Benech-Arnold RL, Wawrzkiewicz M, Steinbach HS (1997) α-Amylase activity in developing *Sorghum* caryopses from sprouting resistant and susceptible varieties. The role of ABA and GAs on its regulation. Ann Bot 79: 13-17

Palmgren MG (1990) An H^+-ATPase assay: Proton pumping and ATPase activity determined simultaneously in the same sample. Plant Physiol 94: 882-886

Park JB (1999) Flavenoids are potent inhibitors of glucose uptake in U937 cells. Biochem Biophys Res Com 260: 568-574

Patrick JW (1989) Solute efflux at plant/micro-organism interfaces. Aust J Plant Physiol 16: 53-68

Patrick JW (1990) Sieve element unloading: Cellular pathway, mechanism and control. Physiologia Plantarum 78: 298-308

Patrick JW (1993) Osmotic regulation of assimilate unloading from seed coats of *Vicia faba*: Role of turgor and identification of turgor-dependent fluxes. Physiologia Plantarum 89: 87-96

Patrick JW (1997) Phloem unloading: Sieve element unloading and post-sieve element transport. Annu Rev Plant Physiol Plant Mol Biol 48: 191-222

Patrick JW, Offler CE (1995) Post-sieve element transport of sucrose in developing seed. Aust J Plant Physiol 22: 761-769

Patrick JW, Offler CE (1996) Post-sieve element transport of photoassimilates in sink regions. J Exp Bot 47 (special ed.): 1165-1177

Patrick JW, Jacobs E, Offler CE, Cram WJ (1986) Photosynthate unloading from seed coats of *Phaseolus vulgaris* L. Nature and cellular location of turgor-sensitive unloading. J Exp Bot 37: 1006-1019

Paz-Vega S (1997) Alternate bearing in the avocado (*Persea Americana* Mill.). CA Avo Soc Yrbk 81: 117-148

Pego JV, Weisbeek PJ, Smeekens SCM (1999) Mannose inhibits *Arabidopsis* germination via hexokinase-mediated step. Plant Physiol 119: 1017-1023

Pennell RI, Lamb C (1997) Programmed cell death in plants. Plant Cell 9: 1157-1168

Perata P, Matsukura C, Vernieri P, Yamaguchi J (1997) Sugar repression of a gibberellin-dependent signalling pathway in barley embryos. Plant Cell 9: 2197-2208

Perata P, Loreti E, Guglielminetti L, Alpi A (1998) Carbohydrate metabolism and anoxia tolerance in cereal grains. Acta Botanica Neerlandica 47: 269-283

Pérez FJ, Gómez M (2000) Possible role of soluble invertase in gibberellic acid berry-sized effect in sultana grape. Plant Growth Regul 30: 111-116

Pfeiffer W, Höftberger M (2000) Oxidative burst in *Chenopodium rubrum* suspension cells: Induction by auxin and osmotic changes. Physiologia Plantarum 111: 144-150

Pharr DM, Stoop JMH, Williamson JD, Studer Feusi ME, Massel MO, Conkling MA (1995) The dual role of mannitol as osmoprotectant and photoassimilate in celery. HortSci 30: 1182-1188

Phillips J (1973) The agricultural and related development of the Tugela basin and its influent surrounds. Town and regional planning commission Natal, Pietermaritzburg. Pp 56-57, 167. ISBN 0-620-00696-X

Phippen WB, Simon JE (1998) Anthocyanins in basil (*Ocimum basilicum* L.). J Agric Food Chem 46: 1734-1738

Platt-Aloia KA, Thomson WW (1980) Aspects of the three-dimensional intracellular organization of mesocarp cells as revealed by scanning electron microscopy. Protoplasma 104: 157-165

Platt-Aloia KA, Thomson WW (1981) Ultrastructure of the mesocarp of mature avocado fruit and changes associated with ripening. Ann Bot 48: 451-465

Platt-Aloia KA, Oross JW, Thomson WW (1983) Ultrastructural study of the development of oil cells in the mesocarp of avocado fruit. Bot Gaz 144: 49-55

Pontis HG (1978) On the scent of the riddle of sucrose. Trends in Biochem 3: 137-139

Pooviah BW (1974) Formation of callose and lignin during leaf abscission in kidney beans. Am J Bot 61: 829-834

Popenoe W (1974) Manual of tropical and subtropical fruits. Hafner Press, New York. Pp 9-78. ISBN 0-02-850280-9

Porter LJ (1989) Tannins. *In*: Methods in plant biochemistry. Vol. 1. Plant phenolics (Ed. Harborne JB). Academic Press, London. Pp 389-419. ISBN 0-12-461011-0

Pospíšilová J, Synková H, Rulcová J (2000) Cytokinins and water stress. Biologia Plantarum 43: 321-328

Prabha TN, Ravindranath B, Pathwardhan MV (1980) Anthocyanins of avocado (*Perisa americana*). J. Food Sci Technol 17: 241. *In*: Anthocyanins in fruits, vegetables and grains (Eds. Mazza G, Miniati E). CRC Press, Boca Raton. P 141. ISBN 0-8493-0172-6

Prasad TK (1996) Mechanisms of chilling-induced oxidative stress injury and tolerance in developing maize seedlings: Changes in antioxidant system, oxidation of proteins and lipids, and protease activities. Plant J 10: 1017-1026

Prata RTN, Williamson JD, Conkling MA, Pharr DM (1997) Sugar repression of mannitol dehydrogenase activity in celery cells. Plant Physiol 114: 307-314

Preiss J (1988) Biosynthesis of starch and its regulation. *In*: The biochemisty of plants – A comprehensive treatise. Vol. 14. Carbohydrates (Ed. Preiss J). Academic Press, San Diego. Pp 181-254. ISBN 0-12-675414-4

Prior RL, Cao G, Martin A, Sofic E, McEwen J, O'Brien C, Lischner N, Ehlenfeldt M, Kalt W, Krewer G, Mainland CM (1998) Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. J Agric Food Chem 46: 2686-2693

Pruvot G, Massimino J, Peltier G, Rey P (1996) Effect of low temperature, high salinity and exogenous ABA on the synthesis of two chloroplastic drought-induced proteins in *Solanum tuberosum*. Physiologia Plantarum 97: 123-131

Purcell PC, Smith AM, Halford NG (1998) Antisense expression of a sucrose non-fermenting-1-related protein kinase sequence in potato results in decreased expression of sucrose synthase transcripts in leaves. Plant J 14: 195-202

Quartacci MF, Pinzino C, Sgherri CLM, Navari-Izzo F (1995) Lipid composition and protein dynamics in thylakoids of two wheat cultivars differently sensitive to drought. Plant Physiol 108: 191-197

Radford JE, White RG (1998) Localization of a myosin-like protein to plasmodesmata. Plant J 14: 743-750

Ramloch-Lorenz K, Knudsen S, Sturm A (1993) Molecular characterisation of the gene for carrot cell wall β-fructosidase. Plant J 4: 545-554

Ranwala AP, Iwanami S-S, Masuda H (1991) Acid and neutral invertases in the mesocarp developing muskmelon (*Cucumis melo* L. cv. Prince) fruit. Plant Physiol 96: 881-886

Rea PA, Sanders D (1987) Tonoplast energization: Two H^+ pumps, one membrane. Physiologia Plantarum 71: 131-141

Rea PA, Poole RJ (1993) Vacuolar H^+ translocating pyrophosphatase. Annu Rev Plant Physiol Plant Mol Biol 44: 157-180

Renz A, Stitt M (1993) Substrate specificity and product inhibition of different forms of fructokinases and hexokinases in developing potato tubers. Planta 190: 166-175

Renz A, Merlo L, Stitt M (1993) Partial purification from potato tubers of three hexokinases which show different organ and developmental specificity. Planta 190: 156-165

Resende MLV, Flood J, Ramsden JD, Rowan MG, Beale MH, Cooper RM (1996) Novel phytoalexins including elemental sulphur in the resistance of cocoa (*Theobroma cacao* L.) to Verticillum wilt (*Verticillum dahliae* Kleb.). Physiol Mol Plant Pathol. 48: 347-359

Ricardo CPP, ap Rees T (1970) Invertase activity during the development of carrot roots. Phytochemistry 9: 239-347

Richardson PT, Baker DA, Ho LC (1982) The chemical composition of cucurbit vascular exudates. J Exp Bot 33: 1239-1247

Richings EW, Cowan AK (2000) Regulation of isoprenoid metabolism and Hass avocado fruit: An overview. SA Avo Grower's Assoc Yrbk 23: 72-78

Richings EW, Cripps RF, Cowan AK (2000) Factors affecting 'Hass' avocado fruit size: Carbohydrate, abscisic acid and isoprenoid metabolism in normal and phenotypically small fruit. Physiologia Plantarum 109: 81-89

Richtmyer NK (1970) The isolation of volemitol and other polyhydric alcohols from avocado seeds. Carbohyd Res 12: 135-138

Robards AW (1982) Cell interactions in plants – a comparative study. *In*: Functional integration of cells in animal tissues (Eds. Pitts JD, Finbow ME). Cambridge University Press, Cambridge. Pp 57-79. ISBN 0-521-24199-5

Robards AW, Lucas WJ (1990) Plasmodesmata. Annu Rev Plant Physiol Plant Mol Biol 41: 369-419

Robbertse PJ, Coetzer LA, Johannsmeier MF, Swart DJ, Köhne JS, Morudu TM (1996) Hass yield and fruit size as influenced by pollination and pollen donor – a joint progress report. SA Avo Grower's Assoc Yrbk 19: 63-67

Robinson NL, Hewitt JD, Bennett AB (1988) Sink metabolism in tomato fruit. I. Developmental changes in carbohydrate metabolizing enzymes. Plant Physiol 87: 727-730

Rochat C, Boutin J-P (1992) Temporary storage compounds and sucrose-starch metabolism in seed coat during pea seed development (*Pisum sativum* L.). Physiol Plant 85: 567-572

Roitsch T (1999) Source-sink regulation by sugar and stress. Current Opinions Plant Biol 2: 198-206

Roitsch T, Tanner W (1996) Cell wall invertase, bridging the gap. Botanica Acta 109: 90-93

Roitsch T, Bittner M, Godt DE (1995) Induction of apoplastic invertase of *Chenopodium rubrum* by D-glucose and a glucose analog and tissue-specific expression suggest a role in sink-source regulation. Plant Physiol 108: 285-294

Roitsch T, Ehneß R, Goetz M, Hause B, Hofmann M, Sinha AK (2000) Regulation and function of extracellular invertase from higher plants in relation to assimilate partitioning, stress responses and sugar signalling. Aust J Plant Physiol 27: 815-825

Ross HA, Davies HV (1992) Purification and characterization of sucrose synthase from the cotyledons of *Vicia faba* L. Plant Physiol 100: 1008-1013

Ross HA, Davies HV, Burch LR, Viola R, McRae D (1994) Developmental changes in carbohydrate content and sucrose degrading enzymes in tuberising stolons of potato (*Solanum tuberosum*). Physiologia Plantarum 90: 748-756

Ross HA, McRae D, Davies HV (1996) Sucrolytic enzyme activities in cotyledons of faba bean. Plant Physiol 111: 329-338

Roy S, Watado AE, Wergin WP (1997) Characterization of the cell wall microdomain surrounding plasmodesmata in apple fruit. Plant Physiol 114: 539-547

Ruan YL, Patrick JW (1995) The cellular pathway of post-phloem sugar transport in developing tomato fruit. Planta 196: 434-444

Ruan YL, Chourey PS (1998) A fibreless seed mutation in cotton is associated with lack of fibre cell initiation in ovule epidermis and alterations in sucrose synthase expression and carbon partitioning in developing seeds. Plant Physiol 118: 399-406

Ruan Y-L, Chourey PS, Delmer DP, Perez-Grau L (1997) The differential expression of sucrose synthase in relation to diverse patterns of carbohydrate partitioning in developing cotton seed. Plant Physiol 115: 375-385

Russell CR, Morris DA (1982) Invertase activity, soluble carbohydrates and inflorescence development in the tomato (*Lycopersicon esculentum* Mill.). Ann Bot 49: 89-98

Salas J, Salas M, Vinuela E, Sols A (1965) Glucokinase of rabbit liver. J Biol Chem 240: 1014-1018

Salerno G, Echeverria E, Pontis H (1996) Activation of sucrose-phosphate synthase by a protein-factor/sucrose-phosphate phosphatase. Cell Mol Biol 42: 665-672

Salisbury JL (1995) Centrin, centrosomes, and mitotic spindle poles. Current Opinions Cell Biol 7: 39-45

Sauer N, Tanner W (1993) Molecular biology of sugar transporters in plants. Botanica Acta 106: 277-286

Schaffer A, Petreikov M (1997a) Sucrose-to-starch metabolism in tomato fruit undergoing transient starch accumulation. Plant Physiol 113: 739-746

Schaffer A, Petreikov M (1997b) Inhibition of fructokinase and sucrose synthase by cytosolic levels of fructose in young tomato fruit undergoing transient starch synthesis. Physiologia Plantarum 101: 800-806

Schaffer AA, Aloni B, Fogelman E (1987a) Sucrose metabolism and accumulation in developing fruit of *Cucumis*. Phytochemistry 26: 1883-1887

Schaffer AA, Sagee O, Goldschmidt EE, Goren R (1987b) Invertase and sucrose synthase activity, carbohydrate status and endogenous IAA levels during *Citrus* leaf development. Physiologia Plantarum 69: 151-155

Schaffer AA, Petreikov M, Miron D, Fogelman M, Spiegelman M, Bnei-Moshe Z, Shen S, Granot D, Hadas R, Dai N, Levin I, Bar M, Friedman M, Pilowsky M, Gilboa N, Chen L (1999) Modification of carbohydrate content in developing tomato fruit. HortSci 34: 1024-1027

Schel JHN, Kieft H, van Lammeren AAM (1984) Interactions between embryo and endosperm during early developmental stages of maize caryopses (*Zea mays*). Can J Bot 62: 2842-2853

Schmülling T, Schäfer S, Romanov G (1997) Cytokinins as regulators of gene expression. Physiologia Plantarum 100: 505-519

Scholefield PB, Sedgley M, Alexander DMcE (1985) Carbohydrate cycling in relation to shoot growth, floral initiation and development and yield in avocado. Scientia Hort 25: 99-110

Schroeder CA (1953) Growth and development of 'Fuerte' avocado fruit. Proc Amer Soc Hort Sci 61: 103-109

Schroeder CA (1958) Growth and development of avocado fruit. C A Avo Soc Yrbk 42: 114-118

Schüssler JR, Brenner ML, Brün WA (1984) Abscisic acid and its relationship to seed filling in soybeans. Plant Physiol 76: 301-306

Schüssler JR, Brenner ML, Brün WA (1991) Relationship of endogenous abscisic acid to sucrose level and seed growth rate of soybeans. Plant Physiol 96: 1308-1313

Scora RW, Bergh B (1990) The origins and taxonomy of avocado (*Persea americana* Mill. Lauraceae). Acta Hort 275: 387-394

Secor J (1987) Regulation of sucrose efflux from soybean leaf discs. Plant Physiol 83: 143-148

Sedgley M, Griffin RA (1989) Sexual reproduction of tree crops. Academic Press, London. Pp 165-167. ISBN 0-12-634470-1

Sephton HH, Richtmyer NK (1963a) The isolation of a second octulose and of a heptose from the avocado: D-*glycero*-L-*galacto*-octulose and D-*glycero*-D-*galacto*-heptose. J Org Chem 28: 1691-1694

Sephton HH, Richtmyer NK (1963b) Isolation of D-*erythro*-L-*gluco*-nonulose from the avocado. J Org Chem 28: 2388-2390

Sephton HH, Richtmyer NK (1966) The isolation of D-*erythro*-L-*galacto*-nonulose from the avocado, together with its synthesis and proof of structure through reduction to D-*arabino*-D-*manno*-nonitol and D-*arabino*-D-*gluco*-nonitol. Carb Res 2: 289-300

Seymour GB, Tucker GA (1993) Avocado. *In*: Biochemistry of fruit ripening (Eds. Seymour GB, Taylor JE, Tucker GA). Chapman and Hall, London. Pp 53-81. ISBN 0-412-40830-9

Sgherri CLM, Pinzino C, Navari-Izzo F (1996) Sunflower seedlings subjected to stress by water deficit: Changes in $O_2^{\cdot-}$ production related to the composition of thylakoid membranes. Physiologia Plantarum 96: 446-452

Sgherri CLM, Maffei M, Navari-Izzo F (2000) Antioxidative enzymes in wheat subjected to increasing water deficit and rewatering. J Plant Physiol 157: 273-279

Shaw PE, Wilson III CW, Knight Jr RJ (1980) High-performance liquid chromatographic analysis of D-manno-heptulose, perseitol, glucose and fructose in avocado cultivars. J Agric Food Chem 28: 379-382

Sheen J (1994) Feedback control of gene expression. Photosynthesis Research 39: 427-438

Sheen J, Zhou L, Jang J-C (1999) Sugars as signalling molecules. Current Opinions Plant Biol 2: 410-418

Simon E, Kracier PF (1966) The blockage of insulin secretion by mannoheptulose [rat, rabbit]. Isr J Med Sci 2: 785-799

Sitrit Y, Riov J, Blumenfeld A (1988) Interference of phenolic compounds with the 1-aminocycloproane-1-carboxylic acid assay. Plant Physiol 86: 13-15

Slater G, Shankman S, Shepherd JS, Alfin-Slater RB (1975) Seasonal variation in the composition of California avocados. J Agric Food Chem 23: 468-474

Smeekens S (1998) Sugar regulation of gene expression in plants. Current Opinions in Plant Biol 1: 230-234

Smeekens S (2000) Sugar-induced signal transduction in plants. Annu Rev Plant Physiol Plant Mol Biol 51: 49-81

Smeekens S, Rook F (1997) Sugar sensing and sugar-mediated signal transduction in plants. Plant Physiol 115: 7-13

Smirnoff N, Cumbes QJ (1989) Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28: 1057-1060

Smith HB (1999) Sucrose synthase and the fruits of its labor. Plant Cell 11: 2261-2262

Sonesson A, Jantzen E, Tangen T (1994) Lipopolysaccharides of *Legionella erythra* and *Legionella oakridgensis*. Can J Microbiol 40: 666-671

Sonnewald U, Brauer M, von Schaewen A, Stitt M, Willmitzer L (1991) Transgenic tobacco plants expressing yeast-derived invertase in either the cytosol, vacuole or apoplast: a powerful tool for studying sucrose metabolism and sink/source interactions. Plant J 1: 95-106

Sowokinos JR, Lulai EC, Knoper JA (1985) Translucent tissue defects in *Solanum tuberosum* L. Plant Physiol 78: 489-494

Steinitz B (1999) Sugar alcohols display nonosmotic roles in regulating morphogenesis and metabolism in plants that do not produce polyols as primary photosynthetic products. J Plant Physiol 155: 1-8

Stephenson AG (1981) Flowers and fruit abortion: Proximate causes and ultimate functions. Annu Rev Ecol Syst 12: 253-279

Steyn EMA, Robertse PJ, Smith D (1993) An anatomical study of ovary-to-cuke development in consistently low-producing trees of 'Fuerte' avocado (*Persea americana* Mill.) with special reference to seed abortion. Sex Plant Reprod 6: 87-97

Stitt M (1996) Plasmodesmata play an essential role in sucrose export from leaves: A step toward an integration of metabolic biochemistry and cell biology. Plant Cell 8: 565-571

Stitt M, Steup M (1985) Starch and sucrose degradation. *In*: Higher plant cell respiration (Eds. Douce R, Day DA). Springer-Verlag, Berlin. Pp 347-390. ISBN 3-540-13935-4

Stommel JR (1992) Enzymic components of sucrose accumulation in the wild type tomato species *Lycopersicon peruvianum*. Plant Physiol 99: 324-328

Stommel JR, Simon PW (1990) Multiple forms of invertase from *Daucus carota* cell cultures. Phytochemistry 29: 2087-2089

Stone JM, Walker JC (1995) Plant protein kinase families and signal transduction. Plant Physiol 108: 51-57

Stoop JMH, Pharr DM (1994) Mannitol metabolism in celery stressed by excess macronutrients. Plant Physiol 106: 503-511

Stumpf PK (1980) Biosynthesis of saturated and unsaturated fatty acids. *In*: The biochemistry of plants – A comprehensive treatise. Vol 4. Lipids: structure and function (Ed. Stumpf PK). Academic Press, New York. Pp 177-204. ISBN 0-12-675404-7

Sturm A (1991) Heterogeneity of the complex N-linked oligosaccharides at specific glycosylation sites of two secreted carrot glycoproteins. Eur J Biochem 199: 169-179

Sturm A (1996) Molecular characterization and functional analysis of sucrose-cleaving enzymes in carrot (*Daucus carota* L.). J Exp Bot (special ed.) 47: 1187-1192

Sturm A (1999) Invertases. Primary structures, functions, and roles in plant development and sucrose partitioning. Plant Physiol 121: 1-7

Sturm A, Chrispeels MJ (1990) cDNA cloning of carrot extracellular β-fructosidase and its expression in response to wounding and bacterial infection. Plant Cell 2: 1107-1119

Sturm A, Tang G-Q (1999) The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. Trends in Plant Sci 4: 401-407

Sturm A, Sebkova V, Lorenz K, Hardegger M, Lienhard S, Unger C (1995) Development- and organ-specific expression of the genes for sucrose synthase and three isoenzymes of acid β-fructofuranosidase in carrot. Planta 195: 601-610

Sturm A, Hess D, Lee H-S, Lienhard S (1999) Neutral invertase is a novel type of sucrose-cleaving enzyme. Physiologia Plantarum 107: 159-165

Sugden C, Donaghy PG, Halford NG, Hardie DG (1999) Two SNF1-related protein kinases from spinach leaf phosphorylate and inactivate 3-hydroxy-3-methylglutaryl-coenzyme A reductase, nitrate reductase, and sucrose phosphate synthase in vitro. Plant Physiol 120: 257-274

Sun J, Loboda T, Sung SS, Black CC (1992) Sucrose synthase in wild tomato, *Lycopersicon chmielewskii*, and tomato sink strength. Plant Physiol 98: 1163-1169

Sung S-JS, Xu D-P, Black CC (1989) Identification of actively filling sucrose sinks. Plant Physiol 89: 1117-1121

Tang G-Q, Lüscher M, Sturm A (1999) Antisense repression of vacuolar and cell wall invertase intransgenic carrot alters early plant development and sucrose partitioning. Plant Cell 11: 177-189

Tauberger E, Hoffmann-Benning S, Fleischer-Notter H, Willmitzer L, Fisahn J (1999) Impact of invertase overexpression on cell size, starch granule formation and cell wall properties during tuber development in potatoes with modified carbon allocation patterns. J Exp Bot 50: 477-486

Tausz M, Wonisch A, Peters J, Jiménez, Morales D, Grill D (2001) Short-term changes in free radical scavengers and chloroplast pigments in *Pinus canariensis* needles as affected by mild drought stress. J Plant Physiol 158: 213-219

Taylor NJ, Cowan AK (2000) The role of cytokinin, auxin and abscisic acid metabolism in the control of Hass avocado fruit size. SA Avo Grower's Assoc Yrbk 23: 79-81

Taylor JS, Bhalla MK, Robertson JM, Piening LJ (1990) Cytokinins and abscisic acid in hardening winter wheat. Can J Bot 68: 1597-1601

Tegeder M, Wang X-D, Frommer WB, Offler CE, Patrick JW (1999) Sucrose transport into developing seeds of *Pisum sativum* L.). Plant J 18: 151-161

Terry BR, Robards AW (1987) Hydrodynamic radius alone governs the mobility of molecules through plasmodesmata. Planta 171: 145-157

Tholakalabavi A, Zwiazek JJ, Thorpe TA (1997) Osmotically-stressed poplar cell cultures: Anthocyanin accumulation, deaminase activity, and solute composition. J Plant Physiol 151: 489-496

Thompson GA, Schulz A (1999) Macromolecular trafficking in the phloem. Trends in Plant Sci 4: 354-360

Thomson WW, Platt-Aloia K (1985) The ultrastructure of the plasmodesmata of the salt glands of *Tamarix* as revealed by transmission and freeze-fracture electron microscopy. Protoplasma 125: 13-23

Thorne JH (1985) Phloem unloading of C and N assimilates in developing seeds. Annu Rev Plant Physiol 36: 317-343

Thorpe MR, Minchin PEH (1996) Mechanisms of long- and short- distance transport from sources to sinks. *In*: Photoassimilate distribution in plants and crops: Source-sink relationships (Eds. Zamski E, Schaffer AA). Marcel Dekker Inc., New York. Pp 261-282. ISBN 0-8247-9440-0

Tietz A, Ludwig M, Dingkuhn M, Dörffling K (1981) Effect of abscisic acid on transport of assimilates in barley. Planta 152: 557-561

Tingwa PO, Young RE (1975a) Studies on the inhibition of ripening in attached avocado (*Persea americana* Mill.) fruits. J Amer Soc Hort Sci 100: 447-449

Tingwa PO, Young RE (1975b) The effect of indole-3-acetic acid and other growth regulators on the ripening of avocado fruits. Plant Physiol 55: 937-940

Toldam-Anderson TB, Hansen P (1997) Growth and development in black currant (*Ribes nigrum*). III. Seasonal changes in sugars, organic acids, chlorophyll and anthocyanins and their possible metabolic background. J Hort Sci 72: 153-169

Tomer E, Gottreich M (1976) Defective ovules in avocado cultivars. J Amer Soc Hort Sci 101: 620-623

Toroser D, Huber SC (1997) Protein phosphorylation as a mechanism for osmotic-stress activation of sucrose-phosphate synthase in spinach leaves. Plant Physiol 114: 947-955

Trębacz K, Fensom DS, Harris A, Zawadzki T (1988) Transnodal transport of ^{14}C in *Nitella flexilis*. III. Further studies on dissolved inorganic carbon movements in tandem cells. J Exp Bot 39: 1561-1573

Trethewey RN, Riesmeier JW, Willmitzer L, Stitt M, Geigenberger P (1999) Tuber-specific expression of a yeast invertase and a bacterial glucokinase in potato leads to an activation of sucrose phosphate synthase and the creation of a sucrose futile cycle. Planta 208: 227-238

Trewavas A, Malhó R (1997) Signal perception and transduction: the origin of the phenotype. Plant Cell 9: 1181-1195

Tsay JS, Kuo CG (1980) Enzymatic activities of starch synthesis in potato tubers of different sizes. Physiologia Plantarum 48: 460-462

Tucker EB (1988) Inositol biphosphate and inositol triphosphate inhibit cell-to-cell passage of carboxyfluorescein in staminal hairs of *Setcreasea purpurea*. Planta 174: 358-363

Tucker EB (1993) Azide treatment enhances cell-to-cell diffusion in staminal hairs of *Setcreasea purpurea*. Protoplasma 174: 45-49

Tucker EB, Boss WF (1996) Mastoporan induced intracellular Ca^{2+} fluxes may regulate cell-to-cell communication in plants. Plant Physiol 111: 459-467

Turgeon R (1984) Efflux of sucrose from minor veins of tobacco leaves. Planta 161: 120-128

Turgeon R, Webb JA (1976) Leaf development and phloem transport in *Cucurbita pepo*: Maturation of the minor veins. Planta 129: 265-269

Turner A, Wells B, Roberts K (1994) Plasmodesmata of root tips: Structure and composition. J Cell Sci 107: 3351-3361

Turner A, Bacic A, Harris PJ, Read SM (1998) Membrane fractionation and enrichment of callose synthase from pollen tubes of *Nicotiana alata* Link et Otto. Planta 205: 380-388

Tymowska-Lalanne Z, Kreis M (1998) The plant invertases: Physiology, biochemistry and molecular biology. Advances in Botanical Research 28: 72-117

Unger C, Hardegger M, Lienhard S, Sturm A (1994) cDNA cloning of carrot (*Daucus carota*) soluble acid β-fructofuranosidase and the comparison with the cell wall isoenzyme. Plant Physiol 104: 1351-1357

Usuda H, Demura T, Shimogawara K, Fukuda H (1999) Development of sink capacity of the "storage root" in a radish cultivar with a high ratio of "storage root" to shoot. Plant Cell Physiol 40: 369-377

Valmayor RV (1967) Cellular development of the avocado from blossom to maturity. The Philippine Agriculturist 50: 907-976

van Bel AJE (1993) Strategies of phloem loading. Annu Rev Plant Physiol Plant Mol Biol 44: 253-281

van Bel AJE, Kempers R (1990) Symplastic isolation of the sieve element-companion cell complex in the phloem of *Ricinus communis* and *Salix alba* stems. Planta 183: 69-76

van Bel AJE, Patrick JW (1984) No direct linkage between proton pumping and photoassimilate unloading from seed coats of *Phaseolus vulgaris* L. Plant Growth Regul 2: 319-326

Van de Venter HA, Currier HB (1977) The effect of boron deficiency on callose formation and 14C translocation in beans (*Phaseolus vulgaris* L.) and cotton (*Gossypium hirsutum* L.). Am J Bot 64: 861-865

Van den Ende W, Van Laere A (1995) Purification and properties of a neutral invertase from the roots of *Cichorium intybus*. Physiologia Plantarum 93: 241-248

Van der Walt M, Davie SJ, Smith DG (1993) Carbohydrate and other studies on alternate bearing Fuerte and Hass avocado trees. SA Avo Growers' Assoc Yrbk 16: 82-85

van Handel E (1968) Direct microdetermination of sucrose. Anal Biochem 22: 280-283

van Niekerk W, Wolstenholme BN, Johnston MA (1999) Mulching and potassium relationships in Hass avocados to increase yield and fruit size. SA Avo Grower's Assoc Yrbk 22: 110-114

Van Rensburg E, Engelbrecht AHP (1986) Effect of calcium salts on susceptibility to browning of avocado fruit. J Food Sci 51: 1067-1070

Veisz O, Galiba G, Sutka J (1996) Effect of abscisic acid on the cold hardiness of wheat seedlings. J Plant Physiol 149: 439-443

Vickery ML, Vickery B (1981) Secondary plant metabolism. Macmillan Press Ltd, London. Pp 157-181, 192-203. ISBN 0-333-27017-7

Vioque A, Albi MA, Vioque B (1981) Role of IAA-oxidase in the formation of ethylene from 1-aminocyclopropane-1-carboxylic acid. Phytochemistry 20: 1473-1475

Vizzotto G, Pinton R, Varanini Z, Costa G (1996) Sucrose accumulation in developing peach fruit. Physiologia Plantarum 96: 225-230

von Schaewen A, Stitt M, Schmidt R, Sonnewald U, Willmitzer L (1990) Expression of a yeast-derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. EMBO J 9: 3033-3044

Wagner W, Wiemken A (1987) Enzymology of fructan synthesis in grasses: Properties of sucrose-fructosyltransferase in barley leaves (*Hordeum vulgare* L. cv. Gerbel). Plant Physiol 85: 706-710

Waigmann E, Turner A, Peart J, Roberts K, Zambryski P (1997) Ultrastructural analysis of leaf trichome plasmodesmata reveals major differences from mesophyll plasmodesmata. Planta 203: 75-84

Waldmann T, Jeblick W, Kauss H (1988) Induced net Ca^{2+} uptake and callose biosynthesis in suspension-cultured plant cells. Planta 173: 88-95

Walker RR, Hawker JS (1976) Effect of pollination on carbohydrate metabolism in young fruits of *Citrullus lanatus* and *Capsicum annum*. Phytochemistry 15: 1881-1884

Walker AJ, Thornley JHM (1977) The tomato fruit: Import, growth, respiration and carbon metabolism at different fruit sizes and temperatures. Ann Bot 41: 977-985

Walker RP, Winters AL, Pollock CJ (1997) Purification and characterization of invertases from leaves of *Lolium temulentum*. New Phytol 135: 259-266

Walker-Simmons M, Kudrna DA, Warner RL (1989) Reduced accumulation of ABA during water stress in molybdenum cofactor mutant of barley. Plant Physiol 90: 728-733

Wang N, Fisher DB (1994) Monitoring phloem unloading and post-phloem transport by microperfusion of attached wheat grains. Plant Physiol 104: 17-27

Wang F, Sanz A, Brenner ML, Smith AC (1993) Sucrose synthase, starch accumulation, and tomato fruit sink strength. Plant Physiol 101: 321-327

Wang F, Smith AC, Brenner M (1994) Temporal and spatial expression pattern of sucrose synthase during tomato fruit development. Plant Physiol 104: 535-540

Wang HL, Offler CE, Patrick JW (1995) Cellular pathway of photosynthate transfer in developing wheat grain. II. A structural analysis and histochemical studies of the transfer pathway from the crease phloem to the endosperm cavity. Plant Cell Environ 18: 373-388

Wang M, Oppedijk B, Lu X, Van Duijn B, Schilperoort R (1996) Apotosis in barley aleurone during germination and its inhibition by abscisic acid. Plant Mol Biol 32: 1125-1134

Warmbrodt RD (1987) Solute concentrations in the phloem and apex of the root of *Zea mays*. Amer J Bot 72: 394-402

Wearie PJ, Kekwick RGO (1975) The fractionation of fatty acid synthetase activities of mesocarp plastids. Biochem J 146: 439-445

Weber H, Borisjuk L, Heim U, Buchner P, Wobus U (1995) Seed coat-associated invertases of Faba bean control both unloading and storage functions: Cloning of cDNAs and cell type-specific expression. Plant Cell 7: 1835-1846

Weber H, Borisjuk L, Wobus U (1996) Controlling seed development and seed size in *Vicia faba*: A role for seed coat-associated invertases and carbohydrate state. Plant J 10: 823-834

Weber H, Borisjuk L, Heim U, Sauer N, Wobus U (1997a) A role for sugar transporters during seed development: Molecular characterization of a hexose and a sucrose carrier in fava bean seeds. Plant Cell 9: 895-908

Weber H, Borisjuk L, Wonus U (1997b) Sugar import and metabolism during seed development. Trends in Plant Sci 2: 169-174

Webster PL, Henry M (1987) Sucrose regulation of protein synthesis in pea root meristem cells. Environ Exp Bot 27: 253-262

Weil M, Rausch T (1990) Cell wall invertase in tobacco crown gall cells. Plant Physiol 94: 1575-1581

Weil M, Krausgrill S, Schuster A, Rausch T (1994) A 17-kDa *Nictotiana tabacum* cell-wall peptide acts as an in-vitro inhibitor of the cell-wall isoform of acid invertase. Planta 193: 438-445

Weise A, Barker L, Kühn C, Lalonde S, Buschmam H, Frommer WB, Ward JM (2000) A new subfamily of sucrose transporters, SUT4, with low affinity/high capacity localized in enucleate sieve elements of plants. Plant Cell 12: 1345-1355

Westgate ME, Boyer JS (1986) Reproduction at low silk and pollen water potentials in maize. Crop Sci 26: 951-956

Westwood JH, Yu X, Foy CL, Cramer CL (1998) Expression of a defence-related 3-hydroxy-3-methylglutaryl CoA reductase gene in response to parasitization by *Orobanche* spp. Mol Plant-Micro Interactions 11: 530-536

Whiley AW, Schaffer BS (1994) Avocado. *In*: CRC handbook of environmental physiology of fruit crops – Vol 2 (Eds. Schaffer B, Anderson PC). CRC Press, Boca Raton. Pp 3-35. ISBN 0-8496-0179-3

Whiley AW, Saranah JB, Wolstenholme BN, Rasmussen TS (1991) Use of paclobutrazol sprays at mid-anthesis for increasing fruit size and yield of avocado (*Persea americana* Mill. cv. Hass). J Hort Sci 66: 593-600

Whiley AW, Rasmussen TS, Saranah JB, Wolstenholme BN (1996) Delayed harvest effect on yield, fruit size and starch cycling in avocado (*Persea americana* Mill.) in subtropical environments. II. The late-maturing cv. Hass. Scientia Horticulturae 66: 35-49

White LM, Secor GE (1961) Microscopic identification of microgram quantities of mannoheptulose and sedoheptulose. Anal Chem 33: 1287-1288

White RG, Baldelt K, Overall RL, Vesk M (1994) Actin associated with plasmodesmata. Protoplasma 180: 169-184

Willenbrink J (1982) Storage of sugars in higher plants. *In*: Encyclopedia of plant physiology. Vol XIII/A. Plant carbohydrates I: Intracellular carbohydrates (Eds. Loewus FA, Tanner W). Springer, Berlin. Pp 684-699. ISBN 3-540-11060-7

Willison JHM (1976) Plasmodesmata: A freeze-fracture view. Can J Bot 54: 2842-2847

Wills RBH, Tirmazi SIH (1982) Inhibition of ripening of avocado with calcium. Scientia Horticulturae 16: 323-330

Winer L, Apelbaum A (1986) Involvement of polyamines in the development and ripening of avocado fruits. J Plant Physiol 126: 223-233

Winter L, Foster JG, Edwards GE, Holtum JAM (1982) Intracellular localization of enzymes of carbon metabolism in *Mesembryanthemun crystallinum* exhibiting C3 photosynthetic characteristics or performing Crassulacean acid metabolism. Plant Physiol 69: 300-307

Winter H, Huber JL, Huber SC (1998) Identification of sucrose synthase as an actin-binding protein. FEBS Lett 430: 205-208

Witt W, Sauter JJ (1995) *In vitro* degradation of starch grains by phosphorylases and amylases from poplar wood. J Plant Physiol 146: 35-40

Wittich PE, Graven P (1995). Histochemical study of the development of the phytomelan layer in the seed coat of *Gasteria verrucosa* (Mill.) H. Duval. Protoplasma 187: 72-78

Wittich PE, Graven P (1998) Callose deposition and breakdown, followed by phytomelan synthesis in the seed coat of *Gasteria verrucosa* (Mill.) H. Duval. Protoplasma 201: 221-230

Wolstenholme BN (1977) A simple climatic classification for tropical and subtropical areas and fruits in South Africa. Crop Production 6: 35-39

Wolstenholme BN (1990) Avocados and health issues. J SA Avo Growers' Assoc 10:1-2

Wolstenholme BN, Whiley AW, Saranah JB (1990) Manipulating vegetative:reproductive growth in avocado (*Persea americana* Mill.) with paclobutrazol foliar sprays. Scientia Hort 41: 315-327

Wrolstad RE (1976) Colour and pigment analyses in fruit products. Agric Exp Station Oregon State University Bulletin No. 624

Wu L-L, Song I, Karuppiah N, Kaufman PB (1993a) Kinetic induction of oat shoot pulvinus invertase mRNA by gravistimulation and partial cDNA cloning by polymerase chain reaction. Plant Mol Biol 21: 1175-1179

Wu L-L, Mitchell JP, Cohen NS, Kaufman PB (1993b) Gibberellin (GA_3) enhances cell wall invertase activity and mRNA levels in elongating dwarf pea (*Pisum sativum*) shoots. Int J Plant Sci 154: 280-289

Wyn Jones RG (1984) Phytochemical aspects of osmotic adaptation. *In*: Recent advances in Phytochemistry. Vol. 18. Phytochemical adaptations to stress (Eds. Timmermann BN, Steelink C, Loewus FA). Plenum Press, New York. Pp 55-78. ISBN 0-306-41720-0

Wyse RE, Zamski E, Tomos AD (1986) Turgor regulation of sucrose transport in sugar beet taproot tissue. Plant Physiol 81: 478-481

Xu D-P, Sung SS, Black CC (1989) Sucrose metabolism in lima bean seeds. Plant Physiol 89: 1106-1116

Xu J, Pemberton GH, Almira EC, McCarty DR, Koch KE (1995) The *Ivr1* gene for invertase in maize. Plant Physiol 108: 1293-1294

Yelle S, Chetelat RT, Dorais M, DeVerna JW, Bennet AB (1991) Sink metabolism in tomato fruit. IV. Genetic and biochemical analysis of sucrose accumulation. Plant Physiol 95: 1026-1035

Yahalom A, Warmbrodt RD, Laird DW, Traub O, Revel J-P, Willecke K, Epel BL (1991) Maize mesocotyl plasmodesmata proteins cross-react with connexin gap junction protein antibodies. Plant Cell 3: 407-417

Yahalom A, Lando R, Katz A, Epel BL (1998) A calcium-dependent protein kinase is associated with maize mesocotyl plasmodesmata. J Plant Physiol 153: 354-362

Young RE, Lee SK (1978) Avocado fruit maturity. CA Avo Soc Yrbk 62: 51-57

Young TE, Gallie DR (2000) Regulation of programmed cell death in maize endosperm by abscisic acid. Plant Mol Biol 42: 397-414

Yu S-M (1999) Cellular and genetic responses of plant to sugar starvation. Plant Physiol 121: 687-693

Zeevart JAD, Swanson CA, Felcows RJ, Ho LL, Shannon JC, Good NE (1979) Regulation of assimilate partitioning. *In*: Partitioning of assimilates: Summary reports of a workshop. May 7-9. Michigan State University, Michigan, USA

Zhang L, Cohn NS, Mitchell JP (1996) Induction of a pea cell wall invertase gene by wounding and its localized expression in phloem. Plant Physiol 112: 1111-1117

Zhou L, Jang J-C, Jones TL, Sheen J (1998) Glucose and ethylene signal transduction crosstalk revealed by *Arabidopsis* glucose-insensitive mutant. Plant Biol 95: 10294-10299

Zhu YJ, Komor E, Moore PH (1997) Sucrose accumulation in the sugar cane stem is regulated by the difference between the activities of soluble acid invertase and sucrose phosphate synthase. Plant Physiol 115: 609-616

Zieslin N, Khayat E (1990) Effects of alternating night temperature and cytokinin application on the activity of enzymes of sucrose metabolism in leaves, young shoots and petals of roses. Plant Cell Physiol 31: 845-849

Zilkah S, Klein I (1987) Growth kinetics and determination of shape and size of small and large avocado fruits cultivar 'Hass' on the tree. Scientia Horticulturae 32: 195-202

Zimmermann MH, Zeigler H (1975) List of sugars and sugar alcohols in sieve-tube exudates. *In*: Encyclopedia of plant physiology – New series. Vol. 1 (Eds. Zimmermann M, Milburn J). Springer-Verlag, Berlin. Pp 480-503. ISBN 3-540-07314-0

Zinselmeier C, Westgate ME, Schussler JR, Jones RJ (1995) Low water potential disrupts carbohydrate metabolism in maize (*Zea mays* L.) ovaries. Plant Physiol 107: 385-391

Zinselmeier C, Jeong B-R, Boyer JS (1999) Starch and the control of kernel number in maize at low water potentials. Plant Physiol 121: 25-35

Zobel A, Nighswander JE (1990) Accumulation of phenolic compounds in the necrotic areas of Austrian and red pine needles due to salt spray. Ann Bot 66: 629-640

Zrenner R, Salanoubat M, Willmitzer L, Sonnewald U (1995) Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.). Plant J 7: 97-107

Zrenner R, Schüler K, Sonnewald U (1996) Soluble acid invertase determines the hexose-to-sucrose ratio in cold-stored potato tubers. Planta 198: 246-252

APPENDICES

APPENDIX A

STANDARD PROCEDURES NOT COVERED IN DETAIL IN MATERIALS AND METHODS

A.1 Wax embedding for light microscopy

Fruit tissue was sectioned into 4×4×4 mm blocks and placed in F.A.A. solution for at least 24 h at 4°C. The samples were then removed from F.A.A. and placed in 50% alcohol solution (Table A.1) for 2 h and in 70% alcohol overnight. The tissue was dehydrated through 85, 95 and 100% alcohol for 2 h at each stage, placed in pure tertiary butanol for 2 h and then put in fresh tertiary butanol overnight The dehydrated material was placed in liquid paraffin: tertiary butanol (1:1) for 24 h. After this period small glass vials were half filled with molten paraffin wax, allowed to cool until the surface solidifies, the sample in the liquid paraffin:tertiary butanol (± equal volume to the paraffin wax) was poured on top of the wax and the vial placed in an oven at 62°C for several h, allowing the sample to sink into the wax slowly. The molten wax was decanted off, replaced with fresh molten paraffin wax and the vial left open in the oven overnight, allowing any remaining solvent to evaporate off. On a clean wooden board brass L-pieces were arranged to give 10×20 mm wells, which were rapidly filled with molten wax. The bottom of the wax block was allowed to cool and the sample carefully positioned with a hot needle, two per block, to give a sample in a 10×10 mm block. The wax block was allowed to cool for a few min, picked up and gently dropped onto the board to release the brass L-pieces and the wax blocks then allowed to cool for several h. The blocks were cut into two and shaved with a scalpel to give a small flat topped four sided pyramid with the sample located at the top. The base of this was melted with a flat metal object and mounted onto wooden stubs.

Table A.1. Alcohol dehydration series for wax embedding.

% alcohol	Water (mL)	ethanol (mL)	tertiary butanol (mL)
50	52	38	10
70	32.5	47.5	20
85	17.5	47.5	35
95	2.2	42.8	55
100	0	25	75

The wax blocks were sectioned to give continuous wax ribbons. These ribbons were picked up with a fine paint brush and placed on a drop of 37% (w/v) formaldehyde on a clean glass slide evenly smeared with a very thin layer of Haupt's adhesive. The slides were then placed on a slide drying bench (Electrothermal, Southend, England) at 40-50°C and allowed to spread evenly, fix and dry overnight.

A.2 Rehydration and mounting of wax embedded sections

Wax embedded sections had to be dewaxed and rehydrated before staining. The slides were immersed twice in clean 100% xylene for 10 min, xylene:ethanol (1:1) for 5 min and then put through an alcohol rehydration series at 100, 80, 70, 50 and 0% (v/v) ethanol, for 10 min at each step. Sections were taken at the 70% (v/v) ethanol stage for alcoholic stains, and at the 0% (v/v) ethanol stage for aqueous stains. Alcoholic stage sections were stained as described in 2.8.2 and a drop of Canada balsam, diluted with xylene, was placed on the section and a glass cover slip gently laid on this, taking care not to trap any air bubbles or to move the cover from side to side and so damage the section. The slide was dried overnight at about 30°C on the drying rack and viewed.

B.3 Epon-Araldite resin embedding for transmission electron microscopy

Fruit tissue was fixed for a minimum of 56 h in 3% (m/v) glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). Fixed material was washed twice for 30 min with 0.05 M sodium cacodylate buffer (pH 7.2) and post-fixed, to stain phospholipids and fats, with 2% (w/v) osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2) for 1 h. The specimen was washed twice for 30 min each time with buffer and block stained, to stain proteins and lipids, with freshly prepared 2% (m/v) uranyl acetate in 2% (v/v) ethanol for 45 min. The specimen was washed with double-distilled water twice for 10 min and dehydrated through 10, 20, 30, 40, 50, 60 and 70% (v/v) ethanol, for 10 min at each level. At 70% (v/v) ethanol the sample was left overnight; the following day the sample was dehydrated (10 min each step) through 80, 90, 100% (v/v) ethanol, with three changes at 100% (v/v) ethanol. This dehydration was followed by two 30 min changes in 100% propylene oxide. The tissue was then embedded in increasing Epon-Araldite resin concentrations (25, 50, 75% (v/v) made up with propylene oxide) for 2 h each time, with 1 mL of DMP added for each mL of resin used. The infiltrated tissue was placed in fresh 75% (v/v) Epon-Araldite resin, 25% (v/v) propylene oxide with DMP and left in the fume cupboard with the vial caps off overnight. The next day the resin was replaced with 100% Epon-Araldite resin with DMP

and left for 24 h (with the caps on). After this period the resin was replaced with fresh 100% resin with DMP for 6 h and the tissue sections removed gently with a sharpened wooden rod and placed in small aluminium dishes containing fresh 100% resin and DMP, and polymerised at 70°C for 48 h.

A.4 Spurr's resin embedding for transmission electron microscopy

For embedding by Spurr's the fixation and dehydration described for Epon-Araldite resin was followed until the resin embedding stage. Once the tissue had been dehydrated to 100% (v/v) ethanol, the tissue was embedded in increasing Spurr's resin concentrations (25, 50, 75% (v/v) made up with 100% (v/v) ethanol) for 2 h each time. The tissue was placed in fresh 75% (v/v) Spurr's, 25% (v/v) ethanol and left in the fume cupboard with the vial caps off overnight. The next day the resin was replaced with 100% Spurr's and left for 24 h (with the caps on). After this period the Spurr's was replaced with freshly made up Spurr's for 6 h and the tissue sections removed gently with a sharpened wooden rod and placed in small aluminium dishes containing freshly made up 100% Spurr's and polymerised at 70°C for 16 h.

B.5 LR White resin embedding for transmission electron microscopy

LR White resin embedding for immunolabelling of callose was adapted from the methods of Elliott (1993), Newman and Hobot (1993) and Enkerli *et al.* (1997).

Fruit was harvested with a 15-20 cm portion of branch attached to the pedicel and placed on ice to reduce possible stress responses involving the synthesis of callose. The fruit was split into seed, endosperm, seed coat and mesocarp tissue, cut into 1×2×3 mm blocks and placed into 0.05 M sodium cacodylate buffer (pH 7.2) containing 4% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde for at least 24 h. The fixed tissue was washed twice for 30 min in 0.05 M sodium cacodylate buffer (pH 7.2), dehydrated through 10, 20, 30, 40, 50 and 60% (v/v) ethanol for 10 min at each stage and then placed in 70% (v/v) ethanol overnight. The next day the tissue was dehydrated at 80, 90, 100 and 100% (v/v) ethanol for 30 min at each step. The tissue blocks were then transferred to increasing amounts of LR White resin in ethanol (1:2 for 2 h; 1:1 for 2 h; 2:1 for 2 h) and then placed in pure resin overnight at room temperature. The next day fresh resin was dispensed into '0'-gauge gelatine capsules (Eli Lilly (S.A.) Pty. Ltd., Isando, R.S.A.) and the tissue blocks placed on top of the resin (taking care to minimize resin carry-over), and allowed to sink to the bottom. The caps were closed and two small holes punched into the top of the capsule

using a 0.5×16 mm needle. Through the lower hole fresh resin was injected (1 mL Terumo® Syringe, Tokyo, Japan) until it flowed out the top. Care was taken to ensure the holes were as small as possible and as close to the highest point of the capsule as possible to ensure minimal air being trapped in the capsule, as aerobic conditions inhibit polymerisation. The capsules were polymerised at 50°C for 24 h. The blocks were stored in sealed vials containing silica desiccant beads at -18°C to preserve immunoreactivity.

A.6 Critical point drying for scanning electron microscopy

Samples dehydrated to 100% (v/v) ethanol were placed in baskets submerged in 100% (v/v) ethanol. The CPD was cooled to -10°C and flushed with liquid CO_2. The Baskets in the ethanol were sealed in the CPD, flushed three times with liquid CO_2 to remove the ethanol, the chamber was flooded with liquid CO_2 and left to stand for 1 h. The chamber was flushed again with liquid CO_2, the CO_2 source closed and the chamber heated to 35°C. The CO_2 gas was leaked slowly from the chamber over a period of 30 min until the chamber pressure reached 0 Kgf cm^{-2}, after which the sample was taken out and stored in a desiccator until mounted.

A.7 Sputter coating of samples of scanning electron microscopy

Mounted dehydrated samples were inserted into the bell jar on the specimen stage of the sputter coater and the target length from the cathode (gold:palladium (40:60)) to the anode (specimen) adjusted to 4 cm. The bell jar was sealed and evacuated to 5×10^{-2} torr, flushed with argon, pumped down and flushed again. The voltage was then turned on to 2.2 kV for 2-3 min and the plasma current maintained at 10 mA by adjusting the argon leak valve. After this period the voltage was turned off, air leaked into the jar and the samples removed and viewed.

A.8 Tricine gels for the electrophoretical separation of membrane proteins

The glass/aluminium plates were clamped together and, in the case of the Mighty Small vertical slab unit, the bottom space sealed with 1% (m/v) agarose at 60°C and allowed to solidify. The running gel (10% acrylamide) was prepared by mixing 5 mL 3 M Tris-HCl buffer (pH 8.45) containing 0.3% (m/v) SDS, 3 mL solution of 49.5% (m/v) acrylamide and 3% (m/v) bis-acrylamide, 50 μL freshly prepared 10% (m/v) ammonium persulfate, 7.5 μL TEMED and 7 mL ultra-pure water, poured to 3 cm from top of plates, overlaid with ultra-pure water and allowed to polymerise for 1 h. The stacking gel was then prepared by mixing 1.5 mL 3 M Tris-HCl buffer

(pH 8.45) containing 0.3% (m/v) SDS, 0.5 mL solution of 49.5% (m/v) acrylamide and 3% (m/v) bis-acrylamide, 30 μL freshly prepared 10% (m/v) ammonium persulfate, 15 μL TEMED and 4 mL ultra-pure water. The water overlying the running gel was discarded, the stacking gel poured in its place, the combs placed in, taking care not to trap air and the gel allowed to set for 30 min. The combs were removed, the wells rinsed with distilled water and the top chamber of the unit (the cathode) filled with 100 mM Tris-HCl buffer (pH 8.25) containing 100 mM tricine and 0.1% (m/v) SDS. The lower chamber (the anode) was filled with 200 mM Tris-HCl buffer (pH 8.9), 5-10 μL membrane extract was loaded into each well and the gel run at 80 V until the proteins had stacked evenly at the base of the stacking gel. Thereafter the voltage was increased to 100 V and the unit run for 30 min after the tracker dye had reached the end of the gel (total run time 2.5-3 h).

A.9 Silver staining of proteins on tricine gels

After separation of proteins, the gels were removed and fixed in a solution consisting of 50% (v/v) methanol, 12% (v/v) acetic acid and 0.5% (v/v) formaldehyde overnight on a gentle shaker (IKA-Vibrax-VXR, IKA® Labortechnik, Staufen, Germany). The gels were then washed 3×20 min with 50% ethanol, soaked in 0.02% (m/v) $Na_2S_2O_3.5H_2O$ for 1 min, washed 3×20 sec in water, soaked in 0.2% (m/v) $AgNO_3$ in 0.75% (v/v) formaldehyde for 23 min, washed 2×20 sec in water and soaked in 6% (m/v) Na_2CO_3, 0.0004% (m/v) $Na_2S_2O_3.5H_2O$ in 0.5% (v/v) formaldehyde until all the expected bands were clearly visible. The stained gels were rinsed in distilled water and the development stopped by placing in 50% (v/v) methanol, 12% (v/v) acetic acid solution for 10 min. The gels were washed in 50% (v/v) methanol for 10 min and observed.

Gels could be preserved for several months by sealing in clear polyethylene bags containing a little 50% (v/v) methanol to prevent drying out.

APPENDIX B

PAPERS PUBLISHED

Campbell T, Richings EW, Cripps RF, Taylor NJ, Cowan AK (2000) The plant SnRK1 complex: sugar sensing and cell metabolism in avocado fruit growth. S Afr J Bot 66: 104-111

Cowan AK, Richings EW, Cripps RF, Cairns ALP (1998) Metabolic control of Hass avocado fruit growth. SA Avo Growers' Assoc Yrbk 21: 48-51

Cowan AK, Cripps RF, Richings EW, Taylor NJ (2001) Fruit size: towards an understanding of the metabolic control of fruit growth using avocado as a model system. Physiologia Plantarum 111: 127-136

Cripps RF, Cowan AK (2000) Sucrose movement and metabolism in small Hass avocado fruit. SA Avo Growers' Assoc Yrbk 23: 63-69

Cripps RF, Richings EW, Taylor NJ, Cowan AK (1999) The Hass small fruit syndrome: solving a 50 million rand per season problem. SA Avo Growers' Assoc Yrbk 22: 1-6

Richings EW, Cripps RF, Cowan AK (2000) Factors affecting 'Hass' avocado fruit size: carbohydrate, abscisic acid and isoprenoid metabolism in normal and phenotypically small fruit. Physiologia Plantarum 109: 81-89

APPENDIX C

RESEARCH PAPERS PRESENTED

1. Cripps R (1999) Sugar metabolism in normal and small 'Hass' fruit. South African Avocado Growers Association Research Symposium. Magoebaskloof, South Africa.
2. Cripps RF, Cowan AK (1999) The role of seed coat ultra-structure and callose in 'Hass' avocado fruit development. 8th Congress of the South African Society of Horticultural Science. Stellenbosch, South Africa.
3. Cripps R, Cowan AK (2000) Solute allocation in 'Hass' avocado – an overview. South African Avocado Growers Association Research Symposium. Nelspruit, South Africa.
4. Cripps RF, Cowan AK (2000) The possible role of callose in symplastic solute transport. 26th Annual Conference of the South African Association of Botanists. Potchefstroom, South Africa.
5. Cripps R, Richings EW, Cowan AK (1998) The distribution and activity of sugar metabolising enzymes in phenotypically normal and small 'Hass' avocado fruits. 25th Annual Conference of the South African Association of Botanists. Cape Town, South Africa.
6. Dennison T, Taylor NJ, Cripps RF, Richings EW, Cowan AK (1999) Factors affecting fruit growth in 'Hass' avocado. 4th World Avocado Congress. Uruapan, Mexico.
7. Richings EW, Cripps RF, Cowan AK (1998) Hormone and sugar signals interact to regulate avocado fruit growth. 16th International Conference on Plant Growth Substances. Chiba, Japan.
8. Richings EW, Cripps RF, Cowan AK (1998) The role of isoprenoid compounds in avocado fruit growth and development: mediated by sugar content and composition. 25th Annual Conference of the South African Association of Botanists. Cape Town, South Africa.