ASPECTS OF AVOCADO FRUIT GROWTH AND DEVELOPMENT: TOWARDS UNDERSTANDING THE 'HASS' SMALL FRUIT SYNDROME

By
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ABSTRACT

Persea americana Mill. cv. Hass is predisposed towards producing a high proportion of undersized fruit. Reasons for phenotypically small 'Hass' fruit are obscure, but it does appear to be aggravated by adverse growing conditions. A detailed study of the metabolic control of avocado fruit growth was carried out to determine the underlying physiological reasons for the appearance of the 'Hass' small fruit phenotype. Furthermore, the application of a mulch was evaluated as a possible management strategy to increase 'Hass' fruit size.

Anatomical and morphological comparisons were made between normal and small 'Hass' fruit in an attempt to characterise the 'Hass' small fruit phenotype. Small fruit always contained a degenerate seed coat and fruit size was closely correlated with seed size. Kinetic analysis of changes in cell number and size during fruit development revealed that growth was limited by cell number in phenotypically small fruit. Analysis of endogenous isopentenyladenine (iP) and abscisic acid (ABA) revealed that ABA concentration was negatively correlated with size of similarly aged fruit. Calculation of the iP:ABA ratio showed a linear relationship with increasing fruit size. Qualitative and quantitative differences in mesocarp sterol composition were observed between normal and phenotypically small fruit.

Both the normal and small-fruit phenotypes were used to probe the interaction between end-products of isoprenoid biosynthesis and activity of mesocarp 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) in the metabolic control of avocado fruit growth. In phenotypically small fruit, a 70% reduction in microsomal HMGR activity was associated with a substantial rise in mesocarp ABA concentration at all stages of development. Application of mevastatin, a competitive inhibitor of HMGR, via the pedicel reduced growth of phenotypically normal fruit and increased mesocarp ABA concentration. These effects were reversed by co-treatment of fruit with either mevalonate, iP or the synthetic cytokinin (CK) analogue, N-(2-chloro-4-pyridyl)-N-phenylurea, but were unaffected by gibberellic acid. Likewise, in vivo application of ABA reduced fruit growth and HMGR activity, and accelerated abscission at all stages of development, effects that were reversed by co-treatment with iP. In contrast, the effect of sterols on mevastatin-induced inhibition of fruit growth was temporally different. Application of either stigmasterol or cholesterol during phase I caused a decline in growth, accelerated fruit abscission and

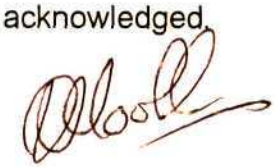
exacerbated the effects of mevastatin whereas during phase II and III, stigmasterol reversed inhibition of fruit growth. Stigmasterol did not however, reverse the inhibitory effect of mevastatin on HMGR activity - presumably as a result of mevastatin-induced increased endogenous ABA. It was therefore concluded that ABA accumulation down-regulates mesocarp HMGR activity and that *in situ* CK biosynthesis modulates the effect of ABA during phase I of fruit growth whereas, both CK and sterols perform this function during the later stages to sustain the developmental programme.

The effect of an altered CK:ABA ratio on solute allocation, cell-to-cell communication and plasmodesmatal structure was investigated in 'Hass' avocado fruits to determine the relationship between a change in hormone balance and expression of phenotypically small fruit. Exogenous application of ABA induced early seed coat senescence and retarded fruit growth, and these effects were negated in fruit co-injected with ABA and iP. The underlying physiological mechanisms associated with ABA-induced retardation of 'Hass' avocado fruit growth included: diminution of mesocarp and seed coat plasmodesmatal branching; gating of mesocarp and seed coat plasmodesmata by deposition of apparently proteinaceous material in the neck region; abolishment of the electrochemical gradient between mesocarp and seed coat parenchyma; and arrest of cell-to-cell chemical communication. In addition, solute allocation in ABA-treated fruit resembled closely that of phenotypically small fruit confirming that elevated ABA concentration had contributed to the decline in postphloem symplastic continuity.

In a field trial in the KwaZulu-Natal midlands, root growth was substantially increased throughout three seasons by the application of a coarse composted pinebark mulch. Mulching resulted in a significant 6.6% increase in mean fruit mass, in spite of 14.7% more fruits per tree. The combined effect was a 22.6% increase in overall yield. Differences in productivity between treatments closely correlated to levels of bark carbohydrate reserves. Data collated during this study to suggest that mulching at least partly ameliorated tree stress included: a reduction in the incidence of premature seed coat senescence and pedicel ring-neck, both of which are considered to be advanced symptoms of the stress syndrome; a lowering of mean foliage temperatures; and a reduction in the degree of photoinhibition during the heat of the day.

DECLARATION

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



Signed: _____

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For my Mum and Dad who have given me so much opportunity, love and support over the years

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CHAPTER 1

GENERAL INTRODUCTION

The avocado (*Persea americana* Mill.) is a member of *Lauraceae* and falls under the valid genus *Persea* (Scora and Bergh, 1990). *Persea americana* originates from central America and Mexico, though its precise origin is obscure due to its long history of utilization (Whiley and Schaffer, 1994). Three distinguishable ecological races, viz. Mexican, Guatamalan and West Indian have been identified and named after their presumed centres of origin (Storey *et al.*, 1986). These races freely hybridize, giving rise to a variety of genotypes with adaptation from cool, semi-arid to hot, humid tropical lowland climates (Whiley and Schaffer, 1994). The 'Hass' cultivar is a hybrid clone, and is derived from the Guatamalan (85 - 90%) and Mexican (10 - 15%) races (Bergh and Ellstrand, 1986). Botanically, the avocado fruit is described as a berry with a thick fleshy pericarp (consisting of three distinct regions viz. exocarp, mesocarp and endocarp) surrounding a single large seed (Valmayor, 1967). The fruit is pyriform or glabose in shape and has a yellow-green to purple-black skin which can be smooth or warty (Whiley and Schaffer, 1994).

By 1991 'Hass' accounted for approximately 35% of avocado fruit production in South Africa and has since become increasingly important to the local industry (van Zyl and Ferreira, 1995). This cultivar is utilized in nearly all sub-tropical avocado producing regions because of its higher yield potential and superior quality fruit. Furthermore, 'Hass' is a late-maturing cultivar and can therefore be used by growers to extend the avocado harvest period, which has obvious economic implications. Unfortunately, this cultivar has a tendency to bear a large number of phenotypically small fruit (less than 200 g at harvestable maturity). The 'Hass' small fruit syndrome is estimated to cost the South African industry between R30 and R40 million in lost revenue each year (Wolstenholme[1], *pers. comm.*), and hence there is some urgency within the industry to reduce the extent of this problem.

[1] B.N. Wolstenholme, Dept. Hort. Science, University of Natal, Pietermaritzburg, South Africa

1.1 AVOCADO FRUIT DEVELOPMENT

The development of fleshy fruit was last comprehensively reviewed twenty years ago (Coombe, 1976) while fruit development in avocado, has been reviewed as recently as 1988 (Bower and Cutting, 1988). Since that time, very little progress has been made in our understanding of fruit developmental programmes and in particular that of avocado. Nevertheless, it is now accepted that fruit morphogenesis can generally be divided into three distinct phases (Gillaspy *et al.*, 1993). The first phase includes ovary development, fertilization and fruit set; the second, cell division, seed formation and early embryo development; and the third, cell expansion and embryo maturation.

1.1.1 Ovary development, fertilization and fruit set

Nothing is known about the molecular signals that control ovary development in avocado although, it would be expected that both spatial and temporal molecular interaction between cells/tissues of the developing structure would occur. In avocado, flowering behaviour is described as "complementary synchronous dichogamy" where each flower opens twice on consecutive days, and is an adaptation to promote cross pollination. At the first opening the flower is functionally pistillate (i.e. the stigma is receptive and no pollen is shed), and at the second opening the flower is functionally staminate (i.e. the stigma is deteriorated and the stamens are pollen shedding) (Whiley and Schaffer, 1994). 'Hass' is classified as having a type "A" dichogamy pattern where flowers open as females in the morning of the first day and as males during the afternoon of the following day (Bergh, 1986). Irradiance (light quality and quantity), temperature and plant water status seem to be important abiotic factors in determining floral function in the avocado (Whiley and Schaffer, 1994). Alterations in photoperiod influence the dichogamy of the floral cycle (Sedgley, 1985) while low temperatures appear to inhibit pollen tube development (Sedgley, 1977; 1979). It is therefore not unreasonable to expect abiotic stimuli to exert an effect on floral development through changes in concentration of endogenous signals. However, the identity of these endogenous signals remains obscure.

Fruit set follows successful completion of pollination and fertilization. Anatomical

studies have revealed that within 3 days of pollination, initiation of endosperm formation has occurred and by 9 days, the endosperm has formed a large cellular body and a pre-embryo of two to six cells is evident (Tomer and Gazit,1979). These early stages of avocado fruit development are schematically illustrated in Figure 1.1. What is immediately evident from the studies of Tomer and Gazit (1979) is that the early stages of cell division appear anticlinal and are followed primarily by periclinal cell divisions. This suggests both spatial and temporal control of cell division in developing avocado fruit. The early anticlinal divisions presumably result in formation of ground tissue to support embryo development, suggesting that formation of a pre-embryo is required as the possible source of chemical trigger(s) needed for extension/expansion growth of developing fruit.

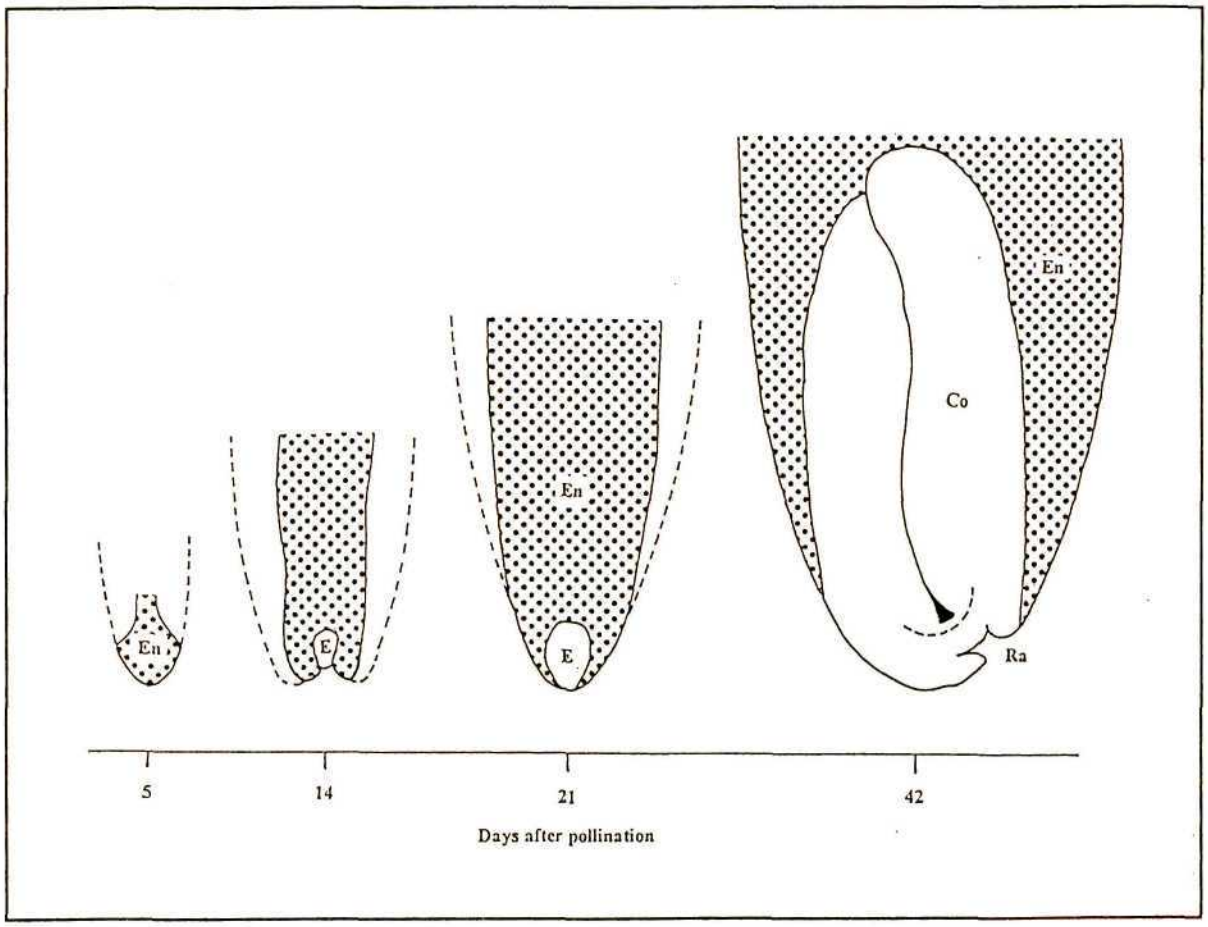

Figure 1.1

Schematic illustration of early stages of avocado fruit development (E = embryo; En = endosperm; Co = cotyledon; Ra = radicle) (adapted from Tomer and Gazit, 1979).

1.1.2 Fruit growth

Avocado fruit growth follows a single sigmoid curve (Valmayor, 1967; Lee and Young, 1983; Muñoz-Perez *et al.*, 1987) in which the lag phase persists for approximately 10 weeks after full bloom. The exponential or rapid growth phase lasts for about 30 weeks after full bloom, although it does depend on cultivar and environment, and is followed by a mature phase during which growth slows.

Initiation of fruit development in angiosperms is considered to involve both auxins and gibberellins (GAs) produced during pollen-tube growth while a secondary stimulus, apparently produced in the developing seed, is required to maintain growth (Lee, 1987). Avocado seeds must therefore play an important role in the development of fruit (Blumenfeld and Gazit, 1970; Gazit and Blumenfeld, 1970; Wolstenholme *et al.*, 1985; Bower and Cutting, 1988). For example, seed-bearing avocado fruits are many times larger than parthenocarpic fruit (Blumenfeld and Gazit, 1974) and a close correlation between seed size and fruit size has been observed in avocado (Wolstenholme and Whiley, 1995). Seeds contribute to fruit growth and development by synthesizing and/or accumulating growth promoting substances and nutrients (Blumenfeld and Gazit, 1970; Cannell, 1985; Wolstenholme *et al.*, 1985).

Cell number and size influence the capacity of developing fruits to import assimilate, and therefore contribute directly to fruit growth (Bohner and Bangerth, 1988a; 1988b). The dynamics of avocado fruit growth can be related to the rate of cell division and cell expansion in mesocarp tissue (Schroeder, 1960). Unlike most sub-tropical fruits, cell division in avocado mesocarp proceeds throughout fruit development (Schroeder, 1953; 1958; Coombe, 1976), albeit at a slower rate during the later stages of this programme. During the exponential growth phase, rate of cell division is at a maximum (Schroeder, 1953). Schroeder (1953) observed that different sizes of horticulturally mature avocado fruit comprised similarly sized cells, and concluded that differences in fruit size appeared to be a consequence of cell number. This latter observation suggests that the impact of cell division on final fruit size of avocado is determined by the number of cell divisions that occur, particularly during the early stages of fruit development.

1.1.3 Regulation of fruit development

Control of fruit size requires maximisation of cell division and expansion during the developmental programme (Valmayor, 1967; Coombe, 1976) and any reduction in availability of required resources will impact on fruit growth. Of the requirements for fruit growth and development, photoassimilate, mineral nutrients and adequate water availability are amongst the most important. In addition, developmental programmes such as fruit growth are known to be co-ordinated, at least in part, by plant hormones which act either directly or indirectly to alter gene expression. However, appreciation of the pleiotropic effects of plant hormones suggests that no single growth regulator can account for a complex process such as fruit morphogenesis (Trewavas, 1980; 1983) and it is now generally accepted that hormones exert multiple control on development through changes in endogenous concentration and via alterations in sensitivity of developing tissue to respective plant hormones (Trewavas, 1982; 1991; Firn; 1986).

With regard to the latter, a quantitative model has recently been proposed to account for the wide range of hormone-induced phenomena that occur during growth and development (Bradford and Trewavas, 1994). In view of this, it becomes apparent that development must be considered the result of intricate spatial and temporal interactions between the resources required for growth and hormonal mediation through the regulation of gene expression (Barendse and Peeters, 1995). Even so, the fruit developmental programme remains obscure. As stated by Gillaspy *et al.* (1993) - "Despite centuries of intensive genetic selection of agriculturally valuable fruit, we still lack most information about how fruits develop, how this development is coordinated with embryonic development and seed formation, and the molecular, cellular, and physiological events that control fruit growth and differentiation."

Figure 1.2 illustrates the temporal changes in phytohormone content and shows that auxins, cytokinins (CKs) and GAs are required for most of the normal course of fruit development, but their function during ovary development prior to fertilization is poorly understood (Gillaspy *et al.*, 1993). Positive growth stimuli, including auxins and GAs, are produced by pollen during pollen tube growth and influence fruit set (Nitsch, 1970). Poor pollination leads to incomplete fruit set which in turn results in undersize fruits or

ovary abortion (Nitsch, 1970). Wolstenholme *et al.* (1985) showed that mesocarp auxin concentrations of 'Fuerte' avocado fruit were highest early in the developmental programme and then steadily decreased towards maturity. Blumenfeld and Gazit (1972) noted high levels of GA activity in seed and seed coat of developing avocados, the latter decreasing with fruit growth, but no measureable GA-like activity in fruit flesh.

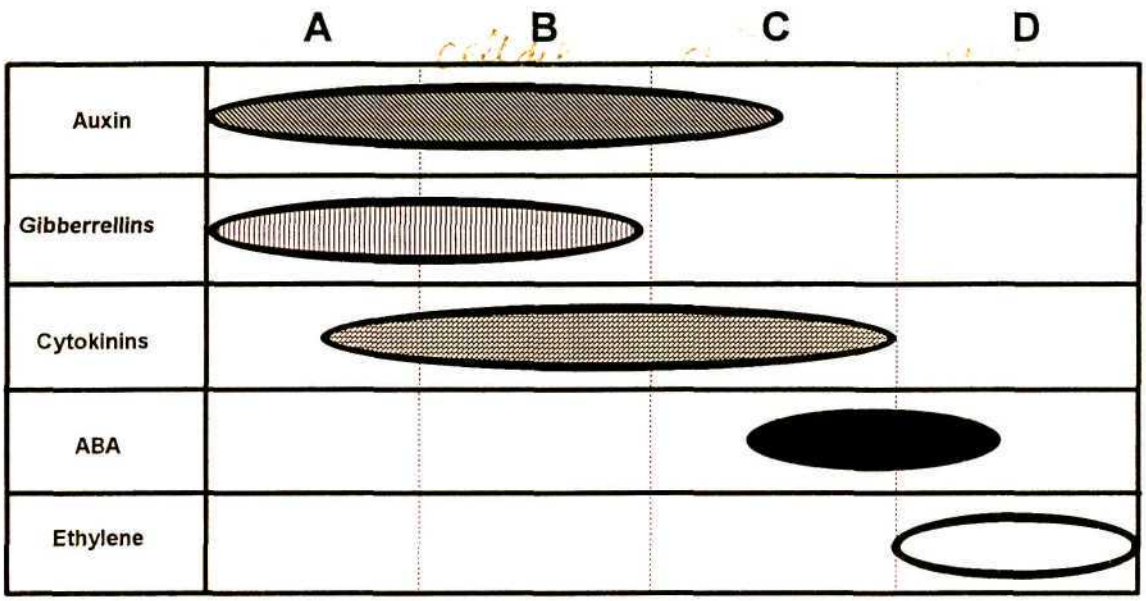

Figure 1.2

Changes in the concentration of plant growth substance during fruit development of the avocado (A = flowering and fruit set; B = cell division; C = cell expansion; and D = ripening). Adapted from Wolstenholme *et al.*, 1985; Blumenfeld *et al.*, 1986; Cutting *et al.*, 1986; Donkin, 1995.

Cell number is a function of the number of mitotic divisions and is balanced in the cell cycle by both differentiation and dedifferentiation. It is generally accepted that CKs contribute to the control of cell division in plants (Lee, 1987), although synergistic relationships between CKs and other promotive plant growth substances (PGS) have been observed (Ferreira *et al.*, 1994), e.g. freshly isolated protoplasts can be stimulated to divide if provided with adequate concentrations of both auxin and CK (Binns, 1994). A good correlation exists between CK concentration in developing *Lycopersicon* seeds and cell division activity in surrounding tissue (Abdel-Rahman, 1975; Bohner and Bangerth, 1988b). In *Arabidopsis*, transcription of *cdc2* (the catalytic subunit of the protein kinase that triggers mitosis) can be both inhibited and induced depending on which hormone is applied. Thus, *cdc2* expression is induced during

auxin-mediated lateral root formation whereas CK-induced *cdc2* expression is associated with primary thickening of roots (Hemerly *et al.*, 1993). *Cdc2* expression is positively correlated with physiological competence for cell division, a process that apparently requires cyclins (the regulatory subunit of the protein kinase that initiates cell division), *cdc2* and mitogen-activated protein (MAP) kinase (Ferreira *et al.*, 1994). Furthermore, expression of cyclin genes and control of cyclin gene expression seems to be necessary for activation of the *cdc2* kinase and, thus, cell division.

Although the precise mechanism determining entry of plant cells into mitosis (M) remains obscure, it is nevertheless evident that negative influences on cell division cycle activity will impact on cell number and ultimately fruit size. CKs might regulate processes either in G_2 (post-DNA synthetic, pre-mitotic period) or in the transition from G_2 to M in the cell cycle (Binns, 1994; Jacobs, 1995). In the absence of CK, cells may accumulate in the G_2 to M transition for a period of time and then leave the cell cycle entirely (Fosket, 1977). As pointed out by Ferreira *et al.* (1994), the G_2 to M transition is crucial for entry into mitosis. If, however, certain conditions are not met, e.g. replication of DNA must have been completed, then differentiation ensues.

MAP kinase is another potential regulator of cell division in plants (Ruderman, 1993; Mizoguchi *et al.*, 1994). This phosphorylating enzyme is part of the cascade triggered by *ras*, a super-family of low-molecular-weight guanine nucleotide-binding proteins involved in control of cell growth and differentiation, cytokinesis and membrane trafficking and characterized by protein prenylation (Schafer and Rine, 1992). Prenylation refers to the covalent modification of a molecule by the attachment of a lipophilic isoprenoid group, and protein prenylation may be important for the regulation of 3-hydroxymethyl-3-glutaryl-CoA reductase (HMGR), the primary enzyme of isoprenoid synthesis (Schafer and Rine, 1992; Clarke, 1992). Although a true *ras* homologue has yet to be identified in plants, recent evidence would appear to suggest that isoprenoid biosynthesis is required early in the fruit developmental programme (Narita and Gruissem, 1989). These authors concluded that growth inhibition and morphological differences caused by blocking HMGR was the direct result of depletion of phytosterols (Gillaspy *et al.*, 1993).

Expression and activity of HMGR are regulated at many levels including transcription, translation, thiol status of the cell, phosphorylation and enzyme stability (Narita and Gruissem, 1989). Degradation of HMGR is relatively slow and requires both a steroidal and non-steroidal derivative of mevalonic acid (MVA). Farnesol has recently been identified as a possible non-steroidal derivative which initiates and promotes degradation of HMGR in animal cells (Correll and Edwards, 1994). In plants, increased ABA concentration has been correlated with reduced HMGR activity in developing endosperm of maize vivipary mutants (Moore and Oishi, 1994).

1.2 FACTORS AFFECTING 'HASS' AVOCADO FRUIT SIZE

Phenotypical aspects such as growth rate, duration of growth and response of plants/plant parts to the environment are co-ordinated by expression of genetic material. Likewise, fruit size, which is also influenced by cultural practises, must ultimately be determined/controlled at the genetic level. Expression of genetic material involves a process termed signal-response-coupling in which the signal may constitute either a chemical or physical stimulus. Physical stimuli (e.g. temperature and water availability) need to be translated into a chemical form (e.g. change in hormone balance) before effective signalling can take place. Once this has occurred, chemical signals interact with receptor proteins inducing a cascade of second messengers which act either directly or indirectly to alter gene expression. There is substantial evidence to suggest that 'Hass' fruit growth is affected by abiotic/biotic pressure including poor climatic conditions, inadequate availability of water and nutrients, changes in supply and composition of photoassimilate and alterations in hormone balance.

1.2.1 Temperature

Mean temperature during fruit ontogeny has a pronounced effect on fruit growth and final fruit size in late-maturing cultivars such as 'Hass' (Whiley and Schaffer, 1994). In South Africa, where avocado is cultivated under more severe conditions, up to 50% of the 'Hass' crop may be undersize in any particular season (Köhne, 1992). A similar, albeit less pronounced, situation has been observed for 'Hass' fruit produced in other avocado growing regions. For example, in subtropical Australia, fruit are on average

30% smaller in a warm coastal environment than those cultivated in a cool highland climate, while fruit produced in southern Florida are much smaller than fruit grown under the cooler conditions of California (Whiley and Schaffer, 1994). Since fruit respiration increase with increasing temperature (Blanke and Whiley, 1995), and 'Hass' has a long period of development on the tree, the small fruit condition in warmer districts might be due to assimilate deficiency as a result of a higher fruit respiration rate (Whiley and Schaffer, 1994).

1.2.2 Water availability

Obviously water and minerals are important in fruit growth and development. Water is generally considered the most important limiting factor to plant growth (Syvertsen, 1985; Smith and Griffiths, 1993) and water deficit stress impacts negatively on productivity and fruit size of avocado (Whiley *et al.*, 1988). Optimal plant water status is therefore vital for maximum fruit growth. Water stress during critical stages of fruit ontogeny results in increased pedicel ring-neck, a symptom associated with premature seed coat senescence (Whiley *et al.*, 1986). Furthermore, avocado fruits acts as reservoirs and under conditions of water deficit, moisture required for leaf growth may be drawn from fruits (Schroeder and Wieland, 1956). Consequently, leaves of avocado trees exert a priority over fruits for water which will again impact on fruit growth and final fruit size (Wolstenholme, 1986).

It is well established that plants exposed to sustained abiotic/biotic pressure accumulate the plant hormone abscisic acid (ABA). Stress-induced accumulation of ABA occurs readily in aerial plant parts when leaf water potential nears a critical threshold value (Milborrow, 1981; Zeevaart and Creelman, 1988). Furthermore, there is increasing evidence in support of root-synthesized ABA accumulating in the xylem of plants growing in drying soil (Davies and Zhang, 1991; Davies *et al.*, 1994). An alternative hypothesis is that leaves export ABA into the phloem which is translocated to the roots and then re-translocated in the xylem, from roots to shoots (Hoad, 1975; 1995). Elevated xylem ABA levels may also impact on fruit growth and development directly by down-regulating activity and/or synthesis of components essential for control

of fruit morphogenesis. With regard to the latter, Barlow and Pilet (1983; 1984) demonstrated that ABA retards completion of the cell cycle by preventing exit from either the G_1 (an interval in the cell cycle during which there is a high rate of RNA formation and protein synthesis) or G_2 (a period of cell growth before entrance into prophase of M) phase (Müller *et al.*, 1994). G_2 is considered a possible point of control in the signal transduction pathway for CK control of the cell cycle (Binns, 1994). This suggests that abiotic/biotic factors which decrease the endogenous CK:ABA ratio either during or immediately after fruit set, impact on cell cycle activity to reduce the number of cell divisions in fruit ontogeny and thus final fruit size.

1.2.3 Nutrient availability

Potassium (K^+), calcium (Ca^{2+}) and boron (B) are important minerals affecting avocado fruit growth (Robertson, 1971; Moore and Hirsch, 1983). B deficiency causes reduced growth of rapidly expanding organs, such as young fruits. Smith *et al.* (1995) demonstrated that application of B increased 'Hass' fruit weight by 15% on trees cultivated in B-deficient soils. Although the exact function of B in plants is unknown, there is increasing evidence that it is required for membrane integrity and function. Apparently B exerts its effect by promoting K^+ uptake and H^+ extrusion (Pollard *et al.*, 1977). Both K^+ and Ca^{2+}, the latter being maintained at low endogenous levels, play major roles in membrane trafficking and in signal-response coupling, i.e. the integration of abiotic and biotic stimuli with changes in intracellular biochemistry and whole-plant physiological responses (Ward *et al.*, 1995).

Nitrogen (N) is critical in maintaining the desired balance between vegetative and reproductive growth in avocado trees (Whiley *et al.*, 1988). An over-supply of N results in excessively luxuriant foliage at the expense of fruit production (Wolstenholme and Whiley, 1989), and fruit yields decline when N is limiting (Embleton *et al.*, 1959). Plant N status appears to affect CK biosynthesis in a number of species (Horgan and Wareing, 1980). Sattelmacher and Marschner (1978b) demonstrated that N enhanced CK formation in roots. Furthermore, N deficiency resulted in reduced CK translocation from roots to aerial parts of the tree (Sattelmacher and Marschner, 1978a). Horgan and

Wareing (1970) suggest that the response of CK biogenesis to plant N status is more pronounced in species which are sensitive to this element. Since avocado trees are highly sensitive to N (Klein and Zilkah, 1986; Whiley *et al.*, 1988), levels of this nutrient may have a marked effect on biosynthesis of CK.

Adequate CK supply to developing fruit is necessary for full morphogenic expression of the fruit developmental programme of many plant species, e.g. apple and tomato (Wareing *et al.*, 1976). The effect of CKs on fruit size may be attributed to the hormones involvement in promoting cell division and/or increasing sink strength. CKs have been shown to promote cell division (Lackie and Dow, 1989; Smith and Wood, 1992) and increase sink strength (Mothes and Engelbrecht, 1961; Richards, 1980; Ronzhina *et al.*, 1995) of a number of plant organs, including fruits.

CKs also interact with other plant hormones, e.g. mitotic activity of meristematic cells requires the presence of both CKs and auxins (Das *et al.*, 1956; Patau *et al.*, 1957; Binns, 1994; Ferreira *et al.*, 1994). However, it is unlikely that auxins have a direct effect on avocado fruit size, as they are involved primarily in control of cell expansion (Cleland, 1971; 1995; Theologis, 1986; Cosgrove, 1986; 1993), a process that is not limiting in avocado (Schroeder, 1953; Valmayor, 1967). CKs could therefore be the limiting factor required for full morphogenic expression of the fruit developmental programme. Although some CK could arise from tRNA breakdown in developing fruits (Maaß and Klämbt, 1981a; 1981b; Letham and Palni, 1983; Roberts and Hooley, 1988), it seems that root-derived CKs imported during anthesis also contributes to the CK pool in sink structures such as developing fruit (Bohner and Bangerth, 1988a; 1988b; Bower *et al.*, 1990; Baker and Allen, 1992; Bernier *et al.*, 1993).

1.2.4 Availability, composition and utilization of photoassimilate

During development of fleshy fruits assimilate supply is required for maintenance of cell division, establishment of sink strength and ultimately fruit size (Ho, 1988; Patrick, 1988). Sink strength is influenced by a number of factors including proximity of sink to source and the relative strength of other sinks. Developing fruits usually attract

assimilate from adjacent leaves, but also import assimilate over considerable distances (Feree and Palmer, 1982). Generally the closer the sink to source, the greater its command on available nutrients (Cook and Evans, 1983), albeit complicated by sink competition (Monselise and Goldschmidt, 1982). For example the panicle in avocado trees is sub-terminal while vegetative shoots are terminal (Hallé *et al.*, 1978), i.e. vegetative and reproductive components are in close proximity. Competition between these structures is therefore intense, particularly in early spring, before developing shoots become sources (Scholefield *et al.*, 1985; Finazzo and Davenport, 1987). Whiley (1994) recorded that 'Hass' fruits from determinate flowering shoots were significantly larger than those from indeterminate (ending in a vegetative bud) flowering shoots, presumably due to less vegetative competition. In addition, inter-fruit competition has a profound effect on fruit size, e.g. Lahav and Kalmer (1977) observed that mean avocado fruit size was reduced in years of abundant yield, i.e. available resources had to be allocated to more sinks.

Nutrient/assimilate accumulation in developing fruits occurs via the vasculature and is driven by gradients of decreasing water potential established by transpirational water loss coupled to fruit photosynthesis during the early stages of fruit ontogeny (Blanke and Lenz, 1989). As shown in Figure 1.3, vascular traces permeate the mesocarp of avocado fruit and coalesce towards the distal end where they enter the seed coat as a single group (Kaiser, 1993). Early senescence of the seed coat substantially reduces the source of nutrients required for fruit growth thus retarding or arresting the process completely (Cutting *et al.*, 1986).

Photoassimilate required for fruit growth is manufactured by leaves, and hence it is important that trees have sufficient foliage to produce the required carbohydrate during the course of fruit development (Cull, 1989). Mature, healthy leaves export the most photoassimilate (Salisbury and Ross, 1978), whereas young developing leaves act as sinks to which material is exported by mature leaves, i.e. leaves pass through a period where they are metabolic sinks before gradually assuming the role as sources for photoassimilate (Salisbury and Ross, 1978; Whiley, 1990; Wolstenholme, 1990).

Although leaves are the major source of photoassimilate, other green plant parts such as young green stems, fruits and flowers may also contribute (Bazzaz *et al.*, 1979; Blanke and Lenz, 1989), although photoassimilate supply by these plant parts is usually very small (Todd *et al.*, 1961; Whiley *et al.*, 1992). Source leaves control timing and supply of carbohydrate (Gifford and Evans, 1981; Wright, 1989), whilst sinks play a key role in the distribution of assimilate (Walker and Ho, 1977).

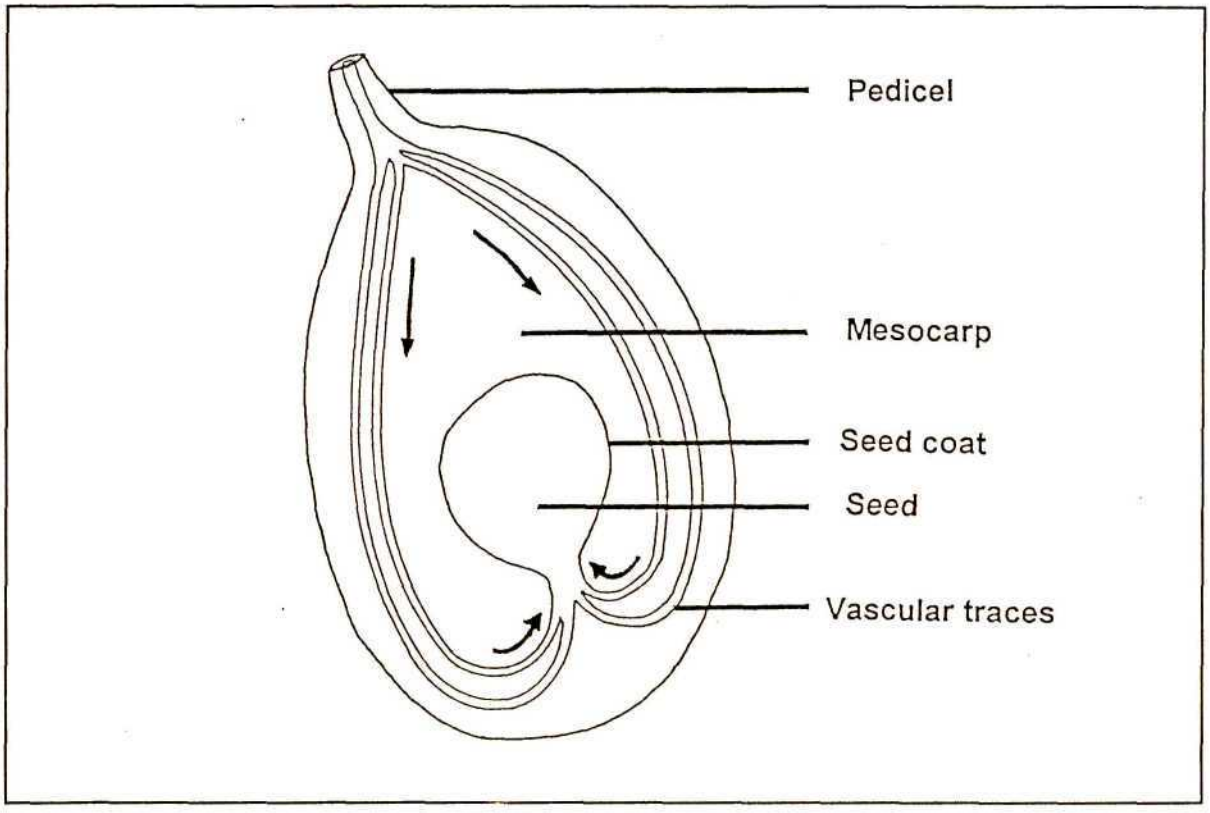

Figure 1.3
Diagramatic representation of longitudinal section of avocado fruit illustrating vasculature and direction of assimilate movement into the fruit (adapted from Kaiser, 1993).

1.3 DEFINING THE 'HASS' SMALL FRUIT SYNDROME

'Hass' avocado produces two distinct populations of fruit, the distinguishing feature being size (Zilkah and Klein, 1987). Phenotypically small fruit are not the result of disease (Kremer-Köhne and Köhne, 1995), and the syndrome is therefore considered a physiological disorder (Blanke and Bower, 1991). As trees age, so the syndrome becomes more pronounced (Cutting, 1993), and it is particularly noticeable in orchards

situated in warmer and/or drier climates (Hilton-Barber, 1992; Whiley and Schaffer, 1994). Therefore, both abiotic stress and ageing appear to be major contributing factors in the determination of 'Hass' avocado fruit size.

Based on a study of growth kinetics, and the determination of fruit shape and size, Zilkah and Klein (1987) concluded that small and large fruit arose due to earlier fruit set of large fruit. However, casual observation has revealed that the small fruit phenotype is always associated with early senescence and or death of the seed coat. Figure 1.4 shows a typical example of a phenotypically small 'Hass' fruit in which premature senescence and death of the seed coat has occurred. Although these fruit are of similar age, growth has clearly been arrested in the small fruit. The question therefore arises: Are small fruit the result of early seed coat senescence and death? or, Is seed coat senescence the result of some other factor which causes growth in these fruit to slow? Furthermore, it is important to appreciate that all fruit, irrespective of final fruit size, will eventually develop degenerate seed coats which can be used as a measure of fruit maturity. This suggests that seed coat senescence is a normal event during the avocado fruit developmental programme and as such, must be genetically co-ordinated. 'Hass' is predominantly of Guatamalan origin, and avocado fruits from natural forest of Guatamala tend to be smaller than fruits from other centres of origin, which suggests that the 'Hass' small fruit syndrome is a genetic disorder (Chandler, 1957). Confirmation of a genetic basis for the small fruit syndome might best be achieved by comparing key aspects of metabolism in small and normal sized fruit.

Aside from the obvious morphological differences between fruit of different cultivars, biochemical studies have revealed differences between 'Hass' and 'Fuerte' which produces substantially larger fruit. For example, 'Hass' fruit displays higher respiration rates and greater transpirational water loss than 'Fuerte' during fruit development and it has been suggested that the higher energy requirement of 'Hass' fruit (or the less efficient fruit photosynthesis) could lead to smaller sized fruit (Blanke and Whiley, 1995). A similar argument has been developed to account for the small fruit syndrome in *Citrus sinensis* cv. Valencia (Blanke and Bower, 1991).

Figure 1.4

Photograph illustrating the relationship between fruit size and seed coat viability. The larger fruit still has a healthy functional seed coat, whereas the seed coat of the smaller fruit appears senescent, dessicated and brown in colour.

1.4 OBJECTIVES

Little is known about the biochemistry, physiology and molecular biology of fruit growth and in particular that of the avocado. If the 'Hass' small fruit syndrome is to be resolved to the benefit of the industry, a greater understanding of fruit growth and development is required. The objectives of this study were to;

(1) Examine the dynamics of 'Hass' avocado fruit growth.

(2) Investigate factors involved in the metabolic control of 'Hass' avocado fruit growth.

(3) Evaluate the significance of (1) and (2) in terms of orchard mulching as a viable short term management strategy.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS

2.1.1 Radiochemicals

DL-*cis,trans*-[2-^{14}C]-abscisic acid (35.2mCi/mmol), [U-^{14}C]-sucrose (23.2 mCi/mmol), DL-3-Hydroxy-3-methyl-3-[^{14}C]glutaryl CoA (HMG-CoA) (58.0 mCi/mmol), [4- ^{14}C]-cholesterol (53.0mCi/mmol), and [^{3}H]-isopentenyladenine were purchased from Amersham International, Buckinghamshire, U.K.

2.1.2 Growth regulators and isoprenoids

Mevastatin (compactin), DL-mevalonic acid lactone (MVAL), (±)-*cis,trans*-abscisic acid (ABA), gibberellic acid (GA_3), 6-(γ,γ-dimethylallylamino)-purine (iP), N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU), 3β-hydroxy-24-ethyl-5,22-cholestadiene (stigmasterol), 5-cholesten-3β-ol (cholesterol), 24α-methyl-5-cholesten-3β-ol (campesterol) and 3β-hydroxy-8.24-lanostadiene (lanesterol) were purchased from Sigma, St Louis, U.S.A. 2'-isopropyl-4'-(trimethylammonium chloride)-5'methyl phenyl piperidine-1'-carboxylate (AMO 1618) was purchased from Calbiochem, Durban, South Africa.

2.1.3 General chemicals

Lucifer yellow-CH (LYCH), 4-amino-antipyrene, p-hydroxybenzoic acid, D-glucose oxidase, peroxidase, amyloglucosidase (Novo 200L) and termamyl were purchased from Sigma, St Louis, U.S.A. Hampt's adhesive solution, safranin, fast green, Canada balsam, tetrabromofluorescein (eosin) and Tween-20 were purchased from BDH Chemicals, Johannesburg, South Africa. Sodium cacodylate, glutaraldehyde, osmium tetroxide, propylene oxide, 2.4.6-tri (dimethylaminomethyl) phenol (DMP-30), epon, lead citrate and uranyl acetate were purchased from Wirsam Scientific, Johannesburg, South Africa.

2.1.4 Solvents

HPLC grade solvents (acetonitrile, ethyl acetate and methanol) were purchased from Burdick and Jackson, Muskegon, U.S.A. All other solvents were of analytical grade and were purchased either from Associated Chemical Enterprises, Johannesburg, South Africa or Saarchem, Krugersdorp, South Africa.

2.2 CHROMATOGRAPHIC MEDIA

Sep-Pak C_{18} cartridges were purchased from Waters Chromatography division, Millipore. Thin layer plates of silica gel (GF_{254}) (20 x 20 cm; 0.2 mm thick) were purchased from Whatman, New Jersey, U.S.A. For high performance liquid chromatography (HPLC) an ODS 2 (Spherisorb) 5 µm C_{18} column (250 x 4.6 mm i.d.) was purchased from Phase Separations Limited, Deeside, U.K., and an ODS 2 (Prodigy) 5 µm C_{18} column (150 x 4.6 mm i.d.) was purchased from Phenomenex, Torrance, California, U.S.A.

2.3 STUDY SITE

The study was conducted on Everdon Estate in the KwaZulu-Natal midlands (30° 16'E and 29°27'S). The orchard was situated in Phillips' Bioclimatic region 3, which is characterised by cool mesic conditions, typical of a "mist-belt" climate.

The mean annual temperature was 17.3°C with a daily temperature range of 12.0° C averaged over a 15 year period (Anon., 1997). Maximum and minimum temperatures in January are 26.1°C and 15.0 °C respectively, with the July maximum and minimum being 19.4°C and 6.7°C. The mean elevation is 1082 m above sea level and the rainfall has averaged 1051.7 mm over an 87 year period (Anon., 1997). In this region, the period from May to August is considered to be ecologically dry, with the remaining months being ecologically humid (Fig. 2.1). Mean, maximum and minimum monthly temperatures for Everdon Estate during the study period are summarised in Figure 2.2.

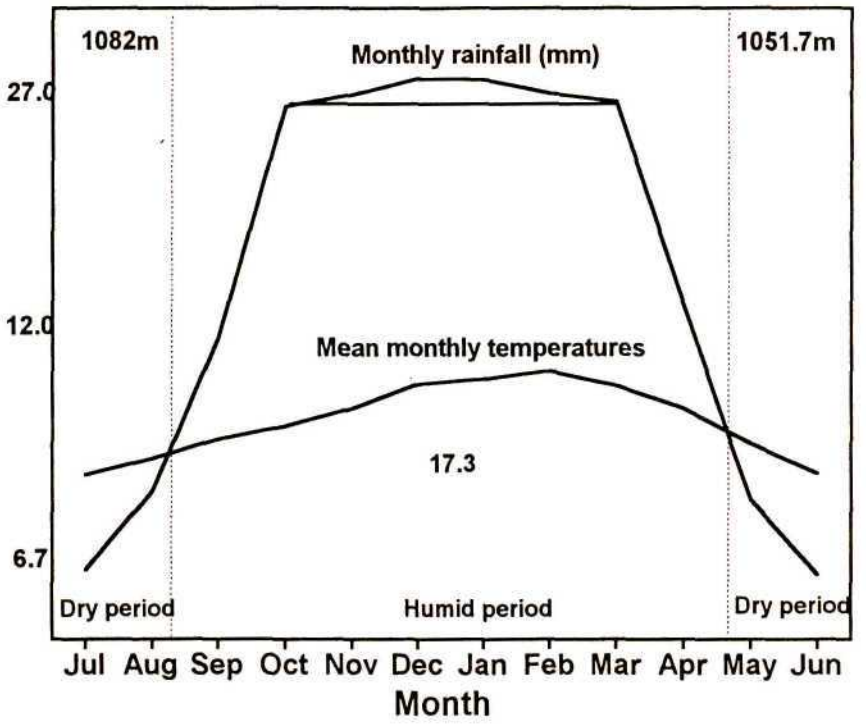

Figure 2.1

Climatograph for Everdon Estate illustrating humid and dry periods. (Rainfall above 100 mm plotted at $^{1}/_{10}$ of value).

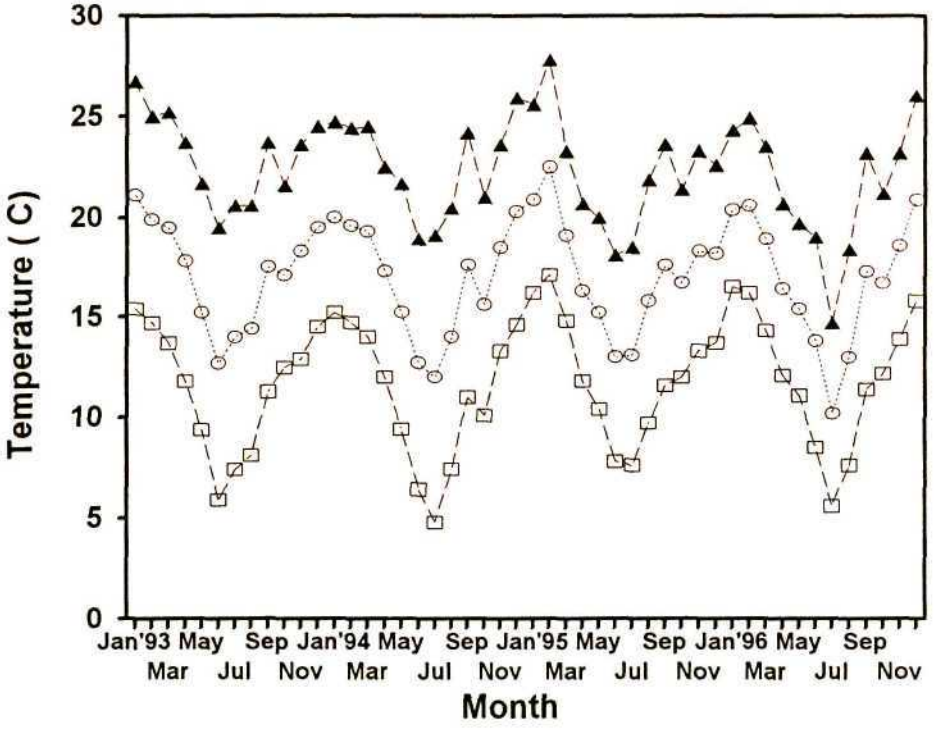

Figure 2.2

Temperature data for Everdon Estate from January 1993 through to December 1996. Maximum (▲), minimum (❑), and mean (○). (Source: I.S.C.W., Pretoria).

Trees were cultivated in a Hutton form soil which is characterised by an orthic A horizon overlying a red apedal B horizon (Fig. 2.3). Typically these soils are medium to heavy textured (with a clay content of 35-55%) and form in well-drained, oxidising environments. They generally have weakly structured, acidic topsoils (MacVicar *et al.*, 1984).

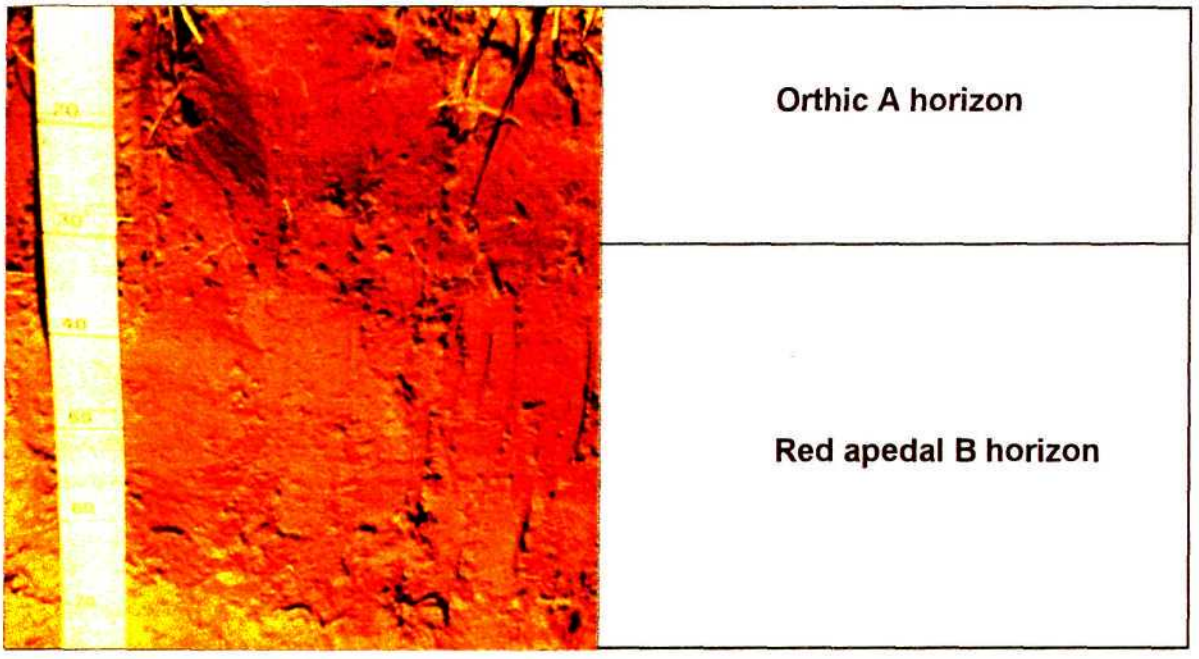

Figure 2.3
Soil profile of Hutton form soil at trial site, on Everdon Estate, KwaZulu-Natal midlands.

No cover crop was planted in the orchard and mechanical weed control was implemented. The orchard was irrigated through a micro-jet system, with two micro-jets per tree, and scheduled according to tensiometer readings. Tensiometers were placed at depths of 30 and 60 cm, and water was applied when the tensiometer pressure reading dropped to -40 kPa.

2.4 PLANT MATERIAL

Six year old 'Hass' trees on clonal 'Duke 7' rootstocks were selected for the trial. Trees were planted in 1988 on gently sloping land with a south-easterly aspect. Tree rows were orientated in a north-westerly to south-easterly direction, i.e. parallel to the slope.

2.5 APPLICATION OF MULCH

A total of 1.5 m^3 coarse pinebark was applied in February 1993 under six trees to a depth of approximately 15 cm. Pinebark was supplied by Kynoch Soil Services (Johannesburg, South Africa) and the specific product used was Gromed coarse potting mix.

2.6 PHENOLOGICAL MEASUREMENTS

Vegetative shoot flushes were estimated by measuring shoot extension. Ten shoots per tree were tagged and marked in 1993 prior to the spring flush. At the end of each major vegetative flush, shoots were marked with a different colour to distinguish between the different flushes. At the end of each month shoot length was measured, and from this shoot flushing periods were estimated.

Root flushes were monitored by visually estimating the area covered by white healthy feeder roots under a newspaper mulch (with an approximate area equal to 1250 cm^3). The newspaper mulch was placed 1 m from the micro-jet nozzle on the south-west side of the trees to avoid direct sunlight. Three measurements per tree were taken at the end of each month. Visual estimates of root flushing were performed using a rating of 0 to 10. Groupings of "poor", "medium" and "good" were chosen, viz. 0 to 2, 3 to 4, and ≥ 5 respectively, as described by Kaiser and Wolstenholme (1994).

2.7 MEASUREMENT OF FRUIT GROWTH AND YIELD

To measure fruit growth, 40 fruits per tree were tagged when fruit were approximately 10 mm in length. Subsequent length and diameter measurements, using digital calipers (Mitutoyo-500, Tokyo, Japan), were taken at regular intervals throughout the growing season. These measurements were fitted to a gompertz curve and an analysis of variance (ANOVA) was performed on each parameter of the equation.

At the end of each season, fruit were harvested and fruit size distributions were recorded for each tree. Fruit size was determined gravimetrically and classified according to the number of fruit per standard 4 kg export carton. Fruits were graded as

follows: Count 10, 366 to 450 g; count 12, 306 to 365 g; count 14, 266 to 305 g; count 16, 236 to 265 g; count 18, 211 to 235 g; count 20, 191 to 210 g; count 22, 171 to 190g; count 24, 156 to 170 g; count 26, 146 to 155 g; and factory grade, <146 g. Total tree yields were calculated by adding the product of the number of fruit per count size and the class centre of all the count sizes.

2.8 MEASUREMENT OF PHYSIOLOGICAL DISORDERS ASSOCIATED WITH SMALL FRUIT

2.8.1 Seed coat viability

To determine the relationship between seed coat viability and fruit size, all fruit from a single eight year old 'Hass' tree on Everdon Estate were harvested in July 1995. These fruits were weighed, and allocated a seed coat viability rating. Broad groupings of "healthy", "degenerate" and "intermediate" were selected, where healthy seed coats were still white and fleshy, degenerate seed coats brown and thin, with the intermediate category falling between these two extremes. Once fruit had been passed through the packhouse, the impact of mulching on seed coat abortion was determined. For this, 10% of fruit in each count were randomly selected and bisected longitudinally, and the presence or absence of a degenerate seed coat was recorded.

2.8.2 Incidence of pedicel ring-neck

To determine the effect of mulching on the incidence of pedicel ring-neck, 100 fruit per tree were randomly harvested, with care being taken to ensure that the fruit were still attached to their pedicels. Before fruit were passed through the packhouse, presence or absence of the ring-neck syndrome was recorded for each fruit.

2.9 CANOPY TEMPERATURE MEASUREMENTS

Using weather-proof infra-red thermometers (IRT's), surface canopy temperatures of two trees per treatment were recorded continuously from November 1994 to May 1996. Insulation and protective foil were applied to the IRT's to reduce temperature effects. The IRT's were mounted 2.5 m from the trees, facing south, on tripod stands at a height of 4.5 m above the ground. IRT's were connected to a Campbell Scientific CR10 data

logger beneath the trees. Simultaneous air temperature measurements were recorded by two thermocouples, and these data were also recorded by the data-logger.

2.10 MEASUREMENT OF CHLOROPHYLL FLUORESCENCE

Using a Hansatech-MK2 Plant Efficiency Analyser (Hansatech Instruments Ltd., Norfolk, U.K.), photochemical efficiency was measured in leaves on trees from mulched and non-mulched treatments. Diurnal comparisons were made between these two treatments, and data were selected for days that showed typical trends. Ten leaves per tree were covered with small light-weight leaf-clips and the leaves allowed to dark adapt for 15 min ('Hass' avocado leaves took at least 8 min to fully dark adapt (Fig. 2.4a)). Thereafter, the sensor unit was attached to each leaf clip and the leaves exposed to 80% red light (with a peak wavelength of 650 nm) from high intensity light-emitting diodes housed in the sensor unit, for 2.5 s.

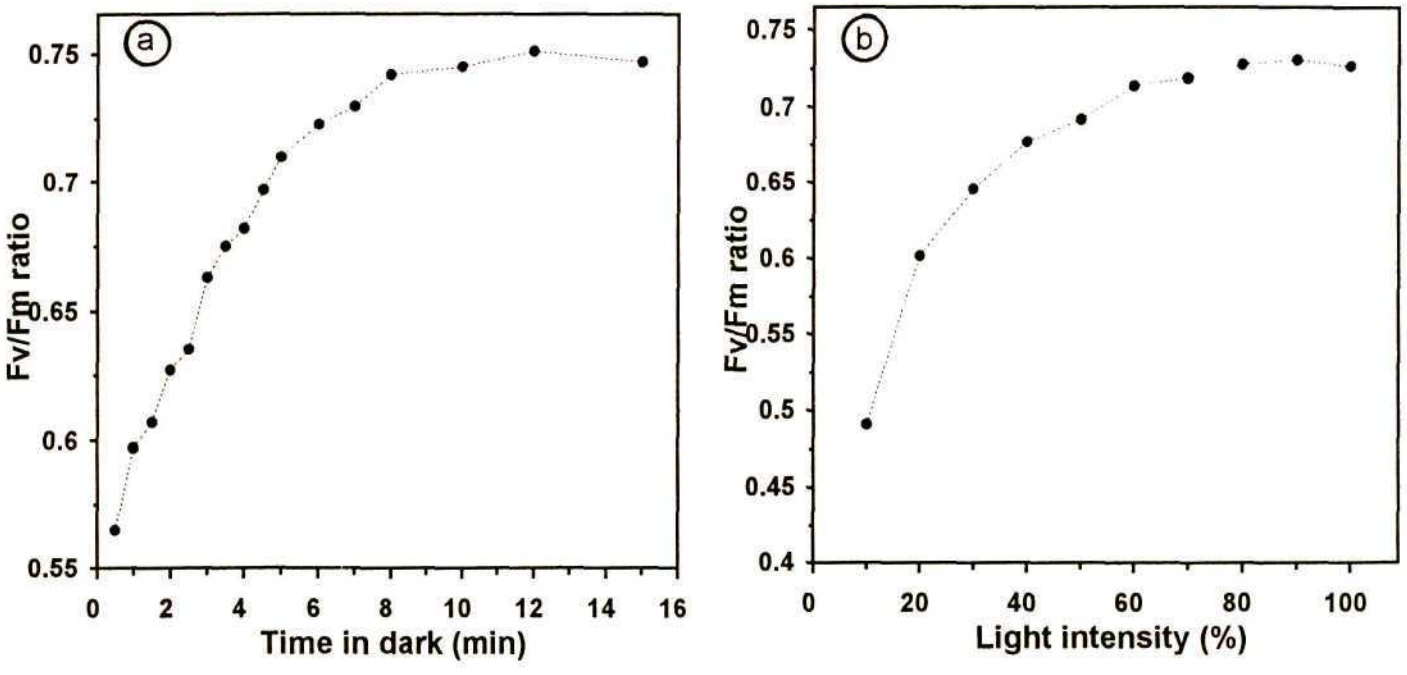

Figure 2.4
Relationship between F_v/F_m and time of dark adaptation (a), and red light intensity (b).

The initial fluorescence level (F_0) is reached immediately upon illumination, and this represents the level of constant fluorescence emission in a completely "dark adapted" plant. If illumination is sufficiently strong (greater than 60% red light ensures light

saturation (Fig. 2.4b)), fluorescence increases from F_0 to its peak (F_m). This increase in chlorophyll fluorescence emission is termed the variable fluorescence component (F_v), i.e. $F_v = F_m - F_0$. The ratio F_v/F_m can then be derived from the values obtained. This value has been shown to be proportional to the quantum yield of photochemistry (Butler and Kitajima, 1975), and correlates with the quantum yield of net photosynthesis (Björkman and Demmig, 1987), which is a measure of the efficiency by which light is utilized by leaves.

2.11 APPLICATION OF CHEMICALS

For application of chemicals, compounds of interest were formulated in Tween 20: acetone:water (1:1:8, by vol.) to a final concentration of 1 mg mL^{-1} and 20 µL of each, or combinations thereof, injected into the pedicel of individual fruits (8 fruits per treatment) using a Hamilton-7105 micro-litre syringe, 55 (phase I), 92 (phase II) and 210 d (phase III) after full bloom, unless otherwise stated. Control fruit were treated with and without Tween 20:acetone:water (1:1:8, by vol.). Following injection, the wound was covered with silicone grease and fruit growth monitored by measuring the increase in both fruit length and diameter using digital calipers (Mitutoyo-500), at the intervals specified in Results (Chapter 4, sections 4.2.1. and 4.2.2). Since identical trends were observed for both fruit length and diameter, only results for % increase in fruit length are shown.

2.12 TRANSPORT OF EOSIN AND [^{14}C]-SUCROSE

Following harvest, 226 d old fruits (3 per treatment) were immediately supplied either a 5 mL solution of eosin or 0.5 mL solution of U-[^{14}C]-sucrose (2 Mbq in distilled water) via the pedicel. After uptake, excess water was added and transport allowed to proceed for 48 h at room temperature. For analysis of the distribution of eosin, fruit was bisected, the stone removed and the two halves photographed. For analysis of the distribution of radioactivity, three 1 g dry weight samples of mesocarp, seed coat and seed from at least three different fruits were extracted in 80% aqueous methanol at 4°C for 24 h. Residual tissue was removed by centrifugation and radioactivity in the supernatant determined, after the addition of 2 mL Picofluor 40, using a Packard Tri-

Carb 1500 liquid scintillation spectrometer programmed for automatic quench correction.

2.13 MICROSCOPY

2.13.1 Fluorescence microscopy

2.13.1.1 Electrophysiological and microinjection procedure

Sections (approximately 2.5 cm in length) of mesocarp/seed coat tissue from freshly-harvested 226 d old 'Hass' fruit were placed in cold (6°C) MES buffer (10 mM NaOH-MES (pH 7.2), containing KCl, $MgCl_2$ and $CaCl_2$ (all 0.5 mM) in 125 mM Mannitol), and allowed to recover for a minimum of 30 min. Prior to electrophysiological and/or microiontophoretic experimentation, 0.25 mm sections, in a Perspex slide well containing MES buffer, were examined under blue light using an Olympus BHWI erect-image UV microscope with a fixed stage and extra-long working distance objectives.

All electrophysiological measurements were made using a WPI Duo-773 electrometer (World Precision Instruments Inc., Sarasota, Florida, U.S.A.) fitted with high impedance, active probes. Inner-filamented glass microelectrodes were made using 1mm diameter pipettes, (WPI Kwik-Fil K100-F3) which were pulled with a Narishige PB-7 Pipette Puller (Narishige Co. Ltd., Tokyo, Japan). Tips were routinely between 0.5 and 1 µm in diameter. Microelectrodes were back-filled with a Lucifer Yellow (5% w:v, in 3 M LiCl) solution, and the shank of the microelectrode filled with 3 M LiCl. Microelectrodes were attached to KCl half cells filled with 3 M LiCl, coupled to a WPI high impedance probe and attached to a WPI PM-10 Piezo controller unit, and to a WPI DC-3 motorised micro manipulator. Once impaled, cell potentials were monitored using the Duo 773 electrometer, to ascertain viability of cells. Cell potentials of the impaled cells were monitored in the dark, by inserting a shutter in the light path to prevent UV exposure and damage to the cells. Once membrane potentials had stabilised (at least -40 mV as prescribed by Farrar *et al.* (1992) and van Bel *et al.* (1996)), impaled cells were reverse iontophoresed, using pulsed current (-2 to -30 nA, for 5 to a maximum of 60 s) in order to inject the dye. Impaled, injected cells were photographed using an Olympus AD PM-10 camera system, (Fujichrome Sensia 400 ASA slide, or Super HVG

200 ASA print film) or the data were recorded using a Panasonic CL-WV 350 video camera, connected to a Panasonic NV-SD3 video recorder. Selected images were captured and saved to disk on a 486 personal computer, fitted with a digital image recording and capture system. All equipment used was supplied by Wirsam Scientific (Port Elizabeth, South Africa).

2.13.1.2 Digital Imaging

Video recordings showing cell-to-cell transport of LYCH were examined and frames of interest captured in digital format. Image files were converted to 300 dpi 256 colour images and a five-colour "pseudocolour" palette based on fluorescence intensity of LYCH concentrations, ranging from black (zero), to aquamarine (low), blue (medium), purple (high), and white (highest), was applied. The computer-enhanced digitized images enabled easy visualisation of the actual distribution of LYCH within avocado mesocarp tissue.

2.13.2 Light microscopy

2.13.2.1 Sample preparation

Whole fruits (during phase I) and three 5 mm^3 tissue samples (during phase II and III), excised from three distinct zones (viz. a zone including the endocarp and seed coat, a zone including the exocarp, and a zone from mesocarp tissue mid-way between the exo- and endocarp, across the equatorial region of each of three randomly selected fruit) were collected at regular intervals throughout the season. Samples were fixed in FAA (formalin:acetic acid:ethanol:water made up in the following proportions 2:1:10:7), then dehydrated in a graded ethanol/*tert*-butanol series (Table 2.1a) and finally embedded in wax (Table 2.1b). Thin sections were prepared using a Reichert rotary microtome, de-waxed and stained with Safranin and Fast Green (Table 2.1c) and examined using an Olympus BH-2 light microscope.

Table 2.1

Summary of the dehydration (a) and wax embedding (b) series and the staining procedure (c) used for sample preparation for the light microscope study.

(a) Dehydration series

	Solution (Water : ethanol : butanol)	Minimum time (hr)	Temperature (°C)
1.	45 : 45 : 10	1	20
2.	30 : 50 : 50	12	20
3.	15 : 50 : 35	1	20
4.	15 : 40 : 55	1	20
5.	0 : 25 : 75	1	20
6.	0 : 0 : 100	2	40
7.	0 : 0 : 100	18	40

(b) Wax embedding series

	Solution	Minimum time (hr)	Temperature (°C)
1.	Butanol : liquid paraffin (50:50)	24	40
2.	Liquid paraffin	12	40
3.	Liquid paraffin + wax pellets	12	40
4.	Liquid paraffin + wax pellets	24	60
5.	Pure molten wax	48	60

(c) Staining procedure

	Solution	Time (s)
1.	xylene/alcohol	60
2.	95% alcohol	30
3.	70% alcohol	30
4.	Safranin stain	24 h
5.	95% alcohol	30
6.	absolute alcohol	60
7.	absolute alcohol	60
8.	xylene/alcohol	60
9.	Fast Green stain	5
10.	xylene/alcohol	30
11.	xylene	60

2.13.2.2 Estimation of cell size and cell number

Detailed studies of cell size and number in the mesocarp tissue were made in transverse section along the diameter axis. The number of cells present in a representative area of 90 000 μm^2 was determined. For cells at the borders, if greater than 50% of cell area was within the designated sample area, the cell was regarded as part of the sample. The number of cells per 90 000 μm^2 was used to estimate apparent cell size. To convert number of cells in sample area to number of cells across fruit, the following expression was used: $n = d\sqrt{x}$; where n, is the number of cells across fruit; d, fruit diameter in mm at the equatorial region; and x, number of cells in sample area. Measurements of mean cell size and number across fruit diameter throughout development were fitted to a general logistic curve and an analysis of variance was performed on each parameter of the resultant cellular development curves.

2.13.3 Electron microscopy

Sample material was fixed in a 3% glutaraldehyde solution containing a 0.05 M sodium cacodylate buffer (pH 7.1) for 24 h. Samples were post-fixed for 4 h in 2% osmium tetroxide and then dehydrated in a graded ethanol series (10 to 100%). Following this, specimens were resin infiltrated using a graded series of epon and a mixture of propylene oxide and DMP-30. Infiltrated specimens were placed into fresh epon resin, and allowed to polymerise in at 70°C for 48 h. Using an LKB Ultratome III ultramicrotome, sections were trimmed, and areas of interest were isolated using a light microscope. Thin (70-90 µm) gold sections were cut using a tungsten-coated glass knife attached to the ultramicrotome, and collected on 200-mesh copper grids. Sections were stained in uranyl acetate, followed by lead citrate and viewed in a Jeol 100-CX (Jeol, Japan) transmission electron microscope at an accelerating voltage of 80 kV.

2.14 HMGR ASSAY

Freeze-dried mesocarp tissue was homogenized in ice-cold 100 mM K-phosphate buffer (pH 7.0) containing 4 mM $MgCl_2$ and 5 mM DTT, the homogenate filtered through 2 layers of Miracloth and centrifuged at 10 000g for 15 min at 2°C. To the supernatant was added 8 mM $CaCl_2$ and the microsomes sedimented at 27 000g for 15 min at 2°C

as described by Cinti *et al.* (1972). The pellet was washed in 150 mM KCl, re-centrifuged at 27 000g for 15 min at 2°C and the microsomes resuspended in a small volume of 100 mM K-phosphate buffer (pH 7.0) containing 50 mM DTT. Approximately 100 µg of microsomal protein (Bradford, 1976) was incubated in a total volume of 300 µL containing 5 mM NADPH and 1.72 nmol [3-^{14}C]-HMG-CoA. Reactions were initiated by addition of substrate and allowed to proceed for 45 min at 30°C. On conclusion of incubation, reactions were terminated by addition of 2 µL of MVAL (100 mg mL^{-1}) and 20 µL HCl (6 N) followed by vortexing, and the MVA lactonized at room temperature for 15 min. Particulate material was removed by centrifugation and the supernatant analysed for [^{14}C]-MVA. Using a modification of the method described by Chappell *et al.* (1995), 700 µL 0.5 M K-phosphate (pH 6.0) followed by 1 mL ethyl acetate was added to the supernatant. After thorough mixing and centrifugation, radioactivity in the ethyl acetate phase was determined by liquid scintillation spectrometry. Alternatively, the ethyl acetate fraction was applied to thin layers of silica gel (GF_{254}) and plates developed to 15 cm in chloroform:acetone (2:1, by vol.) and radioactivity in the MVAL-containing zone (R_f 0.65) determined by liquid scintillation spectrometry. Assays were performed in triplicate, with less than 10% variation between samples and the two methods of analysis.

2.15 CARBOHYDRATE ANALYSIS

Soluble and storage carbohydrate extraction was based on the method described by Rasmussen and Henry (1990). Determination of soluble carbohydrate was performed by tissue extraction in 70% ethanol, whilst determination of insoluble carbohydrate concentration involved enzymatic hydrolysis of the remaining starch.

Three trunk bark disc samples ca. 3 cm^2 were collected from each tree on a bi-monthly basis throughout the duration of the trial. Discs were dried to constant mass in a forced draught oven at 70°C and milled. 50 mg (dry weight) samples were extracted in a 5 ml aliquot of 80% ethanol for 30 min at a temperature of 80°C. This process was repeated three times and the combined extracts were centrifuged (BHG Hermle Z-510) at 3000g for 10 min. The supernatant was decanted and percentage sugar determined (via a

glucose-specific colour reaction followed by a comparison against the glucose standard curve). To the pellet 2.5 ml acetate buffer (pH 5) and 50 µl Termamyl were added, and the samples allowed to incubate at 90°C for 30 min. After cooling to room temperature, 50 µl amyloglucosidase was added and incubated for 20 h at 60°C. Particulate material was removed by centrifugation for 10 min at 3000g, and 100 µl aliquots of the supernatant diluted to 5 ml using glucose oxidase colour solution prepared as follows: Into 2000 ml distilled water dissolve; (1) 24.8 g disodium hydrogen orthophosphate dodecahydrate, (2) 12.4 g sodium dihydrogen orthophosphate-2-hydrate, (3) 4.0 g benzoic acid (dispersed in a small volume of ethanol), (4) 0.2 g 4-amino-antipyrene, (5) 3.0 g p-hydroxybenzoic acid, (6) 0.04 g glucose oxidase, and (7) 0.001 g peroxidase. The solution was incubated in a water bath at 40°C for 15 min, and subsequently cooled to room temperature for a further 60 min. Absorbance was measured at 505 nm using a Metertek SP-850 spectrophotometer, and percentage carbohydrate determined by comparison against a D-glucose standard curve.

2.16 ABA ANALYSIS

For analysis of ABA, aliquots of freeze-dried mesocarp tissue were homogenized in ice-cold methanol/ethyl acetate (50:50, by vol.), containing a known amount of radio labelled ABA (to correct for losses) and diethyldithiocarbamate (200 mg L^{-1}) as an antioxidant, in the presence of insoluble PVP (10% w/w) and extracted for 24 h in darkness at -20°C. The homogenate was centrifuged and the pellet extracted with further methanol/ethyl acetate (50:50, by vol.). The combined supernatants were reduced *in vacuo* and the residue resuspended in 0.5 M K-phosphate buffer (pH 8.5) and partitioned three times against equal volumes of diethyl ether to remove neutral and basic impurities. The pH of the aqueous phase was adjusted to 2.5 and ABA partitioned into diethyl ether (repeated three times). Purified ABA-containing samples were analysed by reversed-phase HPLC. Chromatography was carried out on an ODS 2 (Spherisorb) 5µm C_{18} column (250 x 4.6 mm i.d.) eluted with a linear gradient of 0 to 100% methanol in 1% aqueous acetic acid over 60 min at a flow rate of 1.0 mL min^{-1}. ABA was quantified at 254 nm by peak integration following calibration with authentic standards using a Waters 990 programmable UV-Vis detector.

After quantification, fractions corresponding to authentic ABA were collected and pooled. Pooled fractions were methylated with ethereal diazomethane and further analysed by GC-MS to identify the chemical nature of the peak using a Hewlett-Packard 5890 gas chromatogram fitted with a fused-silica capillary column (12 m x 0.32 mm i.d.) programmed from 120°C at 5°C min^{-1} with He as the carrier gas (1.5 to 2.0 mL min^{-1}). The electron impact mass spectrum of ABA methyl ester is shown in Figure 2.5, which is identical to the published mass spectrum of this compound (Gray *et al.*, 1974; Dörffling and Tietz, 1983).

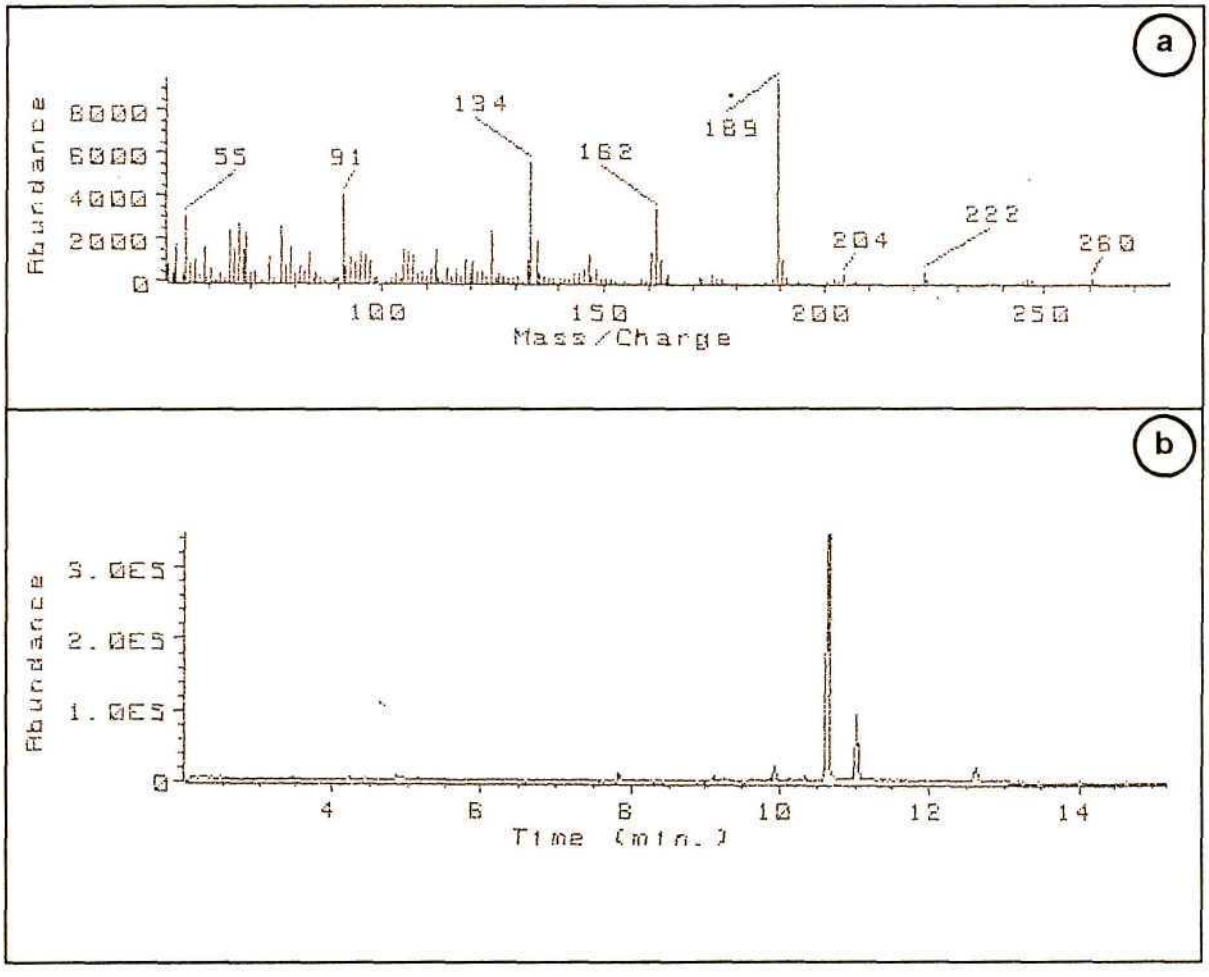

Figure 2.5

(a) Electron impact mass spectrum of authentic ABA methyl ester from 'Hass' avocado mesocarp. (b) Total ion chromatogram of the ABA methyl ester purified from the avocado mesocarp samples.

2.17 iP ANALYSIS

To determine iP concentration, 1 g freeze-dried samples were extracted in 80% methanol containing 0.1 g PVP and BHT (50 mg L^{-1}) as an anti-oxidant and a known amount of radio labelled iP (to correct for losses) in the dark for 24 h at 4°C. Samples were centrifuged at 10 000g for 10 min, and the supernatant reduced to dryness in a Savant vacuum concentrator. The extracts were resuspended in water (adjusted to pH 3 with acetic acid) and loaded onto a pre-conditioned SepPak C_{18} cartridge. The cartridge was first washed with 2 ml 50% methanol and then eluted with 2ml 80% methanol. 0.5 ml aliquots were then dried down in vacuo and used for quantification of iP by radioimmunoassay (RIA).

For quantification by RIA, 100 µl tritiated iP radio tracer, 100µl antibody (for antiserum production, see Cutting *et al.*, 1984) dissolved in 0.1% phosphate buffered saline (PBS) (pH 7.2) bovine serum albumen and 0.5 ml bovine serum in PBS were added to each sample. The mixture was allowed to incubate at 37°C for 30 min. Following this, 0.75 ml 95% $(NH_4)_2SO_4$ was added and the samples allowed to stand for a further 30 min. Thereafter the solution was centrifuged at 4000g for 15 min and the pellet washed in 1.5 ml 55% $(NH_4)_2SO_4$ and re-centifuged at 4000g for a further 15 min. The pellet was then dissolved in 0.25 ml water, and radioactivity determined using a Packard Tri-Carb 1500 liquid scintillation spectrometer programmed for automatic quenching, following the addition of 1 ml Picofluor 40. Samples were processed in triplicate and raw data were analysed using the SecuRia 2200 data reduction radioimmunoassay package (Packard Instrument Company).

2.18 STEROL ANALYSIS

Sterols were extracted from avocado mesocarp tissue (1 g freeze-dried samples) after homogenization in 15 ml methanol, containing known amount of [4-^{14}C]-cholesterol (to correct for losses). To the homogenate was added 6 ml chloroform and 2 ml water, then vortexed and centifuged at 15 000 g for 10 min. To the supernatant was added 6 ml chloroform and 8 ml water, mixed and centrifuged at 15 000 g for a further 10 min. The organic phase was collected and reduced *in vacuo*, and the residue resuspended in

chloroform. Purified sterol-containing samples were analysed by reverse-phase chromatography (Figure 2.6 illustrates the chromatograph for a range of sterol standards). Separations were achieved on a Prodigy column (150 x 4.6 mm i.d.), and the column was eluted with an acetonitrile/methanol (2:1) mixture for 35 min at a flow rate of 1 mL min^{-1}.

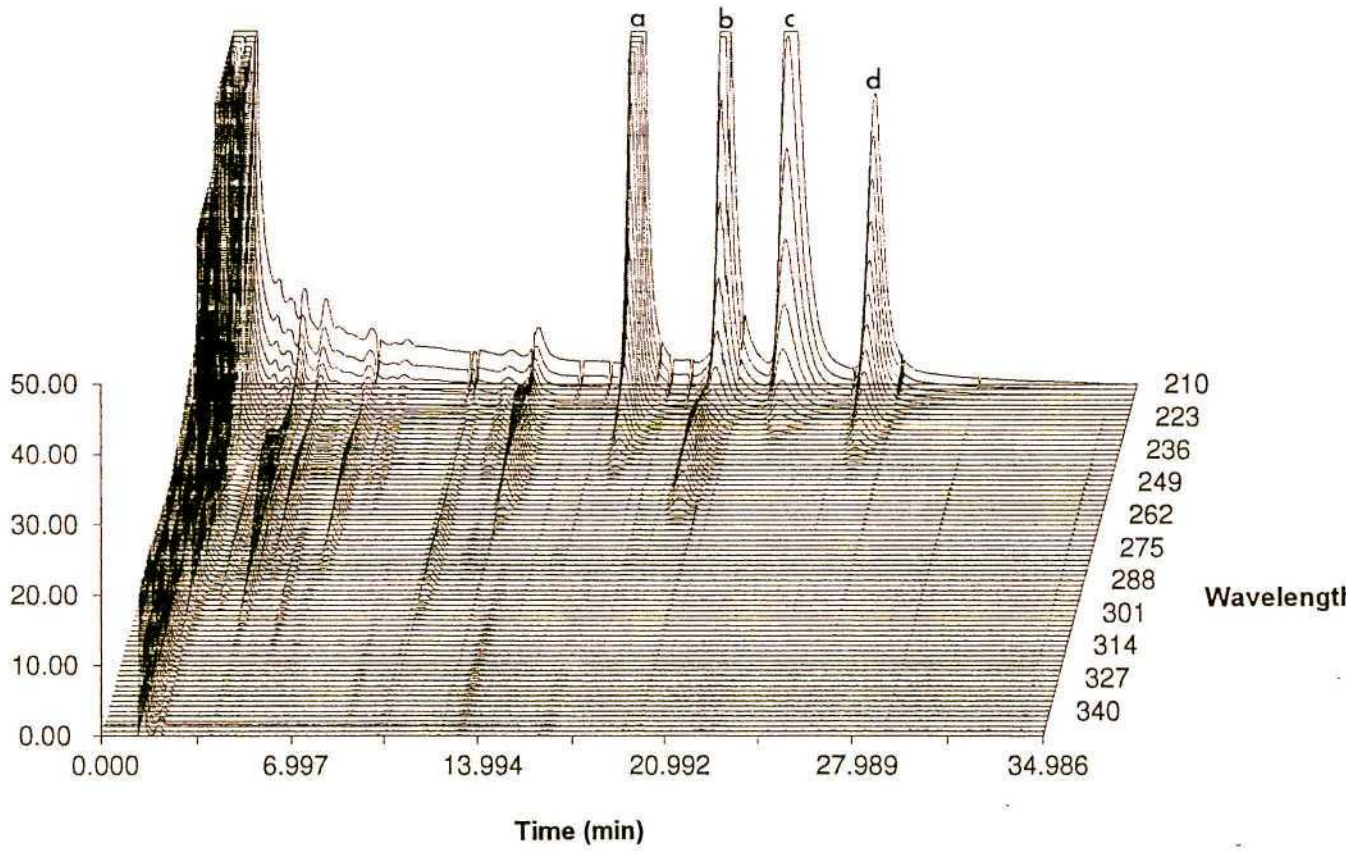

Figure 2.6

Chromatograph of sterol standards observed over a range of wavelengths (a = lanesterol, b = cholesterol, c = stigmasterol, and d = campesterol).

CHAPTER 3

CHARACTERIZATION OF THE 'HASS' SMALL FRUIT PHENOTYPE

3.1 INTRODUCTION

Although Zilkah and Klein (1987) demonstrated that 'Hass' produces two distinct populations of fruit, the small fruit variant remains physiologically ill-defined. In attempting to relate fruit size to differences in the time of fruit set these authors demonstrated that small fruit were set 2-3 days later than large fruit. One explanation for this observation is that fruit which are set earlier may establish sink priority and consequently obtain more assimilate to fuel growth and development (Monselise and Goldschmidt, 1982). Even so, there is evidence to suggest that fruit size of 'Hass' differs from other cultivars because of genetic differences (Chandler, 1957). However, there is no evidence to suggest similar differences between the two populations of 'Hass' fruit. Furthermore, no studies have been carried out to determine whether physiological and biochemical differences exist between the two populations of fruit. This is surprising, given the enormous economic impact that appearance of small fruit has on the 'Hass' avocado industry, particularly in South Africa.

Clearly without a detailed understanding of the physiological basis of the 'Hass' small fruit syndrome, a solution seems unlikely. Thus the present investigation attempts to characterize the 'Hass' small fruit syndrome. This was achieved by: (1) relating final fruit size to morphological aspects such as seed size and seed coat viability; (2) examining the contribution of cell number and cell size to overall size of the fruit; and (3) measuring the endogenous concentration of key regulatory chemicals thought to play a role in fruit growth and development, as a function of final fruit size.

3.2 RESULTS

3.2.1 Fruit growth

Length and diameter measurements were taken throughout two seasons and the growth kinetics of differently sized 'Hass' avocado fruits compared by fitting these measurements to a gompertz curve with the mathematical equation; $y = C \exp \{-\exp [-B (x-M)]\} + A$ (where; A = starting value (mm), B = growth rate (mm day^{-1}), C = total growth (mm), and M = point of inflection (mm)), and the results are presented in Figures 3.1 and 3.2.

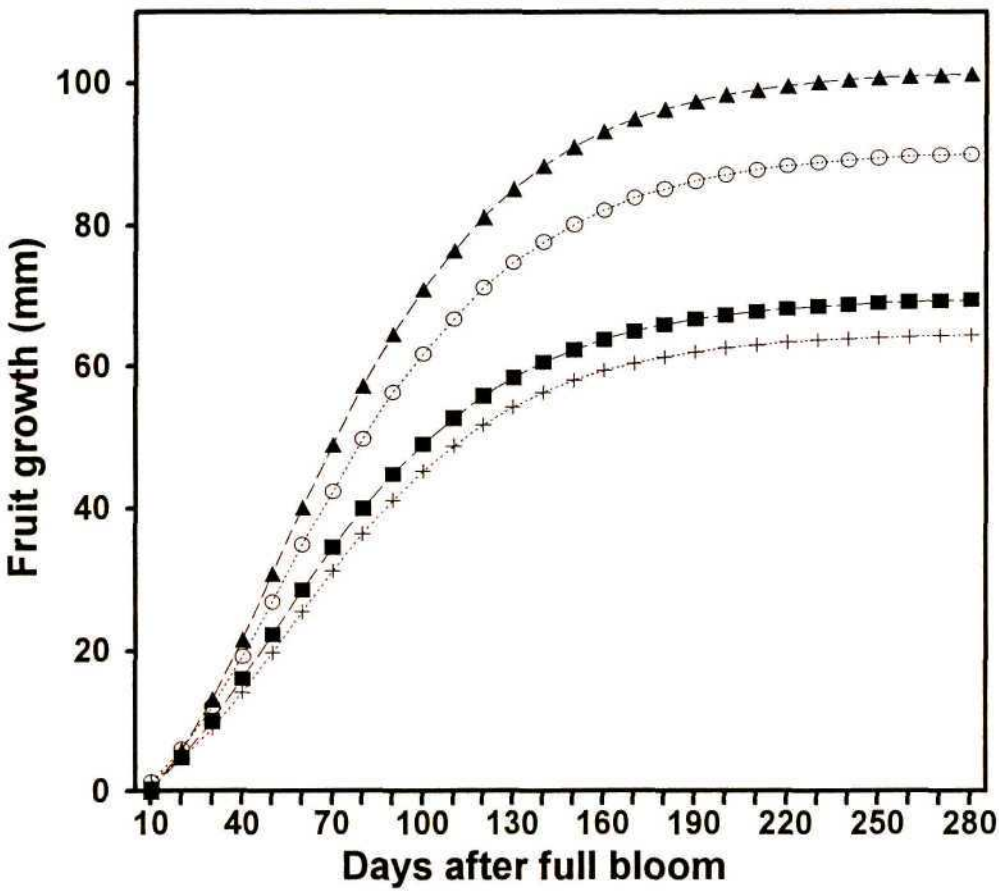

Figure 3.1

'Hass' fruit growth curves for the 1993/1994 season. Full bloom was reached by 12 October 1993. For normal fruit, regression line for the length axis (▲) is represented by y = 110.0 exp {-exp [-0.02297 (x - 51.61)]} - 8.16; and the diameter axis (■) by y = 77.08 exp {-exp [-0.02222 (x - 48.29)]} - 7.07. For phenotypically small fruit, regression line for the length axis (○) is represented by y = 95.84 exp {-exp [-0.02252 (x - 53.86)]} - 5.20; and the diameter axis (+) by y = 67.53 exp {-exp [-0.02336 (x - 54.13)]} - 2.66. (Growth curves were constructed from a total of 82 normal fruits and 92 phenotypically small fruits).

Starting values (*A*) were not significantly different between normal and phenotypically small fruit for both axes (Table 3.1), indicating that all fruit were set at the same time, i.e. 'Hass' trees can produce variable sized fruit even if they are set at the same time.

Total growth (*C*) of normal 'Hass' fruits was significantly ($P \leq 0.01$) greater than phenotypically small fruits (Table 3.1). At the time of harvest in 1993/1994, approximately 284 d after full bloom, normal fruit had grown an average of 14.3 ± 2.3

mm more along the length axis than phenotypically small fruit. Similarly, fruit expansion in the diameter axis was 9.6 ± 3.3 mm more in normal 'Hass' fruit during the same period. Fruit growth measurements for the 1994/1995 season show similar trends (Fig. 3.2): at time of harvest, 255 d after full bloom, normal 'Hass' fruit had grown an average of 8.8 ± 2.4 mm and 6.8 ± 2.7 mm more along the length and diameter axes respectively. Increased mass of fruit was attributed to increased growth in both major axes. The results support work carried out by Zilkah and Klein (1987) who showed that avocado fruit grows proportionately in all directions once fruit shape is established at the early stages of fruit development.

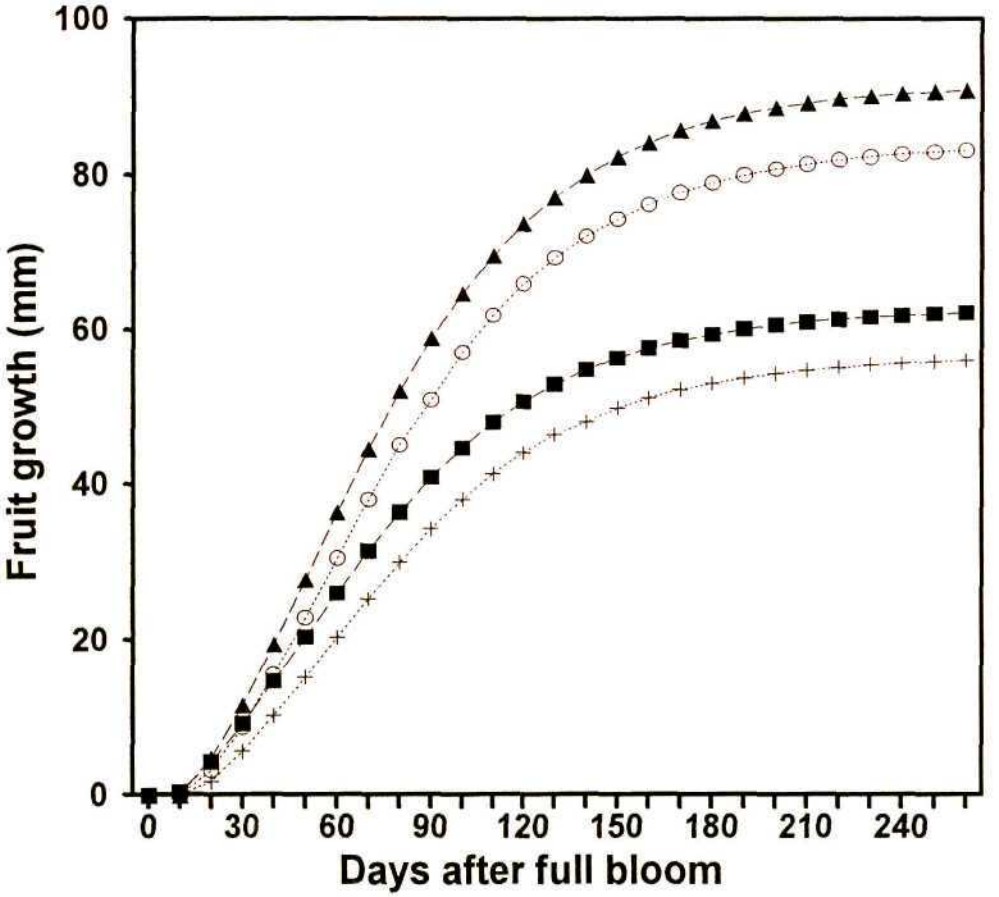

Figure 3.2

'Hass' fruit growth curves for the 1994/1995 season. Full bloom was reached by 8 November 1994. For normal fruit, regression line for the length axis (▲) is represented by y = 98.92 exp {-exp [-0.02281 (x - 49.44)]} - 7.34; and the diameter axis (■) by y = 68.34 exp {-exp [-0.02303 (x - 48.32)]} - 5.72. For phenotypically small fruits, regression line for the length axis (○) is represented by y = 90.14 exp {-exp [-0.02316 (x - 55.23)]} - 6.49; and the diameter axis (+) by y = 61.57 exp {-exp [-0.02279 (x - 54.73)]} - 5.03. (Growth curves were constructed from a total of 73 normal fruits and 71 phenotypically small fruits).

For the gompertz equation, growth up to the point of inflection (*M*) is exponential and thereafter growth slows down. At *M*, fruit size was substantially greater in normal fruit (Table 3.1), i.e. during the period of rapid exponential growth, rate of fruit expansion was greater in normal fruit. Towards the end of the growth period (after the point of inflection), differences in length and diameter of normal and phenotypically small fruit remained approximately constant (Figs. 3.1 and 3.2), i.e. final fruit size was determined early in the fruit developmental programme, before the point of inflection.

Table 3.1

A summary of mean values and standard errors for each growth parameter (*A, B, C,* and *M*) of the gompertz curve for measurements of labelled 'Hass' fruit over 2 seasons. Values are means of 155 normal fruits and 163 phenotypically small fruits.

		x ± SE (x)		
	Parameter	**Normal**	**Small**	**Significance†**
Length	*A*	-7.77 ± 0.97	-5.61 ± 0.83	NS
	B	0.02289 ± 0.00083	0.02280 ± 0.00087	NS
	C	104.83 ± 1.69	93.36 ± 1.48	★★
	M	54.51 ± 1.06	50.66 ± 1.01	★
Diameter	*A*	-6.77 ± 1.68	-3.70 ± 1.47	NS
	B	0.02245 ± 0.0098	0.02311 ± 0.00094	NS
	C	72.96 ± 1.84	64.93 ± 1.88	★★
	M	54.41 ± 1.03	48.30 ± 1.05	★★

† Using an F-test, NS denotes parameters are not significantly different; ★ denotes parameters are significantly different ($P \leq 0.05$); and ★★ denotes parameters are significantly different ($P \leq 0.01$).

3.2.2 Seed size and fruit growth

To determine whether 'Hass' fruit size was dependant on seed size, a range of differently sized fruits were weighed and the corresponding seed weights also recorded. Seedless avocado fruits are many times smaller than seed-bearing fruits (Fig. 3.3a) and results show that there was a positive correlation between seed and fruit size (Fig. 3.3b).

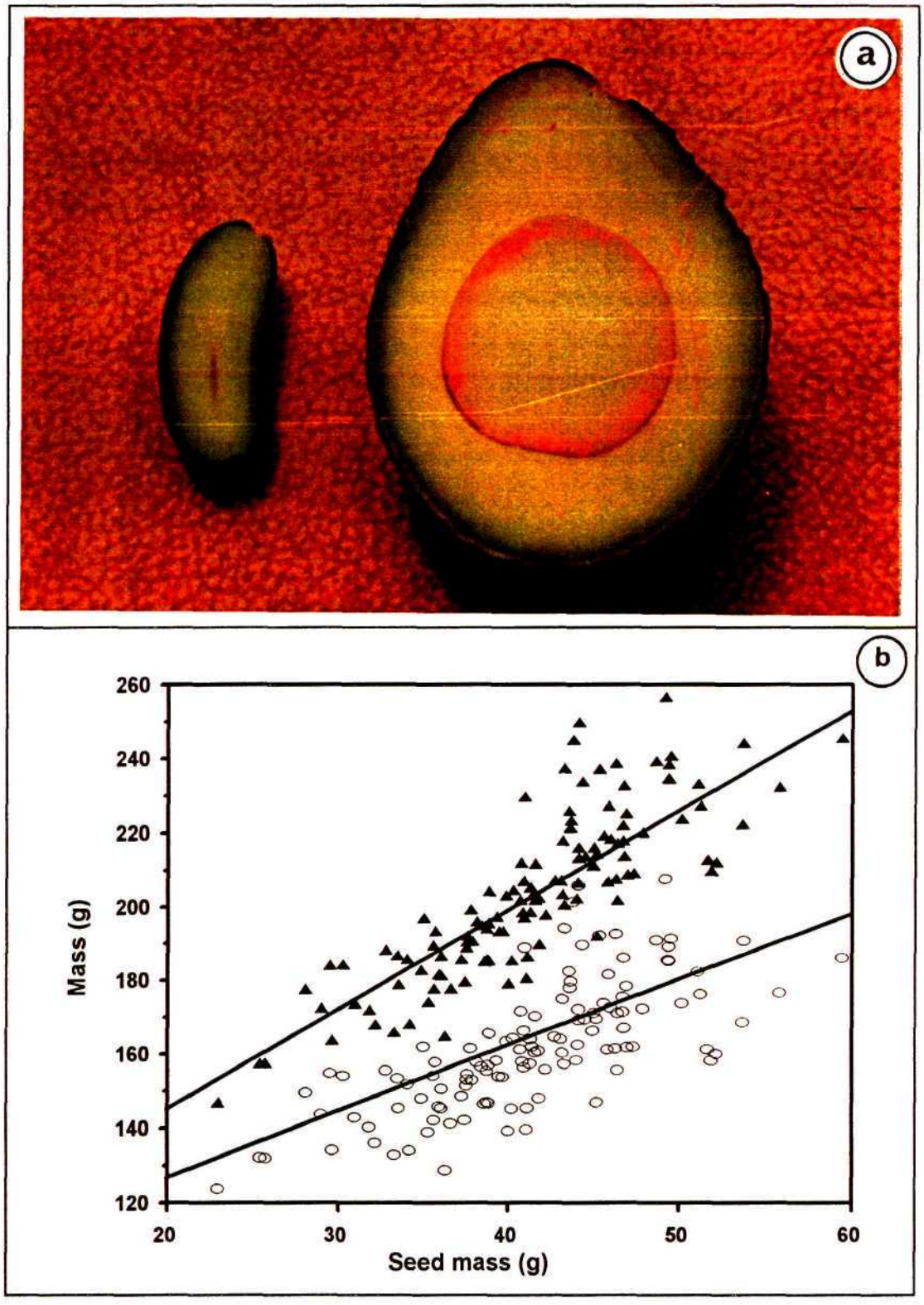

Figure 3.3

Photograph illustrating the importance of the seed to avocado fruit growth (a), and linear regression to illustrate the effect of seed size on fruit size (b). Linear regression line for whole fruit weight (▲) is represented by y = 2.881x + 84.266 (R^2 = 71.6%), and for flesh weight (○) by y = 1.881x + 84.266 (R^2 = 51.8%). Regression was performed on a sample population of 120 fruits.

By extrapolation, linear regression equations for whole fruit weight and flesh weight had the common intercept of 84.27 ± 12.01 g. As shown in Fig. 3.3b the slope coefficient for whole fruit weight was greater than that of fruit flesh weight alone (2.88 ± 0.17 compared to 1.88 ± 0.17). Thus, it can be concluded that the increase in whole fruit weight was dependant on the percentage increase in seed weight and was less affected by the percentage increase in flesh weight, i.e. larger fruit generally had a higher seed:fruit flesh ratio. In other words, although an increase in fruit size was accompanied by an increase in mesocarp weight, there was a proportionally greater increase in seed weight.

3.2.3 Seed coat senescence and fruit growth

3.2.3.1 Seed coat senescence and fruit size

To determine whether a relationship existed between 'Hass' avocado fruit size and seed coat senescence, a range of differently sized fruits were weighed and then sectioned longitudinally to examine the seed coats after removal of the seed. Fruits were allocated a seed coat viability rating ranging from healthy and functional to senescent and dessicated (Fig. 3.4a). Results show that a reasonably good relationship existed between fruit size and seed coat viability of fruit at maturity (Fig. 3.4b). Fruit less than 100 g fresh weight had completely degenerate seed coats or seed coats that showed signs of the onset of senescence, whereas larger (normal) fruits had a greater proportion of seed coats that appeared to be healthy and functional.

3.2.3.2 Seed coat structure

In the early stages of avocado fruit development, before the small fruit condition is evident, the outer seed coat boundary appears as a continuous lignified layer of fairly large and irregular stone cells (Fig. 3.5a). Adjacent to this layer is the endocarp (innermost portion of the mesocarp) which is characterised by two or three layers of partly lignified sclerenchyma cells (Fig. 3.5a). The inner seed coat boundary is thicker than the outer seed coat boundary and is less regular in appearance. It is tightly adhered to the seed, which is characterised by relatively small and tightly packed parenchyma cells (Fig. 3.5a). Between the two seed coat boundaries are several layers of parenchyma, which collectively make up the pachychalazal (seed coat) tissue.

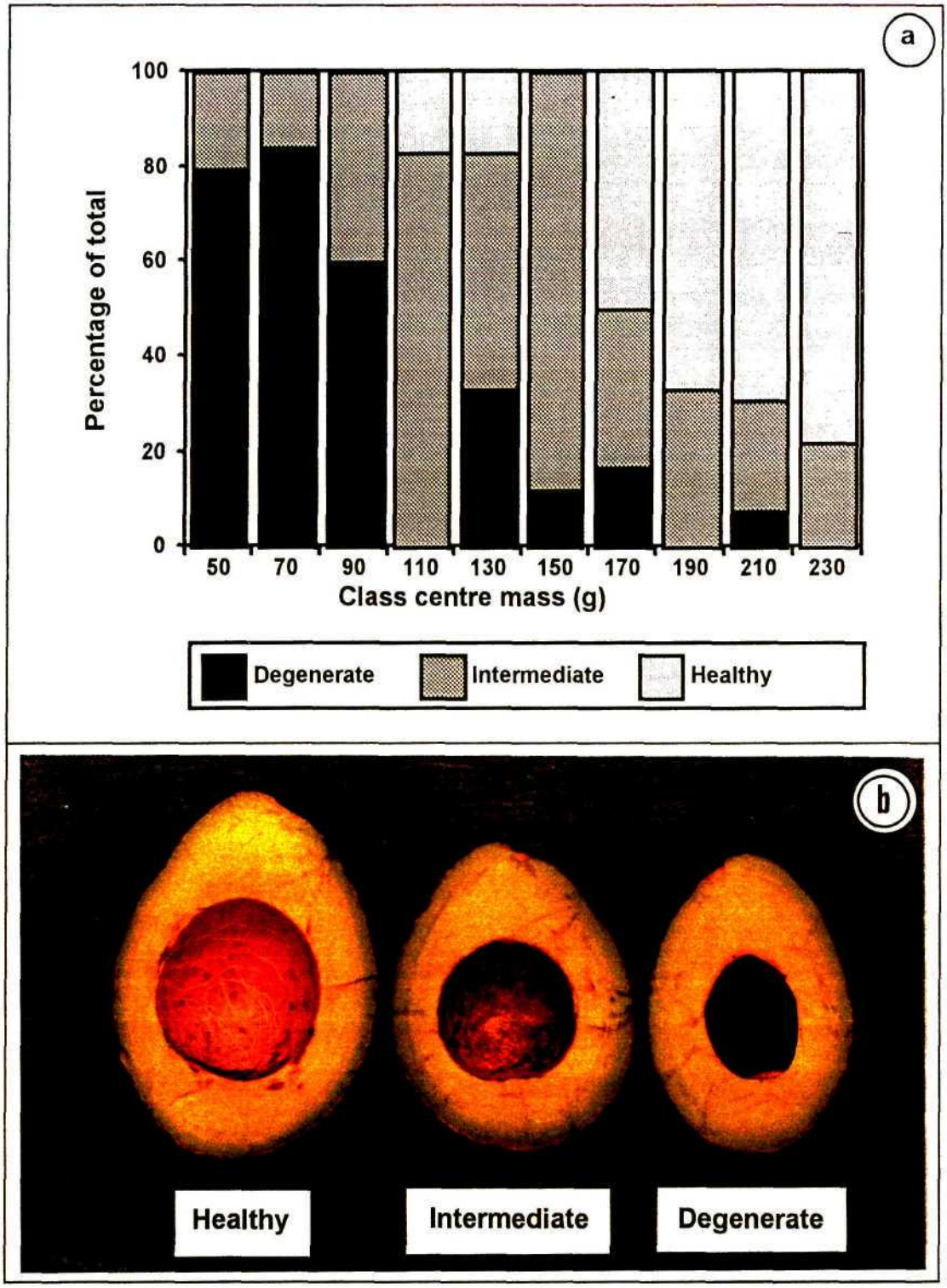

Figure 3.4

Relationship between seed coat viability and fruit size in 'Hass' avocado (a). Similarly aged fruit of harvestable maturity from 8 year old trees were sectioned longitudinally and the seed coats examined after removal of the seed (b). "Healthy" seed coats were turgid and white/pale yellow in colour, "degenerate" seed coats appeared senescent, dessicated and brown in colour. Seed coats of "intermediate" appearance were neither "healthy" nor "degenerate" but showed signs of dehydration and onset of senescence.

During the course of normal fruit development, the pachychalazal tissue increases in thickness as the parenchyma cells continue to divide (Fig. 3.5d).

Large fruits have well-developed vascular systems within the pachychalazal tissue, indicating that the seed coat forms a physiological connection between the seed and mesocarp. Xylem, viz. vessel elements and tracheids, become lignified and have thickened secondary walls (Fig. 3.5b). In longitudinal section, annular-ringed and spiral-form secondary wall thickening is evident (Fig. 3.5c). Presumably this imparts strength to the conducting tissue while allowing for expansion of the cells. Phloem tissue appears as a collection of irregularly shaped cells making up sieve elements, companion cells and parenchyma (Fig. 3.5b). This network of conducting tissue branches frequently, so towards the end of fruit development many vascular bundles permeate the seed coat tissue.

In contrast, small fruit, with degenerate or senescent seed coats, typically show a considerable degree of seed coat cell and tissue degeneration (Fig. 3.5e). The parenchyma tissue between the two seed coat boundaries is broken down. The dry seed coat becomes heavily lignified and there is evidence of tannin- and phenolic-accumulation. Unlike functional seed coats which adhere tightly to the seed (Fig. 3.5d), degenerate seed coats become physically separated from the seed (Fig. 3.5e).

The most obvious ultra-structural difference between functional and degenerate seed coat tissue is the state of their cell walls and membranes. In aborted seed coat tissue, cell wall breakdown was evident (Fig. 3.6a), whereas in healthy seed coat tissue, cell walls were rigid and organised (Fig. 3.6b). In degenerate seed coat cells, the cell membrane pulls away from the cell wall (Fig. 3.6a), in contrast to functional seed coat tissue where the cell membrane is still intact and closely bound to the cell wall (Fig. 3.6b). Furthermore, movement of rough endoplasmic reticuli across cell wall via plasmodesmata was common in healthy seed coat tissue and absent in degenerate seed coat tissue (Fig. 3.6b).

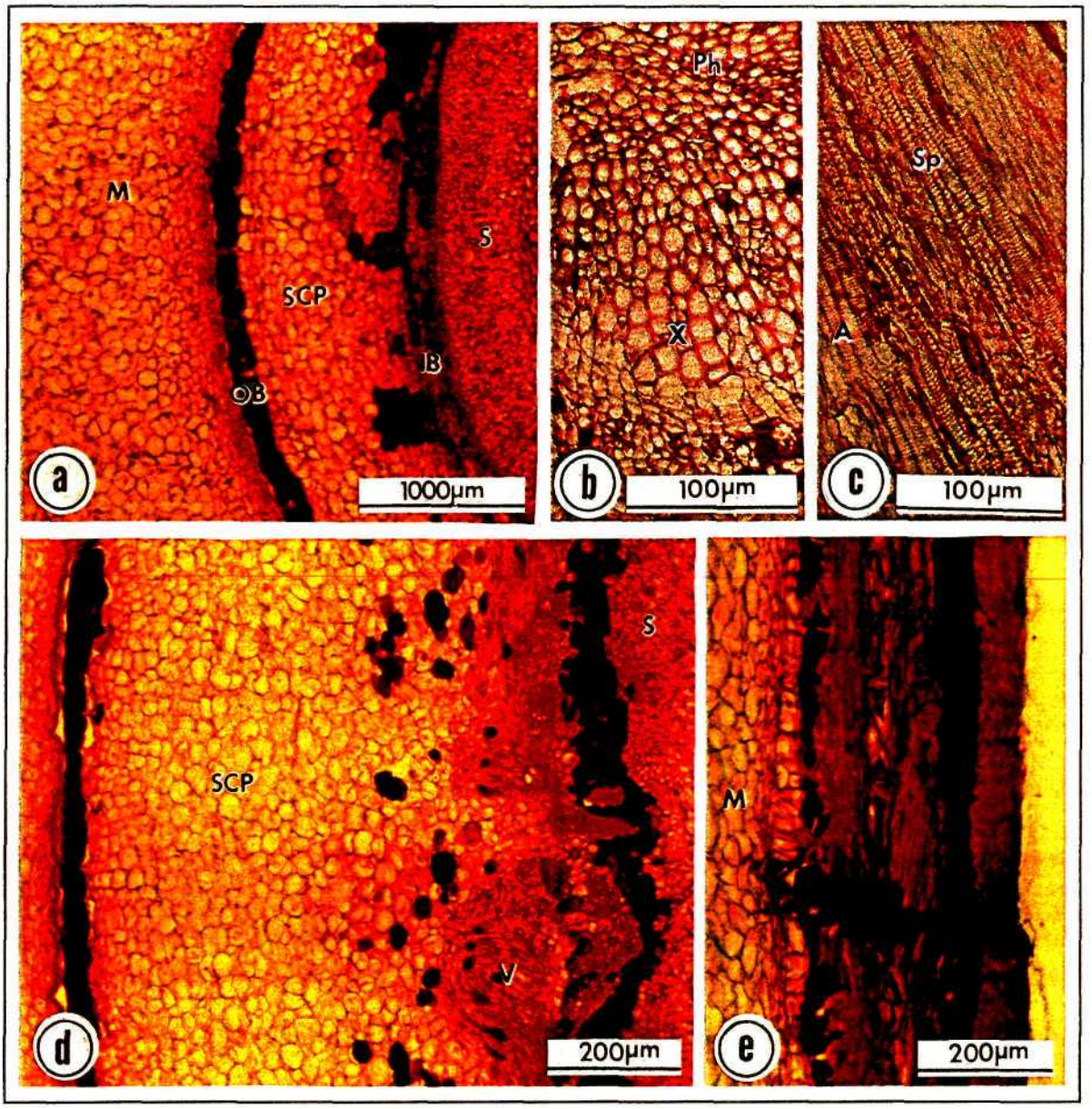

Figure 3.5

Light micrographs of transverse sections of developing 'Hass' avocado fruits illustrating gross structural changes to the seed coat. (a) 30 d after full bloom; (b) vascular system permeating functional seed coat tissue, 161 d after full bloom; (c) LS illustrating annular-ringed and spiral-form secondary wall thickening of xylem conducting elements, 161 d after full bloom; (d) large fruit, 161 d after full bloom; (e) small fruit, 161 d after full bloom. (S = seed tissue; SCP = seed coat parenchyma; IB = inner seed coat boundary; OB = outer seed coat boundary; M = mesocarp; V = vascular system; Ph = phloem; X = xylem; A = annular-ringed; Sp = spiral form).

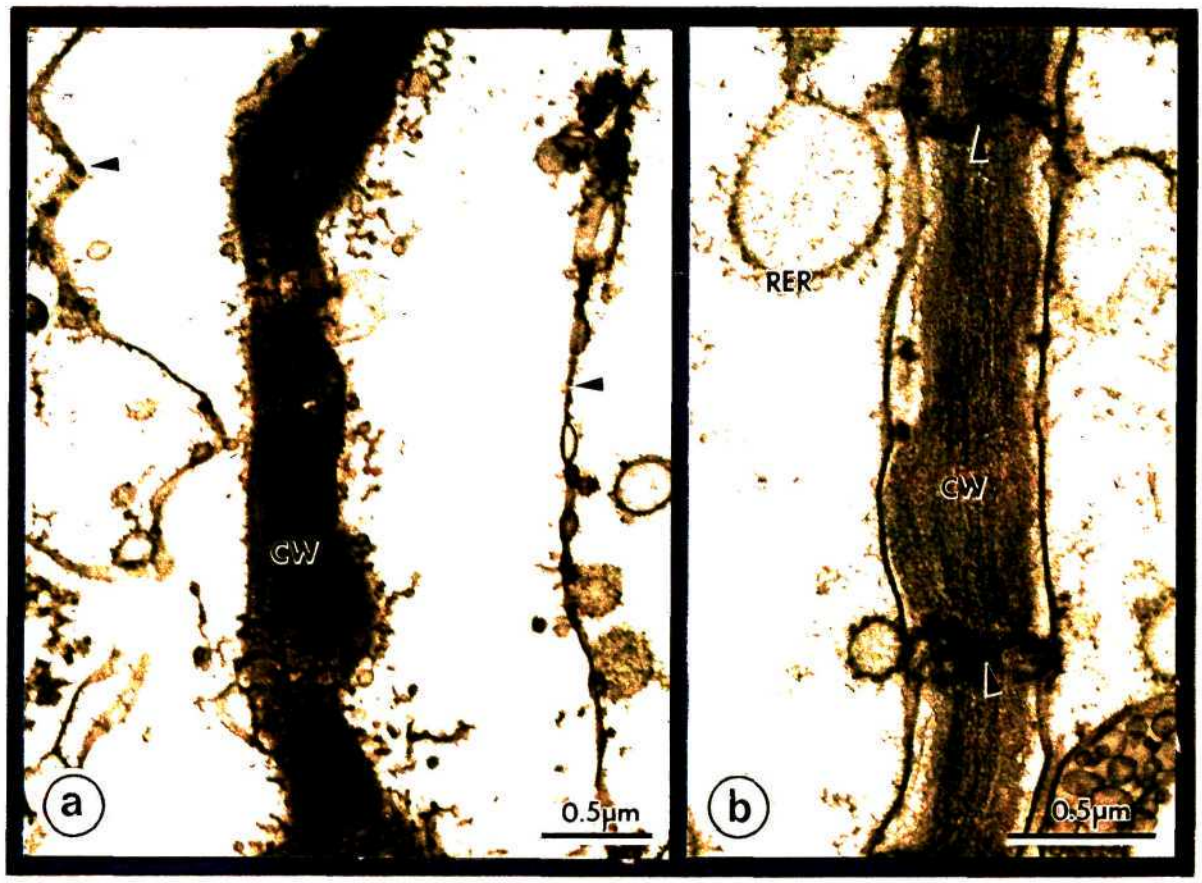

Figure 3.6

Transmission electron micrographs of 'Hass' avocado seed coat tissue, 161 d after fruit set. (a) Small fruit with degenerate seed coat (arrows indicate where plasma membrane has pulled away from the cell wall (CW)). (b) Large fruit with functional seed coat (RER = rough endoplasmic reticulum; arrow = plasmodesmata).

3.2.4 Cell size vs cell number

Measurements of cellular development included mean rate of cell division and mean rate of cell expansion. These measurements were fitted to a general logistic curve which has the following mathematical equation; $y = (a + b).r^x$ (where; a = asymptote, $a+b$ = starting value, and r = rate). The resultant trends are illustrated in Figures 3.7 and 3.8.

There were no significant differences between normal and phenotypically small fruit for the parameter representing rate of cell expansion (Table 3.2). This supports work done by Schroeder (1953) who found that different sizes of horticulturally mature 'Fuerte' fruit had the same average cell size. In contrast, the parameter representing rate of cell

division was significantly different between normal and phenotypically small fruit ($P \leq 0.01$) (Table 3.2). Anatomically then, the limiting factor for growth in small 'Hass' fruit was a reduction of cell division in the mesocarp tissue. By the end of fruit development there were an average of 84.5 ± 2.1 more cells across the fruit diameter of normal fruits.

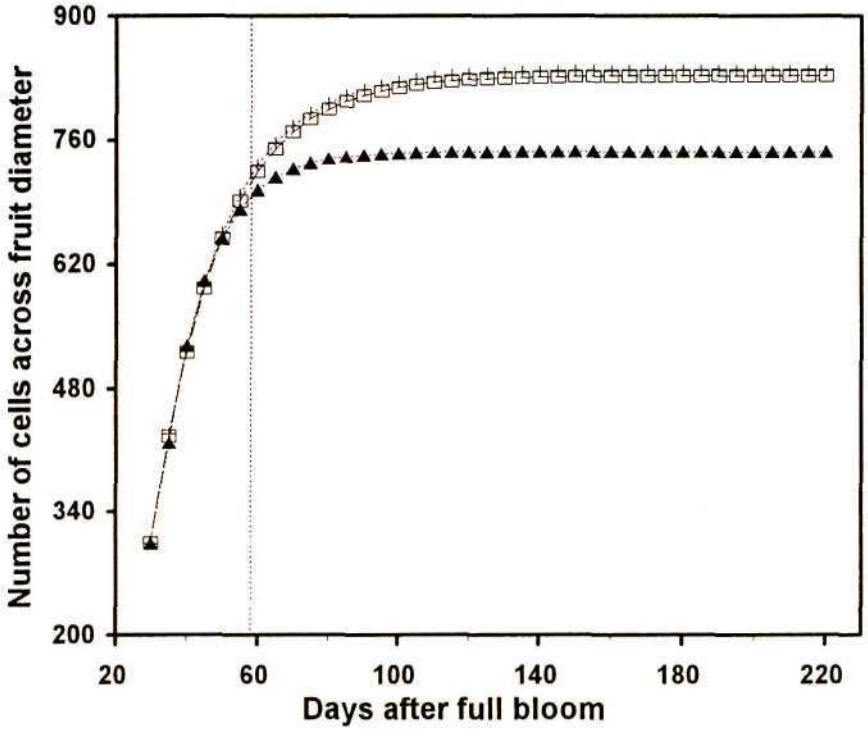

Figure 3.7

Estimated changes in mean equatorial mesocarp cell number during the course of 'Hass' fruit development. The vertical line at 57 d after full bloom represents time at which fruit with degenerate seed coats were first recorded. Regression line for normal fruit (□) is represented by $y = 833.9 - 2560.5(0.9488)^x$; fruit with healthy seed coats (+) by $y = 839.6 - 2661.8(0.9480)$; and fruit with degenerate seed coats (▲) by $y = 746.9 - 5576.3(0.9222)^x$. (Curves were calculated from a total of 54 measurements per treatment at each time interval).

When comparing *r* for cell size and cell number measurements of fruit with degenerate seed coats and those with functional ones, it is apparent that cell size is not a function of seed coat degeneration (Fig. 3.8; Table 3.2). Seed coat degeneration has a marked effect on rate of cell division, with rate being significantly greater ($P \leq 0.01$) in fruits with functional seed coats (Table 3.2). From the time at which fruits with aborted seed coats were first recorded, 57 d after full bloom, rate of cell division in fruits with degenerate seed coats slowed markedly, whereas in fruits with healthy seed coats, cell division continued for at least a further 50 d (Fig. 3.7).

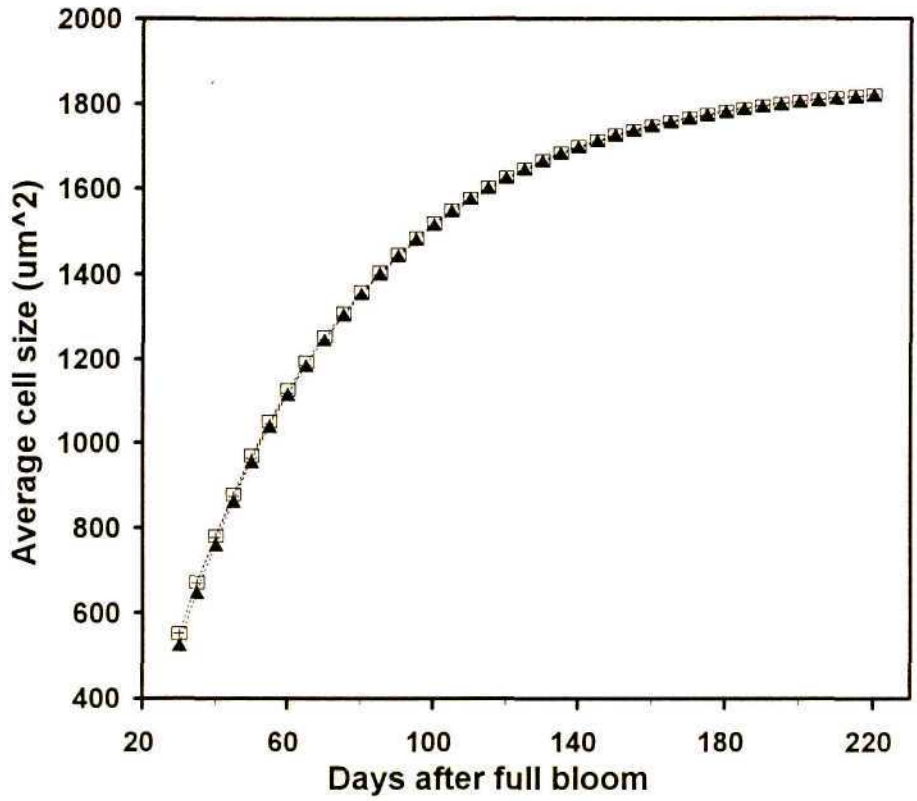

Figure 3.8

Mean mesocarp cell size during the course of 'Hass' avocado fruit development. Regression line for normal fruit (❑) is represented by $y = 1854.5 - 2330.0(0.9808)^x$; fruit with healthy seed coats (+) by $y = 1859.5 - 2311.3(0.9812)^x$; and fruits with degenerate seed coats (▲) by $y = 1852.9 - 2390.8(0.9806)^x$ (Curves were calculated from a total of 54 measurements per treatment at each time interval).

Table 3.2

Summary of mean values for the parameter representing rate (*r*) of the general logistic curve for cell size and cell number measurements. Values are means of 54 measurements at each time interval.

	Rate (*r*)	
	Cell number	Cell size
Normal fruit	0.9488[a†]	0.9808[a]
Small fruit	0.9233[b]	0.9814[a]
Healthy seed coat	0.9480[a]	0.9812[a]
Degenerate seed coat	0.9222[b]	0.9806[a]

The LSD (1%) between treatments is 0.0018 and 0.0008 for cell number and cell size measurements respectively. †Treatments having different letters are significantly different between treatments ($P \leq 0.01$).

3.2.5 Fruit size and concentration of iP and ABA

To determine what relationship, if any, exists between iP and ABA and expression of the 'Hass' avocado small fruit phenotype, mesocarp iP and ABA concentrations of fruit at harvestable maturity were measured by RIA and HPLC respectively. The results presented in Figure 3.9 show that phenotypically small fruit contained substantially less iP than similarly aged large fruit and that with an increase in fruit size there is a concomitant increase in iP concentration. A similar effect of fruit size on fruit ABA content was observed. Normal 'Hass' fruit contained substantially less ABA than phenotypically small fruit (Fig. 3.10). The combined effect was an elevation in the mesocarp iP:ABA ratio with increasing fruit size (Fig. 3.11).

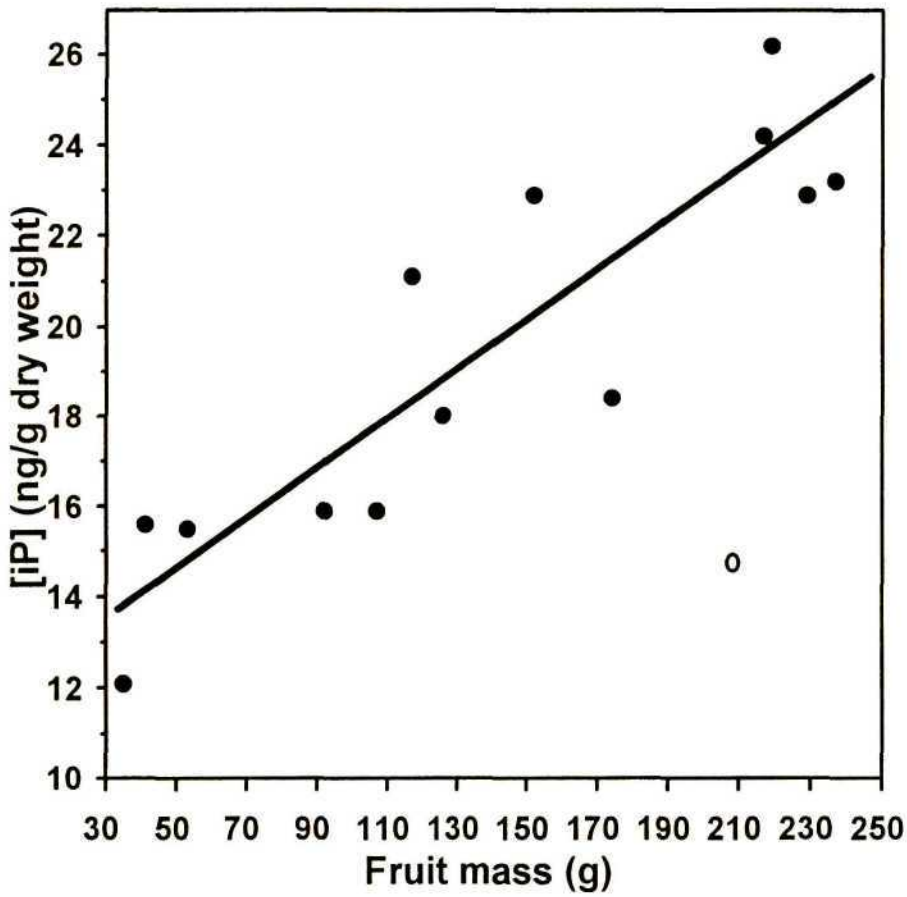

Figure 3.9
Relationship between fruit size and mesocarp ABA concentration. Regression line is represented by the equation $y = 18.62 + [-27.3/(1 - 0.0407x)]$, $R^2 = 85.0\%$.

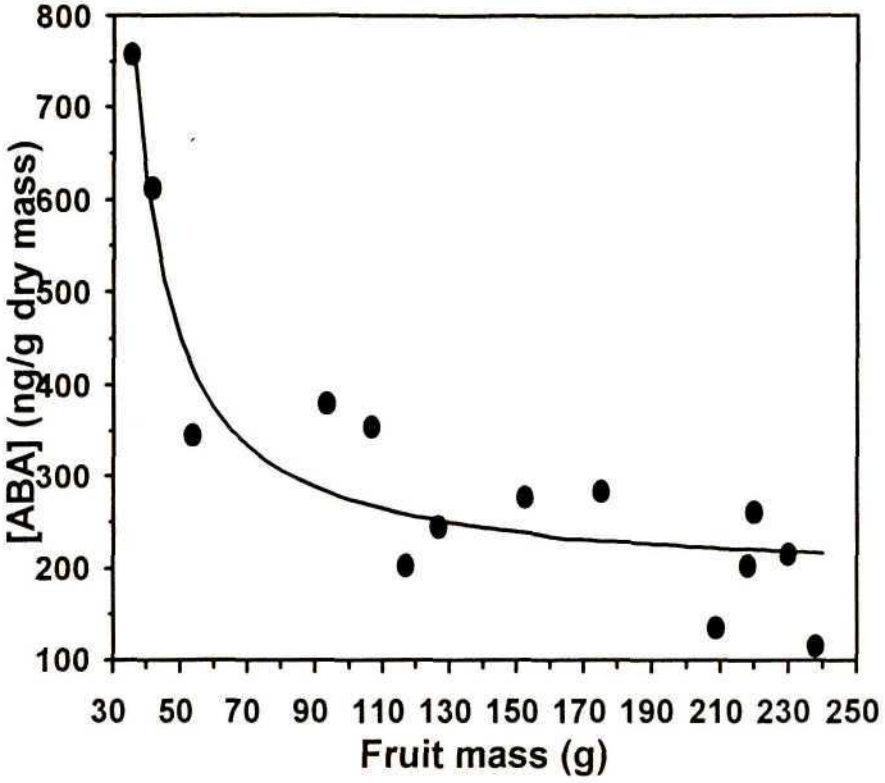

Figure 3.10

Relationship between fruit size and mesocarp iP concentration. Regression line is represented by the equation y = 0.053x + 12.07, R^2 = 79.6% (o represents a high residual point and was excluded from the regression analysis).

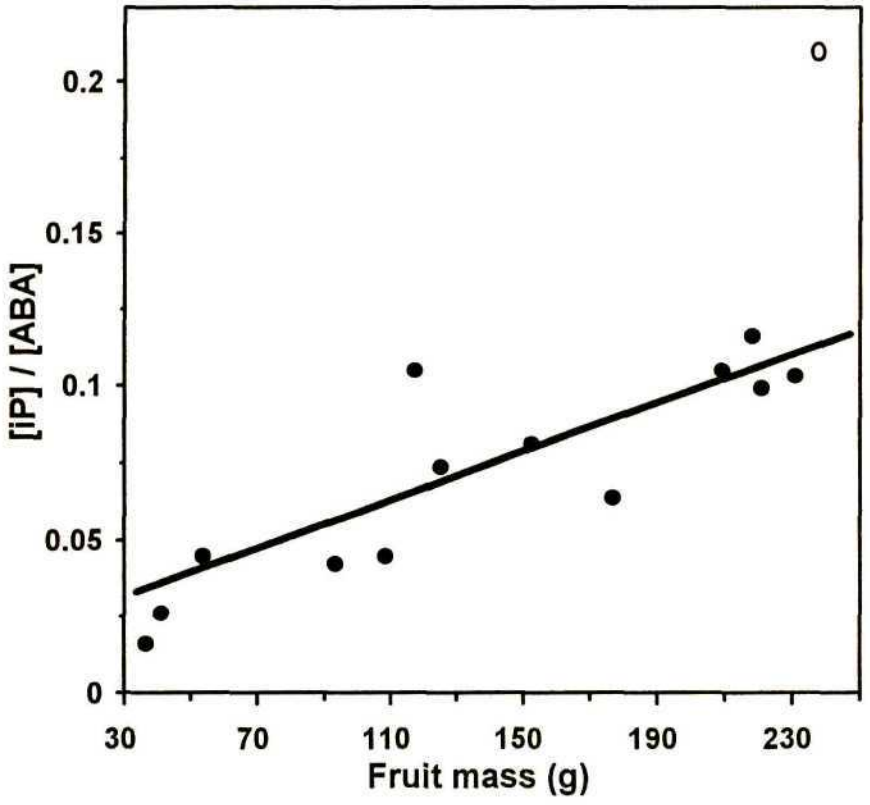

Figure 3.11

Relationship between fruit size and mesocarp iP:ABA ratio. Regression line is represented by the equation y = 0.00278x + 0.01103, R^2 = 76.2% (o represents a high leverage point and was excluded from the regression analysis).

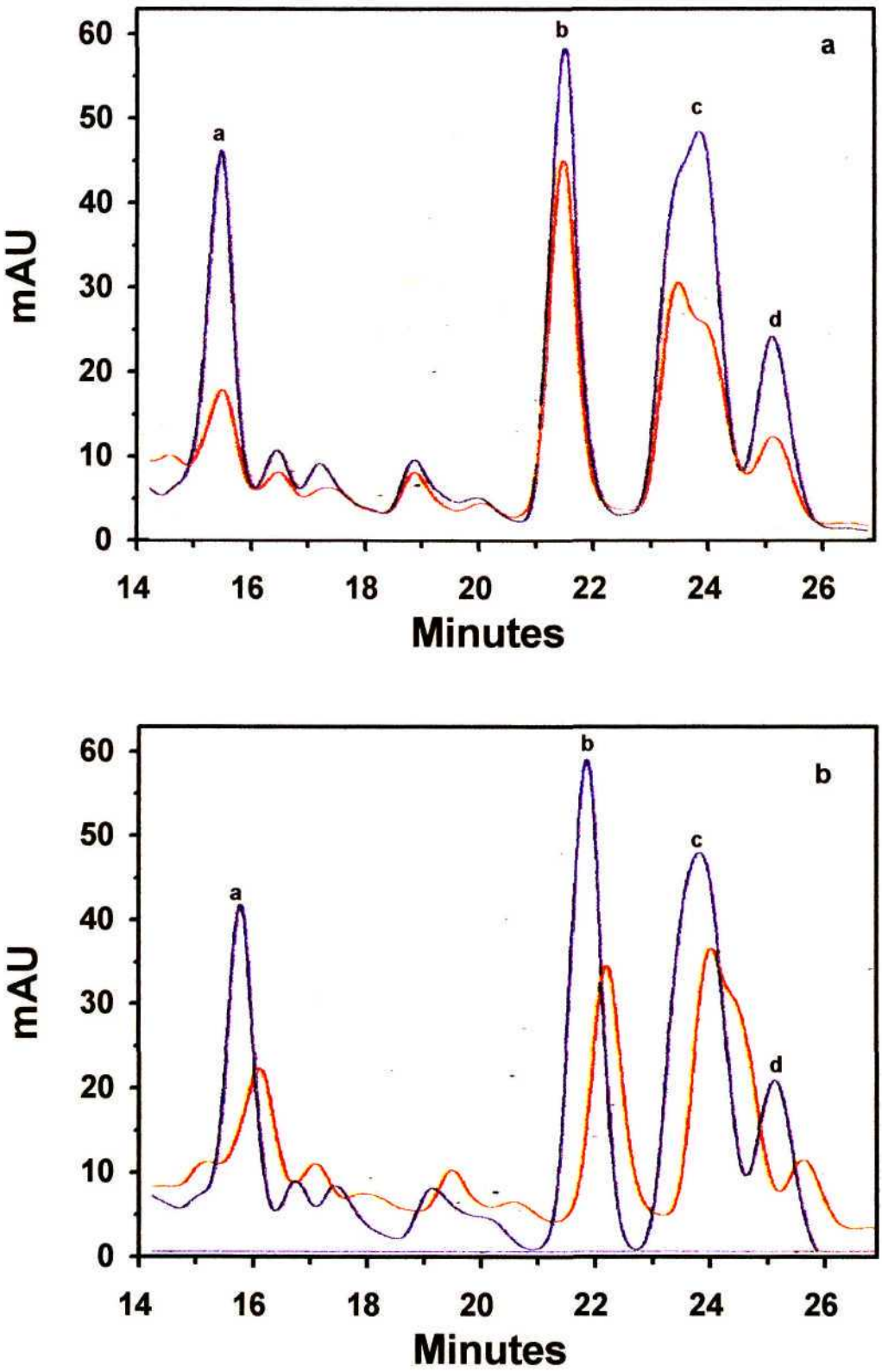

Figure 3.12

Chromatograph of sterols in the mesocarp of phenotypically small (blue) and normal (red) 'Hass' avocado fruit, 55 d (a) and 146 d (b) after full bloom.

3.2.6 Fruit size and sterols

Sterols of mesocarp of phenotypically small and normal fruit were analysed by reverse phase HPLC and data are illustrated in Figure 3.12 (a and b). Results show that four major sterol-like components were resolved, and one of these (labelled C) clearly comprised more than one compound. Although identification of these sterols was unfortunately not determined in the present study, it is evident that small fruit contained higher concentrations of phytosterols A, B, C and D on two consecutive sampling dates (Fig. 3.12a and b), indicating that these products of isoprenoid metabolism are not limiting in small 'Hass' fruit. Even so, sterol accumulation has been associated with increased ageing and onset of membrane maturity (Stalleant and Geuns, 1994), processes that would be expected to occur in small 'Hass' fruit in which cell division ceases early and is followed by seed coat senescence and the onset of horticultural maturity.

3.3 SUMMARY

(1) Phenotypically small 'Hass' fruit (less than 100g) always contained a degenerate seed coat, and fruit size was closely correlated to seed size.

(2) Final 'Hass' avocado fruit size is a function of mesocarp cell number and not mesocarp cell size.

(3) Fruit size was positively correlated with CK concentration and negatively correlated with ABA concentration, and mesocarp sterol complement showed qualitative and quantitative differences between phenotypically small and normal fruit. Mesocarp iP:ABA ratio was linearly correlated with increasing fruit size.

CHAPTER 4

ROLE OF ISOPRENOIDS AND 3-HYDROXY-3-METHYLGLUTARYL COENZYME A IN THE METABOLIC CONTROL OF AVOCADO FRUIT GROWTH

4.1 INTRODUCTION

For plant growth and development, synthesis of isoprenoids is fundamental because the pathway supplies compounds which are essential for full morphogenic expression. This class of compounds is of structural significance, e.g, carotenoids and the side chain chlorophylls and plastoquinone for photosynthesis, the side chain of ubiquinone for respiration, sterols for membrane structure and phytoalexins for defence. The pathway also supplies several regulatory molecules including ABA, brassinosteroids, gibberellins and the side chain of CKs (Fig. 4.1) that contribute to control of both temporal and spatial events during higher plant ontogeny. Despite this, surprisingly little information is available concerning regulation of isoprenoid biosynthesis in plants and plant parts, particularly developing fruit.

While controversy still surrounds the subcellular site of MVA metabolism (Campos and Boronat, 1995; Chappell, 1995a; 1995b), it is generally agreed that reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) is potentially a major point of regulation of isoprenoid biosynthesis in plants (Bach, 1987; Gray, 1987; Gondet *et al.*, 1992; Moore and Oishi, 1994; Chappell *et al.*, 1995). HMG-CoA arises from the sequential condensation of three acetyl-CoA units (Chappell, 1995a) (Fig. 4.1). The conversion of HMG-CoA to mevalonate (Fig. 4.1) is irreversible, and is considered to be the rate limiting step for sterol metabolism in mammals (Goldstein and Brown, 1990). Whether HMGR plays a similar rate-limiting role in controlling plant isoprenoid biosynthesis remains unresolved (Bach *et al.*, 1991; Choi *et al.*, 1992). In plants, increased ABA concentration, associated with stress, has been correlated with decreased HMGR activity in developing endosperm of maize vivipary mutants (Moore and Oishi, 1994). Using tomato as a model system, Narita and Gruissem (1989) demonstrated that HMGR expression and activity is required during early fruit development. Furthermore, these authors showed that *in vivo* inhibition of HMGR

during early fruit development disrupted the process whereas inhibition during the later, expansion stage had no significant effect. Since ripening was apparently unaffected it was concluded that inhibition of HMGR reduced the MVA pool required for phytosterol biosynthesis.

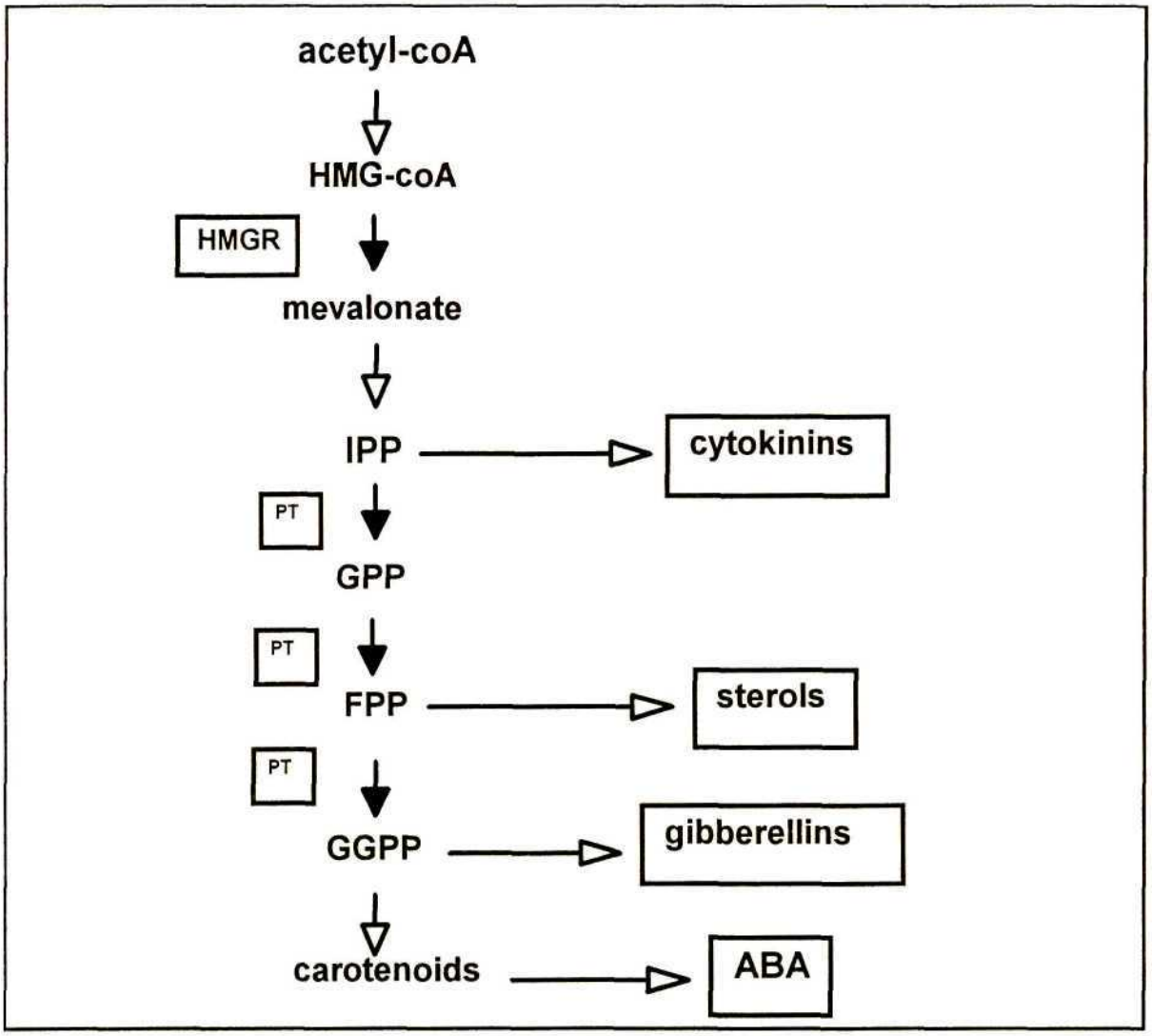

Figure 4.1

Schematic representation of the isoprenoid biosynthetic pathway. Light arrows indicate multiple steps or reactions (IPP = isopentenyl diphosphate; GPP = geranyl diphosphate; FPP = farnesyl diphosphate; GGPP = geranylgeranyl diphosphate; and PT = prenyltransferase-controlled reaction).

The small fruit condition is closely correlated to a low CK:ABA ratio (Fig. 3.11). It is therefore proposed that a decline in the CK:ABA ratio lessens sink strength of developing organs by influencing HMGR and cell division cycle activity to reduce final fruit size. This hypothesis is supported by evidence which shows that ABA retards cell

division cycle activity (Müller *et al.*, 1994) and inhibits HMGR activity (Russell and Davidson, 1982; Moore and Oishi, 1994) in several higher plant tissue systems. In order to examine the interrelationship between HMGR, isoprenoid growth regulators and the small fruit phenotype in 'Hass' avocado, mevastatin was used to specifically inhibit *in vivo* HMGR activity during phase I, II and III of the developmental programme. Supplementation with products of the isoprenoid biosynthetic pathway, and similarly derived plant hormones, was performed to reveal which isoprenoids were the most limiting during fruit growth and development.

4.2 RESULTS

4.2.1 Inhibition of fruit growth by mevastatin and effect of sterols

Injection of mevastatin, a competitive inhibitor of HMGR, through the pedicel during either phase I or phase II retarded avocado fruit growth and development by 60% (Figs. 4.2 and 4.3).

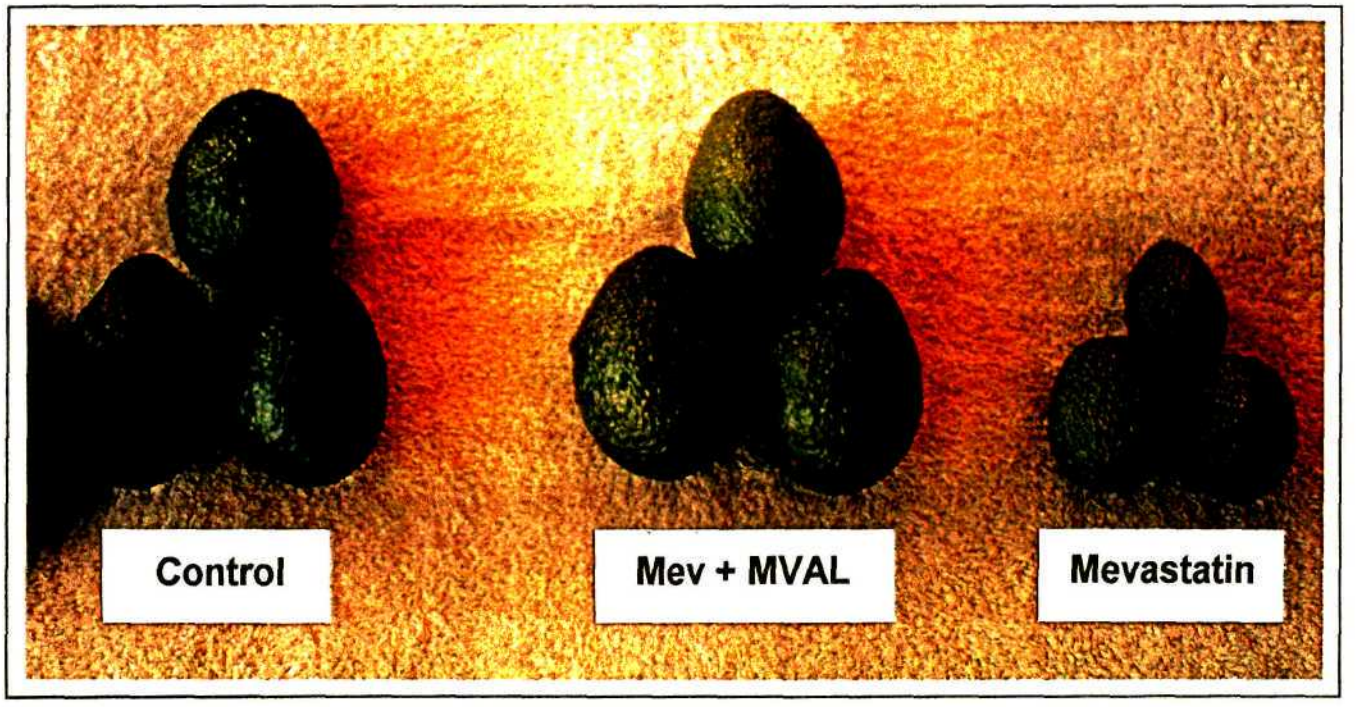

Figure 4.2

Photograph illustrating the effect of mevastatin on 'Hass' avocado fruit growth, and the reversal of this effect by co-injection with MVAL. 20 ug of mevastatin was injected into each fruit.

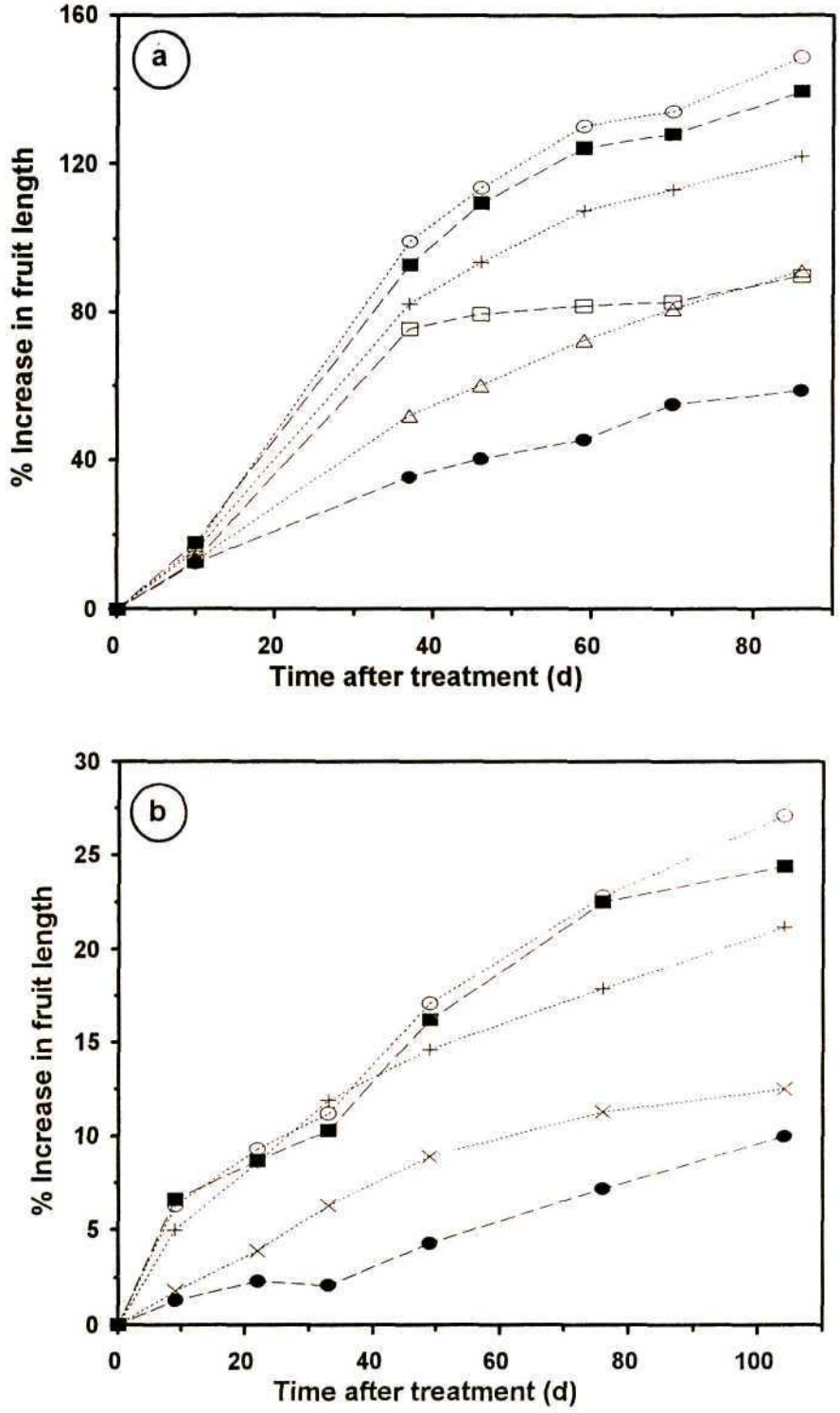

Figure 4.3

Effect of mevastatin, stigmasterol and cholesterol on 'Hass' avocado fruit growth. Compounds of interest were applied during the 1994/95 season. 20 µL Tween 20:acetone:water (1:1:8, by vol.) via the pedicel at concentrations of 1 µg µL^{-1} (a) 55 d (Phase I) and (b) 92 d (Phase II) after full bloom, and growth monitored as % increase in fruit length. Each value represents the mean of 8 determinations. SE (diff) = 9.0 (a) and 0.9 (b). Control (○); mevastatin (●); mevastatin + MVAL (■); stigmasterol (+); mevastatin + stigmasterol (x); cholesterol (Δ); stigmasterol + cholesterol (❑).

In both experiments, mevastatin-induced retardation of fruit growth was reversed by co-injection with MVAL resulting in recovery of the normal phenotype. Sterols reduced avocado fruit growth when applied either in phase I or phase II (Figs. 4.3 a and b). A combination of cholesterol and stigmasterol, administered during phase I, also reduced fruit growth and eventually arrested the process (Fig. 4.3a), causing 50% fruit abscission 70 d after treatment. Although stigmasterol retarded avocado fruit growth to the same extent when applied in phase II, it partially reversed the inhibitory effect of mevastatin (Fig. 4.3b).

4.2.2 Effect of plant growth regulators on mevastatin-induced inhibition of fruit growth

To determine the relationship between the plant growth regulators, iP and ABA, and expression of the 'Hass' avocado small fruit phenotype, a single 20 µg dose of ABA was administered via the pedicel to large fruit in the linear phase of rapid growth, either in the presence or absence of iP, and the effect of each treatment compared with respect to untreated large and phenotypically small fruit at harvest. Application of exogenous ABA to large fruit during the linear phase of rapid growth caused fruit growth to slow and induced seed coat senescence (Fig. 4.4). When ABA was co-injected with an equal concentration of iP the deleterious effects of ABA were negated, suggesting that an imbalance in the CK:ABA ratio may be pivotal in determination of phenotypic expression.

Results presented in Figure 4.5a show that mevastatin-induced retardation of 'Hass' avocado fruit growth during phase I (55 d after full bloom), could be completely reversed by co-injection with either MVAL, iP or the CK analogue CPPU. GA_3 and stigmasterol by comparison, had little or no effect.

As shown in Figure 4.5b, neither CK, stigmasterol or GA_3 markedly influenced the 'normal' course of 'Hass' avocado fruit development when applied during phase I, although towards conclusion of this growth period both GA_3- and stigmasterol-treated fruit showed a slowing of growth. Likewise, AMO-1618, a purported inhibitor of kaurene synthase activity (Dennis *et al.*, 1965) and sterol biosynthesis (Douglas and Paleg,

1972), did not markedly affect fruit growth, although it did cause growth to slow towards the end of the experimental time frame. Exogenously applied ABA, however, reduced fruit growth substantially and caused 90% fruit abscission within 50 d of application. Co-injection with iP reversed the growth-retarding effect of ABA (Fig. 4.5b) and reduced the incidence of fruit abscission compared to that observed in control treatments (Fig. 4.7).

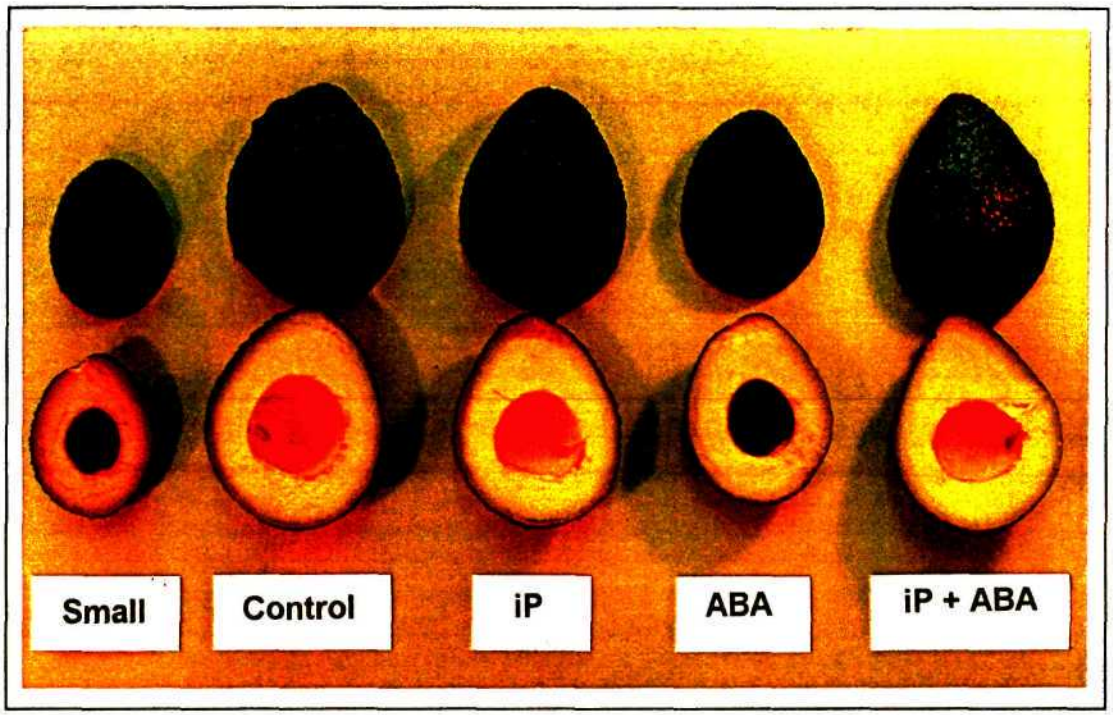

Figure 4.4

Photograph comparing small and large 'Hass' fruit with fruit pre-treated with ABA, iP and ABA + iP, and illustrating the effect of these plant growth substances on seed coat senescence.

During phase II (146 d after full bloom), treatment of fruit with iP countered the growth retarding effect of mevastatin (Fig. 4.5c). iP alone, however, did not increase fruit growth during this phase.

In phase III (210 d after full bloom), mevastatin reduced growth by 50% (Fig. 4.6a) whereas treatment with iP stimulated this process (Fig. 4.6b). Surprisingly, only iP completely reversed the growth retarding effect of mevastatin, although co-injection of mevastatin with either MVAL or stigmasterol reduced the effect of this inhibitor (Fig. 4.6a). ABA reduced avocado fruit growth by 50% and this effect was reversed in fruits co-treated with iP.

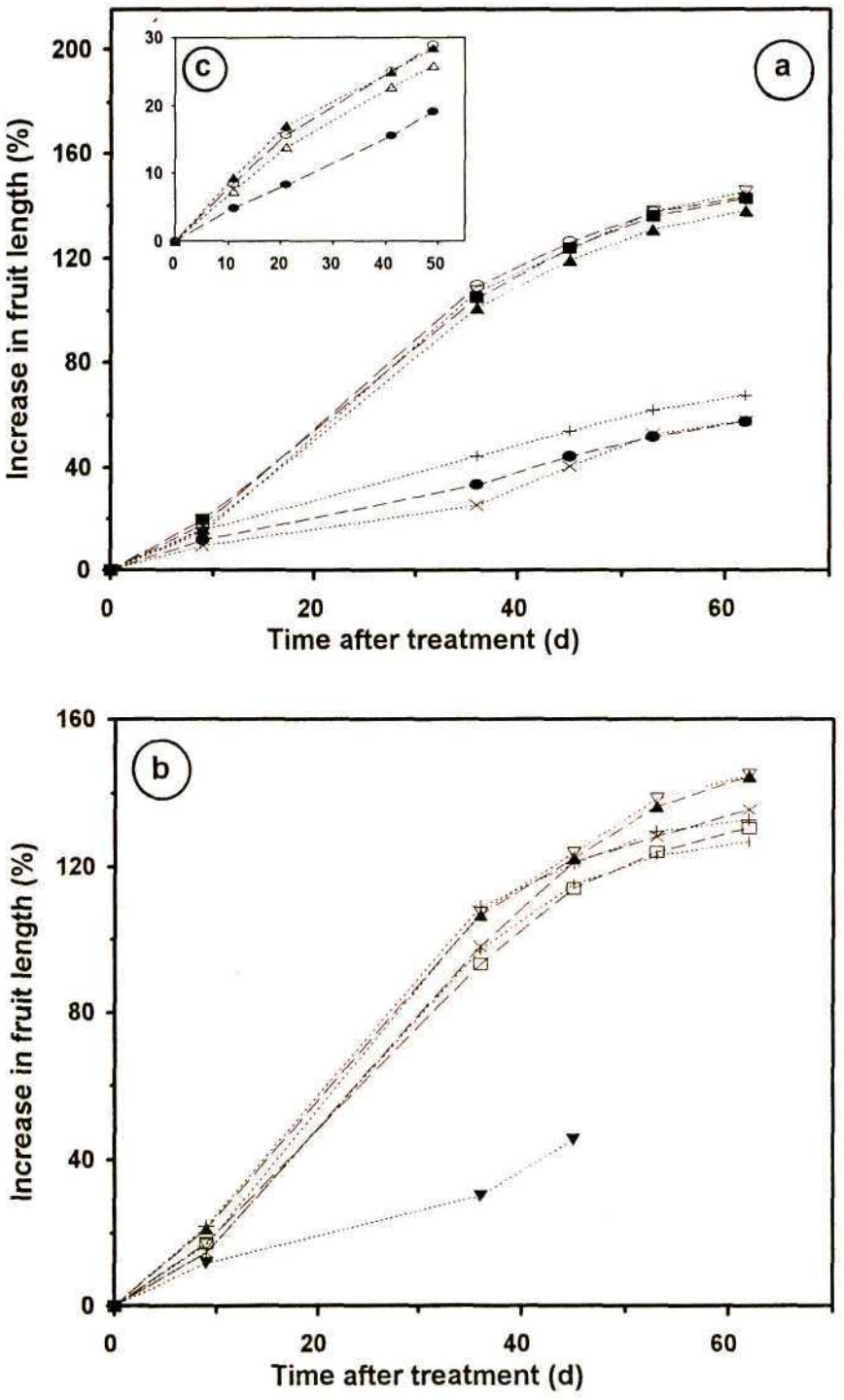

Figure 4.5

Influence of isoprenoid growth regulators on mevastatin-induced inhibition of 'Hass' fruit growth. (a and c), mevastatin treated; and (b), control. Compounds of interest were applied in phase I (a, 55 d after full bloom), phase II (b, 146 d after full bloom) and phase III (c, 210 d after full bloom) of the 1995/96 season, and growth monitored as a % increase in fruit length. Each value represents the mean of 8 determinations. SE (diff) = 6.0 (a and b), and SE (diff) = 3.5 (c). Control (○); mevastatin (●); MVAL (■); mevastatin + iP (△); iP (▲); ABA (▼); iP + ABA (❑); AMO 1618 (I); CPPU (V); GA_3 (+); stigmasterol (x).

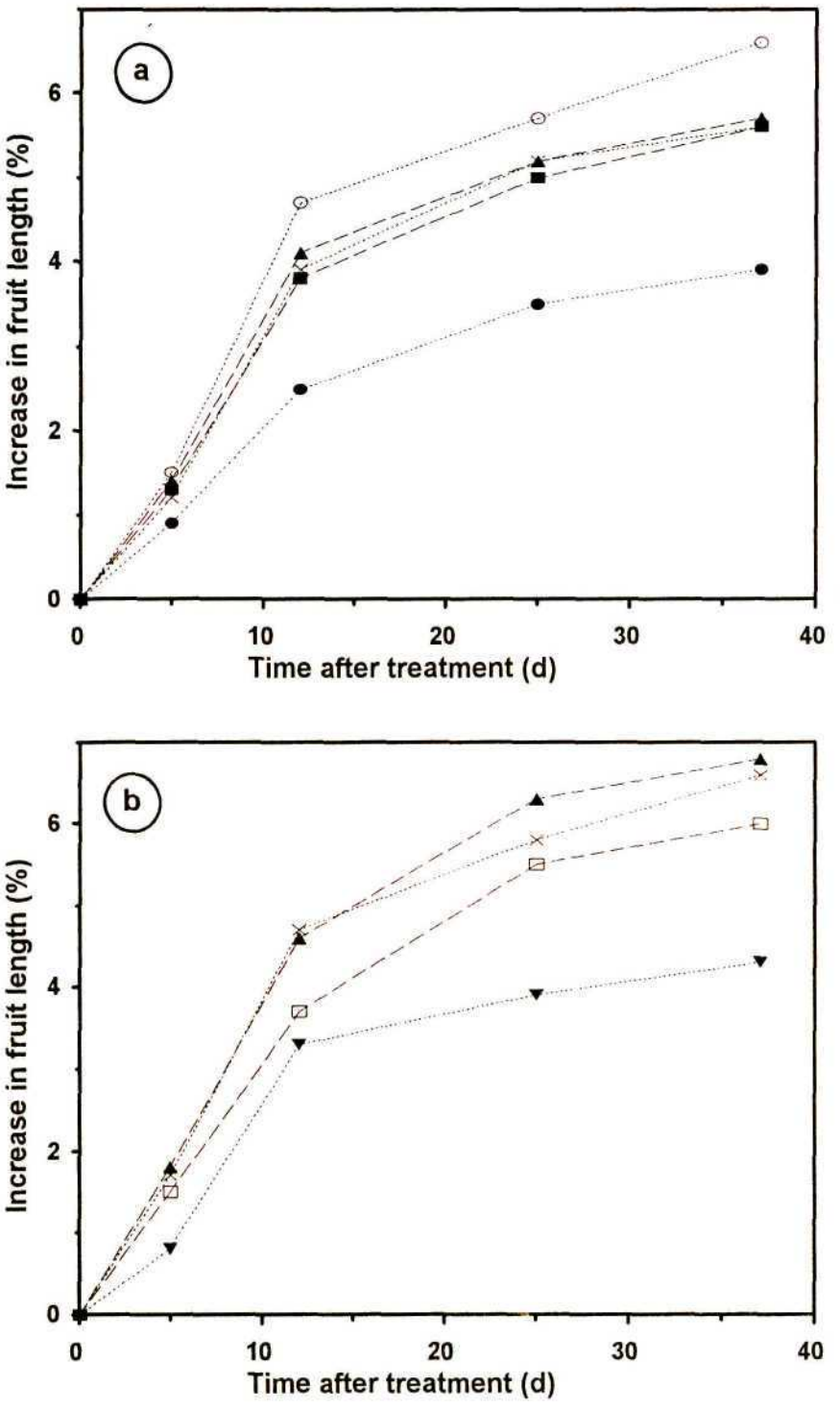

Figure 4.6

Effect of iP, MVAL and stigmasterol on growth of mevastatin-treated fruit (a), and effect of iP, ABA and stigmasterol on growth of control fruit (b) during phase III. Chemicals were applied in 20 µL Tween 20:acetone:water (1:1, by vol.) via the pedicel 210 d after full bloom (phase III) during the 1995/96 season at concentrations of 1 µg μL^{-1} and growth monitored as a % increase in fruit length. Determinations are the mean of 8 fruits per treatment. SE (diff) = 1.4. Control (○); mevastatin (●); MVAL (■); iP (▲); stigmasterol (x); ABA (▼); iP + ABA (□).

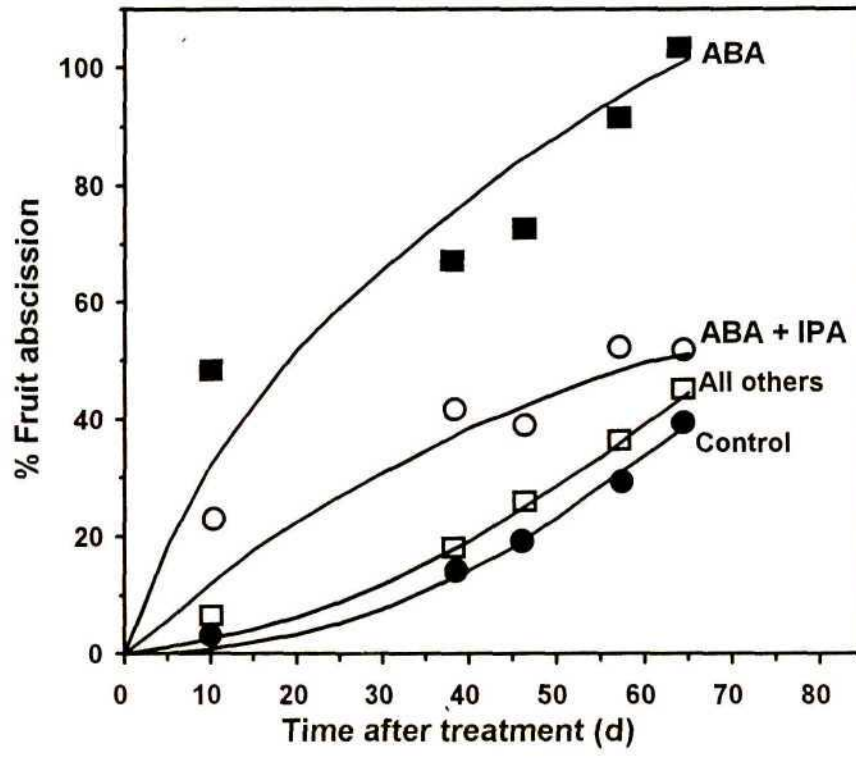

Figure 4.7

Comparison between the effect of ABA, ABA + iP, and all other treatments on 'Hass' avocado fruit abscission following pedicel injection of compounds during phase I of development. Experimental conditions were as described for Figure 4.5. Control (●); ABA (■); ABA + iP (○); all others (□).

4.2.3 Microsomal HMGR activity of mevastatin treated and non-treated fruit

In an attempt to further elucidate the proposed link between CKs, sterols, and the synthesis of MVA, HMGR activity in fruits treated with or without mevastatin in phase I, II and III was determined and the results are presented in Figure 4.8.

During the course of 'Hass' avocado fruit development, activity of microsomal HMGR remained unchanged (Fig. 4.8a). Although a similar trend was observed for small fruit, specific activity of microsomal HMGR was approximately 30% that of untreated and control fruit of comparable age (Fig. 4.8c). Fruit pretreated with mevastatin in either phase I, II or III showed a substantial reduction in HMGR activity (Fig. 4.8d), with levels similar to those observed for small fruit. Likewise, ABA treatment of fruit in phase III reduced *in vivo* HMGR activity by 70% to 1.41 ± 0.27 nmol h^{-1} mg^{1} protein. Unfortunately, insufficient samples, due to fruit abscission, precluded a comprehensive assessment of the effect of ABA on *in vivo* HMGR activity during avocado fruit

development. Even so, co-injection of ABA with iP during phase III partially restored HMGR activity (cf. 2.15 ± 0.24 versus 6.35 ± 0.92 nmol h^{-1} mg^{-1} protein in untreated fruit). HMGR activity was unaffected in fruits co-injected with MVAL and mevastatin (Fig. 4.8e), whereas stigmasterol was inhibitory and exacerbated the effect of mevastatin on enzyme activity (Figs. 4.8f and g). Treatment of fruit with iP did not affect HMGR activity significantly during the course of fruit development (Fig. 4.8h). At all stages of fruit growth iP treatment reversed the inhibitory effect of mevastatin (Fig. 4.8i).

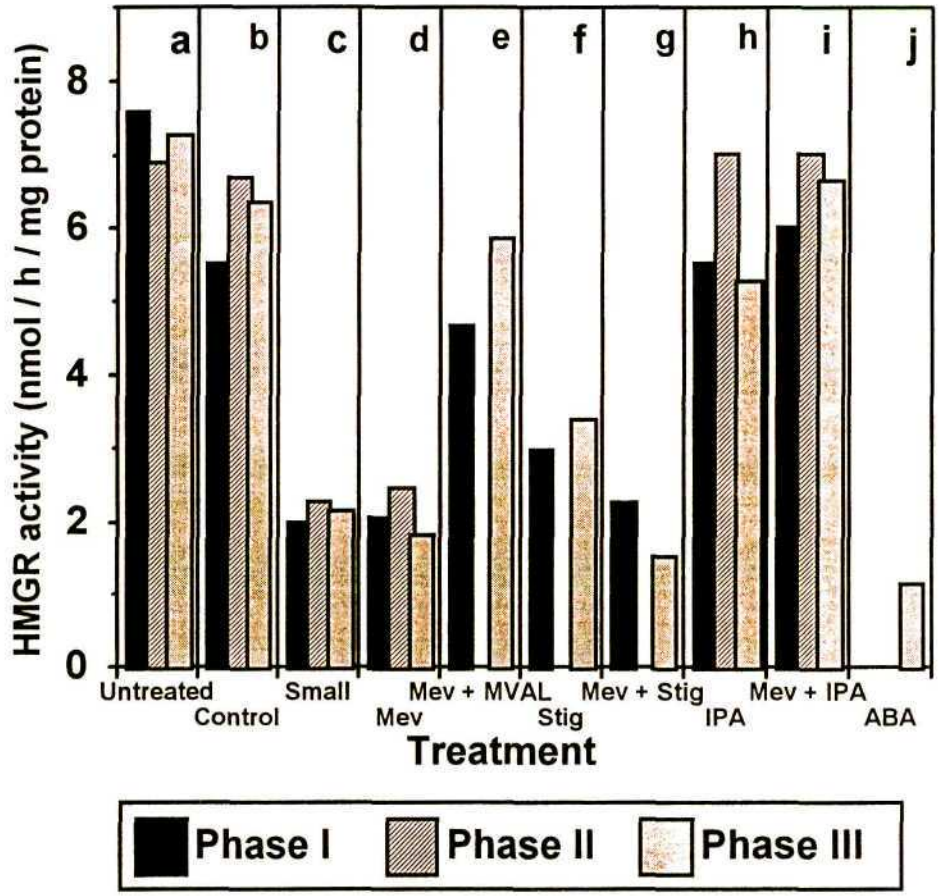

Figure 4.8

HMGR enzyme activity in developing 'Hass' avocado fruit and fruit pretreated with mevastatin and/or iP and/or stigmasterol. Batches of fruit (8 per treatment were injected with 20 µL of mevastatin and/or MVAL and/or iP and/or stigmasterol (all 1 µg $µL^{-1}$) 55 (phase I), 146 (phase II) and 210 (phase III) *d after full bloom and the fruit harvested 40 d later. HMGR activity was determined* in Ca^{2+}-sedimented microsomal membranes derived from freeze-dried mesocarp tissue as described in Section 2.14. Each value is the mean of three to six determinations. SE (diff) = 0.63. (a), Untreated; (b), control; (c), small fruit; (d), mevastatin; (e), mevastatin + MVAL; (f), stigmasterol; (g), mevastatin + stigmasterol; (h), iP; (i), mevastatin + iP.

4.2.4 Effect of mevastatin on mesocarp ABA content

Analysis of ABA in mesocarp from small fruit and fruit pretreated with or without mevastatin and/or MVAL, iP, and stigmasterol revealed the trends shown in Table 4.1. ABA concentration declined over the normal course of avocado fruit growth and development. By comparison, mesocarp ABA content of small fruit increased and at all stages of growth, small fruit contained substantially more ABA than fruit from control treatments. Mevastatin treatment significantly enhanced ABA concentration at all stages of fruit growth while co-injection of this inhibitor with either MVAL or iP reversed the effect. MVAL resulted in a return to basal ABA concentration at all stages of fruit growth. In contrast, exogenous application of iP reduced basal ABA content by >50% during the early stage of fruit growth but was only 50% as effective as MVAL during the later stages of this process. Stigmasterol reduced mevastatin-induced ABA accumulation by 30% in fruits treated in phase I and by more than 50% when co-injected with mevastatin in phase III.

Table 4.1

ABA content of mesocarp tissue from developing small fruit and fruit pretreated with mevastatin, MVAL, stigmasterol and iP. Batches of fruit (8 fruit per treatment) were injected via the pedicel with 20 µL solutions of Tween 20:acetone:water (1:1:8, by vol.) containing mevastatin, mevastatin + MVAL , mevastatin + stigmasterol and mevastatin + iP (all 1 µg µL^{-1}) 55 (phase I), 146 (phase II) and 210 (phase III) d after full bloom. Fruits were harvested between 50 and 100 d after application of chemicals and ABA content determined as described in Chapter 2. Data are the mean of at least three determinations ($LSD_{(5\%)}$ = 109).

Treatment	Time after full bloom (d) 163	216	290
	[ABA] ng g^{-1} dry weight (%)†		
Control	293 (100)^{b‡}	122 (100)^{a}	109 (100)^{a}
Small fruit	636 (217)^{d}	818 (670)^{d}	755 (693)^{c}
Mevastatin	669 (228)^{d}	390 (320)^{c}	673 (617)^{c}
Mevastatin + MVAL	330 (113)^{b}	161 (132)^{a,b}	111 (102)^{a}
Mevastatin + iP	141 (48)^{a}	264 (216)^{b}	249 (228)^{b}
Mevastatin + Stigmasterol	446 (152)^{c}	ND	315 (289)^{b}

†Percent relative to control‡ At each time interval, values followed by different letters are significantly different ($P \leq 0.05$). ND = not determined.

4.3 SUMMARY

(1) Isoprenoid biosynthesis is intimately involved in the regulation of 'Hass' avocado fruit growth, and a role for the key regulating enzyme of this pathway, HMGR, was confirmed.

(2) Application of ABA or mevastatin (a competitve inhibitor of HMGR) reduced 'Hass' fruit growth and increased mesocarp ABA concentration.

(3) Mevastatin-induced inhibition of fruit growth was reversed by stigmasterol during phase II and III but not during phase I.

(4) Down-regulation of HMGR by mevastatin and ABA was reversed by co-treatment with either MVAL or CKs and CKs respectively.

CHAPTER 5

CYTOKININ AND ABSCISIC ACID MEDIATION OF SYMPLASTIC SOLUTE TRANSPORT IN DEVELOPING AVOCADO FRUIT

5.1 INTRODUCTION

The limiting parameter for growth of phenotypically small fruit appears to be cell number, and the observed reduction in cell division occurred coincident with increased mesocarp ABA concentration and reduced HMGR activity. Since ABA-induced retardation of fruit growth, and inhibition of HMGR activity were negated by co-treatment with iP, a relationship between activity of HMGR and endogenous ABA and CK concentration in the metabolic control of 'Hass' avocado fruit growth was suggested. Similarly, previous reports on phytohormone regulation of HMGR suggested that a change in hormone balance during development could impact on growth through modulation of HMGR (Bach and Lichtenthaler, 1983; Brooker and Russell, 1979; Moore and Oishi, 1994; Russell and Davidson, 1982). Although changes in hormone content of phenotypically small fruit presumably result from alterations in metabolism and transport of affected bioactive molecules, the underlying stimulus responsible for these changes has yet to be elucidated. One possibility might be the supply of photoassimilate required for cell growth and differentiation during fruit ontogeny, particularly as both ABA and iP are products of MVA metabolism.

Formation of MVA is considered to involve condensation of three units of acetyl-CoA to HMG-CoA, which is then reduced to MVA by HMGR. In developing fruit the bulk of newly formed acetate is derived from sugars, imported from source tissue (Ho, 1988). In avocado, source tissues include photosynthetically active leaves and fruitlets (Coombe, 1976; Thorne, 1985; Blanke and Lenz, 1989; Blanke and Whiley, 1994).

Additionally, stored tree reserves are mobilized during periods of organ growth to sustain development of these structures (Kozlowski, 1992). Phloem is the most likely path of solute movement in dicotyledonous species and in developing fruit, phloem unloading occurs in the testa (Thorne, 1985). However, in avocado the seed is exotestal (i.e. completely pachychalazal) and enclosed by a highly vascularized seed coat (Steyn *et al.* 1993). It is the developing pachychalaza (i.e. the seed coat) that supplies photo assimilate, mineral nutrients and water to the expanding mesocarp.

Two possible paths exist for the uptake of sugars from the seed coat in developing avocado fruit. Firstly, active transport via the plasma membrane and secondly, passive transport via plasmodesmata. Plasmodesmata are dynamic structures in which pore size, and hence molecular exclusion limit, is up- or down-regulated by processes involving callose deposition and removal from the annulus, and by structural modifications to the central lipoprotein core (Lucas *et al.*, 1993; Morris, 1996). A variety of agents are considered to be involved in the regulation of transport via plasmodesmata including: Ca^{2+} release, initiated by the inositol triphosphate-diacylglycerol (IP_3/DAG) second messenger system (Robards and Lucas, 1990); phosphorylation of the callose synthesizing enzyme (Lucas *et al.*, 1993); and, plant hormones (Morris, 1996). Since many hormone responses appear to be mediated by the IP_3/DAG signal transduction system it has been suggested that hormones like ABA, that are transported in the phloem, might act to modulate symplastic phloem loading and unloading by influencing protein phosphorylation (Morris, 1996).

Casual observation has revealed that a characteristic of phenotypically small 'Hass' fruit is early senescence of the seed coat. The question therefore arises: Are phenotypically small fruit a consequence of early seed coat senescence, or is the abortion of seed coat function a response to some other factor induced by a reduction in HMGR activity and diminished cell division cycle activity?

It has recently been demonstrated that transgenic tobacco plants that constitutively express a yeast insoluble-acid invertase gene develop symptoms which are characteristic of the onset of early leaf senescence (Ding *et al.*, 1993). Ultrastructural analysis of these transgenic plants revealed that development of secondary plasmodesmata was inhibited in the greenish-yellow sectors of affected leaves. Based on these observations, Ding *et al.* (1993) hypothesized that secondary plasmodesmata differ from primary plasmodesmata in being able to traffic regulatory molecules that are involved in the coordination of development and physiological function. In addition to these structural changes, biochemical and physiological studies showed, accumulation of carbohydrates, a decline in photosynthesis and increased respiration in leaves of transgenic tobacco expressing the yeast invertase gene (von Schaewen *et al.*, 1990). Since these alterations in metabolism can also contribute to accelerated leaf senescence, it was further suggested that inhibition of secondary plasmodesmatal development may be the consequence of any change in carbon catabolism (Ding *et al.*, 1993).

The terms "feast" and "famine" have been used to describe developmental trends in response to changes in carbohydrate concentration and composition in higher plants (Koch, 1996). Carbohydrate availability affects carbohydrate allocation through altered gene expression and may therefore be crucial in the metabolic control of fruit growth. For example, sugar availability strongly affects cell differentiation and cell cycle activity in higher plants (Ballard and Wildman, 1963; Webster and Henry, 1987). Furthermore, carbohydrate supply is critical for kernel set in maize (Zinselmeier *et al.*, 1995), fruit size of tomato (Klann *et al.*, 1996), and utilization of sugars in developing leaves, seed and fruit is strongly dependent on Suc metabolizing enzymes (Klann *et al.*, 1993; 1996; Miller and Chourey, 1992; Morris and Arthur, 1984; Ohyama *et al.*, 1995) that are encoded by sugar responsive genes (Koch *et al.*, 1992). This information suggests that availability of sugars and the composition thereof, are crucial for fruit development. Furthermore, plant HMGR kinase, responsible for regulating the activity of HMGR, has

been classified as a member of the Suc nonfermenting-1 (SNF-1) family of protein kinases (Barker *et al.*, 1996). SNF-1 represents a primary target of the Gluc repression pathway in budding yeast, and Gluc repression of metabolism involves a signal transduction pathway that links perception of Gluc concentration with repression and/or derepression of Gluc-repressible genes (Thevelein, 1994). For example, down-regulated genes function in gluconeogenesis and respiration while those up-regulated, function in glycolysis and storage carbohydrate breakdown. SNF-1 is integral to this pathway and Gluc-repressible genes cannot be switched on in response to Gluc deprivation in the absence of SNF-1 activity (Celenza and Carlson, 1989; Gancedo, 1993). Thus, it is tempting to suggest that avocado mesocarp HMGR is likewise modulated by the effects of carbohydrate concentration and composition on HMGR kinase activity.

This chapter describes experiments that were carried out to determine the effect of fruit size on the endogenous iP and ABA concentration and to establish the effects of an altered CK:ABA ratio on symplastic solute transport, mesocarp cell-to-cell communication and plasmodesmata structure/function in developing 'Hass' avocado fruit.

5.2 RESULTS

5.2.1 Carbohydrate cycling and fruit growth

To determine fluctuations in stored carbohydrate reserves relative to timing of the major phenological events, bark starch levels were determined on a monthly basis. Results show that a period of rapid decline of stored starch coincided with early fruit growth (Fig. 5.1), i.e. stored carbohydrate reserves were mobilized during this period to sustain the energy expensive event of fruit growth. Towards the end of the fruit growth period, trees again accumulated stored carbohydrate reserves (Fig. 5.1).

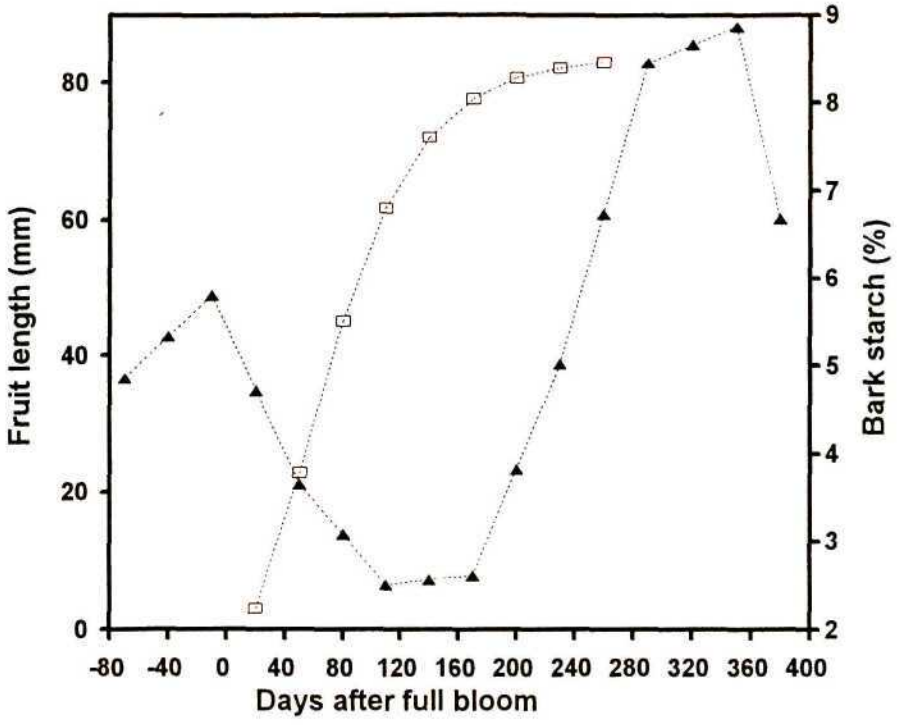

Figure 5.1

Relationship between fruit growth and storage carbohydrate mobilization in 'Hass' avocado trees (▲) in relation to fruit growth (❑). SE (diff) = 0.25.

5.2.2 Path of assimilate movement and solute allocation

Phloem-translocated carbohydrate drives growth of developing sinks. Since both ABA and CK are translocated in the phloem it was of interest to determine the effect of an increase in concentration of these growth substances on solute allocation in developing avocado fruit. To examine the functional significance of the seed coat and vasculature, the major path of sugar movement was established by application of an aqueous solution of tetrabromofluorescein (eosin) via the pedicel. Figure 5.2 illustrates the pattern of eosin distribution in developing avocado fruit. Eosin cannot permeate membranes and is therefore restricted to the syplastic pathway of solute flux. As expected, dye was restricted to the vasculature, which permeates the mesocarp and coallesces at the chalaza, i.e. the basal region where the nucellus and integuments fuse, coincident with the funiculus. From the chalazal region, eosin appeared to enter the seed (data not shown) and permeate seed coat tissue via vascular traces. No lateral diffusion of eosin from vascular traces in either mesocarp or seed coat tissue was observed suggesting that bulk solute movement into the developing fruit was apoplastic and occurred along the continuum: pedicel vasculature → mesocarp vasculature → chalaza → seed; or seed coat → seed → mesocarp.

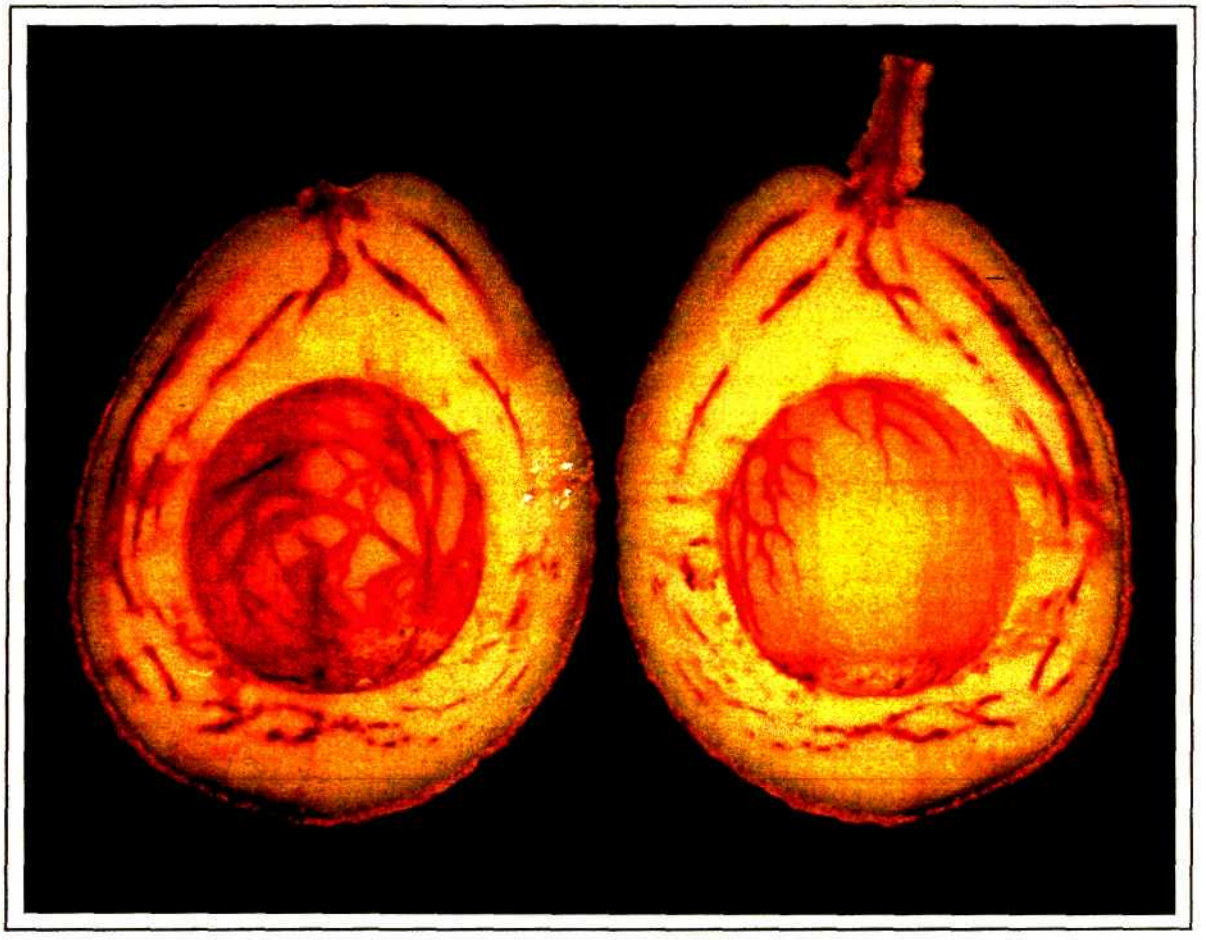

Figure 5.2
Photograph illustrating the pattern of eosin distribution in developing 'Hass' avocado fruit.

A more detailed examination of this path and the effect of applied ABA and iP on solute movement was made by monitoring the accumulation of radioactivity, from pulsed [^{14}C]-sucrose, in seed, seed coat and mesocarp tissue. As shown in Figure 5.3, the distribution of accumulated radioactivity was essentially similar for untreated control-fruit, iP-treated fruit and fruit co-treated with equal amounts of ABA and iP. Interestingly, the bulk of radioactivity was associated with seed coat tissue in all treatments. Both phenotypically small fruit and ABA-treated fruit preferentially accumulated radioactivity in the seed, suggesting that only the path via the seed coat had been affected by ABA and expression of the small-fruit phenotype. The pattern of distribution of radioactivity in ABA-treated fruit was restored to that of the control by co-treatment with iP. The relatively high proportion of radioactivity in mesocarp of small fruit and ABA-treated fruit probably reflects reduced transport capacity, a suggestion

supported by the observation that uptake of [^{14}C]-sucrose by these fruit was <10% that of the control.

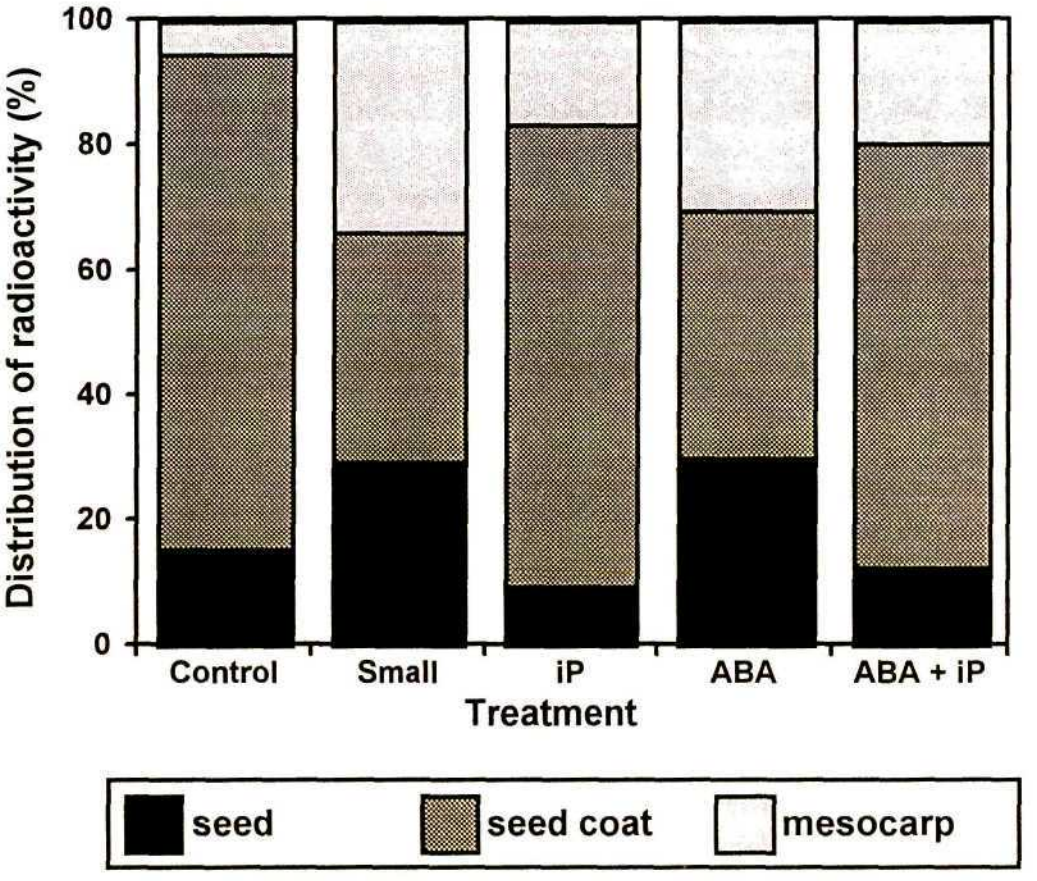

Figure 5.3

Effect of ABA, iP and stigmasterol on distribution of radioactivity in the seed, seed coat and mesocarp tissue of 226 day old 'Hass' avocado fruit pulsed with [^{14}C]-sucrose (2 Mbq in 0.5 mL distilled H_2O) followed by water via the pedicel and incubated for 48 h at room temperature.

5.2.3 Membrane potential of seed coat and mesocarp parenchyma

In attempting to define the cellular pathway of post-phloem sugar transport in developing avocado fruit, the membrane potential (E_m) of seed coat and mesocarp parenchyma was determined. Although measurement of E_m is extremely difficult (van Bel and Kempers, 1990), with due precaution cells were successfully impaled and after sealing of the plasma membrane (indicated by voltage stabilization) values were recorded and are shown in Table 5.1. It is evident that an electrical potential gradient exists between seed coat and mesocarp parenchyma and that this gradient is maintained in fruit pretreated with iP. However, in response to exogenous ABA the

gradient is flattened suggesting reduced transport of sugars between these tissues. Interestingly, co-treatment of ABA with iP was unable to restore the E_m between seed coat and mesocarp tissue. Since the steepness in E_m between neighbouring cells (tissues) reflects symplastic connectivity, i.e. the steeper the gradient the poorer the symplastic connectivity (van Bel and Kempers, 1990), the present results indicate good symplastic connectivity between avocado seed coat and mesocarp tissue.

Table 5.1

Plasma membrane electrical potentials (+SE) of tissues of developing 'Hass' avocado fruit pre-treated *in vivo* with or without ABA, iP and iP + ABA. Number of E_m measurements in brackets.

Treatment	Membrane potential (E_m) (mV) Seed coat parenchyma	Mesocarp parenchyma	Difference
Control	-44 ± 7 (9)	-56 ± 6 (8)	12
iP	-46 ± 5 (11)	-61 ± 7 (3)	15
ABA	-45 ± 5 (8)	-47 ± 5 (8)	2
iP + ABA	-45 ± 8 (11)	-48 ± 6 (7)	3

5.2.4 Iontophoresis of Lucifer Yellow in avocado mesocarp

As shown in Figure 5.4a, labelling of impaled mesocarp cells by post-injection of LYCH by iontophoresis substantiated the claim for a high degree of symplastic connectivity in control fruit. Even so, one of the major limitations of using fluorescence microscopy is that image visualization is difficult, often as a result of low level fluorescence from insignificant injected fluorochrome volumes, or masking effects due to autofluorescence from surrounding cells/tissues. Thus, pseudocolour images, based on the intensity of fluorescence, were generated by digitizing video pictures and these are presented in Figure 5.4b. The enhanced ease of interpretation clearly indicates that most of the LYCH had diffused radially from the injected cell by 2 min. Similar observations were made for seed coat tissue (data not show) in which radial diffusion of LYCH was extremely rapid.

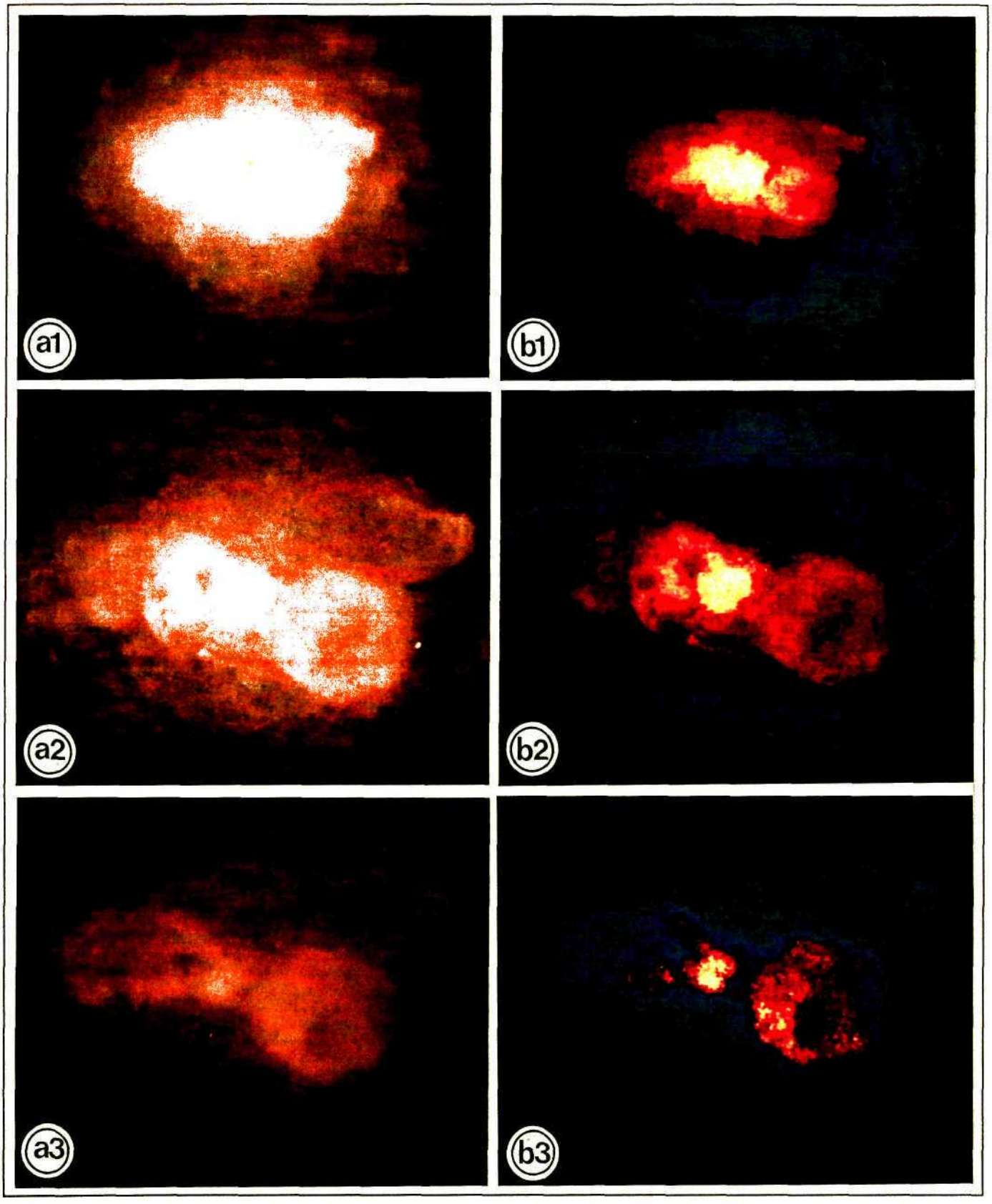

Figure 5.4

Microiontophoresis of LYCH in mesocarp of 'Hass' avocado fruit. (a1) to (b1) Fluorescence micrographs of hand-cut sections of avocado mesocarp parenchyma cells reverse microiontophoresesd with LYCH as described in Materials and Methods, immediately (a1), 1 min (a2) and 2 min (a3) after time of injection. (B1) to (b3) Digitized pseudocolor images of (a). A five-colour "pseudocolour" palette based on fluorescence intensity of LYCH concentrations, ranging from black (zero), to aquamarine (low), blue (medium), purple (high), and white (highest), was applied.

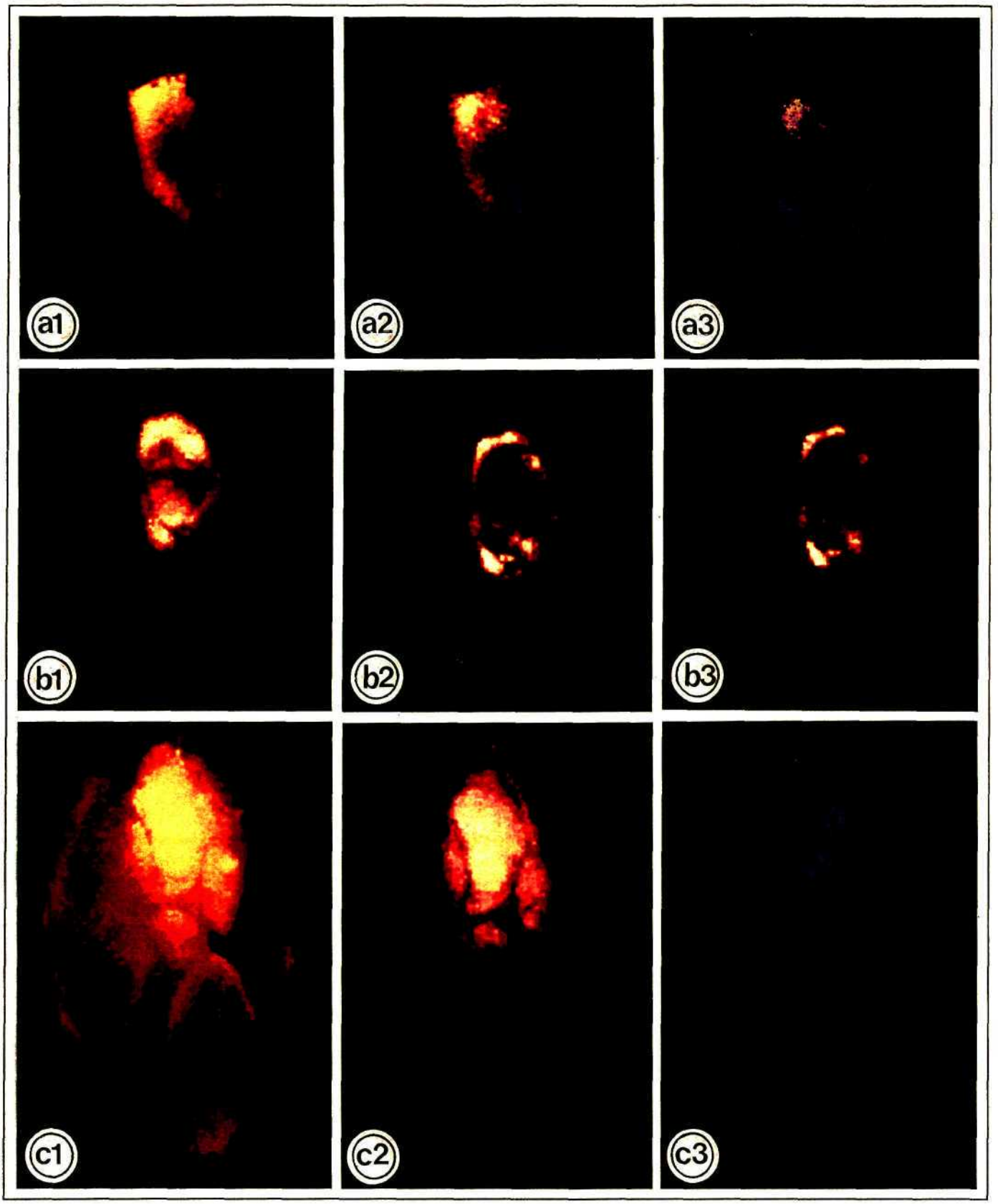

Figure 5.5

Microiontophoresis of LYCH in mesocarp parenchyma of 'Hass' avocado fruits pre-treated with ABA, iP, and ABA + iP. Digitized images of fluorescence micrographs of hand-cut sections of mesocarp from fruit pre-treated with iP and microiontophoresed with LYCH immediately (a1), 1 min (a2) and 2 min (a3) after injection. Digitized images of fluorescence micrographs of mesocarp parenchyma from fruit pre-treated with ABA, microiontophoresed with LYCH, immediately (b1), 2 min (b2) and 6 min (b3) after injection. Digitized images of fluorescence micrographs of mesocarp parenchyma from fruit pre-treated with ABA + iP and microiontophoresed with LYCH immediately (c1), 1 min (c2) and 2 min (c3) after injection.

Pre-treatment of avocado fruit with exogenous iP did not affect mesocarp cell-to-cell communication as depicted by the pseudocolour images presented in Figure 5.5a. By comparison, pre-treatment of fruit with exogenous ABA retarded mesocarp cell-to-cell transfer significantly and LYCH was contained within the injected cell for periods in excess of 6 min (Fig. 5.5b). Cell-to-cell communication in mesocarp from fruit co-treated with iP and ABA was rapid and resembled that observed in control and iP-treated tissue (Fig. 5.5c)

5.2.5 Plasmodesmata structure/function in avocado mesocarp

The principal results of plasmodesmatal ultrastructure in mesocarp of developing small and normal 'Hass' avocado fruit and fruits pre-treated with iP, ABA, and iP+ABA are illustrated in Figures 5.6a to 5.8b.

Figure 5.6a shows that plasmodesmatal aggregates occur within primary pitfields in mesocarp of developing 'Hass' avocado fruit. Both branched and unbranched plasmodesmata are evident (Fig. 5.6a & b) and these are usually associated with rough endoplasmic reticulum (RER, Fig. 5.6b). As illustrated in Figure 5.6c, plastids appear intact and well preserved with no evidence of senescence. Longitudinal (Fig. 5.6b) as well as transverse views (Fig. 5.6d) show that plasmodesmata contain a clearly defined desmotubule, surrounding a cytoplasmic annulus of variable electron density. All plasmodesmata in mesocarp tissue appeared to have slightly constricted outer plasmodesmatal orifices, which could be argued as neck constrictions (Gunning, 1975). In many cases, as shown in Figures 5.6b, c & d, the plasmodesmata are associated with large, complex-structured median cavities, that seem to cross link many plasmodesmata. Median cavities occur in the middle lamella region and plasmodesmata are sometimes branched on one side only (Fig. 5.6c). In many instances, plasmodesmata are highly convoluted (Fig. 5.6e). Similarly the plasma membrane is convoluted.

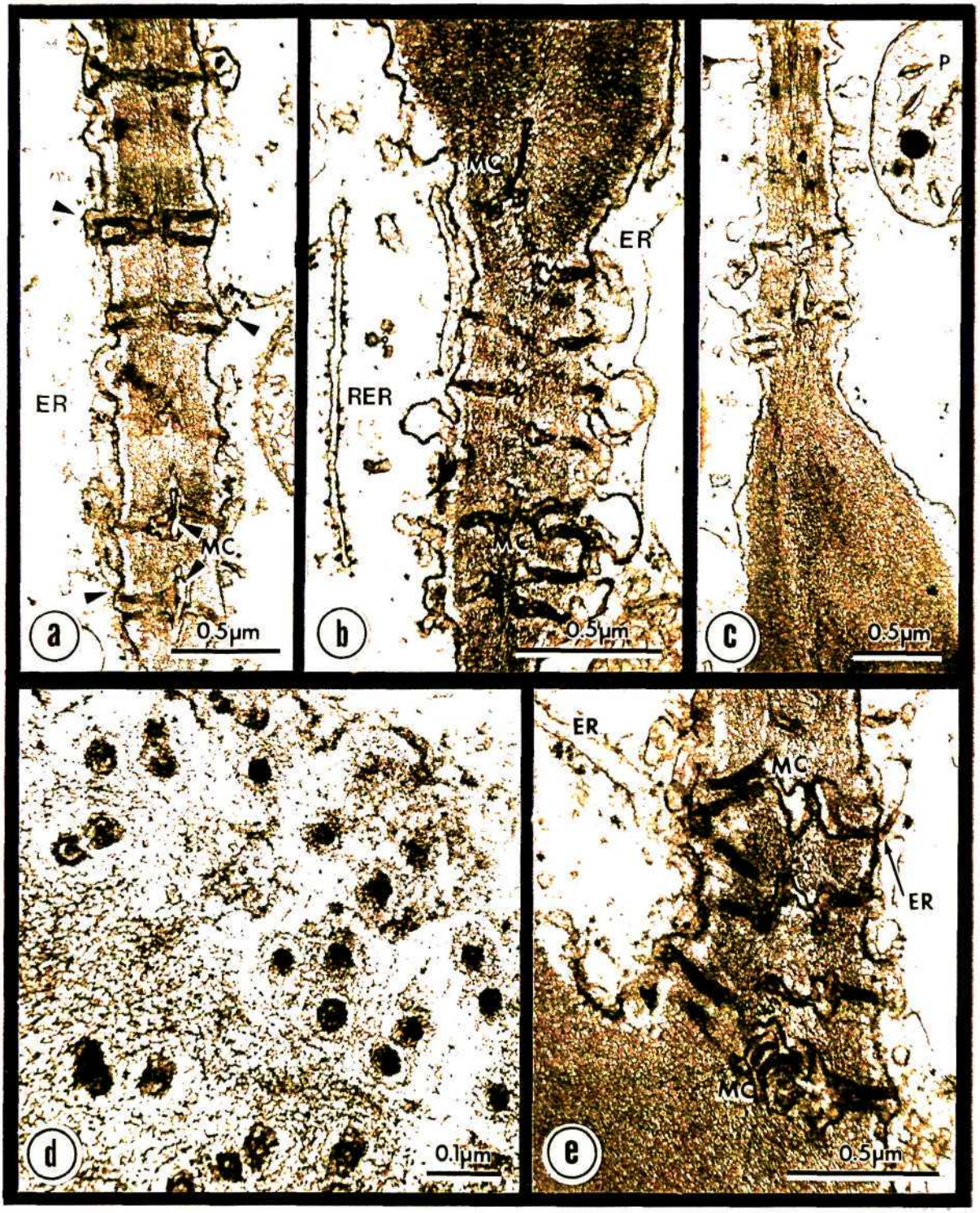

Figure 5.6

Ultrastructure of untreated "normal" 'Hass' fruit plasmodesmata. (a) Transection through the tangential wall between two mesocarp cells, showing mostly branched plasmodesmata, associated with large median cavities (MC) in the middle lamella interface between the two common cell walls. Note the close conformation between the endoplasmic reticulum (ER) and the outer orifice of the plasmodesmata (darts). (b) Transection of a common radial wall between two mesocarp cells, but close to the mesocarp-seed coat interface. Unbranched and branched plasmodesmata, associated with ER and rough ER (RER), occur commonly within these cells. (c) Shows part of two adjacent mesocarp cells (P = plastid). (d) Transection through part of a plasmodesmatal pit field in the radial wall of an inner mesocarp cell. Note the tight conformational structure of the plasmodesmata and the desmotubule with central rod. Most plasmodesmata have an electron-lucent cytoplasmic sleeve in these sections. (e) Shows part of a plasmodesmata pit field in the tangential wall of inner mesocarp cell, with highly convoluted MC. Note ER in close association with plasmodesmata.

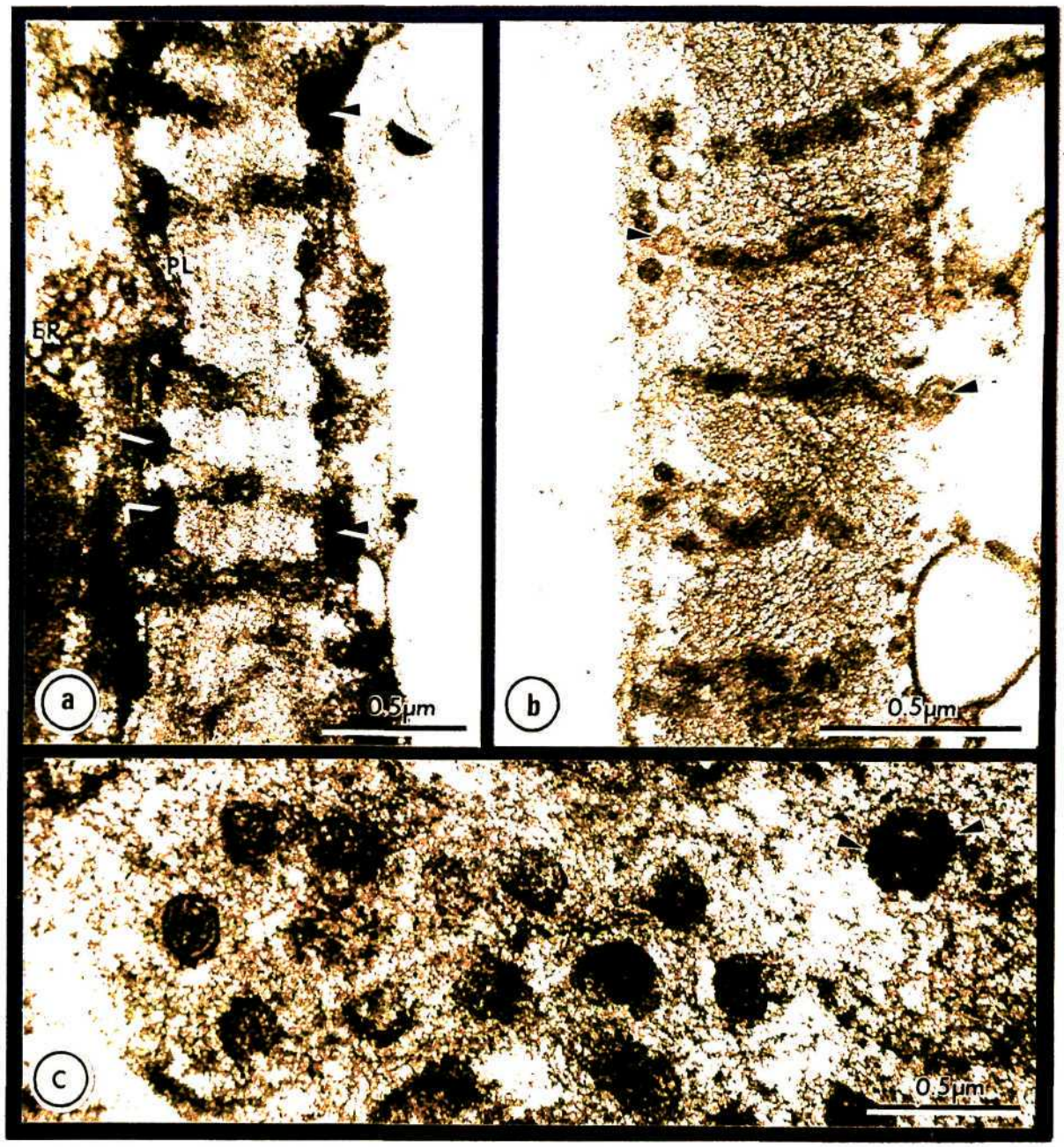

Figure 5.7

Ultrastructure of plasmodesmata in mesocarp of ABA-treated and phenotypically small 'Hass' avocado fruit. (a) Transection of a tangential wall between mesocarp cells of ABA-treated fruit. Note unbranched and branched plasmodesmata, which are all occluded by granular electron-dense material, which appears to form a tight collar, plugging the outer orifices of the plasmodesmata (darts). (b) Transection of a tangential wall between mesocarp cells of small fruit. Plasmodesmata appear "plugged" similar to those from ABA-treated tissue (darts). (c) Transection through part of plasmodesmatal pitfield in the radial wall of an inner mesocarp cell from ABA-treated fruit, illustrating collar-like structure on outer surface (darts).

Figure 5.7 shows that striking structural changes were manifested in sections prepared from mesocarp of fruit pre-treated with ABA. Furthermore, structural similarities between small (Fig. 5.7b) and ABA-treated (Fig. 5.7a) 'Hass' fruit were apparent. The plasma membrane, as illustrated in Figure 5.7a, lacks the highly convoluted appearance evident in control tissue (cf. Figs. 5.6 a, b & c). Most plasmodesmatal orifices appear to be 'plugged' by a granular electron-dense substance, which forms a collar-like structure when viewed in transverse (darts, Fig. 5.7c) or plug-like formations (darts, Fig. 5.7a) on the outer surface of the plasmodesmata. The complexity of median cavities in avocado mesocarp tissue is again apparent, indicating a high degree of interconnectivity of the plasmodesmata.

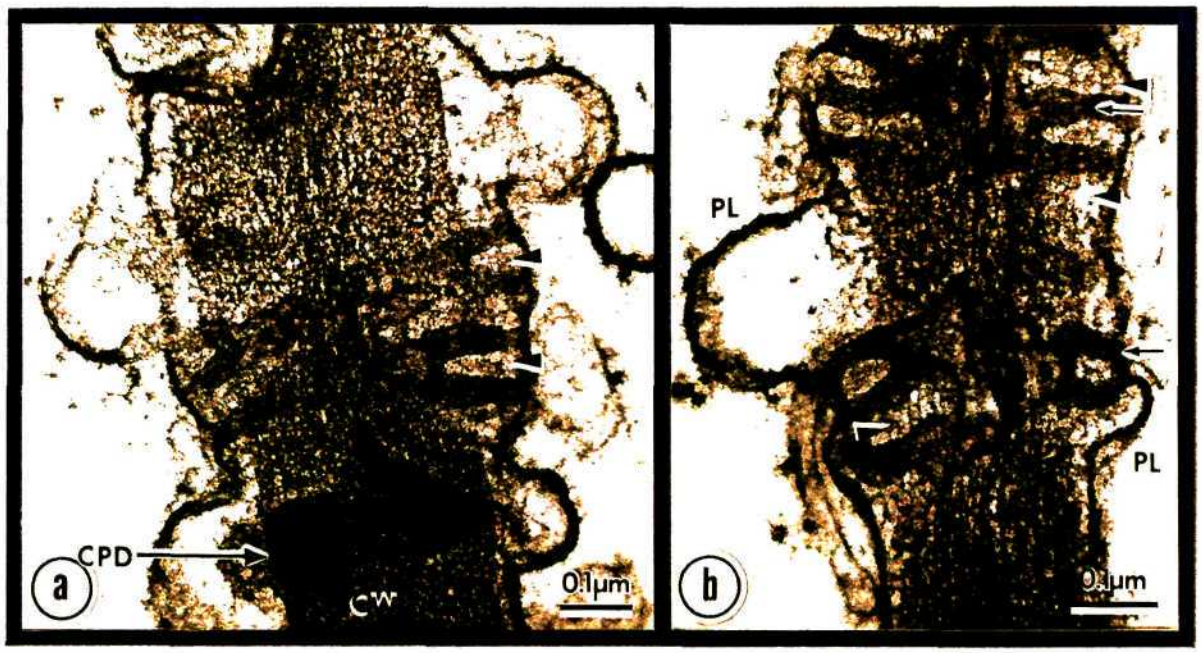

Figure 5.8

Ultrastructure of plasmodesmata in mesocarp of iP- and iP + ABA- treated 'Hass' avocado fruit. (a) Transection of tangential wall between mesocarp cells of iP-treated fruit. Plasmodesmata are separated by an electron-lucent wall region (darts). Commonly and of interest, are the large electron-dense (possibly coalesced) plasmodesmata (CPD). (b) Transection showing tangential wall between mesocarp cells of iP + ABA treated tissue. Note the wide electron-lucent outer wall zone (darts), between concomitant plasmodesmata (arrows).

Figure 5.8a shows plasmodesmata from the tangential wall of a mesocarp cell from iP-treated avocado fruit. The plasmodesmata appear less electron-dense than those in control tissue and are complex, cross-linked and multi-branched in appearance.

Plasmodesmata from mesocarp of iP-treated fruit do not appear to be constricted. A striking feature of iP-treated avocado tissues, are large, electron-dense regions, possibly coalesced plasmodesmata (CPD), which occur within otherwise normal-looking plasmodesmatal pit fields. Outer wall regions are more electron-lucent than in control tissues (darts). Plasmodesmata in mesocarp of fruit co-injected with iP and ABA are occluded by globular electron-dense material at the plasmodesmatal orifices (arrows) and others lack this material (Fig. 5.8b). Of interest is the absence of neck constrictions in unoccluded plasmodesmata.

5.3 SUMMARY

(1) Solute transport was similarly affected in phenotypically small and in normal fruit injected with ABA, whilst co-treatment with ABA plus iP restored solute transport to sinks in a manner similar to that in control fruit and/or in iP-treated fruit.

(2) An electrical potential gradient was observed between seed coat and mesocarp parenchyma cells of normal 'Hass' fruit. This gradient is reduced by the application of ABA, and this reduction was unaffected by co-treatment with ABA plus iP.

(3) Plugging of plasmodesmata in avocado mesocarp was observed in phenotypically small and ABA-treated fruit, but not in control and iP-treated fruit. When iP was co-injected with ABA, the ABA effects were negated or reversed.

CHAPTER 6

STRESS AND THE 'HASS' SMALL FRUIT SYNDROME: ALLEVIATION THROUGH MULCHING

6.1 INTRODUCTION

Previous chapters have revealed that a complex of interrelated factors are apparently involved in the control of fruit size, and the results suggest that the 'Hass' small fruit phenotype is induced by a low CK:ABA ratio, which through a cascade of events reduces HMGR activity and retards fruit development. A reduction of the CK:ABA ratio is considered to be initiated by abiotic/biotic plant stress (Chapin, 1991). In drying soils, xylem ABA concentration increases (Davies and Zhang, 1991; Davies *et al.*, 1994), which presumably leads to a lowering of the CK:ABA ratio in leaves and developing fruits. During conditions of water stress, ABA acts on stomata in the leaf epidermis to cause closure or inhibit the opening of stomata (Wartinger *et al.*, 1990; Tardieu *et al.*, 1992a; 1992b; Davies *et al.*, 1994). Since the amount of CO_2 required for photosynthesis is balanced against available water, a limitation in supply of CO_2 as a result of elevated xylem ABA levels, is transducted into mechanistic down-regulation of carbon fixation (Cowan, 1994). Thus under sustained abiotic/biotic pressure, photoassimilate required to drive fruit growth and development becomes limiting.

Furthermore, water availability also affects the efficiency of light utilization by leaves (Flore and Lakso, 1989; Thomas and Strain, 1991; Schaffer *et al.*, 1994). Under conditions of water stress, a decrease in the efficiency of photosynthetic conversion, a phenomenon known as photoinhibition takes place (Powles, 1984; Adams *et al.*, 1987; Björkman and Schäfer, 1989; Demmig-Adams and Adams, 1992). Photoinhibition is a response that apparently protects photosynthetic pigments and electron transport apparatus from severe photooxidative destruction (Demmig-Adams *et al.*, 1988; Krause *et al.*, 1995). Photoinhibition is associated with a loss of productivity (Ögren and Sjöström, 1990), and so under prolonged periods of water stress, avocado yields may be reduced.

Symptoms of the 'Hass' small fruit syndrome are aggravated by incorrect cultural practise and are more prevalent under adverse growing conditions. The present study has highlighted a correlation between the 'Hass' small fruit phenotype and early seed coat senescence, a physiological response that is apparently stress-induced (Whiley *et al.*, 1986). Early senescence of the seed coat substantially reduces supply of nutrients, assimilates and plant hormones required for fruit development, thus retarding fruit growth considerably (Cutting *et al.*, 1986).

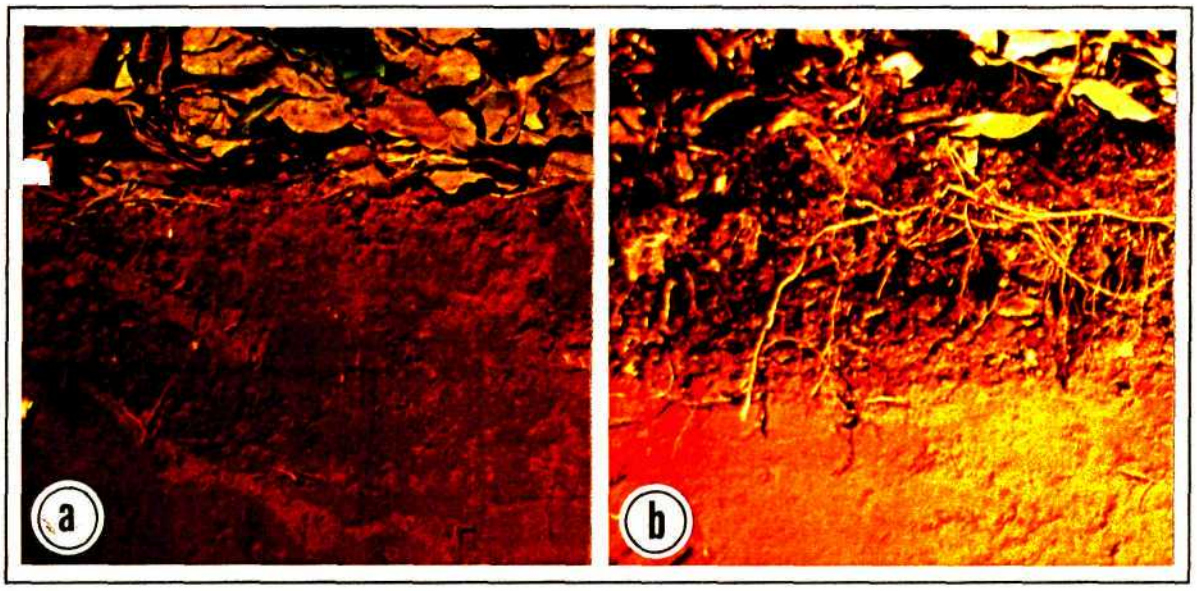

Figure 6.1

Photograph illustrating the control (a) and coarse composted pinebark mulch (b) treatments. 1.5m^3 pinebark was applied under each tree to a depth of 15 cm.

This study proposed mulching as a strategy to alleviate stressful growing conditions and increase fruit size. This strategy is based on the avocado evolving as a "litter-feeder" and its adaptation to soils with a high humic content. Coarse composted pinebark was chosen for this study (Fig. 6.1) because of its excellent physical properties and relatively slow speed of breakdown. The application of a mulch to the orchard floor creates edaphic conditions that avocado trees are more acclimatized to. It was hoped that this would reduce the occurrence of small fruit by eliminating the confounding effects of either stress-induced ABA accumulation and/or feedback regulation of photosynthesis. In addition, photochemical efficiency of leaves from mulched trees might be improved resulting in enhanced tree productivity. The purpose

of this research was to determine whether mulching could be employed as an effective method of improving growing conditions and elevating overall fruit productivity. The effect of mulching on various phenological events and its impact on efficiency of light utilization and accumulation of stored carbohydrate was also assessed. Finally, the effect of mulching on yield and fruit size, as well as its economic significance was also evaluated.

6.2 RESULTS

6.2.1 Canopy temperatures

In order to compare transpirational activity of mulched and non-mulched trees, canopy temperatures were monitored using infra-red thermometers for two seasons, and the results are presented in Figures 6.2 and 6.3.

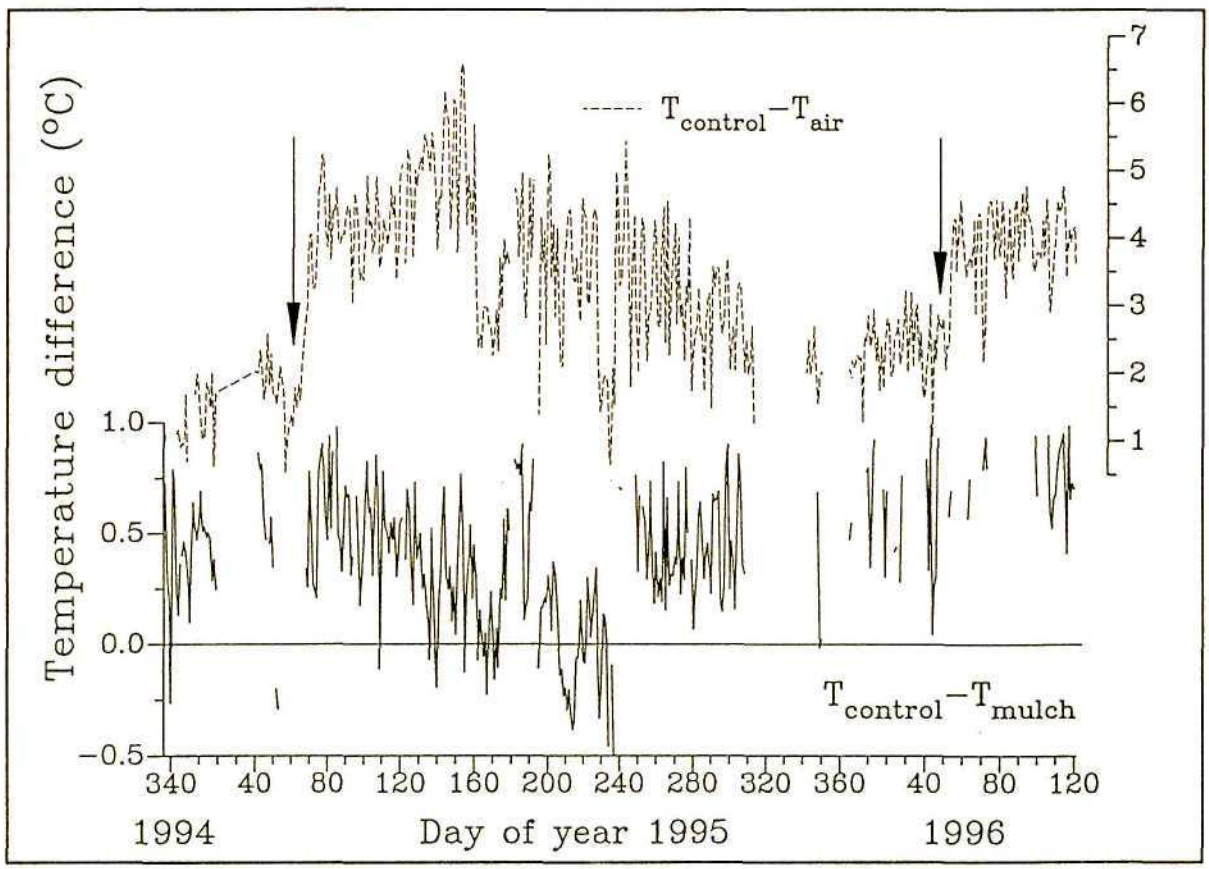

Figure 6.2

Comparison of seasonal fluctuations in canopy temperatures (minus air temperature) of trees in the presence or absence of a pinebark mulch. Data are the means of measurements recorded every 15 sec between 0800H and 1600H. Arrows indicate onset of rapid increase in canopy temperature of non-mulched or control trees ($T_{control}$ - T_{air}).

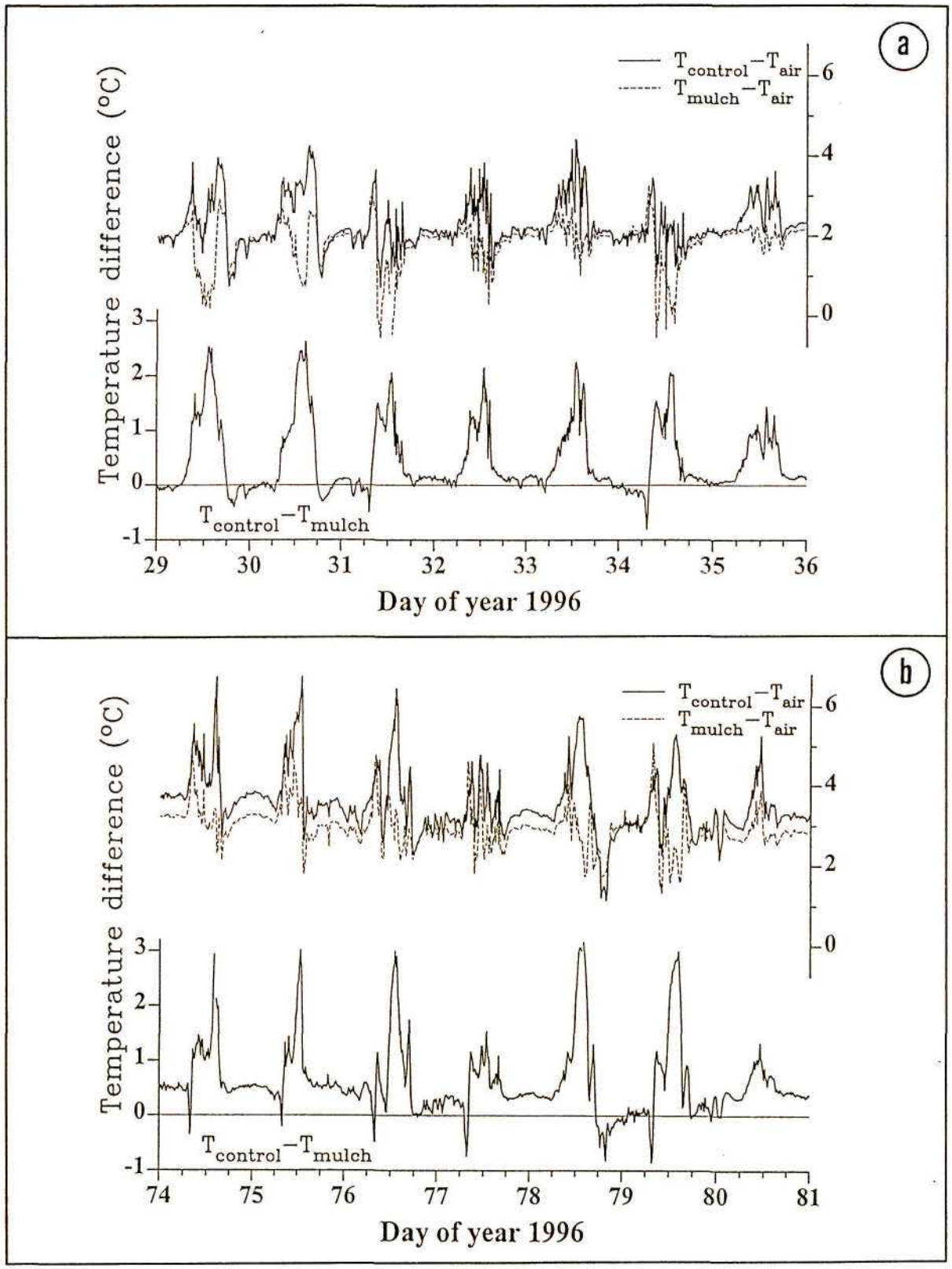

Figure 6.3

Comparison of diurnal fluctuations of canopy temperatures of mulched and control trees ($T_{control}$ - T_{mulch}) before (a) and after (b) the period of rapid increase in canopy temperatures. Data are the means of measurements recorded every 15 sec.

Seasonal fluctuation in canopy temperatures ($T_{control}$ - T_{air}) show a flux ranging from ca. 1°C through to 6°C (Fig. 6.2). For two consecutive seasons a dramatic rise in foliage temperature coincided with the onset of fruit drop, apparently a response by avocado trees associated with stress (Whiley *et al.*, 1988). The period of maximum canopy temperature coincided with the physiological window associated with seed coat senescence, approximately 106 and 95 days after full bloom for the 1994/95 and 1995/96 seasons respectively (assuming that full bloom was reached by mid-November in each season). For the majority of the sampling period foliage temperatures of mulched trees were lower than non-mulched control trees ($T_{control}$ - T_{mulch} was positive) indicating increased transpirational activity by mulched trees (Fig. 6.2).

By comparing diurnal fluctuations of foliage temperatures immediately before and during the period of rapid increase in canopy temperature ($T_{control}$ - T_{air}), differences between the two treatments were apparent. $T_{control}$ - T_{mulch} was greater during the period of rapid rise in foliage temperature (Fig. 6.3b) than immediately before it (Fig. 6.3a), i.e. differences in canopy temperature between control and mulched trees was greater during this critical stress period. Interestingly, night leaf temperatures of control trees were greater than leaves from mulched trees during the period of high canopy temperatures (Fig. 6.3b), whereas before the time of rapid rise in foliage temperatures, no differences in night temperatures between leaves from mulched and non-mulched trees were recorded (Fig. 6.3a).

6.2.2 Measurement of chlorophyll fluorescence

To compare photochemical efficiency of foliage from mulched and non-mulched trees, a plant efficiency analyser was used to determine the rate of variable to maximum chlorophyll fluorescence emission (F_v/F_m ratio). Typically, throughout the season, efficiency of light utilization (the F_v/F_m ratio) by leaves on both treatments had values ranging from 0.75 to 0.85 early in the morning (Fig. 6.4 a to d). During the period of greatest abiotic pressure (viz. the heat of the day) this value dropped, afterwhich the F_v/F_m ratio returned to its original level in the evening. The magnitude of this drop varied throughout the season with the greatest flux in the F_v/F_m ratio occurring 78 d

after fruit set. At all time periods, the magnitude of this drop was reduced on trees growing under mulched conditions, suggesting elevated photochemical efficiency of leaves from mulched trees.

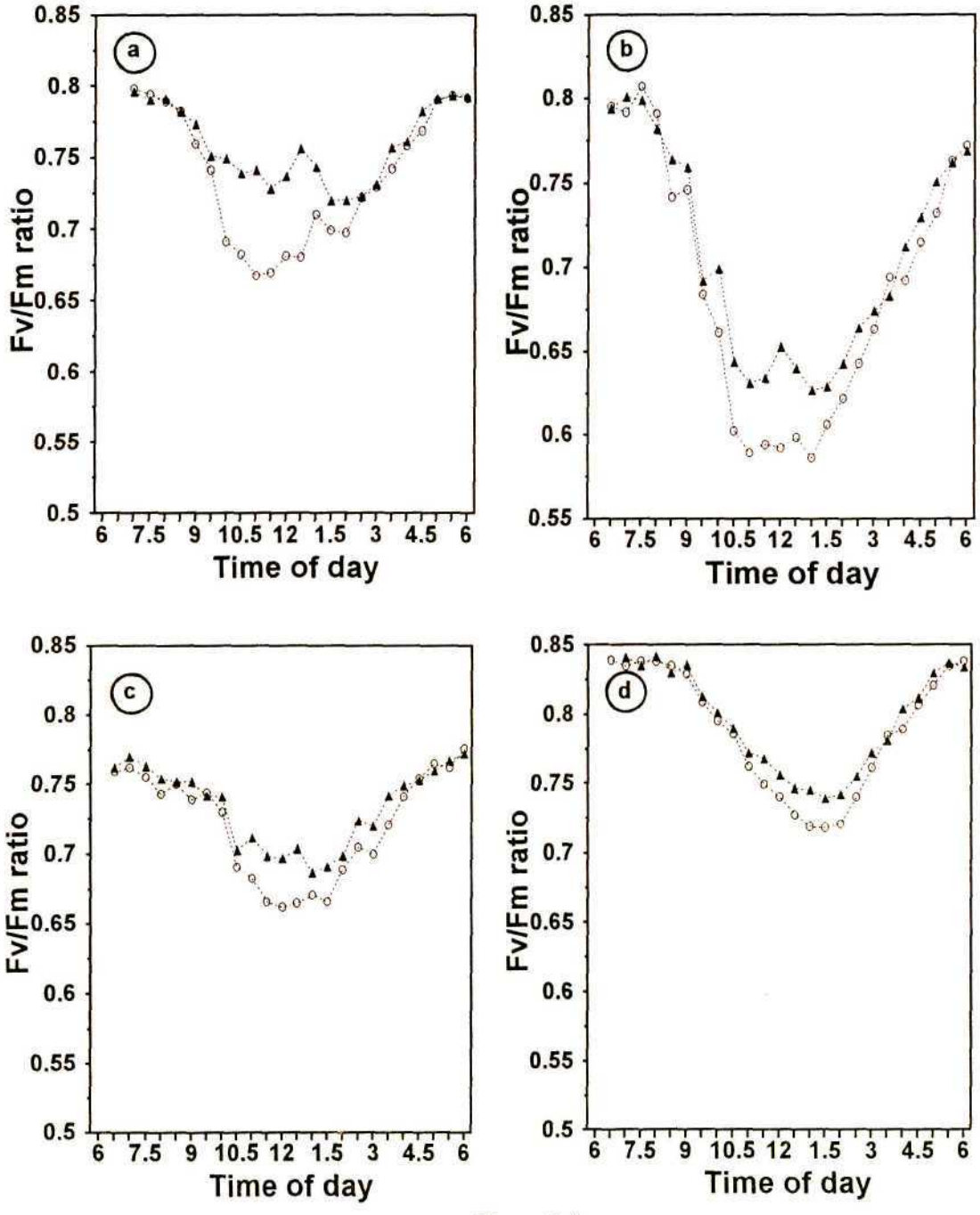

Figure 6.4

Typical diurnal fluctuation in F_v/F_m ratio of 'Hass' trees on the pinebark mulch (▲) and control (○) treatments, 36 (a), 78 (b), 113 (c) and 176 (d) days after full bloom. Values are means of 10 measurements per treatment at each time interval. SE (diff) = 0.0031 (a), 0.0046 (b), 0.0037 (c) and 0.0028 (d).

6.2.3 Physiological disorders associated with small fruit

Mulching with pinebark significantly ($P \leq 0.05$) reduced the incidence of fruit with degenerate seed coats by 38.6% over two seasons (Table 6.1). Mulching also reduced the incidence of fruit with pedicel ring-neck by 57.1%, 45.9% and 28.7% for the first, second and third seasons respectively (Table 6.1).

Table 6.1

Effect of mulching on the incidence of pedicel ring-neck and seed coat abortion. To determine the impact of mulching on seed coat abortion, 10% of harvested fruit were randomly selected and bisected longitudinally, and the absence or presence of a degenerate seed coat was recorded. To ascertain the effect of mulching on pedicel ring-neck, 100 fruit per tree were randomly selected and presence or absence of the syndrome was recorded.

		1993/94	1994/95	1995/96	Overall
Pedicel ring neck	Control	17.5±2.2	13.3±2.7	9.4±1.6	13.4±2.4
	Mulch	7.5±2.4	7.2±1.9	6.7±1.3	7.1±1.8
	% decrease	57.1**	45.9**	28.7^{NS}	47.0**
Degenerate seed coat	Control	ND	31.4±4.2	19.4±3.2	25.4±3.7
	Mulch	ND	13.9±2.4	17.3±3.0	15.6±2.8
	% decrease	ND	55.7**	10.8^{NS}	38.6*

NS denotes not significantly different; ⋆ denotes a significant decrease ($P \leq 0.05$); ⋆⋆ denotes a significant decrease ($P \leq 0.01$). (ND = not determined).

6.2.4 Carbohydrate cycling

6.2.4.1 Starch cycling

The concentration of bark starch for both the mulch and control treatments showed marked seasonal changes throughout the sampling period, with a flux ranging between ca. 2% and 9% (Fig. 6.5). In each season, accumulation of starch reserves started in autumn, with the cessation of shoot growth, and continued throughout winter. Maximum levels of starch occurred in early spring and from then decreased sharply until the following autumn (Fig. 6.5).

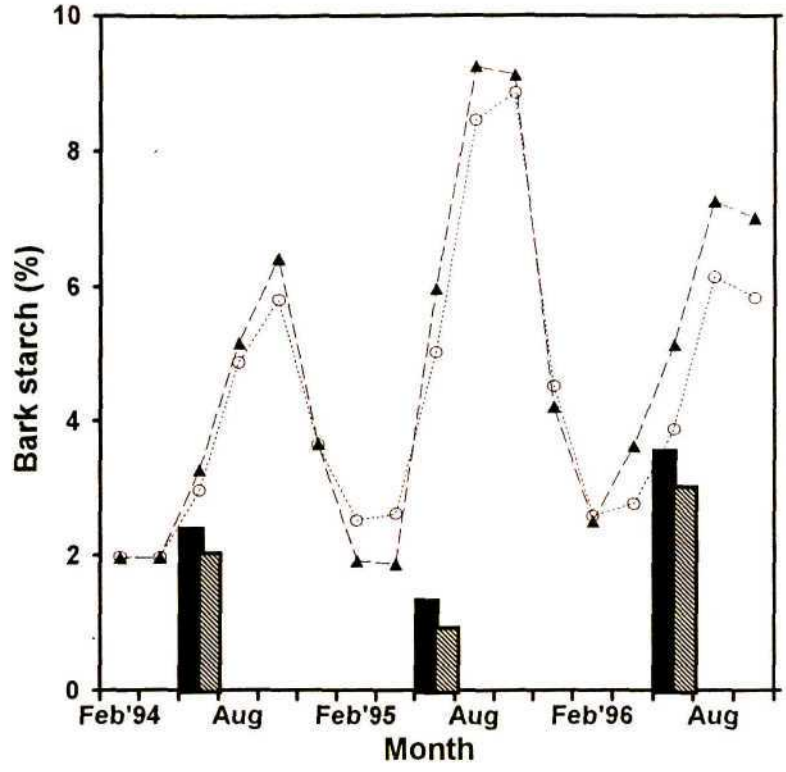

Figure 6.5

Bark starch cycling in trees on the mulch (▲) and control (○) treatments. Histograms show relative yields for the mulch (solid) and control (striped) treatments each season. SE (diff) = 0.25.

These fluctuations follow the general pattern noted by Whiley *et al.* (1996a and b) in sub-tropical Queensland, but with a slightly greater amplitude. Although flux periods of starch levels were consistent in each season, amount of starch accumulated and depleted varied from season to season. Seasons of depressed yield followed a period of low starch accumulation during the previous winter and were followed by high levels of starch accumulation the ensuing season (Fig. 6.5).

6.2.4.2 Sugar cycling

Trunk bark sugar levels fluctuated rhythmically throughout the sampling period (Fig. 6.6), and the amplitude of these fluxes were less than that of fluctuations in starch levels. In all seasons, peak sugar levels were reached towards the end of winter. Thereafter, rapid depletion took place, coinciding with the periods of flowering, fruit set and early fruit growth. Minimum levels of sugar occurred during late summer (Fig. 6.6), which roughly corresponded with the time of least concentration of starch. For the majority of the sampling period, percentage bark sugar content was slightly higher in the mulched trees, i.e. there was a larger pool of immediately accessible carbohydrates available to trees growing on the pinebark mulch.

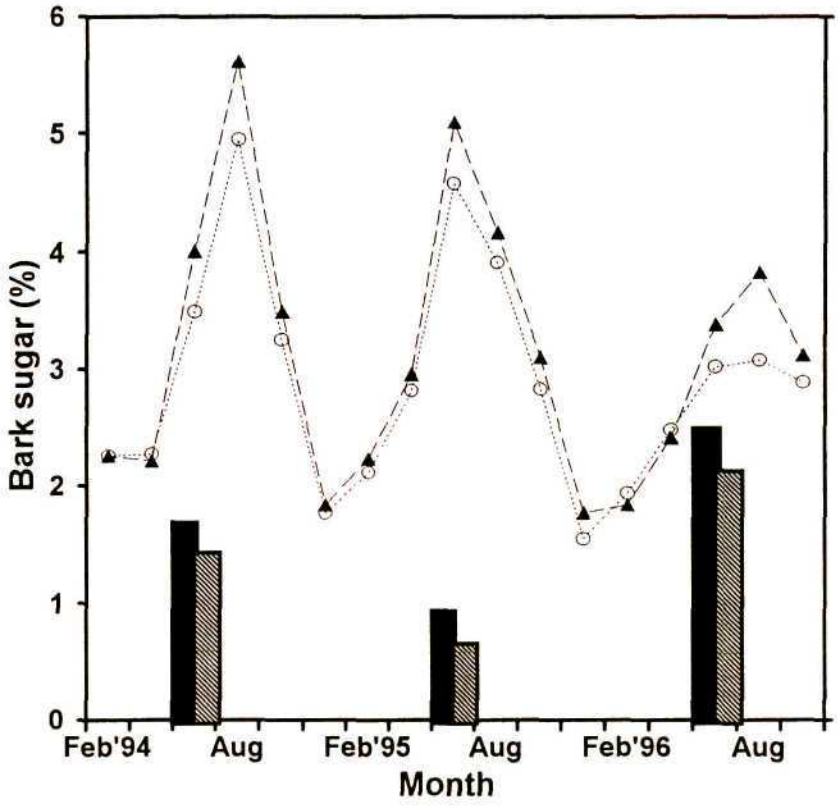

Figure 6.6

Bark sugar cycling in trees on the mulch (▲) and control (○) treatments. Histograms show relative yields of the mulch (solid) and control (striped) treatments for each season. SE (diff) = 0.23.

6.2.5 Phenology

6.2.5.1 Shoot flushing

No differences in flushing periods were observed between the mulch and control treatments. Typically, trees entered the spring flush in late August / early September and shoot extension had ceased by December / January. This spring flush coincided with the onset of fruit set and early fruit growth. A second period of shoot extension (the summer flush) was measured within two months after the cessation of the spring flush. Surprisingly, the summer flush was very weak compared to the spring flush (Fig. 6.7). In each season mean shoot extension during the spring flush was slightly greater on mulched trees than on control trees (118 mm cf. 113 mm in 1993/94, 101 mm cf. 91 mm in 1994/95, and 149 mm cf. 125 mm in 1995/96).

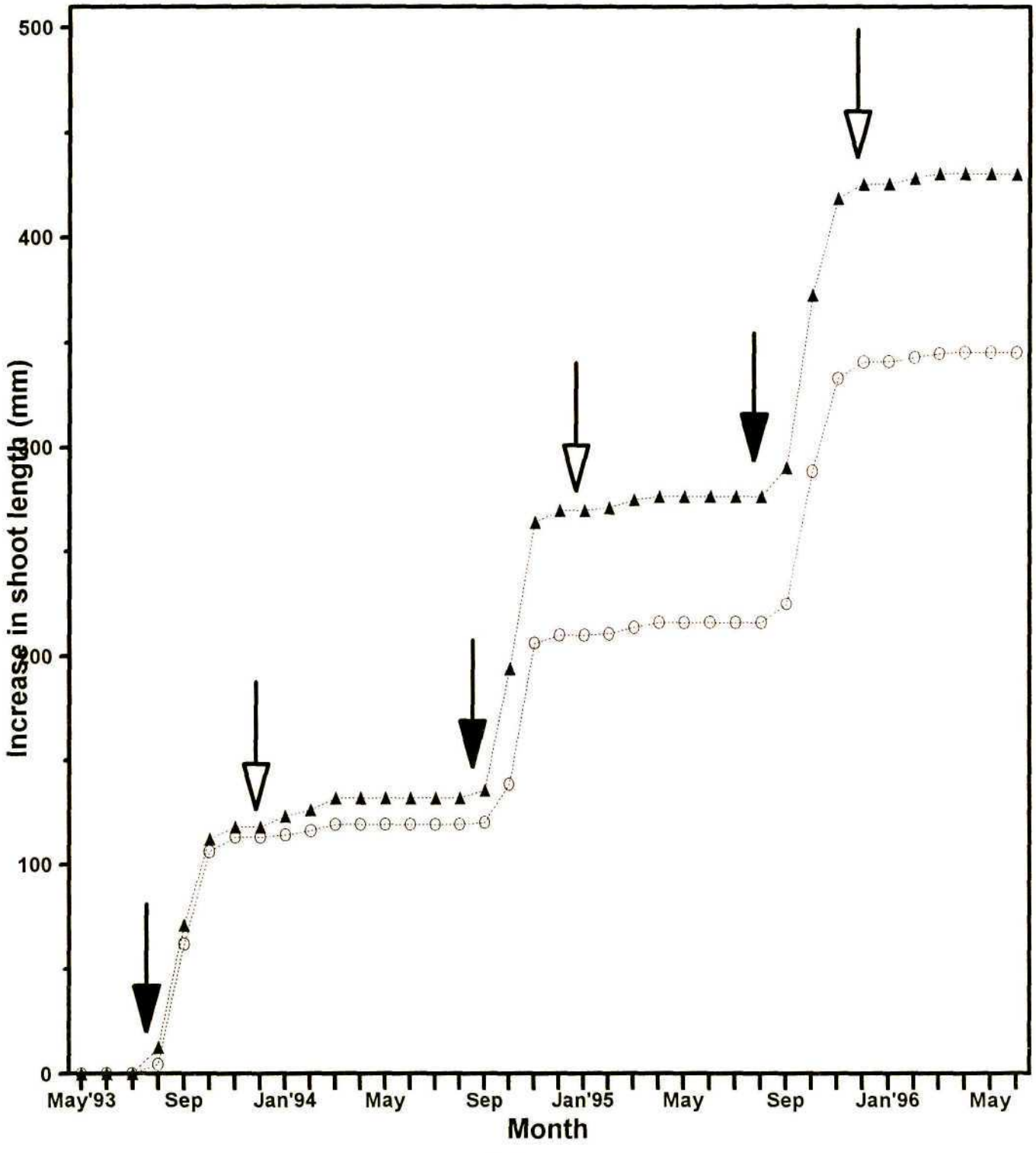

Figure 6.7

Vegetative flushing periods of shoots on the mulch (▲) and control (○) treatments. Solid arrows indicate start of spring flush and light arrows indicate start of summer flush. Measurements are means of 10 shoots per tree.

6.2.5.2 Root flushing

Root flushing periods occurred at approximately the same time, but in the mulch treatment were slightly earlier and for a more prolonged period (Fig. 6.8). Decline in root activity coincided with flowering. Root activity in the mulch treatment was always more intense than in the control (Fig. 6.8). In the mulch treatment, root growth fell into the "medium" category for the majority of each season, whereas in the control mainly "poor" root growth was recorded. For a substantial part of each season root activity was allocated a "good" rating in mulched trees (Fig. 6.8). A small flush of surface root activity was observed each winter, this being earlier in onset and more pronounced in mulched trees.

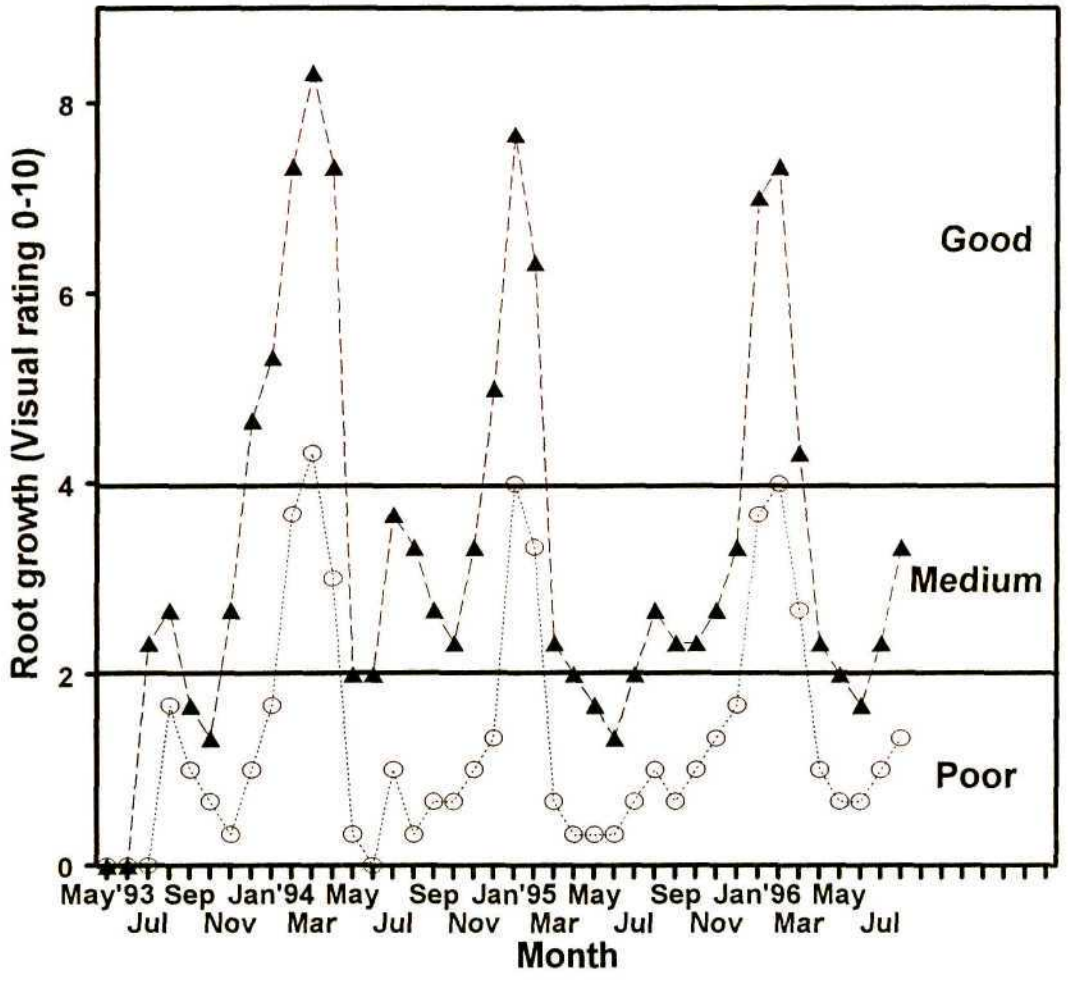

Figure 6.8

Root flushes for the mulch (▲) and control (○) treatments as determined by a visual rating where there is no root growth for a rating of 0 and extensive root growth for a rating of 10. Values are the mean of 6 measurements per treatment.

6.2.5.3 Flowering, fruit set and fruit drop

There were no differences in periods of flowering, fruit set and fruit drop between the mulch and control treatments. Periods of these phenological events are summarised in Figure 6.9. Reproductive phenological events occurred one month later in the 1994/95 and 1995/96 seasons, and this might be related to the colder winters experienced during these seasons (Fig. 2.2), with the resultant delay in floral bud break.

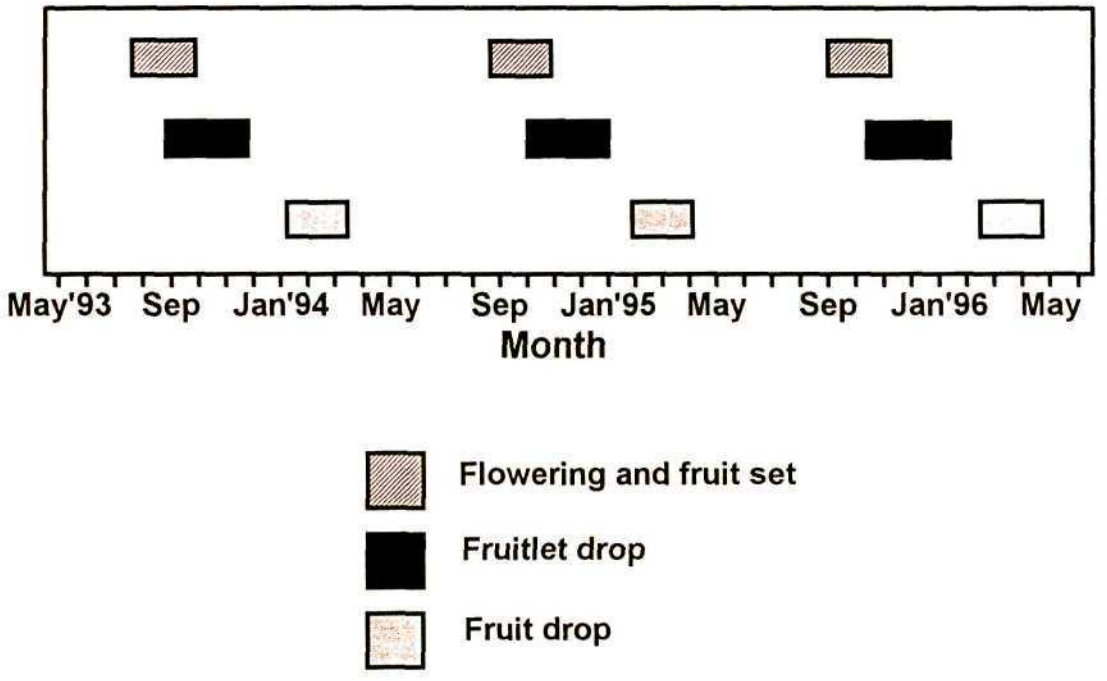

Figure 6.9
Schematic representation of periods of reproductive phenological events.

Fruit retention was strong for the 1993/94 and 1995/96 seasons, probably due to a favourable leaf : fruit ratio after completion of the spring drop. This contributed to the greater number of fruit and higher yields in these seasons. Typically, fruit drop is most intense in the first six weeks after fruit set, e.g. a total of 70.6% and 67.4% of the fruit tagged in spring 1993 and 1994 respectively, had abscised within four weeks. Fruitlet drop for the 1994/95 season was more intense than in the other two seasons, and consequently yields were lower for this crop.

6.2.6 Yield and fruit size

Overall productivity was significantly ($P \leq 0.01$) increased by mulching with composted pinebark, and this positive response was achieved in three successive seasons (Table 6.2). Over the three year duration of the trial, mulched trees produced an average of 22.0 ± 1.2 kg more than control trees, representing a 22.6% increase in yield. Harvest results also confirm the biennial bearing nature of cropping in avocado trees. A heavy crop in 1993/94 was followed by a relatively light crop in 1994/95, with high yields for the following season (Table 6.2). Furthermore, the positive effects of pinebark mulching on overall productivity was more pronounced during a season of low yield (1994/95) (Table 6.2). Control trees show a typical fruit size distribution of the 'Hass' cultivar with many fruit in the count size range of 22 to 26, and a high proportion of factory grade avocados (Figs. 6.10a to d).

Table 6.2

Summary of the effects of pinebark mulching on 'Hass' avocado productivity. Figures are means of six trees. ★★ denotes a significant ($p \leq 0.01$) increase in response to mulching.

	Control	Mulch	Percentage increase
1993/1994			
Mean fruit mass (g)	198.0	221.3	11.8**
Fruit number / tree	509	540	6.1
Yield (kg / tree)	101	119	18.5**
1994/1995			
Mean fruit mass (g)	178.2	199.2	11.8**
Fruit number / tree	262	333	27.2**
Yield (kg / tree)	47	67	42.2**
1995/1996			
Mean fruit mass (g)	216.1	220.4	2.0
Fruit number / tree	698	814	16.6**
Yield (kg / tree)	151	179	18.9**
Overall			
Mean fruit mass (g)	203.1	216.5	6.6**
Fruit number / tree	509	540	14.7**
Yield (kg / tree)	100	122	22.6**

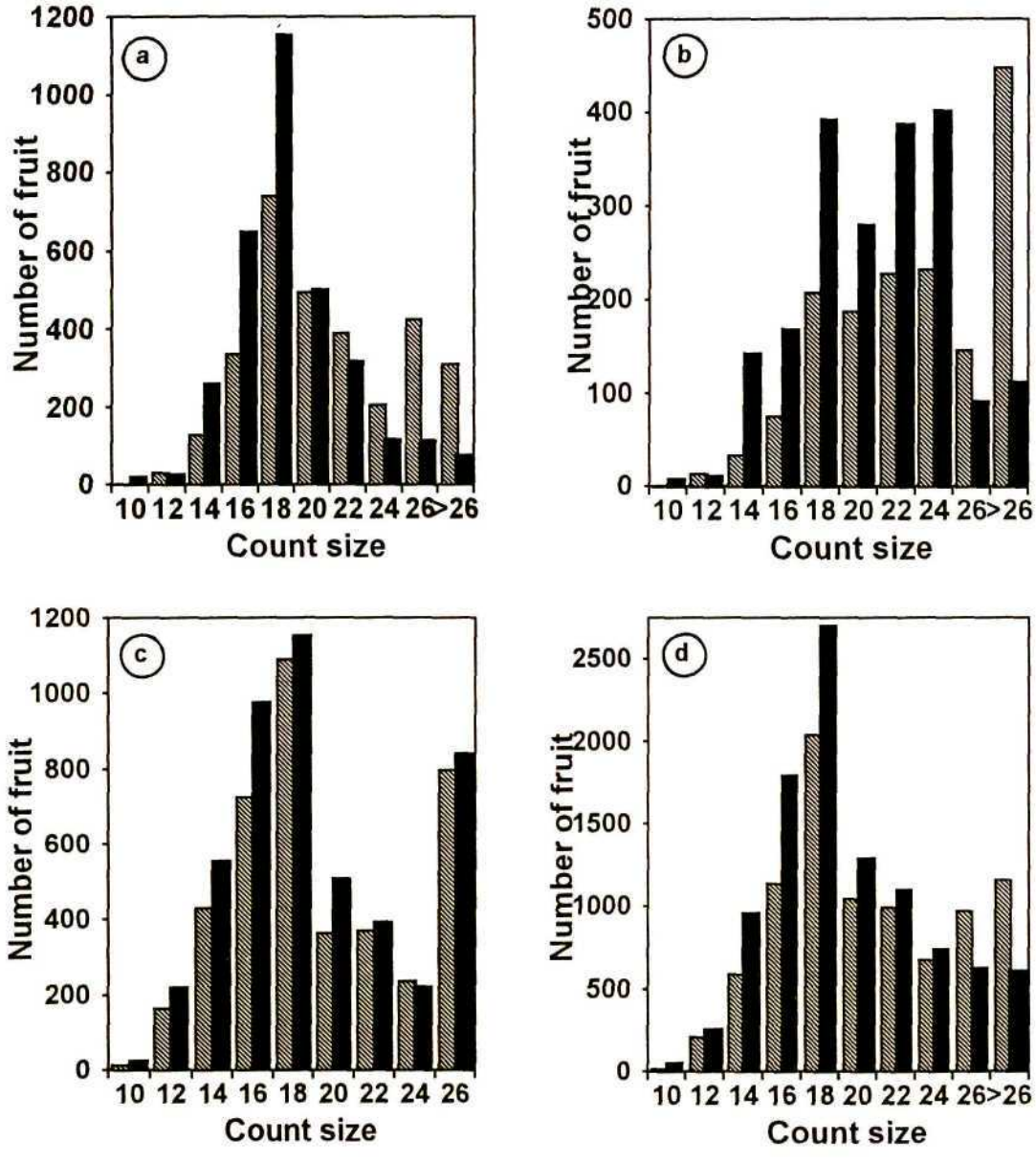

Figure 6.10

'Hass' fruit size distributions for the 1993/94 (a), 1994/95 (b) and 1995/96 (c) seasons, and a total for all three seasons (d). Solid histograms represent the mulch treatment and striped histograms represent the control.

Mulching with pinebark resulted in fruit size being significantly ($P \leq 0.01$) increased by an average 13.4 ± 1.2 g, representing an overall shift of one count in favour of larger fruit. Furthermore, this average 6.6% increase in fruit size was achieved in spite of a 14.7% increase in the number of fruit per tree. It was noteworthy however that significant differences were obtained in the two seasons when yields were lower, and not in the 1995/96 season of very high yield.

6.2.7 Economic significance

The increase in mean fruit mass coupled with elevated yields in response to mulching resulted in an increase in the number of fruit that meet export requirements of fruit size (Table 6.3), a beneficial response since the South African avocado industry is predominantly export-orientated. Over the three season duration of the trial, mulching increased the number of fruit that are considered highly suitable for export (counts 14 - 18) by 45.0%, and in addition the number of fruit that are acceptable for export (counts 10 - 12; 20 - 22) by 20.0%. During the same period the number of fruit that are deemed unsuitable for export was reduced by 29.0% in the mulch treatment (Table 6.3).

Table 6.3

Summary of the effects of pinebark mulching on export potential related to fruit size. Counts 14 - 18 were considered to be highly suitable for export; counts 10 - 12 and 20 -22 were considered to be acceptable for export; and counts ≥ 24 were considered to be not suitable for export. Figures are mean numbers of fruits per category per tree.

	Control	Mulch	Percentage difference[†]
1993/1994			
Suitable	200	344	+ 72.0
Acceptable	152	145	- 4.6
Not suitable	157	51	- 67.5
1994/1995			
Suitable	53	117	+ 120.8
Acceptable	71	114	+ 60.6
Not suitable	138	102	- 26.1
1995/1996			
Suitable	374	447	+ 19.5
Acceptable	152	190	+ 25.0
Not suitable	172	177	+ 2.9
Overall			
Suitable	209	303	+ 45.0
Acceptable	125	150	+ 20.0
Not suitable	155	110	- 29.0

[†]**Figures preceded by a positive sign indicate an increase by mulching, and figures preceded by a negative sign indicate a decrease by mulching.**

The increased yield and mean fruit size coupled with improved export potential as a result of mulching, means that financial rewards to avocado producers could be considerably boosted, although costs of the mulch would have to be off-set. Considering that the half-life of composted pinebark is regarded as five years (Wolstenholme *et al.*, 1996), and that the initial costs of the pinebark were off-set within two seasons (Table 6.4), the application of pinebark or similar mulches provides avocado growers with another option of increasing profitability.

Table 6.4

Breakdown of costs of and extra revenue generated by the application of a pinebark mulch on Everdon Estate, KwaZulu-Natal midlands.

1993/94	*Cost of pinebark + transport = R26 300 / ha*			(-R26 300)
	Return / ha (On farm)			
	Control	**Mulch**	**Extra revenue**	
1993/94	R34 700	R47 800	R13 100	(-R13 200)
1994/95	R16 300	R30 100	R13 800	(+R600)
1995/96	R70 500	R85 400	R14 900	(+R15 500)

6.3 SUMMARY

(1) Root growth was substantially increased by the application of a coarse composted pinebark mulch.

(2) Mulching decreased the incidence of fruit with degenerate seed coats, lowered mean canopy temperatures, improved photosynthesis and increased starch and glucose availability.

(3) The mulch treatment proved to be cost-effective.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 GENERAL DISCUSSION

Under South African conditions, 'Hass' avocado is predisposed to producing a high proportion of phenotypically small fruit which cannot be marketed. The large number of small fruit costs the South African avocado industry between R30 and R40 million per season. Although substantial investment in management strategies have been made by growers and researchers, the underlying reasons for the appearance of phenotypically small 'Hass' fruit have remained obscure.

Recourse to the literature revealed that surprisingly little information was available concerning the metabolic control of avocado fruit growth and in particular processes that might be affected by abiotic/biotic pressure and cultural practise. Furthermore, no attempt had been made to characterise the 'Hass' small fruit phenotype either biochemically, physiologically or using molecular technology. It was therefore reasoned that without a basic knowledge and understanding of the major metabolic processes contributing to growth and development of avocado fruit, it was unlikely that an appropriate management strategy could be adapted and implemented with confidence. In an attempt to address these issues, the present study has characterised the 'Hass' small fruit phenotype, demonstrated the role of isoprenoid metabolism and symplastic transport in the metabolic control of avocado fruit growth, and examined the contribution of tree stress to appearance of the small fruit phenotype. Finally, mulching was evaluated as a potential orchard management strategy to reduce or eliminate the high incidence of 'Hass' small fruit under South African conditions.

7.1.1 Characterization of the 'Hass' small fruit phenotype

Zilkah and Klein (1987) first studied details of the growth of 'Hass' avocado fruit. This study showed that 'Hass' typically produces two distinct populations of fruit, the distinguishing feature being size. These authors analysed measurements of small and large fruit length and diameter using a hyperbolic function and concluded that final fruit

size was dictated by the time of fruit set, with large fruit being set approximately 2 to 3 d earlier than small fruit. They also showed that length and diameter measurements of small fruits approached those of large fruits, i.e. by extrapolation from the growth curves, small fruit during the initial stages of development eventually reached the same size as large fruit. In contrast, the present study showed that small fruit never reached the same size as large fruit. An asymptotic function (i.e. a gompertz equation) proved to be the line of best fit for the data (a regression coefficient of 0.998 for the asymptotic function cf. to 0.937 for the hyperbolic function used by Zilkah and Klein (1987)). A weakness of the study by Zilkah and Klein (1987) was that fruit growth was only monitored for the first 140 d after full bloom. Had the authors used an extended sampling period, the growth data thus obtained would have revealed that small fruit never reach the same size as large fruit. An advantage of the present study is that growth was measured throughout fruit development, up to the time of harvest (viz. 284 and 255 d in the first and second seasons respectively).

Although the author appreciates that final fruit size is influenced by time of fruit set (Zilkah and Klein, 1987), this study has shown that even fruit which are set at the same time also produce different sized fruit, i.e. some other factor must play a role in influencing fruit size. The time of seed coat senescence is also important in the determination of final fruit size (see Section 3.2.3.1). Casual observation revealed that the small fruit phenotype was always associated with early senescence and/or death of the seed coat. Growth was arrested earlier in phenotypically small fruit and progressively later in fruit of increasing size. Thus, and as illustrated in Figure 7.1, seed coat senescence and/or death, and cessation of growth can occur at any stage during 'Hass' avocado fruit ontogeny. Furthermore, it is important to appreciate that all fruit, irrespective of final fruit size, will eventually develop degenerate seed coats. This study has demonstrated the presence of a physiological window (time period in development) 60-90 d after full bloom, when 'Hass' fruit first becomes susceptible and expresses the small fruit phenotype. Appearance of small fruit during this critical period, assuming the fruit is retained on the tree, will yield small fruit at time of harvest. Since the emergence of small fruit is less frequent as growth and development proceeds, it is clear that the time of seed coat senescence determines final fruit size (Fig. 7.1).

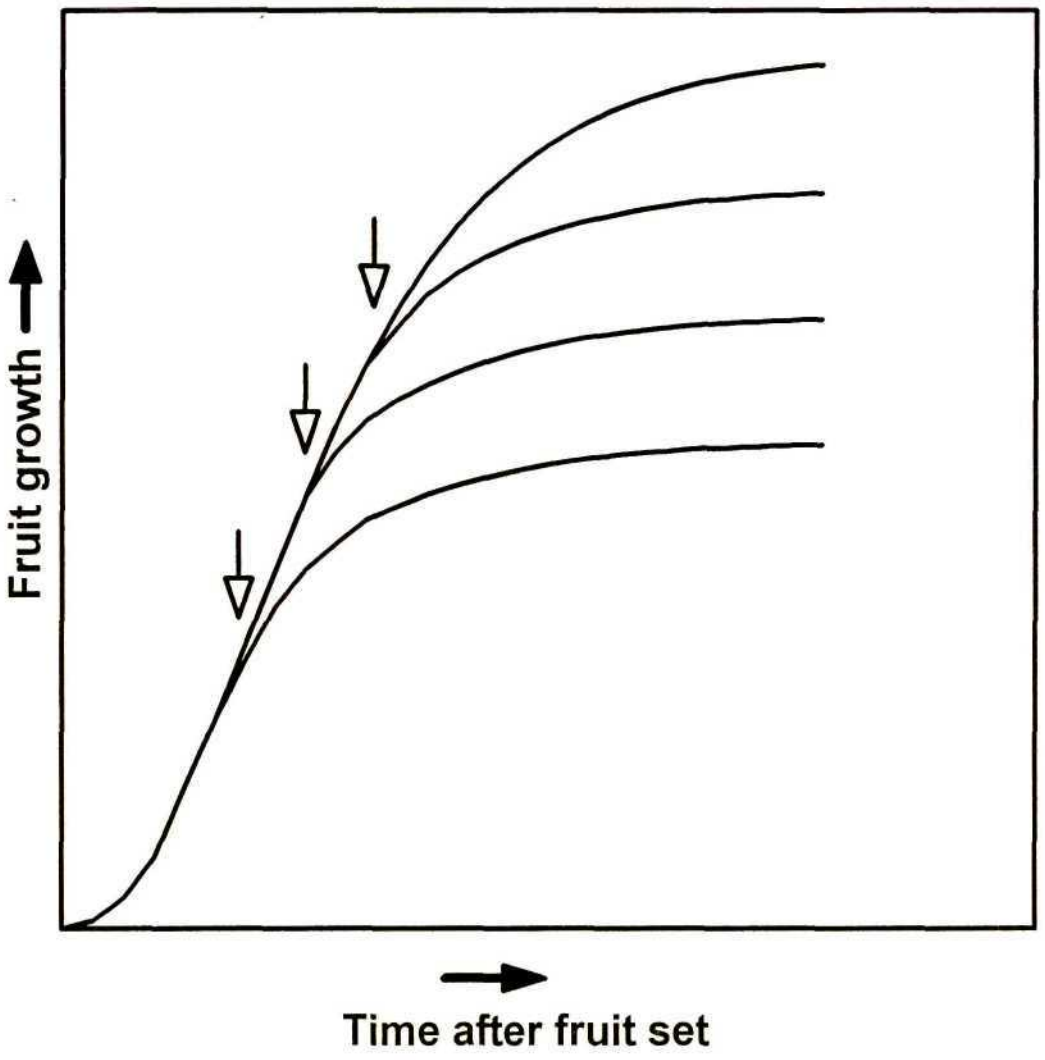

Figure 7.1

Proposed relationship between final 'Hass' avocado fruit size and time of onset of seed coat senescence as a function of growth. Arrows indicate the time at which seed coat senescence is induced.

7.1.2 Metabolic control of 'Hass' avocado fruit development

7.1.2.1 HMGR activity and the 'Hass' small fruit syndrome

When the present study was initiated nothing was known about the metabolic control of avocado fruit growth. However, several reports had implicated HMGR activity and phytosterols in the control of fruit development (Narita and Gruissem, 1989; Gillaspy *et al.*, 1993) and at least one study had suggested an essential role for stigmasterol in the support of plant cell division (Haughan *et al.*, 1987). Since cell division in avocado proceeds throughout development, it was hoped that by using a specific inhibitor of

HMGR the interaction between isoprenoid growth regulators, phytosterols and HMGR in the control of 'Hass' fruit growth and development could be elucidated.

Although deprivation of MVA and sterols is reported to increase HMGR half life, high levels of sterol enhance the rate of HMGR degradation (Correll and Edwards, 1994). Thus, it was not unexpected that treatment with stigmasterol (and/or cholesterol) would reduce *in vivo* HMGR activity and fruit growth. Co-injection of mevastatin with stigmasterol however, caused fruit to respond differently in phases I, II and III. In phase I, stigmasterol reduced fruit growth and accelerated abscission whereas, fruit treated in phase II showed partial recovery from mevastatin-induced inhibition of growth and rates of abscission closely resembled those of control treatments. In phase III however, stigmasterol alone did not affect fruit growth and reversed the growth retarding effect of mevastatin to the same extent as MVAL. Even so, stigmasterol did not reverse mevastatin-induced inhibition of HMGR, presumably due to mevastatin-induced ABA accumulation. Likewise, the ABA content of small fruit resembled closely that of mevastatin-treated fruit and in these, HMGR activity was substantially reduced.

Earlier studies on regulation of higher plant cytosolic HMGR suggested hormonal mediation of enzyme activity (Russell and Davidson, 1982). These authors demonstrated *in vivo* ABA-, stigmasterol- and cholesterol-inhibition of enzyme activity. When added to reaction mixtures *in vitro* however, these products of isoprenoid biosynthesis had no effect on enzyme activity. It was therefore concluded that hormonal control was not allosteric but exerted via some unknown phosphorylation system. Similar conclusions were reached in studies on the effect of endogenous ABA on HMGR activity during seed maturation. *Vivipary* mutants of maize which are defective in ABA biosynthesis and the V*p*1 mutant which is defective in an ABA response element, all show enhanced HMGR activity relative to wild-type siblings (Moore and Oishi, 1994). Since the V*p*1 gene product is involved in ABA signal transduction during seed development, it was proposed that HMGR activity during seed maturation is regulated via a V*p*1-dependent signal transduction pathway that is affected by reduced ABA.

Mevastatin-induced ABA accumulation in avocado mesocarp was both surprising and interesting. First, this observation supports plastid-localized ABA synthesis (Zeevaart and Creelman, 1988) as mevastatin and its structural analogues are unable to inhibit chloroplast isoprenoid synthesis (Bach and Lichtenthaler, 1983; Bach, 1987). Second, the result might suggest that a product(s) of cytosolic isoprenoid biosynthesis is responsible for regulating ABA formation in or by chloroplasts. Two possible candidates include CKs and phytosterols. iP reversed the inhibitory effects of mevastatin at all stages of avocado fruit development. Similarly, inhibition of tobacco cell growth by lovastatin (a mevastatin analogue) was reversed by CKs (Crowell and Salaz, 1992). Furthermore, iP and its hydroxylated derivative zeatin replaced the essential role of MVA in initiating DNA replication in the cell cycle (Siperstein, 1984). Since CK biosynthesis is purported to involve prenylation of the purine moiety catalysed by isopentenyl transferase, a process in which dimethylallylpyrophosphate (DMAPP) is added to AMP at position N^6 (Binns, 1994), the above observations might suggest that inhibition of HMGR limits the MVA pool available for synthesis of DMAPP (isomerization of IPP) and hence *in situ* CK biosynthesis. iP also reversed the inhibitory effects of ABA. The role of ABA in plant stress responses and its ability to retard developmental processes (Zeevaart and Creelman, 1988) suggests that it is a likely candidate to influence fruit growth under adverse conditions and thereby contribute to down-regulation of developmental programmes.

Several studies have intimated a cell-cycle-regulating function for ABA because exogenous ABA inhibits nucleic acid and protein synthesis (Owen and Napier, 1988). Meyers *et al.* (1990) showed that exogenously applied ABA consistently inhibited cell division in cultures of maize kernels. More recently, Müller *et al.* (1994) obtained evidence to suggest that ABA functions to reduce cell division cycle activity by retarding completion of the cell cycle. Stress-induced accumulation of ABA might therefore be expected to exert an effect on fruit growth during the early stages, when cell division cycle activity is at a maximum.

Fruit enlargement is correlated with both cell division and expansion, developmental

processes requiring a significant level of sterol biosynthesis (Narita and Gruissem, 1989; Chappell, 1995a). It is well established that ABA increases permeability of lipid membranes (Stillwell and Hester, 1984; Bach, 1986; Stillwell *et al.*, 1989; Purohit *et al.*, 1992; Bürner *et al.*, 1993) and that phytosterols inhibit these ABA-induced perturbations (Stillwell *et al.*, 1990). ABA also appears to inhibit HMGR activity (Russell and Davidson, 1982; Moore and Oishi, 1994). In light of these observations it is tempting to suggest that stress-induced initiation of the ABA signal transduction pathway depresses HMGR activity limiting synthesis of both CKs and phytosterols to reduce cell division cycle activity in affected 'Hass' fruits. Furthermore, accumulation of ABA during fruit growth might be sufficient to induce 'lipid melting' in affected membranes (e.g. seed coat), causing onset of senescence and cessation of fruit development, processes that would be reversed in the presence of sufficient sterol. This proposal is supported by the observation that fruit treated with stigmasterol in the presence of mevastatin, an inhibitor of HMGR that induces ABA accumulation, show a decline in endogenous ABA concentration and recovery of growth.

7.1.2.2 ABA and 'Hass' avocado fruit size

Results from the present investigation provide good evidence to support an interaction between fruit (mesocarp) ABA concentration and expression of the 'Hass' avocado small-fruit phenotype. Mesocarp ABA concentration of mature fruit was negatively correlated to fruit size and exogenous application of ABA during the linear phase of rapid growth caused early seed coat senescence and retarded fruit growth.

The ability of ABA to induce expression of the 'Hass' small fruit phenotype at all stages of fruit growth and development suggests that an altered endogenous ABA concentration is a major contributing factor. However, the mechanism involved remains to be elucidated. Several studies have revealed that the ABA content of young fruit is high and declines during the course of development (Fraser *et al.*, 1995; Guinn, 1982). Likewise, the present study showed that the ABA concentration of developing 'Hass' fruit is initially high and declines with increasing fruit age. Although the physiological significance of elevated ABA levels during the initial stages of fruit growth remains

unresolved, there is evidence to support a role for ABA in photosynthate unloading from phloem in developing grains, seeds and fleshy fruits. For example, application of ABA to wheat and barley grains promoted import of recently fixed photo assimilate (Dewdney and McWha, 1979; Tietz *et al.*, 1981). Furthermore, Schüssler *et al.* (1984) demonstrated that testa of large seeded genotypes of soybean had higher ABA content than small-seeded genotypes and suggested that the additional ABA acted to increase sieve element unloading into the testa apoplast. Likewise, ABA enhanced the uptake of sugar into, vacuoles of apple fruit flesh (Beruter, 1983; Yamaki and Asakura, 1991), sugar beet root tissue discs (Saftner and Wyse, 1984) and increased the sugar content of developing citrus fruit (Kojima *et al.*, 1995).

The diminution in concentration of free ABA during the course of fruit development presumably occurs due to catabolism and more specifically, due to formation of ABA-glucose ester and ether derivatives (Harris and Dugger, 1986; Hirai and Koshimizu, 1983; Loveys and Milborrow, 1984). Conjugation of ABA to ABA-glucose ester is irreversible and appears to be a means whereby ABA is sequestered in an inactive form in the vacuole (Zeevaart and Creelman, 1988). While the biosynthesis and regulation of hormone conjugates has yet to be elucidated (Semdbner *et al.*, 1994), changes in glucose concentration could impact on activity of the glucosyltransferase responsible for ABA glucosylation, which is purported to take place at the cytosolic face of the tonoplast (Kaiser *et al.*, 1985), and hence the concentration of free ABA.

7.1.2.3 Mediation of symplastic solute transport in developing 'Hass' fruit

The antagonistic effects of ABA and CKs in the regulation of plant organ senescence are well documented (Beevers, 1976; Biswal and Biswal, 1988; Noodén, 1988; Smart, 1994). In addition, CKs appear to antagonize many other physiological processes thought to be mediated, all or in part by ABA. For example, ABA-induced stomatal closure and leaf and fruit abscission are reversed by exogenous application of CK while CK-mediated release of seed dormancy contrasts with ABA inhibition of germination (Salisbury, 1994). Although the biochemical and molecular basis for this antagonism remains to be elucidated, a possible cause of ABA-induced seed coat senescence and

cessation of avocado fruit growth may be a reduction in the supply of photoassimilate required for the maintenance of cell division and cell expansion.

In addition, the low level of iP in phenotypically small fruit might suggest that the small fruit syndrome arises as a result of impaired CK biosynthesis. Sugars have been shown to promote CK synthesis (Koch, 1996) and both sugars and CKs delay senescence (Smart, 1994). Alternatively, the small fruit phenotype may be a consequence of tree stress and an associated rise in fruit ABA levels which would be expected to accelerate the onset of senescence. To evaluate the antagonism between CKs and ABA in the metabolic control of avocado fruit growth, the effect of these hormone applied either singly or in combination on syplastic solute flow and plasmodesmatal structure was investigated.

7.1.2.3.1 ABA and iP, and symplastic solute transport

The distribution of radioactivity in small 'Hass' fruit and fruit pre-treated with ABA, following pulsed application of [^{14}C]sucrose, was similar. Thus, when compared to control and iP-treated fruit, incorporation of label into seed coat tissue was reduced by 50% whereas the amount of [^{14}C] associated with the seed increased two-fold. Measurement of seed coat and mesocarp E_m revealed an electrical potential gradient between seed coat and mesocarp tissue that was abolished in fruit pre-treated with ABA. Furthermore, microiontophoretic and detailed ultrastructural studies of mesocarp and seed coat tissue revealed that plasmodesmata in ABA-treated tissue were gated by electron dense material deposited at the annuli, a phenomenon that was negated by co-injection of fruit with iP. Although the pattern of [^{14}C] allocation from pulsed [^{14}C]sucrose, and cell-to-cell transport of LYCH were similar for control fruit and fruit pre-treated with iP and iP plus ABA, co-injection of iP with ABA did not reverse the apparent ABA-induced membrane depolarization of mesocarp parenchyma suggesting operation of a second, plasma membrane-localized pathway, that is unaffected by iP but sensitive to ABA.

Hartung *et al.* (1980) demonstrated that exogenous ABA caused plasma membrane

hyperpolarization, increased soluble sugar content (measured as glucose units) and reduced glucose-induced changes in membrane potential in *Lemna gibba*. These authors suggested that stimulation of invertase activity could have caused the increase in glucose concentration.

ABA is purported to stimulate soluble acid invertase activity, at least in developing soybean seeds (Ackerson, 1985). Acid invertase, a glycosylated enzyme responsible for the hydrolysis of sucrose (Wagner and Wiemken, 1987), is located both in the vacuole and apoplast and is thought to increase the sucrose gradient between source and sink tissue to facilitate phloem unloading (Ruffner *et al.*, 1990). Several studies have revealed that increased expression of extracellular (insoluble) acid invertase reduces plant organ growth (Dickinson *et al.*, 1991; Heineke *et al.*, 1992; von Schaewen *et al.*, 1990), whereas Klann *et al.* (1996) showed that expression of an antisense soluble acid invertase in tomato results in sucrose accumulation, decreased hexose sugar concentration and reduced fruit. It has recently been observed that insoluble acid invertase activity of avocado seed tissue is substantially higher in the small-fruit phenotype and that mesocarp soluble and insoluble acid invertase activity of this phenotype is reduced (Cowan[1], *pers. comm.*). In addition, expression of the small-fruit phenotype caused a reduction in seed and seed coat sucrose concentration without affecting total fruit hexose concentration although, glucose accumulated in the seed and fructose in the mesocarp (Cowan[1], *pers. comm.*). Together, these observations suggest a change in the sucrose gradient between seed coat, and seed and mesocarp tissue. Since symplastic continuity in both seed coat and mesocarp tissue is arrested and the E_m gradient between seed coat and mesocarp parenchyma is abolished by exogenous ABA and expression of the small-fruit phenotype, it is suggested that the seed assumes dominance over the mesocarp for available sugar. Whilst measurements of E_m obtained using avocado tissues were not high, it must be remembered that this is mature, pre-climacteric tissue and would thus not be expected to yield high values such as those obtained in leaf mesophyll cells (van Bel *et al.*, 1996). Nevertheless, it is generally accepted that the more negative E_m becomes, the more physiologically active cells are assumed to be in terms of cell-to-cell

[1] A.K. Cowan, Dept. Hort. Sci., University of Natal, Pietermaritzburg, South Africa

transport. Thus, our results appear to indicate that seed coat and mesocarp tissue are within the same electrophysiological (and by implication, structurally-connected) continuum.

7.1.2.3.2 Plasmodesmatal structure/function in response to ABA and iP

Analysis of 'Hass' avocado plasmodesmatal ultrastructure revealed three distinct changes induced by pre-treatment of fruit with ABA that were reversed or negated by co-injection of ABA and iP. Firstly, median cavities were clearly evident in continuous branched plasmodesmata in control and iP-treated fruit. Plasmodesmatal branching was apparently reduced in fruit that had been pre-treated with ABA whereas this effect was negated in fruit co-injected with ABA and iP. A correlation between arrested branched plasmodesmatal development and onset of accelerated senescence has been established for leaf tissue (Ding *et al.*, 1993). Thus expression of the 'Hass' small fruit phenotype which is associated with senescence of the seed coat, implies that development and structure/function of plasmodesmata may be crucial in the determination of final fruit size. Since primary plasmodesmata are formed cytokinetically during cell plate assembly and secondary plasmodesmata arise *de novo*, post-cytokinesis, in pre-existing cell walls (Lucas *et al.*, 1993), it is concluded that avocado mesocarp and seed coat plasmodesmata are morphological modifications of simple primary plasmodesmata. As stated by Ehlers and Kollmann (1996), primary plasmodesmata may undergo morphological change but will not develop into plasmodesmata of a truly secondary origin. Whether CKs play a role in mediating branching of primary plasmodesmata is currently unknown.

Secondly, the data illustrates the presence of electron-dense, particulate material associated with the neck region of plasmodesmata in mesocarp and seed coat tissue of small, ABA- and ABA + iP-treated avocado fruit. Coupled with microiontophoretic analysis, these data suggest that application of ABA stimulated gating by inducing deposition of globular (and assumedly proteinaceous) plasmodesmatal-localized material. The absence of this material in control and iP-treated tissues, as well as its apparent reduction in ABA + iP-treated material, suggests that it is formed in response to exogenous ABA application.

It is essential that plasmodesmata are dynamic to accommodate larger or smaller trafficked molecules in cell-to-cell chemical communication. Robards and Lucas (1990) suggest that up- or down-regulation of plasmodesmatal pore size is achieved by either modification of the central lipoprotein core or, by deposition of callose near the cytoplasmic annulus. Thus, a central role for ABA in the regulation of plasmodesmatal pore diameter, as a means of either controlling or curtailing molecular trafficking in developing avocado fruit seems very plausible. Since integrity of the seed coat and associated mesocarp is essential for maintenance of sink strength throughout fruit development, ABA-induced down-regulation of plasmodesmatal pore size may cause loss of sink strength and induce early seed coat senescence.

Finally, the plasma membrane adjacent to the primary pitfields appears highly convoluted in mesocarp from control and iP-treated fruit. In contrast, the plasma membrane in mesocarp of fruit pre-treated with ABA lacks this convoluted appearance suggesting ABA-induced diminution of membrane activity. A similar response was observed in seed coat tissue. Interestingly, injection of fruit with ABA + iP reversed this effect but only on one side of the plasma membrane/cell wall complex of adjacent cells, i.e. opposite occluded plasmodesmata. Although the physiological significance of this observation has yet to be established, it does suggest that ABA affects both symplastic and apoplastic solute flux in avocado mesocarp.

7.1.2.4 An integrated model for the metabolic control of avocado fruit growth

Based on the findings of this study a model presented in Figure 7.2 is proposed to explain appearance of phenotypically small 'Hass' fruit. The model is consistent with recent reports that ABA retards cell division cycle activity (Meyers *et al.*, 1990; Müller *et al.*, 1994) whereas CKs promote this process and do so by regulating the G_2 to mitosis transition, i.e. stimulating tyrosine dephosphorylation and activation of $p34^{cdc2}$-like H1 histone kinase (Zhang *et al.*, 1996). Similarly, withdrawal of CK causes cessation of the cell cycle and cells accumulate in M, S and G_1 (Mander and Hanke, 1996). An imbalance in the CK:ABA ratio, through reduced CK synthesis or increased ABA, might therefore be expected to impact on avocado fruit cell division activity and

sink strength, particularly as growth of phenotypically small 'Hass' fruit is limited by cell number, not cell size.

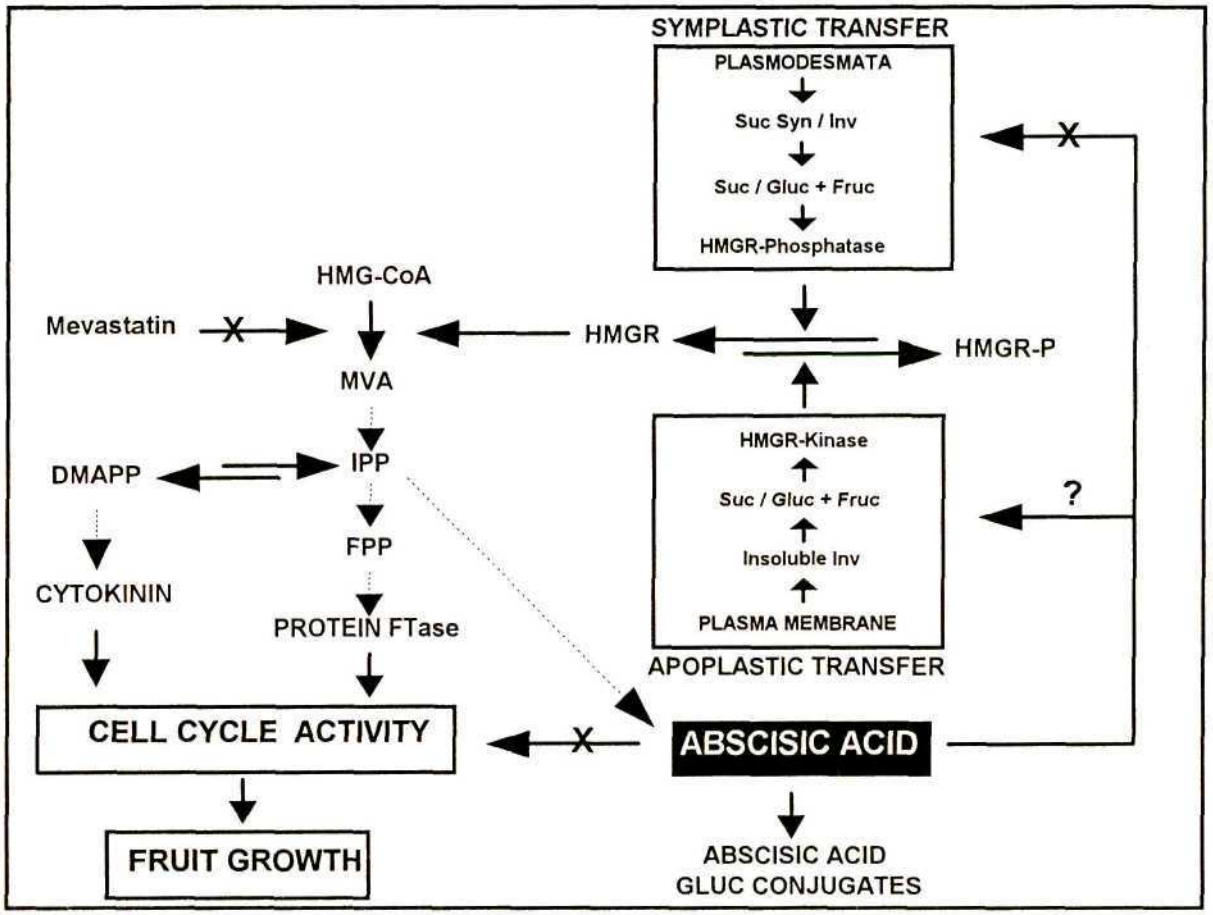

Figure 7.2

Proposed interaction between ABA and iP, and processes thought to be involved in mediating the appearance of 'Hass' avocado small fruit phenotypes. X denotes inhibition.

Assuming CK is derived *in situ* by isoprenylation of purine, inhibition of isopentenyl diphosphate synthesis will limit the amount of dimethylallyl pyrophosphate available for CK biosynthesis. Isopentenyl diphosphate is formed from MVA, the product of the reaction catalysed by HMGR, and inhibition of HMGR by mevastatin is reversed by both MVAL and CK (Bach, 1987; Crowell and Salaz, 1992). HMGR is subject to regulation by phytochrome, reaction end product feedback and post-translational modification (Bach, 1987). The latter process is a well documented regulatory system in mammalian cells where enzyme activity is inactivated by a reversible phosphorylation mechanism involving an AMP- or ADP-stimulated kinase (Chappell, 1995b). Biochemical evidence

for the existence of plant HMGR kinase activity, with similar properties to AMP kinases, includes the purification and characterization of HRK-A (Ball *et al.*, 1994) and HMGR kinase-B (MacKintosh *et al.*, 1992) from cauliflower and other species. More recently, a protein kinase capable of phosphorylating *Arabidopsis* HMGR was isolated from barley endosperm (Barker *et al.*, 1996). Inhibition of HMGR activity is known to impact on mammalian cell division cycle activity (Sinensky and Logel, 1985; Jakobisiak *et al.*, 1991). Similar findings have been obtained for higher plants using cultured tobacco cells (Crowell and Salaz, 1992). In the latter instance, inhibition of HMGR activity and cell growth was attributed to reduced CK biosynthesis.

Recent information suggests that in addition to CK, pyrophosphorylated intermediates in isoprenoid synthesis are equally important. Thus isoprenylation of Rab and GTP-binding proteins has been shown (Morehead *et al.*, 1995; Biermann *et al.*, 1996; Yalovsky *et al.*, 1996) and farnesyl protein transferase (FPTase), biochemically characterized in tomato (Schmitt *et al.*, 1996) and pea (Qian *et al.*, 1996). More importantly however, inhibition of FPTase by manumycin completely blocked mitosis when added at the S stage but not when added at G_2 (Qian *et al.*, 1996). This observation suggests that FPTase is required for cell division cycle activity and that it modulates progression of the cycle through S and in the transition from G_1 to S. Furthermore, it has been demonstrated that mutations that confer enhanced response to ABA (*era1* mutants) arise due to perturbed farnesylation of a protein(s) that negatively regulates ABA signaling (Cutler *et al.*, 1996). Thus, the appearance of an ABA-supersensitive *Arabidopsis* phenotype. Whether a similar perturbation is responsible for the appearance of phenotypically small 'Hass' fruit is currently under investigation. Nevertheless, the accumulated information strongly suggests that the aforesaid molecular responses could be manifestations of altered HMGR activity.

Analysis of a partially purified protein kinase from developing barley endosperm has revealed *in vitro* phosphorylation of HMGR, confirming it to be HMGR kinase (Barker *et al.*, 1996). These authors also presented convincing evidence to support the hypothesis that barley HMGR kinase is a member of the sucrose nonfermenting-1

protein kinase family. This latter observation indicates that higher plant HMGR kinase may be mediated by carbohydrate status.

Carbohydrate-modulated enzymes are thought to be regulated by a hexose sensor comprising phosphorylated glucose and fructose and a putative plasma membrane signal (Koch, 1996). The concentration of each component is determined by the mechanism of sugar uptake. For example, hydrolysis of symplastically imported sucrose by soluble invertase generates more substrate for the hexose sensor than does sucrose synthase whereas uptake of sugar, hydrolysed extracellularly, requires expression of an energy-coupled plasma membrane hexose carrier that may be sterol-modulated (Grandmougin-Ferjani *et al.*, 1997) and ABA-sensitive. Availability of carbohydrate might also impact on plasmodesmatal pore size to promote pathway switching from symplastic to apoplastic transfer. Tentative evidence in support of this phenomenon has recently been obtained from studies of the pathway of postphloem sugar transport in developing tomato fruit (Ruan and Patrick, 1995; Patrick and Offler, 1996). Results from the present investigation therefore suggest that sugar transport pathway switching in 'Hass' avocado may be a response to altered CK:ABA ratio and that accumulation of ABA arrests both symplastic and apoplastic sugar transport causing early seed coat senescence, loss of sink strength and reduced fruit growth. Taken together, the complexity of expression of the 'Hass' small fruit phenotype and the potential role of ABA in this process indicates operation of multiple signalling pathways that are influenced by hormone balance and sugar concentration and composition.

7.1.3 Tree stress and the small fruit syndrome

Water is considered to be the most important limiting factor to plant growth (Syvertsen, 1985; Smith and Griffiths, 1993), and water stress has been shown to lead to reduced avocado yield and fruit size (Whiley *et al.*, 1988). In drying soils, ABA biosynthesis by roots is enhanced and a decline in soil water status has been correlated with an increase in xylem ABA concentration (Davies and Zhang, 1991; Tardieu *et al.*, 1992a; 1992b; Davies *et al.*, 1994). Plants which are exposed to conditions of water stress

have been shown to accumulate ABA in leaves but more specifically in the region surrounding the stomatal guard cells (Davies and Mansfield, 1983). Associated with this increase in ABA is rapid stomatal closure brought about by osmotic adjustments in the two opposing guard cells surrounding each stoma (Zeevaart and Creelman, 1988; Hartung and Slovik, 1991). This mechanism is thought to involve a signal transduction system in which ABA interacts with the plasma membrane to induce stimulus-response coupling and involves changes in cytosolic [Ca^{2+}], [K^+] and pH, and initiation of second messengers (Hetherington and Quatrano, 1991). ABA also exerts an effect on the enzymes that regulate proton movement across the outer membrane of guard cells (Anderson *et al.*, 1994). Stomatal aperture regulates transpirational water loss and CO_2 uptake from the atmosphere (Zhang and Davies, 1991). Stomatal pore size impacts on photochemical efficiency via its effect on photosynthesis (Demmig-Adams and Adams, 1988) and leaf temperatures (via its effect on energy dissipation (Raschke, 1960)), both of which were monitored during this study.

The surface temperature of a leaf is the tangible manifestation of its energy balance and therefore is affected by abiotic and biotic factors. The most prominent of the latter are the stomates which, in closing, limit the amount of energy that can be dissipated by transpiration, and consequently cause leaf temperatures to increase (Raschke, 1960). These observations led Tanner (1963) to postulate that the surface temperature of the leaf may be used to assess the water status of the canopy, i.e. the degree of water stress. For the majority of the present study period leaf canopy temperatures of control trees were higher than those from mulched trees, which implies that mulched trees experienced conditions of reduced water stress. Interestingly, for two consecutive seasons there was a rapid rise in the canopy temperature (relative to air temperature) approximately 60 to 90 d after full bloom. The rapid increase in temperature during this period coincided with fruit drop, which is apparently a stress related phenomenon (Whiley *et al.*, 1988). Furthermore, the time at which small fruit first became evident (and presumably when seed coat abortion first took place) occurred during this critical period, and this provides further evidence that the 'Hass' small fruit syndrome is exacerbated by tree stress.

Light stress results not from high light *per se*, but rather from a build up of excess photochemical energy which cannot be utilized photochemically or quenched non-photochemically (Björkman and Schäfer, 1989; Krause *et al.*, 1995). An excess of light can arise when the ratio of photon flux density (PFD) relative to photosynthesis is high. This ratio increases either through an increase in the PFD or, due to a reduction in photosynthesis at constant PFD, e.g. in response to water stress (Demmig-Adams and Adams, 1992). A reduction in the yield of chlorophyll fluorescence, indicative of thermal dissipation, has been reported for several plant species experiencing water stress under natural conditions (Adams *et al.*, 1987; Björkman and Schäfer, 1989). Demmig-Adams *et al.* (1988) showed that leaves of *Nerium oleander* exhibited reduced photochemical efficiency when water was witheld from the plants and the PFD kept constant. In the present study, measurement of photosynthetic energy conversion by avocado leaves revealed there to be marked differences between mulched and non-mulched trees. Mulched trees consistently showed a reduction in chlorophyll fluorescence during the heat of the day. Photoinhibition has been related to a loss of productivity in some tree species (Ögren and Sjöström, 1990), and might explain the observed differences in productivity between mulched and non-mulched trees.

7.1.4 Mulching: A strategy to increase 'Hass' fruit size

As previously mentioned, 'Hass' is sensitive to abiotic/biotic pressure and the small fruit syndrome appears to be aggravated by tree stress (Köhne, 1992; Whiley and Schaffer, 1994). The current study evaluated mulching as a practical management strategy to alleviate tree stress and increase mean fruit size. Avocado trees, being of rainforest origin, are natural "litter-feeder" adapted to growing in soils with a high humic content (Broadbent and Baker, 1974a; 1974b).

The proposed interactive effects of mulching on avocado tree physiology are summarised in Figure 7.3. Avocado trees produce the majority of their roots in the top 20 to 40 cm of soil (Whiley, 1994), a region of low hydraulic conductivity. Water in this zone is subject to evaporative forces, drainage and is also bound by matric forces operating within and between soil particles, factors that compete with roots for available

water (Boyer, 1985; Passioura, 1988). Mulching improves soil-water relations, the availability of nutrients and other resources, such that trees are less likely to experience stress.

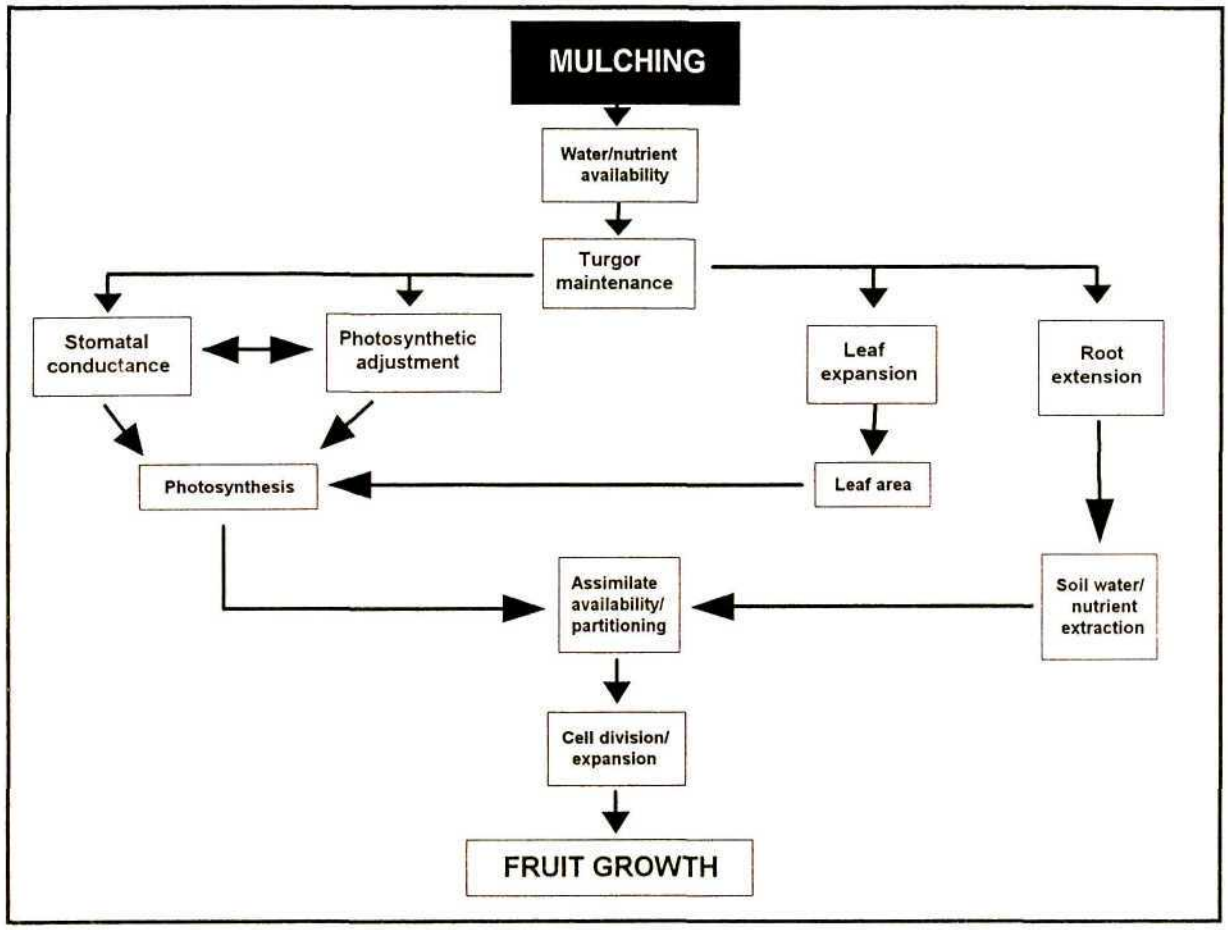

Figure 7.3

Mode of action of mulching in control of turgor maintenance, water/nutrient extraction and assimilate partitioning during avocado fruit growth and development.

The present study illustrated that changes in the CK:ABA ratio is the trigger that directly elicits reduced growth and it is a low resource environment that initiates the stress response system (Chapin, 1991). With respect to the small fruit syndrome, the following cascade of events might be expected to constitute the stress signal-response mechanism as a result of low resource availability; (1) reduced root activity; (2) evapotranspiration in excess of water (nutrient) uptake; (3) elevated xylem ABA; (4) reduced stomatal conductance; (5) down regulation of photosynthesis; (6) availability

of photoassimilate declines; (7) rise in fruit respiration; (8) enhanced ABA synthesis *in situ* in developing fruit; (9) down regulation of HMGR and CK biosynthesis; and (10) retarded cell division cycle activity. Although this sequence of events is illustrated as a consequence of poor root health, it is accepted that all abiotic/biotic factors that decrease the endogenous CK:ABA ratio will impact on developing structures similarly.

Anatomical investigations in this study revealed that the limiting factor in avocado fruit development is cell division. The chemical signal involved is in all probability a change in CK balance. It is proposed that the seed is the most important source of CKs in early fruit ontogeny although roots may assume significance as the source of CKs during the later stages of fruit development, provided roots are actively growing. When availability of resources for growth and development is limited, water movement through the vascular system is reduced. Consequently, availability of root-derived CKs to developing fruit is reduced. Mulched soils contain a large amount of available water at field capacity (Gregoriou and Rajkumar, 1984) and the increased availability of water, coupled with improved root health should, together with adequate assimilate/nutrient supply, sustain fruit growth and development and reduce the incidence of small fruit by eliminating the confounding effects of either stress-induced ABA accumulation and/or feedback regulation of photosynthesis.

7.1.5 Impact of mulching on fruit growth and phenophysiology

Mean fruit mass was significantly increased in mulched trees, and this was achieved in spite of a greater number of fruit per tree. This response is particularly significant since problems of fruit size principally arise in trees with heavy crops, as resources available for fruit growth have to be allocated to more sinks (Lahav and Kalmer, 1977). Mulching appeared to have a greater effect on yield during an "off" year. Assuming that assimilate supply to growing fruits in a season of low yield is limiting, any improvement in resource accumulation and distribution to developing fruits as a result of mulching should considerably enhance fruit productivity. Yields were shown to be closely related to bark carbohydrate levels. Both soluble (immediately available) and insoluble (storage) carbohydrate content of the bark samples were determined. Starch is

considered to be the only important storage carbohydrate in avocado trees and the main repository is the trunk and major scaffold branches (Scholefield *et al.*, 1985). Consequently direct determination of trunk starch levels provided an accurate measure of seasonal fluctuations in storage reserves. Soluble carbohydrates, e.g. glucose and sucrose, are mobile and constitute a pool for immediate use by the trees (Cull, 1989).

Trees from both treatments showed a marked biennial bearing cycle, with alternate heavy and relatively light crops. It appears that alternate bearing in 'Hass' avocado is closely related to storage carbohydrate levels in the tree, as also observed by Whiley *et al.* (1996b) for 'Hass'. Scholefield *et al.* (1985) showed similar trends in 'Fuerte' avocados in temperate southern Australia. Whiley and Schaffer (1994) noted that avocado trees growing in mild Mediterranean or generally cool and dry climates, store greater amounts of reserve carbohydrates. This is borne out by Scholefield's (1985) studies on 'Fuerte' in southern Australia, where trunk starch concentrations reached ca. 18% in winter and fell to ca. 3% by late summer. This study also found that high starch levels resulted in high yields, followed by low accumulation and low yields. However, the position is more complicated, and Whiley *et al.* (1996a) note the much lower trunk starch cycle flux in the summer rainfall subtropics of Queensland, with peak levels in 'Hass' of ca. 6 to 8% in winter. The current study found winter peaks in the 6 to 9% range, similar to Queensland. Unlike bark starch reserves, there was no obvious correlation between sugar content and yield, i.e. it appears that immediately available carbohydrates had little effect on the biennial bearing nature of 'Hass' trees in this study.

Whiley *et al.* (1996a), in a study of delayed harvest of 'Hass' in relation to starch cycling, noted that peak starch concentrations were reduced by heavy fruiting, and that pre-flowering peaks in shoots or trunks were directly correlated to the next seasons yield. However, this relationship did not occur in the early maturing 'Fuerte' cultivar (Whiley *et al.*, 1996b), which is more vigorous. They also point out that crop size can be strongly affected by climatic aberrations and poor flowering. They conclude that the relative importance of stored as compared to current carbohydrate differs in different

climates, with trees in the summer rainfall subtropics being relatively more dependant on current photosynthate than trees in cool, dry areas, which appear to be more dependant on stored carbohydrate.

Differences in levels of trunk bark starch reserves were also evident between the mulch and control treatments. The amplitude of bark starch cycling was greater in mulched trees throughout the sampling period, i.e. starch accumulation and depletion was greater in all three seasons. Furthermore, this enhanced ability by mulched trees to accumulate storage carbohydrate was achieved in spite of a larger crop load on these trees. Since accumulation of storage carbohydrate was consistently greater in mulched trees, this implies that these trees are better equipped to recover from the critical energy expensive periods of flowering, fruit set and early fruit growth. Trees under these improved edaphic conditions can therefore support a greater crop load for several successive seasons, without adversely affecting levels of storage carbohydrate. A study by Kaiser and Wolstenholme (1994) on late-hanging of 'Hass' showed that avocado trees in a cool, mesic area in KwaZulu-Natal have an ability to recover from periods of high energy demand, but also stressed the importance of careful management under these conditions. Mulching possibly enhanced the ability of the trees to recover from periods of extreme starch depletion.

The level of assimilate available for growth and development of fruits is partly dependent on the activity of the vegetative tree component (Hansen, 1989). Since intensity of shoot growth was greater on the mulch treatment, it is possible that the increased fruit size on mulched trees could be related to a greater availability of photoassimilate produced by the leaves. An interesting feature was that a second shoot flush period was not pronounced at the trial site. This unusual shoot phenology at Everdon Estates was also noted by Kaizer and Wolstenholme (1994), and is contrary to observations by Whiley *et al.* (1988), who stated that a strong summer flush is necessary to provide the "fuel" for fruit growth and the following seasons flowering and fruit set period. A possible reason for this is that the cool mesic conditions of the Natal midlands are not ideal for avocado production, bearing in mind the avocado's highland

tropical to sub-tropical origin, and so the trees have a different phenotypic expression. A second shoot flush may not be necessary, for energy requirements are less demanding under these cooler conditions as respiration rates are lower. A contrary view might be that this research has highlighted a problem with orchard management at Everdon in heavy cropping years, viz. that insufficient summer flushing occurs. Since avocado leaf longevity is short (up to 10-12 months, according to Whiley and Schaffer (1994)), the trees would have gone into autumn and winter with predominantly aged and less efficient spring flush leaves. This would have aggravated any tendency towards alternate bearing. Active encouragement of the summer flush by nitrogen fertilization would have resulted in the important leaf renewal, accompanied by greater carbohydrate build-up, that predisposes the tree to more regular bearing.

7.2 CONCLUSIONS AND FUTURE PROSPECTS

This study has demonstrated that 'Hass' fruit which start growing at the same time tend to produce variable sized fruit at maturity, and confirmed that a healthy, functional seed coat is essential for fruit growth. However, we still do not know whether phenotypically small fruit are the consequence of early seed coat senescence or whether abortion of the seed coat is a response to diminished cell division cycle activity and/or sink strength. This study also demonstrated that an increase in fruit size was associated with a rise in the CK:ABA ratio and that this ratio impacts on seed coat senescence, HMGR activity, solute allocation, cell-to-cell communication and plasmodesmata structure of developing avocado fruit. Although it is recognised that concentrations of these hormones influence fruit growth and development, it is still unclear where the site(s) of synthesis are, i.e. whether they are synthesised *in situ* or imported into the fruit.

There is a need to positively identify the primary stress stimuli (e.g. inadequate water and/or nutrient supply, high light and high temperatures) that contribute to production of large numbers of phenotypically small fruit. Although it is appreciated that yield and fruit size are under the control of many interacting factors, and crop failures can be caused by climatic extremes and poor flowering, *inter alia*, this study has shown that

mulching, through creating a more mesic root environment, has the potential to substantially increase 'Hass' avocado productivity. It is notable that these results were obtained in a relatively low stress (more mesic) environment and in a well managed orchard. Logically, one would expect greater increases in more stressful environments, provided that fruit number (crop load) is in balance with the ability of the canopy to supply photosynthate. It is also noteworthy that this study was conducted predominantly during a drought period, so there is a need to test mulches under a variety of conditions before fully assessing their practical value.

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APPENDIX

PUBLISHED ARTICLES

EFFECT OF COMPOSTED PINEBARK MULCHING ON *Persea americana* MILL. CV. HASS FRUIT GROWTH AND YIELD IN A COOL SUBTROPICAL ENVIRONMENT

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KEY WORDS Mulching, avocado, root activity, fruit growth, fruit size

ABSTRACT

The 'Hass' cultivar is important to the South African avocado industry as it is late maturing and is preferred by overseas consumers. However, it produces a variable percentage of undersized fruit, which cannot be exported. Mulching was investigated as a possible method of increasing fruit size through improved root activity and reduced tree stress. In a field trial at Everdon Estate, Howick, root growth was increased throughout two seasons by the application of a coarse composted pinebark mulch beneath the tree canopy. All measured fruits, in both the length and diameter axes, fitted a Gompertz growth curve. Rate of fruit growth and total growth were significantly greater on the mulch treatment. Over two seasons, the mulch treatment resulted in a significant 11.8% increase in mean fruit mass, in spite of 16.7% more fruits per tree. The combined effect was a 30.4% increase in yield.

INTRODUCTION

Since the South African avocado industry is largely export orientated, cultivars such as 'Hass', which are preferred by overseas consumers, are important to the industry (Cutting, 1993). This cultivar is also late maturing and is therefore useful for extending the harvesting season. Unfortunately, 'Hass' trees have a tendency to bear large numbers of undersize fruit, and fruit size is on average much smaller than in other major commercial varieties such as 'Fuerte', 'Pinkerton', 'Edranol' and 'Ryan'. Up to 50% of the 'Hass' crop may be undersize (less than 200g or counts of more than 20 fruits per standard 4kg export carton) in any particular season (Köhne, 1992), and in 1994 this problem was estimated to have cost the South African industry R30 million in lost revenue.

The phenomenon is not restricted to diseased and/or unhealthy trees. Even healthy 'Hass' trees produce a significant proportion (5 to 25%) of small fruit (Kremer-Köhne & Köhne, 1995), which are unsuitable for export. The small fruit problem is thus physiological and also occurs in trees without pathogen involvement. The problem is exacerbated by the onset of symptoms of pedicel ring-neck and early seed coat senescence, pollen incompatibility and poor cultural practices. The problem becomes more pronounced with tree age (Cutting, 1993) and is particularly noticeable in orchards situated in warmer and/or drier climates (Hilton-Barber, 1992; Whiley & Schaffer, 1994). Both stress and ageing are therefore major determinants of 'Hass' avocado fruit size.

Fruit size is fundamentally determined by genome, so the long term and ultimate approach is to discover or breed new large-fruited, black-skinned cultivars. Unfortunately, breeding and testing new cultivars is time-consuming and does not resolve the problem immediately. There is thus a requirement for an interim solution.[1] We hypothesized that the application of a mulch could be a practical short term solution to promote root health, ameliorate stressful growing conditions and reduce the extent of the problem. This strategy is based on the avocado evolving in a highland tropical to subtropical environment, and adaptation to soils with a litter layer and a high humic content. Reinforced mulching (in addition to natural litter fall) simulates rainforest floor conditions, thus providing roots with improved and more natural edaphic growing conditions. Improved root growth should impact positively on a cascade of physiological events promoting cell division in fruits, and prolonging seed coat viability. It is well known that premature seed coat abortion contributes to smaller fruit size (Blumenfeld & Gazit, 1974: Steyn, Robbertse & Smith, 1993).

Any layer of plant material that occurs naturally or is applied to the soil can be considered a mulch (Turney & Menge, 1994). The benefits derived from mulching include, increased water and nutrient availability (Gregoriou & Rajkumar, 1984), improved soil structure and porosity (Gallardo-Laro & Nogales, 1987) and a narrowing in the diurnal soil temperature range (Gregoriou & Rajkumar, 1984). In addition, mulching creates a suppressive environment for *Phytophthora cinnamomi* thus reducing the impact of this phytopathogen (Turney & Menge, 1994).

The objective of this trial was to evaluate the effect of a composted pinebark mulch on 'Hass' fruit growth and yield, over two seasons, in a cool, humid, high rainfall environment in the KwaZulu-Natal midlands.

MATERIALS AND METHODS

Treatment

This study was conducted on six year old (in 1993) 'Hass' trees on clonal 'Duke 7' rootstock, on Everdon Estate, near Howick, in the Kwazulu-Natal midlands (30°16'E and 29°27'S). The orchard is situated in Phillips' Bioclimatic region 3, which is characterised by cool mesic conditions, typical of a "mist-belt" climate. Mean maximum and minimum temperatures range from 26.1 to 15.0C in January and 19.4 to 6.7C in July; mean annual rainfall is 1052mm and altitude is ca. 1082m. Orchards receive standard cultural treatment, including micro-jet irrigation based on tensiometers, and management efficiency is excellent. The soil is an oxisol of the Hutton form, dystrophic, with a high clay content of ca. 50%. A total of 1.5m^3 of coarse composted pinebark (Gromed® coarse potting mix) was applied in February 1993 under the canopy of six trees to a depth of approximately 15cm, and these trees were compared with six adjacent unmulched trees.

Data collection

The data collection period for phenological events spanned from May 1993 through to October 1995. Root flushes were monitored by visually estimating the area covered by white healthy feeder roots under a newspaper mulch (Whiley, Saranah, Cull & Pegg, 1988) (with an approximate area equal to 1250cm^2). The newspaper mulch was placed 1m from the micro-jet nozzle on the south-west side of the tree, so as to avoid direct sunlight. Three readings per treatment were taken at the end of each month. Visual estimates of root flushing were performed using a rating of 0 to 10. Kaiser & Wolstenholme's (1994), groupings of "poor", "medium" and "good" were chosen, viz. 0 to 2, 3 to 4, and ≥5 respectively.

For the purpose of measuring fruit growth, 40 fruits per tree were tagged at the beginning of each season, when all fruit were approximately 10mm in length. Subsequent length and diameter measurements, using digital calipers, were taken at regular intervals throughout the growing season. The number of fruits measured per tree gradually declined through the growing season because of fruit abscission, so that at harvest an average of 15 fruits per tree were measured. Using GENSTAT (1994), fruit measurements were fitted to a Gompertz curve which has the following mathematical equation;

$$y = C \exp \{-\exp [-B (x-M)]\} + A$$

where; x = time from fruit set (days)

y = fruit measurement (mm)

A = starting value

B = growth rate

C = total growth

M = point of inflection

At the end of each season the trials were harvested, and fruit size distributions were recorded for each tree. Fruit size was determined gravimetrically and classified according to the number of fruit per standard 4kg export carton. Fruits were graded as follows: Count 10, 366 to 450g; count 12, 306 to 365g; count 14, 266 to 305g; count 16, 236 to 265g; count 18, 211 to 235g; count 20, 191 to 210g; count 22, 171 to 190g; count 24, 156 to 170g; count 26, 146 to 155g; and factory grade, <146g. Total tree yields were calculated by adding the product of the number of fruit per count size and the class centre of all the count sizes.

RESULTS AND DISCUSSION

Root flushing

Root activity in the mulch treatment was always more intense than in the control. In the mulch treatment root growth fell into the "medium" category for the majority of the season, whereas in the control mainly "poor" root growth was recorded. For a substantial part of the season (December 1993 through to April 1994, and December 1994 through to March 1995) root activity was allocated a "good" rating in the mulched treatment (Fig. 1). Root flushing periods followed a similar pattern, but in the mulch treatment were two to four weeks earlier and continued for a longer period (Fig. 1).

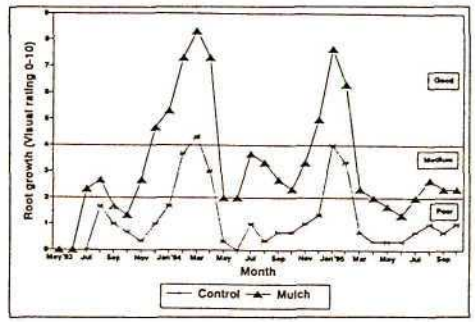


FIGURE 1. Root flushes as determined by a visual rating where there is no root growth for a rating of 0 and extensive root growth for a rating of 10.

Avocado trees are adapted to growing in soils with a thick litter layer and a high organic content, and avocado roots, being "litter feeders" with a high oxygen requirement (Moore-Gordon, Wolstenholme & Levin 1995), thrive under such edaphic conditions. Although healthy trees shed large numbers of leaves (which are relatively short-lived for an evergreen tree), application of the composted pinebark mulch reinforced rain-forest floor conditions, resulting in the more intense and prolonged surface feeder root activity. The rhizotron studies of Whiley (1994) are more representative of root activity at depth, and have indicated the potential for new root growth through winter in deep, cool, high organic matter krasnozem soils in the high rainfall areas of S.E. Queensland. Whether such root activity, at depths of up to 1m, occurs under the climate and edaphic environment of Everdon is unknown, although the soils are substantially similar.

Fruit growth

Each fruit measured had a regression coefficient (R^2) value greater than 0.99 indicating an extremely good fit. This was the case for both length-ways and diameter measurements. Results of an analysis of variance on each parameter of the Gompertz curve equation are summarized in Tables 1 and 2. Figures 2 and 3 give a graphic presentation of the resultant growth curves.

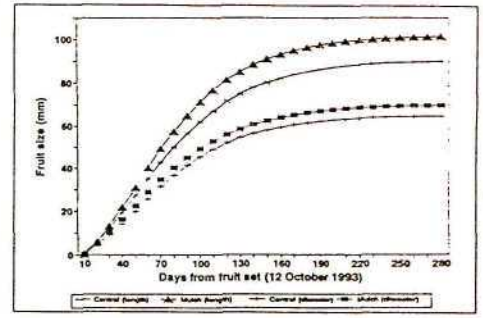


FIGURE 2. 'Hass' fruit growth curves for the 1993/1994 season. Regression line for the mulch treatment (length axis) is represented by y = 110.0 exp {-exp [-0.02297 (x - 51.61)]} - 8.16: the control (length axis) by y = 95.84 exp {-exp [-0.02252 (x - 53.86)]} - 5.20; the mulch treatment (diameter axis) by y = 77.08 exp {-exp [-0.02222 (x - 48.29)]} - 7.07; and the control (diameter axis) by y = 67.53 exp {-exp [-0.02336 (x - 54.13)]} - 2.66. (Growth curves were constructed from a total of 82 fruits on the mulch treatment and 92 fruits on the control).

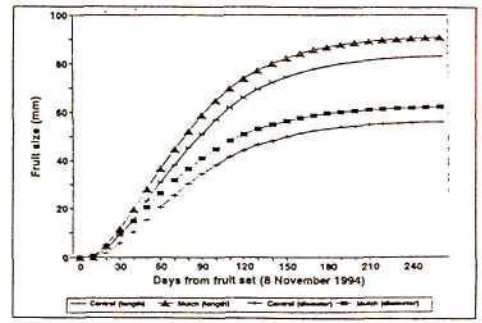


FIGURE 3. 'Hass' fruit growth curves for the 1994/1995 season. Regression line for the mulch treatment (length axis) is represented by y = 98.92 exp {-exp [-0.02281 (x - 49.44)]} - 7.34; the control (length axis) by y = 90.14 exp {-exp [-0.02316 (x - 55.23)]} - 6.21; the mulch treatment (diameter axis) by y = 68.34 exp {-exp [-0.02303 (x - 48.32)]} - 5.72; and the control (diameter axis) by y = 61.57 exp {-exp [-0.02279 (x - 54.73)]} - 5.03. (Growth curves were constructed from a total of 43 fruits on the mulch treatment and 39 fruits on the control).

TABLE 1. Summary of mean values for each growth parameter (where *A* is the starting value, *B* is the growth rate, *C* is the total growth and *M* is the point of inflection) of the Gompertz curve for length measurements on 'Hass' fruit. Values are expressed in mm. *Using an F-test, NS denotes parameters are not significantly* different; and ★★ denotes parameters are significantly different at the 1% significance level.

Parameter	Control	Mulch	Significance
1993/1994			
A	-7.07	-8.16	NS
B	0.02252	0.02297	NS
C	95.84	110.10	★★
M	48.29	51.61	NS
1994/1995			
A	-6.21	-7.34	NS
B	0.02316	0.02281	NS
C	90.14	98.92	★★
M	55.23	49.44	★★

TABLE 2 Summary of mean values for each growth parameter (where *A* is the starting value, *B* is the growth rate, *C* is the total growth and *M* is the point of inflection) of the Gompertz curve for diameter measurements on 'Hass' fruit. Values are expressed in mm.

Parameter	Control	Mulch	Significance
1993/1994			
A	-2.66	-7.07	★
B	0.02336	0.02222	NS
C	67.53	77.08	★★
M	54.13	48.29	★
1994/1995			
A	-5.03	-5.72	NS
B	0.02279	0.02303	NS
C	61.57	68.34	★★
M	54.73	48.32	★★

Using an F-test, NS denotes parameters are not significantly different; ★ denotes parameters are significantly different at the 5% significance level; and ★★ denotes parameters are significantly different at the 1% significance level.

It is possible that those fruit which set early may monopolize available resources at the expense of smaller fruits. Hence for a valid comparison of fruit growth dynamics on different treatments it is important that all fruits are initially the same size. For the first year of study, the starting value (*A*) for the length axis was not significantly different between treatments (Table 1), indicating that all fruit were tagged at approximately the same length. In the diameter axis, the starting value (*A*) was significantly greater ($P \leq 0.05$) for control fruit for the 1993/1994 season (Table 2), so if anything, control fruit may initially have been slightly stronger sinks. Fruit growth measurements during the second season confirm that starting values (*A*) were not significantly different between treatments; this was the case for both length and diameter measurements (Tables 1 and 2). This implies that differences in fruit growth rates between the mulch and control treatments could be attributed to factors during the fruit growth period, after the fruitlets had been tagged.

Total fruit growth (*C*) was significantly different ($P \leq 0.01$) between treatments for the first season's measurements. At the time of harvest, approximately 284 days after fruit set, fruit from the pinebark mulch treatment had grown an average of 14.3 ± 2.3mm more along the length axis than fruit from the control. Similarly, fruit expansion in the diameter axis was 9.6 ± 3.3mm more in the mulch treatment during the same period (Fig. 2). Fruit growth measurements for the 1994/1995 season showed similar trends: at the time of harvest, 255 days after fruit set, fruit from the pinebark treatment had grown an average of 8.8 ± 2.4mm and 6.8 ± 2.7mm more along the length and diameter axes, respectively (Fig. 3).

These results show that mulching with pinebark led to an overall increase in fruit growth and ultimately increased average final fruit size in both seasons. Furthermore, the results suggest that increased average fruit mass at harvest in the mulch treatment was not solely attributed to increased growth in one direction, but rather increased growth in both major axes. The results support work done by Zilkah & Klein (1987) in Israel, who showed that 'Hass' avocado fruits grow proportionately in all directions from the fruit shape established at fruit set. This result does not preclude the well known observation that fruit shape of the same cultivar can vary in different areas, i.e. that a genetic x environment interaction does exist.

The cause of differences in the growth curves of the two fruit populations is not related to the parameter measuring growth rate (*B*) as there were no significant differences between treatments for this parameter in both axes (Tables 1 and 2). Instead, differences in average fruit size at harvest may be sought in the parameter representing the point of inflection (*M*), which occurred later in the mulch treatment (Tables 1 and 2). Since growth is exponential up to the point of inflection, fruit in the mulch treatment grew exponentially for a longer period. This implies that fruit in the mulch treatment grew faster initially, until a point was reached when difference in fruit length between the treatments remained approximately constant.

Yield and fruit size distribution

The control trees showed a typical fruit size distribution for the 'Hass' cultivar with many fruit in the count size range of 22 to 26 (small fruits), and a high proportion of factory grade avocados (Figs 4 and 5). Mulching with pinebark had the effect of shifting the overall count size distribution in favour of large fruit, i.e. the mulch treatment yielded fewer small fruit and more large fruit (Figs 4 and 5).

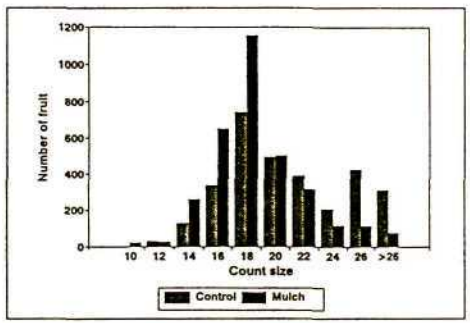
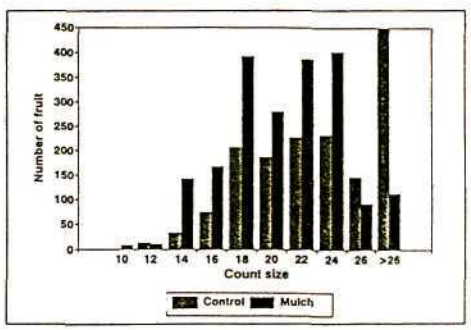


FIGURE 4. Overall 'Hass' fruit size distribution at harvest for the 1993/1994 season.



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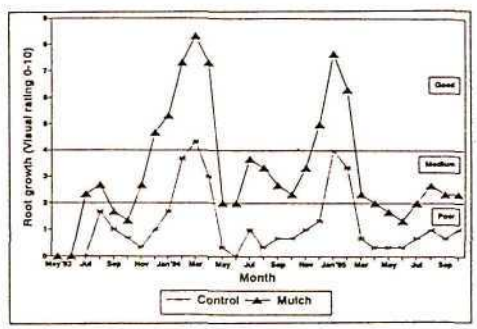

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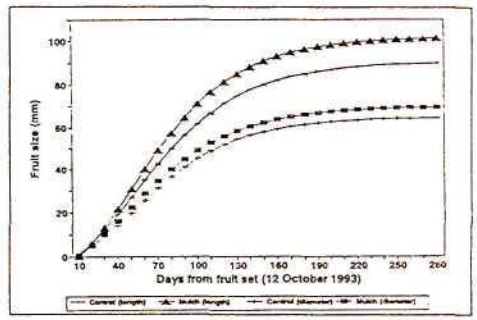

FIGURE 2. 'Hass' fruit growth curves for the 1993/1994 season. Regression line for the mulch treatment (length axis) is represented by $y = 110.0 \exp\{-\exp[-0.02297 (x - 51.61)]\} - 8.16$; the control (length axis) by $y = 95.84 \exp\{-\exp[-0.02252 (x - 53.86)]\} - 5.20$; the mulch treatment (diameter axis) by $y = 77.08 \exp\{-\exp[-0.02222 (x - 48.29)]\} - 7.07$; and the control (diameter axis) by $y = 67.53 \exp\{-\exp[-0.02336 (x - 54.13)]\} - 2.66$. (Growth curves were constructed from a total of 82 fruits on the mulch treatment and 92 fruits on the control).

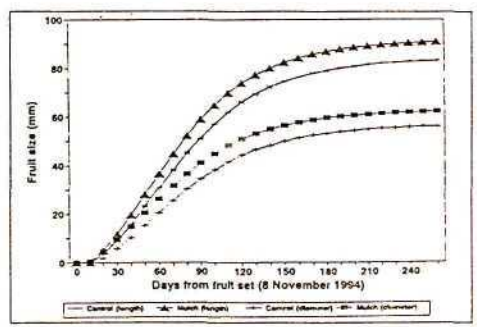

FIGURE 3. 'Hass' fruit growth curves for the 1994/1995 season. Regression line for the mulch treatment (length axis) is represented by $y = 98.92 \exp\{-\exp[-0.02281 (x - 49.44)]\} - 7.34$; the control (length axis) by $y = 90.14 \exp\{-\exp[-0.02316 (x - 55.23)]\} - 6.21$; the mulch treatment (diameter axis) by $y = 68.34 \exp\{-\exp[-0.02303 (x - 48.32)]\} - 5.72$; and the control (diameter axis) by $y = 61.57 \exp\{-\exp[-0.02279 (x - 54.73)]\} - 5.03$. (Growth curves were constructed from a total of 43 fruits on the mulch treatment and 39 fruits on the control).

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Metabolic Control of Avocado Fruit Growth[1]

Isoprenoid Growth Regulators and the Reaction Catalyzed by 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase

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The effect of isoprenoid growth regulators on avocado (*Persea americana* Mill. cv Hass) fruit growth and mesocarp 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity was investigated during the course of fruit ontogeny. Both normal and small-fruit phenotypes were used to probe the interaction between the end products of isoprenoid biosynthesis and the activity of HMGR in the metabolic control of avocado fruit growth. Kinetic analysis of the changes in both cell number and size revealed that growth was limited by cell number in phenotypically small fruit. In small fruit a 70% reduction in microsomal HMGR activity was associated with an increased mesocarp abscisic acid (ABA) concentration. Application of mevastatin, a competitive inhibitor of HMGR, reduced the growth of normal fruit and increased mesocarp ABA concentration. These effects were reversed by co-treatment of fruit with mevalonic acid lactone, isopentenyladenine, or *N*-(2-chloro-4-pyridyl)-*N*-phenylurea, but were not significantly affected by either gibberellic acid or stigmasterol. However, stigmasterol appeared to partially restore fruit growth when co-injected with mevastatin in either phase II or III of fruit growth. In vivo application of ABA reduced fruit growth and mesocarp HMGR activity and accelerated fruit abscission, effects that were reversed by co-treatment with isopentenyladenine. Together, these observations indicate that ABA accumulation down-regulates mesocarp HMGR activity and fruit growth, and that in situ cytokinin biosynthesis modulates these effects during phase I of fruit ontogeny, whereas both cytokinins and sterols seem to perform this function during the later phases.

HMGR catalyzes the irreversible conversion of HMG-CoA to MVA, the committed step in isoprenoid biosynthesis in all eukaryotic organisms (Goldstein and Brown, 1990). For plant growth and development, synthesis of isoprenoids is fundamental because the pathway supplies compounds that are essential for full morphogenic expression. This class of compounds is of structural significance, e.g. carotenoids and the side chain of chlorophylls and plastoquinone for photosynthesis, the side chain of ubiquinone for respiration, sterols for membrane structure, and phytoalexins for defense. The pathway also supplies several regulatory molecules, including ABA, brassinosteroids, GAs, and the side chain of CKs, which contribute to the control of both temporal and spatial events during higher plant ontogeny. Despite this, surprisingly little information is available concerning regulation of isoprenoid biosynthesis in plants and plant parts, particularly developing fruit. Whereas controversy still surrounds the subcellular site of MVA metabolism (Campos and Boronat, 1995; Chappell, 1995a, 1995b), it is generally agreed that reduction of HMG-CoA is potentially a major point of regulation of isoprenoid biosynthesis in plants (Bach, 1987; Gray, 1987; Gondet et al., 1992; Moore and Oishi, 1994; Chappell et al., 1995).

Using tomato as a model system, Narita and Gruissem (1989) demonstrated that HMGR expression and activity are required during early fruit development. Furthermore, these authors showed that in vivo inhibition of HMGR during early fruit development disrupted the process, whereas inhibition during the later expansion stage had no significant effect. Since ripening was apparently unaffected, it was concluded that inhibition of HMGR reduced the MVA pool required for phytosterol biosynthesis, that phytosterols were produced during early fruit development, and that fruit expansion and ripening were independent of HMGR activity.

Why phytosterols? In an attempt to address this question, Gillaspy et al. (1993) produced a comprehensive overview of the potential regulatory networks operating in the metabolic control of fruit development, including cell division, expansion, and differentiation. Although the arguments did little to cement a direct role for phytosterols in metabolic control of fruit growth and development, it was suggested that intermediates in isoprenoid biosynthesis (e.g. farnesyl diphosphate and geranylgeranyl diphosphate) could be important components in this program. Thus, it was concluded that cell proliferation during fruit ontogeny may be an ideal system with which to dissect the regulatory interactions between synthesis of isoprenoids and signal transduction pathways that lead to differentiation.

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Abbreviations: AMO 1618, 2′-isopropyl-4′-(trimethylammonium chloride)-5′ methyl phenyl piperidine-1′-carboxylate; ANOVA, analysis of variance; CK, cytokinin; CPPU, *N*-(2-chloro-4-pyridyl)-*N*-phenylurea; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; iP, 6-(γ,γ-dimethylallylamino)-purine; MVA, mevalonic acid; MVAL, mevalonic acid lactone.

Development of avocado (*Persea americana* Mill.) fruit, like that of most fleshy fruits, follows a single sigmoid curve with a lag period of approximately 10 weeks (phase I) followed by a growth phase of about 30 weeks (phase II), dependent on cultivar and environment, and finally a mature phase (phase III) during which growth slows (Valmayor, 1967). Unlike most fruits, cell division in avocado mesocarp tissue is not restricted to phase I, but proceeds throughout ontogeny (Schroeder, 1953), albeit at a slower rate during the latter stages. Thus, avocado presents an ideal system with which to study the role of isoprenoids in the metabolic control of fruit growth from fruit set to maturity.

The avocado cv Hass produces a large number of phenotypically small fruit. Results from our recent investigations show that the incidence of the small-fruit variant correlates with sensitivity of cv Hass trees to abiotic/biotic pressure, and that environmental perturbations affect crop yield (i.e. fruit quality and quantity) seemingly through modulation of the CK:ABA ratio (C.S. Moore-Gordon, A.K. Cowan, and B.N. Wolstenholme, unpublished data). In short, we propose that a decline in the CK:ABA ratio lessens sink strength of developing organs by influencing HMGR and cell division cycle activity to reduce final fruit size. This hypothesis is supported by evidence that shows that ABA retards cell division cycle activity (Müller et al., 1994) and inhibits HMGR activity (Russell and Davidson, 1982; Moore and Oishi, 1994) in several higher plant tissue systems.

To examine the interrelationship between HMGR, isoprenoid growth regulators, and the small-fruit phenotype, we used mevastatin to specifically inhibit in vivo HMGR activity in normal avocado fruit during phases I, II, and III of the developmental program. Supplementation with products of the isoprenoid biosynthetic pathway and similarly derived plant hormones revealed that CKs were the most important limiting factors during Hass avocado fruit growth and development.

MATERIALS AND METHODS

Isotopes, Isoprenoid Compounds, and Inhibitors

DL-[3-^{14}C]HMG-CoA (58.0 mCi/mmol) was purchased from Amersham. Mevastatin (compactin), MVAL, ABA, GA_3, iP, CPPU, stigmasterol, and cholesterol were purchased from Sigma. AMO 1618 was purchased from Calbiochem.

Plant Material and Application of Chemicals

Experiments were conducted during the 1994–1995 and 1995–1996 seasons using 7-year-old trees of avocado (*Persea americana* Mill. cv Hass) propagated on clonal Duke 7 rootstocks in an orchard on the Everdon Estate in the KwaZulu-Natal midlands, South Africa.

For application of chemicals, compounds of interest were formulated in Tween 20:acetone:water (1:1:8, v/v) to a final concentration of 1 mg mL^{-1}, and 20 μL of each or combinations thereof were injected into the pedicels of individual fruits (eight fruits per treatment) using a 1-μL syringe (7105, Hamilton Co., Reno, NV) 55 d (phase I), 92 d (phase II), and 210 d (phase III) after full bloom, unless stated otherwise. Control fruit were treated with and without Tween 20:acetone:water (1:1:8, v/v). Following injection, the wound was covered with silicone grease, and fruit growth was monitored by measuring the increase in both the fruit length and diameter using digital calipers (Mitutoyo-500, Mitutoyo Corp., Tokyo, Japan) at the intervals specified in "Results." Since identical trends were observed for both the fruit length and fruit diameter, only results for percentage increase in fruit length are shown.

Estimation of Cell Size and Cell Number

Whole fruits (during phase I) and three 5-mm^3 tissue samples (during phases II and III), excised from three distinct zones (viz. a zone including the endocarp and seed coat, a zone including the exocarp, and a zone from mesocarp tissue midway between the exo- and endocarp, across the equatorial region of each of the three randomly selected fruit), were fixed in formalin:acetic acid:95% ethanol:water (2:1:10:7, v/v), dehydrated in a graded ethanol/*tert*-butanol series, and embedded in wax. Thin sections were prepared using a rotary microtome (Reichert, Vienna, Austria), dewaxed and stained with Safranin and Fast Green (Merck, Darmstadt, Germany), and examined using a light microscope (BH-2, Olympus). The number of cells present in a representative area of 90,000 μm^2 was determined. For cells at the borders, if greater than 50% of cell area was within the designated sample area, the cell was regarded as part of the sample. The number of cells per 90,000 μm^2 was used to estimate apparent cell size. To convert the number of cells in the sample area to the number of cells across the fruit, the following expression was used: $n = d\sqrt{x}$, where n is the number of cells across the fruit, d is the fruit diameter in millimeters at the equatorial region, and x is the number of cells in the sample area.

HMGR Assay

Freeze-dried mesocarp tissue was homogenized in an ice-cold 100 mM potassium phosphate buffer (pH 7.0) containing 4 mM $MgCl_2$ and 5 mM DTT, and the homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged at 10,000*g* for 15 min at 2°C. Microsomes were prepared by adding 8 mM $CaCl_2$ to the 10,000*g* supernatant and the membranes sedimented at 27,000*g* for 15 min at 2°C, as described by Cinti et al. (1972). The pellet was washed in 150 mM KCl and recentrifuged at 27,000*g* for 15 min at 2°C, and the microsomes were resuspended in a small volume of 100 mM potassium phosphate buffer (pH 7.0) containing 50 mM DTT. Approximately 100 μg of the microsomal protein (Bradford, 1976) was incubated in a total volume of 300 μL containing 5 mM NADPH and 1.72 nmol [3-^{14}C]HMG-CoA.

Reactions were initiated by addition of the substrate and allowed to proceed for 45 min at 30°C. At the end of the incubation period, reactions were terminated by the addition of 2 μL of MVAL (100 mg mL^{-1}) and 20 μL of HCl (6

N) followed by vortexing, and the MVA was lactonized at room temperature for 15 min. Particulate material was removed by centrifugation and the supernatant analyzed for [^{14}C]MVA. Using a modification of the method described by Chappell et al. (1995), 700 μL of 0.5 M potassium phosphate (pH 6.0) followed by 1 mL of ethyl acetate was added to the supernatant. After thorough mixing and centrifugation, radioactivity in the ethyl-acetate phase was determined by liquid scintillation spectrometry. Alternatively, the ethyl-acetate fraction was applied to thin layers of silica gel (GF_{254}) and plates developed to 15 cm in chloroform:acetone (2:1, v/v), and radioactivity in the MVAL-containing zone (R_F 0.65) was determined by liquid scintillation spectrometry. Assays were performed in triplicate, with less than 10% variation between samples and the two methods of analysis.

Determination of ABA Content

For analysis of ABA, aliquots of freeze-dried mesocarp tissue were homogenized in ice-cold methanol:ethyl acetate (50:50, v/v), containing a known amount of radiolabeled ABA (to correct for losses) and diethyldithiocarbamate (200 mg L^{-1}) as an antioxidant, in the presence of insoluble PVP (10%, w/w) and extracted for 24 h in darkness at −20°C. The homogenate was centrifuged and the pellet extracted with further methanol:ethyl acetate (50:50, v/v). The combined supernatants were reduced in vacuo, and the residue was resuspended in 0.5 M potassium phosphate buffer (pH 8.5) and partitioned three times against equal volumes of diethyl ether to remove neutral and basic impurities. The pH of the aqueous phase was adjusted to 2.5 and ABA partitioned into diethyl ether (repeated three times). Purified ABA-containing samples were analyzed by reversed-phase HPLC. Chromatography was carried out on a 5-μm C_{18} column (250 × 4.6 mm i.d., ODS 2, Spherisorb, Phase Separations, Inc., Clwyd, UK) eluted with a linear gradient of 0 to 100% methanol in 1% aqueous acetic acid over 60 min at a flow rate of 1.0 mL min^{-1}. ABA was quantified at 254 nm by peak integration following calibration with authentic standards using a programmable UV-visible light detector (model 990, Waters).

Data Analysis

Treatment effects on fruit growth and differences in cell size and number were analyzed using Genstat (Rothamsted Experimental Station, UK), compared by ANOVA and *F* tests used to determine the level of significance ($P < 0.01$). All other data are the mean of at least four independent measurements and were either compared by ANOVA and SE (difference) generated ($P < 0.05$) or presented as the mean ± SE for a treatment.

RESULTS

Inhibition of Avocado Fruit by Mevastatin and Effect of Sterols

Injection of mevastatin, a competitive inhibitor of HMGR, through the pedicel during either phase I or phase II retarded avocado fruit growth and development by 60% (Fig. 1). In both experiments, mevastatin-induced retardation of fruit growth was reversed by co-injection with MVAL, resulting in recovery of the normal phenotype. Sterols reduced avocado fruit growth when applied either in phase I or phase II (Fig. 1, A and B). A combination of cholesterol and stigmasterol, administered during phase I, also reduced fruit growth and eventually arrested the process (Fig. 1A), causing 50% fruit abscission 70 d after treatment. Although stigmasterol retarded avocado fruit growth to the same extent when applied in phase II, it partially reversed the inhibitory effect of mevastatin (Fig. 1B).

Cell Number: The Limiting Factor in Avocado Fruit Growth

To determine whether cell size and/or cell number was limiting during development of phenotypically small cv Hass avocado fruit, measurements of cell number and cell size were taken throughout the course of this program. The data were computed using a general logistic curve and an ANOVA performed on each parameter in the nonlinear regression. The resultant trends are shown in Figure 2. The mean equatorial mesocarp cell number was significantly higher in control fruit (Fig. 2A), whereas there was no significant difference between mean mesocarp cell size of the small and control fruit (Fig. 2B).

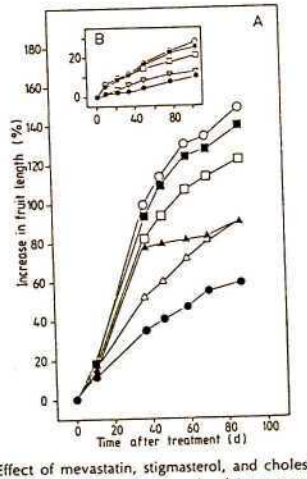

Figure 1. Effect of mevastatin, stigmasterol, and cholesterol on cv Hass avocado fruit growth. Compounds of interest were applied during the 1994–1995 season in 20 μL of Tween 20:acetone:water (1:1:8, v/v) via the pedicel at concentrations of 1 μg μL^{-1} either 55 d (A, phase I) or 92 d (B, phase II) after full bloom, and growth was monitored as percentage increase in fruit length. Each value represents the mean of eight determinations. SE (difference) in A ≤ 9.0; SE (difference) in B ≤ 0.9. O, Control; ●, mevastatin; ■, mevastatin plus MVAL; ▽, mevastatin plus stigmasterol; □, stigmasterol; △, cholesterol; and ▲, stigmasterol plus cholesterol.

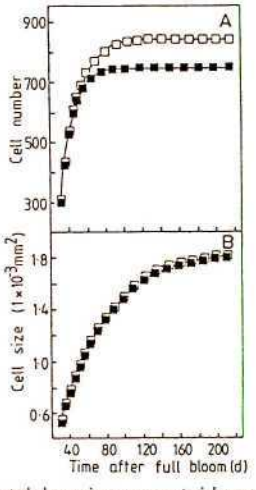

Figure 2. Estimated change in mean equatorial mesocarp cell number and cell size of small (■) and large (□) cv Hass avocado fruit throughout development. A, Cell number was estimated as described in "Materials and Methods" and regression lines for large and small fruit are represented as $y = 833.9 - 2560.5(0.9466)^x$ and $y = 746.9 - 5576.3(0.9222)^x$, respectively. B, For average cell size, regression lines for large and small fruit were $y = 1854.5 - 2330.0(0.9808)^x$ and $y = 1852.9 - 2390.8(0.9806)^x$, respectively. Regression lines were calculated from 54 measurements per treatment at each time interval.

Effect of Plant Growth Regulators on Mevastatin-Induced Inhibition of Fruit Growth

Results presented in Figure 3A show that mevastatin-induced retardation of cv Hass avocado fruit growth during phase I (55 d after full bloom) could be completely reversed by co-injection with MVAL, iP, or the cytokinin analog CPPU. GA_3 and stigmasterol, by comparison, had little or no effect. As shown in Figure 3B, CK, stigmasterol, or GA_3 did not markedly influence the "normal" course of cv Hass avocado fruit development when applied during phase I, although toward the conclusion of this growth period both GA_3- and stigmasterol-treated fruit showed a slowing of growth relative to the control. Likewise, AMO 1618, a purported inhibitor of kaurene synthase activity (Dennis et al., 1965) and sterol biosynthesis (Douglas and Paleg, 1972) did not markedly affect fruit growth, although it did cause growth to slow toward the end of the experimental period. Exogenously applied ABA, however, reduced fruit growth substantially and caused 90% fruit abscission within 50 d of application. Co-injection with iP reversed the growth-retarding effect of ABA (Fig. 3B) and reduced the incidence of fruit abscission to that observed in control treatments (Fig. 4).

During phase II (146 d after full bloom), treatment of fruit with iP countered the growth-retarding effect of mevastatin (Fig. 3C). Isopentenyladenine alone, however, had little or no effect on fruit growth during this phase.

In phase III (210 d after full bloom) mevastatin treatment reduced growth by 50% (Fig. 5A), whereas treatment with iP did not markedly affect this process (Fig. 5B). Surprisingly, only iP completely reversed the growth-retarding effect of mevastatin, although co-injection of mevastatin with either MVAL or stigmasterol reduced the effect of this inhibitor (Fig. 5A). ABA reduced avocado fruit growth by 50%, and this effect was reversed in fruits co-treated with iP.

Microsomal HMGR Activity of Mevastatin-Treated and Nontreated Avocado Fruit

In an attempt to further elucidate the proposed link between the CKs, the sterols, the small-fruit phenotype, and the synthesis of MVA, HMGR activity in small fruit and fruits treated with or without mevastatin in phases I, II, and III was determined and the results are presented in Figure 6.

During the course of cv Hass avocado fruit development, activity of microsomal HMGR remained unchanged (Fig. 6A). Although a similar trend was observed for small fruit, specific activity of microsomal HMGR was approximately 30% that of untreated and control fruit of a comparable age (Fig. 6C). Fruit pretreated with mevastatin in phase I, II, or III showed a substantial reduction in HMGR activity (Fig. 6D), with levels similar to those observed for small fruit.

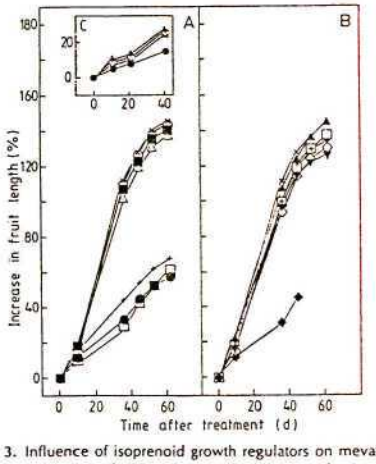

Figure 3. Influence of isoprenoid growth regulators on mevastatin-induced retardation of cv Hass avocado fruit growth. A and C, Mevastatin-treated. B, Control. Compounds of interest were applied in phase I (A and B, 55 d after full bloom) and phase II (C, 146 d after full bloom) of the 1995–1996 season, and fruit growth was measured as described in Figure 1. Each value represents the mean of eight determinations. SE (difference) in A ≤ 6.0; SE (difference) in B ≤ 6.0; SE (difference) in C ≤ 3.5. O, Control; ●, mevastatin; □, stigmasterol; +, GA_3; ×, CPPU; ■, MVAL; △, iP plus mevastatin; ▲, iP; ♦, ABA; ▼, AMO 1618; ◇, ABA plus iP.

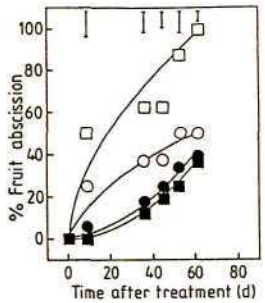

Figure 4. Comparison of the effect of ABA, ABA plus iP, and all other treatments on cv Hass avocado fruit abscission following pedicel injection of compounds during phase I of development. Experimental conditions were as described for Figure 3. ○, ABA plus iP; □, ABA; ●, all other treatments; ■, control.

Likewise, ABA treatment of fruit in phase III reduced in vivo HMGR activity by 70% to 1.41 ± 0.27 nmol h^{-1} mg^{-1} protein. Unfortunately, insufficient samples due to fruit abscission precluded a comprehensive assessment of the effect of ABA on in vivo HMGR activity during avocado fruit development. Even so, co-injection of ABA with iP during phase III partially restored HMGR activity (2.15 ± 0.24 versus 6.35 ± 0.92 nmol h^{-1} mg^{-1} protein in untreated fruit). HMGR activity was unaffected in fruits co-injected

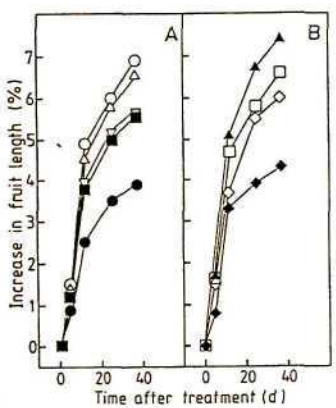

Figure 5. Effect of iP, MVAL, and stigmasterol on growth of mevastatin-treated fruit (A) and effect of iP, ABA, and stigmasterol on growth of control fruit (B) during phase III. Chemicals were applied in 20 μL of Tween 20:acetone:water (1:1:8, v/v) via the pedicel 210 d after full bloom (phase III) during the 1995–1996 season at concentrations of 1 μg μL^{-1}, and fruit growth was monitored as described in "Materials and Methods." Determinations are the mean of eight fruits per treatment. SE (difference) ≤ 1.4. ○, Control; ●, mevastatin; ■, mevastatin plus MVAL; ▽, mevastatin plus stigmasterol; △, mevastatin plus iP; ♦, ABA; ◇, ABA plus iP; □, stigmasterol; ▲, iP.

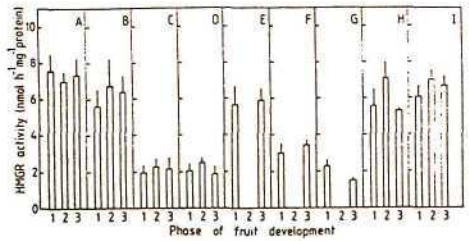

Figure 6. HMGR enzyme activity in developing cv Hass avocado fruit and fruit pretreated with mevastatin and/or iP and/or stigmasterol. Batches of fruit (eight per treatment) were injected with 20 μL of mevastatin and/or MVAL and/or iP and/or stigmasterol (all 1 μg μL^{-1}) 55 d (phase I), 146 d (phase II), and 210 d (phase III) after full bloom, and the fruit were harvested 40 d later. HMGR activity was determined in Ca^{2+}-sedimented microsomal membranes derived from freeze-dried mesocarp tissue, as described in "Materials and Methods." Each value is the mean ± SE of three to six determinations. A, Untreated; B, control; C, small fruit; D, mevastatin; E, mevastatin plus MVAL; F, stigmasterol; G, mevastatin plus stigmasterol; H, iP; I, mevastatin plus iP.

with MVAL and mevastatin (Fig. 6E), whereas stigmasterol was inhibitory and exacerbated the effect of mevastatin on enzyme activity (Fig. 6, F and G). Treatment of fruit with iP did not affect HMGR activity significantly during the course of fruit development (Fig. 6H). At all stages of fruit growth iP treatment reversed the inhibitory effect of mevastatin (Fig. 6I).

Effect of Mevastatin on Avocado Mesocarp ABA Content

Analysis of ABA in mesocarp from small fruit and fruit pretreated with or without mevastatin and/or MVAL, iP, or stigmasterol revealed the trends shown in Table I. ABA concentration declined over the normal course of avocado fruit growth and development. By comparison, mesocarp ABA content of small fruit increased, and at all stages of growth small fruit contained substantially more ABA than fruit from the control treatments. Mevastatin treatment significantly enhanced ABA concentration at all stages of fruit growth, whereas co-injection of this inhibitor with either MVAL or iP reversed the effect. MVAL resulted in a return to basal ABA concentration at all stages of fruit growth. In contrast, exogenous application of iP reduced basal ABA content by >50% during the early stage (phase I) of fruit growth, but was only 50% as effective as MVAL during the later stages (phases II and III) of this process. Stigmasterol reduced mevastatin-induced ABA accumulation by 30% in fruits treated in phase I, and by more than 50% when co-injected with mevastatin in phase III.

DISCUSSION

The occurrence of a substantial percentage of phenotypically small cv Hass fruit is common to all avocado-producing regions. Even healthy trees in well-managed orchards produce a significant number of small fruit, usu-

Table I. *ABA concentration of mesocarp tissue from developing small fruit and fruit pretreated with mevastatin, MVAL, stigmasterol, and iP*

Batches of fruit (eight fruit per treatment) were injected via the pedicel with 20-μL solutions of Tween 20:acetone:water (1:1:8, v/v) containing mevastatin, mevastatin plus MVAL, mevastatin plus stigmasterol, and mevastatin plus iP (all 1 μg μL^{-1}) 55 d (phase I), 146 d (phase II), and 210 d (phase III) after full bloom. Fruits were harvested between 50 and 100 d after application of chemicals and ABA content was determined as described in "Materials and Methods." Data are the mean of at least three determinations ($LSD_{(5\%)}$ = 10.9).

Treatment	Time after Full Bloom		
	163 d	216 d	290 d
	μg $^{-1}$ dry wt (%)[a]		
Control	29.3 (100)a[b]	12.2 (100)a	10.9 (100)a
Small fruit	63.6 (217)d	81.8 (670)d	75.5 (693)c
Mevastatin	66.9 (228)d	39.0 (320)c	67.3 (617)c
Mevastatin + MVAL	33.0 (113)b	16.1 (132)a,b	11.1 (102)a
Mevastatin + iP	14.1 (48)a	26.4 (216)b	24.9 (228)b
Mevastatin + stigmasterol	44.6 (152)c	ND[c]	31.5 (289)b

[a] Percent relative to control. [b] At each time interval, values followed by different letters are significantly different ($P \leq 0.05$). [c] ND, Not determined.

ally characterized by early seed coat senescence and cessation of fruit growth 50 to 60 d after full bloom. We hoped to exploit this phenotypic variant and gain insight into the interaction between isoprenoid growth regulators, phytosterols, and HMGR activity in the metabolic control of avocado fruit growth and development.

Results from the present investigation provide convincing evidence for involvement of sterols, CKs, ABA, and HMGR in the metabolic control of avocado fruit growth. In this regard, several interesting observations were made. First, growth of phenotypically small cv Hass fruit was limited by cell number, and not by cell size, and these fruit showed reduced HMGR activity and elevated endogenous ABA during each phase of development. Second, in vivo inhibition of HMGR by mevastatin resulted in reduced fruit growth and increased fruit ABA concentration, irrespective of time of application after fruit set. Third, phytosterols did not appear limiting in phase I of cv Hass fruit growth. During phases II and III, however, stigmasterol reversed, albeit partially, the growth-retarding effect of mevastatin. Fourth, retardation of avocado fruit growth by mevastatin was reversed by co-injection with iP and the CK analog CPPU, in addition to MVAL. Only MVAL completely reversed mevastatin-induced inhibition of HMGR activity. MVAL was also more effective at reversing the mevastatin-induced increase in ABA than was iP, particularly during the later stages of fruit growth. This observation might account for the inability of iP to completely reverse ABA inhibition of HMGR in phase III. Retardation of fruit growth by exogenous ABA, and ABA induction of fruit abscission were reversed following co-injection with iP. Together, these findings support our proposed interaction between CKs, ABA, sterols, and HMGR, in which an increase in endogenous ABA causes down-regulation of HMGR enzyme activity and fruit growth, typified by the occurrence of phenotypically small fruit.

Several reports have implicated phytosterols in the control of fruit development (Narita and Gruissem, 1989; Gillaspy et al., 1993), and at least one study has suggested an essential role for stigmasterol in the support of plant cell division (Haughan et al., 1987). Although deprivation of MVA and sterols is reported to increase HMGR half-life, high levels of sterol enhance the rate of HMGR degradation (Correll and Edwards, 1994). Thus, it was not unexpected that treatment with stigmasterol (and/or cholesterol) would reduce in vivo HMGR activity and fruit growth.

Co-injection of mevastatin with stigmasterol, however, caused fruit to respond differently in phases I, II, and III. In phase I stigmasterol reduced fruit growth and accelerated abscission, whereas fruit treated in phase II showed partial recovery from mevastatin-induced inhibition of growth, and rates of abscission closely resembled those of control treatments. In phase III, however, stigmasterol alone did not affect fruit growth, but reversed the growth-retarding effect of mevastatin to the same extent as MVAL. Even so, stigmasterol did not reverse mevastatin-induced inhibition of HMGR, presumably due to mevastatin-induced ABA accumulation. Likewise, the ABA content of phenotypically small fruit resembled that of mevastatin-treated fruit and HMGR activity was substantially reduced.

Earlier studies on regulation of higher plant cytosolic HMGR suggested hormonal mediation of enzyme activity (Russell and Davidson, 1982). The authors demonstrated in vivo ABA, stigmasterol, and cholesterol inhibition of enzyme activity. When added to reaction mixtures in vitro, however, these products of isoprenoid biosynthesis had no effect on enzyme activity. It was therefore concluded that hormonal control was not allosteric, but was exerted via some unknown phosphorylation system. Similar conclusions were reached in studies on the effect of endogenous ABA on HMGR activity during seed maturation. *Vivipary* mutants of maize, which are defective in ABA biosynthesis, and the *Vp1* mutant, which is defective in an ABA response element, all show enhanced HMGR activity relative to wild-type siblings (Moore and Oishi, 1994). Since the *Vp1* gene product is involved in ABA signal transduction during seed development, it was proposed that HMGR activity during seed maturation is regulated via a *Vp1*-dependent signal transduction pathway that is affected by reduced ABA.

Mevastatin-induced ABA accumulation in avocado mesocarp was both surprising and interesting. First, this observation supports plastid-localized ABA synthesis (Zeevaart and Creelman, 1988), since mevastatin and its structural analogs are unable to inhibit chloroplast isoprenoid synthesis (Bach and Lichtenthaler, 1983; Bach, 1987). Second, the existence of an alternative pathway that does not involve MVA synthesis, similar to that proposed recently by Schwender et al. (1996), cannot be ignored. In this pathway, isopentenyl diphosphate is formed intrachloroplastically via condensation of pyruvate and glyceraldehyde phosphate. Third, the result might suggest that a product(s) of cytosolic isoprenoid biosynthesis is responsible for regulating ABA formation in or by chloroplasts. Two possible candidates include CKs and phytosterols.

Isopentenyladenine reversed the inhibitory effects of mevastatin at all stages of avocado fruit development. Similarly, inhibition of tobacco cell growth by lovastatin (a mevastatin analog) was reversed by CKs (Crowell and Salaz, 1992). Furthermore, iP and its hydroxylated derivative zeatin replaced the essential role of MVA in initiating DNA replication in the cell cycle (Siperstein, 1984). Since CK biosynthesis is purported to involve prenylation of the purine moiety catalyzed by isopentenyl transferase, a process in which dimethylallylpyrophosphate is added to AMP at position N^6 (Binns, 1994), the above observations might suggest that inhibition of HMGR limits the MVA pool available for synthesis of dimethylallylpyrophosphate (isomerization of isopentenyl diphosphate) and, hence, in situ CK biosynthesis. Similar conclusions were reached by Crowell and Salaz (1992), who suggested that CK biosynthesis is more sensitive to HMGR inhibition than biosynthesis of other essential isoprenoids. Further support for this proposal comes from the observation that CPPU, a CK analog, was as effective as iP at overriding the inhibitory effect of mevastatin on avocado fruit growth.

Isopentenyladenine also reversed the inhibitory effects of ABA. The role of ABA in plant stress responses and its ability to retard developmental processes (Zeevaart and Creelman, 1988) suggest that it is a likely candidate to influence fruit growth under adverse conditions and thereby contribute to down-regulation of fruit development and emergence of small-fruit phenotypes. However, ABA concentration is high and declines during the normal course of fruit growth (Table I). Thus, an alternative interpretation might be related to CK homeostasis, which is purportedly regulated by a substrate-inducible (specifically iP) oxidase (Motyka et al., 1996). High concentrations of ABA during the early phase of fruit ontogeny may therefore be necessary to modulate CK synthesis, possibly at the level of HMGR, and hence cell proliferation.

Several studies have intimated a cell-cycle-regulating function for ABA because exogenous ABA inhibits nucleic acid and protein synthesis (Owen and Napier, 1988). Meyers et al. (1990) showed that exogenously applied ABA consistently inhibited cell division in cultures of maize kernels. More recently, Müller et al. (1994) obtained evidence to suggest that ABA functions to reduce cell-division cycle activity by retarding completion of the cell cycle. In addition, water deficit in developing endosperm of maize has also been reported to inhibit cell division (Artlip et al., 1995). Stress-induced accumulation of ABA might therefore be expected to exert an effect on fruit growth during the early stages, when cell-division cycle activity is at a maximum.

Avocado fruit enlargement in phases II and III is correlated with both cell division and expansion, developmental processes that require a significant level of sterol biosynthesis (Narita and Gruissem, 1989; Chappell, 1995a). It is well established that ABA increases permeability of lipid membranes (Stillwell and Hester, 1984; Bach, 1986; Stillwell et al., 1989; Purohit et al., 1992; Bürner et al., 1993) and that phytosterols inhibit these ABA-induced perturbations (Stillwell et al., 1990). ABA also appears to inhibit HMGR activity (Russell and Davidson, 1982; Moore and Oishi, 1994). In light of these observations it is tempting to suggest that stress-induced initiation of the ABA signal transduction pathway depresses HMGR activity, limiting synthesis of both CKs and phytosterols to reduce cell-division cycle activity in affected cv Hass fruits. Furthermore, accumulation of stress-induced ABA during fruit growth might be sufficient to induce "lipid melting" in affected membranes (e.g. seed coat), causing onset of senescence and cessation of fruit development, processes that would be reversed in the presence of sufficient sterol. This proposal is supported by the observation that fruit treated with stigmasterol in the presence of mevastatin, an inhibitor of HMGR that induces ABA accumulation, show a decline in endogenous ABA concentration and partial recovery of growth.

In conclusion, possible sources that may contribute to elevated fruit ABA concentration and, hence, reduced fruit growth in cv Hass avocado include the xylem/phloem continuum (i.e. stress-induced root- and leaf-derived ABA) and the developing fruit itself (i.e. in situ ABA biosynthesis). We have recently demonstrated more efficient incorporation of label from [1-^{14}C]Glc into ABA than from [2-^{14}C]MVAL in mesocarp of cv Hass avocado (J.C.G. Maurel and A.K. Cowan, unpublished data), which might indicate a novel source of carbon for ABA biosynthesis. In addition to reducing HMGR activity and fruit growth, in vivo application of ABA via the pedicel inhibits seed coat and mesocarp cell-cell communication (A.K. Cowan, C.E.J. Botha, R.H.M. Cross, C.S. Moore-Gordon, and I. Bertling, unpublished data). Whether this effect of ABA is linked to down-regulation of HMGR directly or indirectly remains to be investigated. The recent demonstration that plant HMGR kinase is related to Suc nonfermenting-1 protein kinase (Barker et al., 1996), a gene essential for release from Glc repression, suggests that isoprenoid metabolism, carbohydrate status, and fruit growth are indeed interrelated processes that could contribute to development of small-fruit phenotypes. This is particularly so given that ABA stimulates acid invertase activity (Ackerson, 1985) and transgenic tomato fruit, expressing a constitutive antisense acid invertase gene, show increased Suc concentration and decreased fruit size (Klann et al., 1996).

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The Hass Small-Fruit Problem: Role of Physiological Stress and its Amelioration by Mulching

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ABSTRACT

The Hass cultivar is important to the South African avocado industry because it matures late and is preferred by overseas consumers. However, it produces a variable percentage of undersized fruit that cannot be exported. Mulching was investigated as a possible method of increasing fruit size through improved root activity and reduced tree stress. In a field trial at Everdon Estate, Howick, feeder root growth was greatly increased throughout two seasons by the application of a coarse composted pine bark mulch beneath the tree canopy. Over two seasons, the mulch treatment resulted in a significant 11,8 % increase in mean fruit mass, in spite of 16,7 % more fruits per tree. The combined effect was a 30,4 % greater yield, in spite of a high level of management and a relatively mesic environment. The probable explanation for this increase is that mulching ameliorates overall plant stress. Mulching reduced the incidence of premature seed coat abortion and pedicel ring-neck, both of which are associated with plant water stress. Furthermore, mulching reduced foliage temperatures during stress periods, indicating a reduction in plant water stress during these critical periods.

INTRODUCTION

Because the South African avocado industry is largely export oriented, cultivars such as Hass, which are preferred by overseas consumers, are important to the industry (Cutting, 1993). This cultivar is also late maturing and is therefore useful for extending the harvesting season. Unfortunately, Hass trees have a tendency to bear large numbers of undersize fruit, and fruit size is on average much smaller than in other major commercial varieties such as Fuerte, Pinkerton, Edranol and Ryan. Up to 50 % of the Hass crop may be undersize (less than 200 g or counts of more than 20 fruits per standard 4 kg export carton) in any particular season (Köhne, 1992), and in 1994 this problem was estimated to have cost the South African industry R30 million in lost revenue.

The phenomenon is not restricted to diseased and/or unhealthy trees. Even healthy Hass trees produce a significant proportion (5–25 %) of small fruit (Kremer-Köhne & Köhne, 1995) unsuitable for export. The small-fruit problem is physiological and occurs in trees without pathogen involvement (Blanke & Bower, 1991). It is exacerbated by the onset of symptoms of pedicel ring-neck and early seed coat senescence, and is aggravated by poor cultural practices. The problem becomes more pronounced with tree age (Cutting, 1993) and is particularly noticeable in orchards situated in warmer and/or drier climates (Hilton-Barber, 1992; Whiley & Schaffer, 1994). Stress and ageing are therefore both major determinants of Hass avocado fruit size.

Fruit size is fundamentally determined by genome, so the long-term and ultimate approach is to discover or breed new large-fruited black-skinned cultivars. Unfortunately, breeding and testing new cultivars is time-consuming and does not resolve the problem immediately. There is therefore a requirement for an interim solution. We hypothesized that the application of a mulch could be a practical short-term solution to promote root growth and health, ameliorate stressful growing conditions and reduce the extent of the problem. This strategy is based on the avocado having evolved in a tropical to subtropical highland rainforest environment, and adaptation to soils with a litter layer and a high humic content. Reinforced mulching (in addition to natural litter fall) simulates rainforest floor conditions, thus providing roots with improved and more natural edaphic growing conditions. Improved root growth should impact positively on a cascade of physiological events promoting cell division in fruits, and prolonging seed coat viability. It is well known that premature seed coat abortion contributes to smaller fruit size (Blumenfeld & Gazit, 1974; Steyn *et al.*, 1993).

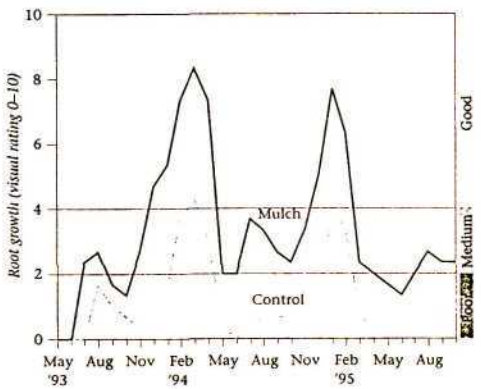

Figure 1
Root flushes as determined by a visual rating where:
0= no root growth; 10 = extensive root growth

Any layer of plant material that occurs naturally or is applied to the soil can be considered a mulch (Turney & Menge, 1994). The benefits derived from mulching include increased water and nutrient availability (Gregoriou & Rajkumar, 1984), improved soil structure and porosity (Gallardo-Laro & Nogales, 1987) and a narrowing in the diurnal soil temperature range (Gregoriou & Rajkumar, 1984). In addition, mulching creates a suppressive environment for *Phytophthora cinnamomi*, thus reducing the impact of this phytopathogen (Turney & Menge, 1994). All of the above benefits of mulching serve to reduce the impact of environmental stress on the tree.

The objective of this study was to investigate whether mulching could be a practical cultural method of increasing mean Hass fruit size through improved root activity and reduced tree stress.

MATERIALS AND METHODS

Treatment

This study was conducted on six-year-old (in 1993) Hass trees on clonal Duke 7 rootstock at Everdon Estate, near Howick, in the Kwazulu-Natal midlands (30° 16' E and 29° 27' S). The orchard is situated in Phillips' Bioclimatic region 3, which is characterized

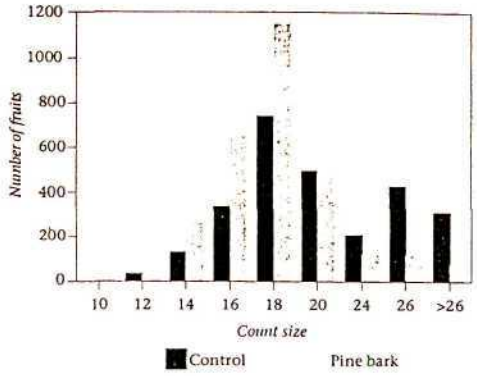

Figure 2
Overall Hass fruit size distribution at harvest for the 1993/1994 season

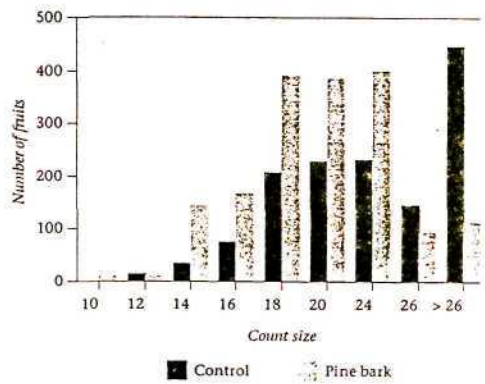

Figure 3
Overall Hass fruit size distribution at harvest for the 1994/1995 season

by cool mesic conditions, typical of a 'mist-belt' climate. Mean maximum and minimum temperatures range from 26,1 and 15,0 °C in January to 19,4 and 6,7 °C in July. Mean annual rainfall is 1 052 mm and altitude is ca. 1 080 m. Orchards receive standard cultural treatment, including microjet irrigation based on tensiometers, and management efficiency is excellent. The soil is an oxisol of the Hutton form, dystrophic, with a high clay content of ca. 50 %. A total of 1,5 m^3 of coarse composted pine bark (Gromed® coarse potting mix) was applied in February 1993 under the canopy of six trees to a depth of approximately 15 cm, and these trees were compared with six adjacent unmulched trees.

Data collection

The data collection period for phenological events spanned from May 1993 through to October 1995. Root flushes were monitored by visually estimating the area covered by white healthy feeder roots under a newspaper mulch (Whiley *et al.*, 1988) with an approximate area of 1 250 cm^2. The newspaper mulch was placed 1 m from the microjet nozzle on the south-west side of the tree, so as to avoid direct sunlight. Three readings per treatment were taken at the end of each month. Visual estimates of root flushing were performed using a rating of 0–10. Kaiser & Wolstenholme's (1994), groupings of 'poor', 'medium' and 'good' were chosen, viz. 0–2, 3–4, and 5 respectively.

At the end of each season the trials were harvested, and fruit size distributions were recorded for each tree. Fruit size was determined gravimetrically and classified according to the number of fruit per standard 4 kg export carton. Fruits were graded as follows:

- Count 10: 366–450 g
- Count 12: 306–365 g
- Count 14: 266–305 g
- Count 16: 236–265 g
- Count 18: 211–235 g
- Count 20: 191–210 g
- Count 22: 171–190 g
- Count 24: 156–170 g
- Count 26: 146–155 g
- Factory grade: < 146 g

Total tree yields were calculated by adding the product of the number of fruit per count size and the class centre of all the count sizes.

Monitoring of tree stress

Seed coat viability

To determine the relationship between seed coat viability and fruit size, all fruit from a single eight-year-old Hass tree on Everdon Estate was harvested on 21 July 1995, at the time of initial fruit harvest. These fruits were weighed and allocated a seed coat viability rating. Broad groupings of 'healthy', 'degenerate' and 'intermediate' were selected (healthy seed coats were still white and fleshy, degenerate seed coats, brown and thin, and the intermediate category falling between these two extremes).

Incidence of pedicel ring-neck

To determine the effect of mulching on the incidence of pedicel ring-neck, 100 fruits per tree were randomly harvested, with care being taken to ensure that the fruit were still attached to their pedicels. Before fruits were passed through the packhouse, the presence or absence of the ring-neck syndrome was recorded for each fruit.

Foliage temperature

Using weather-proof infra-red thermometers (IRTs), surface canopy temperatures of two trees per treatment were recorded continuously from November 1994. Insulation and reflective foil were applied to the IRTs to reduce temperature effects. The IRTs were mounted 2,5 m from the trees, facing south, on tripod stands at a height of 4,5 m above the ground. IRTs were connected to a Campbell Scientific CR10® data-logger beneath the trees. Simultaneous air temperature measurements were recorded by two thermocouples, and these data were also fed into the data-logger.

RESULTS AND DISCUSSION

Root flushing

Root activity in the mulch treatment was always more intense than in the control. In the mulch treatment root growth fell into the 'medium' category for most of the season, whereas in the control mainly 'poor' root growth was recorded. For a substantial part of the season (December 1993 through to April 1994, and December 1994 through to March 1995) root activity was allocated a 'good' rating in the mulched treatment (figure 1). Root flushing periods followed a similar pattern, but in the mulch treatment they occurred two to four weeks earlier and continued longer (figure 1).

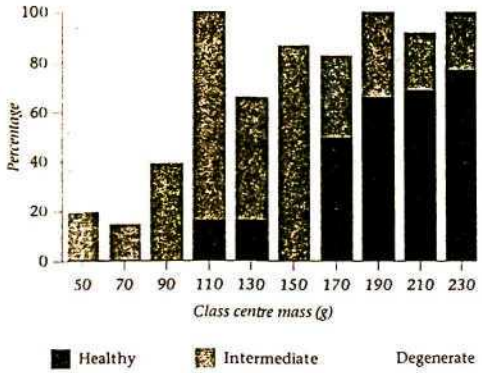

Figure 4
Allocation on a percentage basis of seed coat viability into categories of 'healthy', 'intermediate' and 'degenerate'

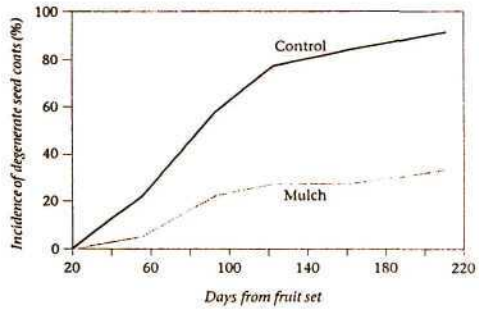

Figure 5
Incidence of fruits having degenerate seed coats from the mulch and control treatments on a percentage basis (values were calculated from 18 fruits per treatment at each time interval)

Avocado trees are adapted to growing in soils with a thick litter layer and a high organic content, and avocado roots, being 'litter feeders' with a high oxygen requirement (Moore-Gordon *et al.*, 1995), thrive under such edaphic conditions. Although healthy trees shed large numbers of leaves (which are relatively short-lived for an evergreen tree), application of the composted pine bark mulch reinforced rain-forest floor conditions, resulting in the more intense and prolonged surface feeder root activity. The rhizotron studies of Whiley (1994) are more representative of root activity at depth, and have indicated the potential for new root growth through winter in deep, cool, high organic matter krasnozem soils in the high rainfall areas of S.E. Queensland. It is not known whether such root activity, at depths of up to 1 m, occurs under the climate and edaphic environment of Everdon, but the soils are substantially similar.

Yield and fruit size distribution

The control trees showed a typical fruit size distribution for the Hass cultivar with many fruit in the count size range of 22–26 (small fruits), and a high proportion of factory-grade avocados (figures 2 and 3). Mulching with pine bark had the effect of shifting the overall count size distribution towards large fruits, i.e. the mulch treatment yielded fewer small fruits and more large fruits (figures 2 and 3).

Average fruit mass was significantly ($P \leq 0,01$) increased in response to pine bark mulching. Fruits from the mulch treatment were on average 23,3 g ± 1,2 g heavier than control fruit after one year of the treatment, representing an 11,8 % increase in mass, in spite of more fruits per tree (table 1). Harvest results for the 1994/1995 season confirm that the pine bark treatment resulted in a significant ($P \leq 0,01$) increase in fruit size, with fruit from this treatment being on average 21,0 g ± 1,4 g heavier (11,8 %) than control fruit (table 1).

Seasonal effects on mean fruit size are also evident, with fruits being 10 % smaller in the control and 10 % smaller in mulched trees in the second season. This might be attributed to the shorter fruit growth period during the second season (255 days in 1994/1995 compared to 284 days in 1993/1994). Since avocado fruit expansion proceeds throughout fruit development (Schroeder, 1953), albeit at a slower rate during the later stages, a prolonged period of fruit growth would be expected to result in larger fruit size.

Assimilate supply to a fruit will depend on the extent of competition from other established fruit sinks (Monselise & Goldschmidt, 1982), and so fewer sinks should yield larger fruit. Table 1 shows that increase in fruit size was achieved in spite of a 6,1 % and 27,2 % increase in number of fruits per tree in the mulch treatment in the first and second seasons respectively. This supports the hypothesis that mulching has altered endogenous physiological conditions in favour of increased fruit growth. The increased fruit size coupled with increase in number of fruits per tree resulted in an overall 18,5 % increase in yield at the end of the first year, and a 42,2 % increase in yield for the second season (table 1), i.e. an average increase in yield of 30,4 % over the two seasons.

Table 1
Summary of the effects of pine bark mulching on average fruit mass, number of fruits per tree and total yield. Figures are means of six trees. *Denotes a significant ($P \leq 0,01$) increase by mulching.

		Control	*Mulch*	*% increase*
1993/1994	Mean fruit mass (g)	198,0	221,3	11,8*
	Fruit number/tree	509	540	6,1
	Yield (t/ha)	20,16	23,88	18,5*
1994/1995	Mean fruit mass (g)	178,2	199,2	11,8*
	Fruit number/tree	262	333	27,2*
	Yield (t/ha)	9,32	13,26	42,2*

Seed coat viability

Results show that there is a good correlation between Hass fruit size and the extent of seed coat degeneration. Smaller fruits had a higher proportion of degenerate seed coats, while larger fruits had a higher percentage of healthy ones (figure 4). The practice of mulching reduced the incidence of fruit with degenerate seed coats (figure 5). Assuming that seed coat degeneration is a consequence of plant stress, this implies that mulching reduces plant stress, probably through improved water uptake as a result of increased water availability and increased root absorbing surface. Maintenance of seed coat health means that the seed still has the capacity to import minerals and assimilate, and other factors necessary for fruit growth: this partly explains why fruit growth was enhanced by mulching.

Incidence of pedicel ring-neck

The practice of mulching reduced the incidence of fruit with pedicel ring-neck by 57,1 % and 45,9 % for the first and second seasons respectively (table 2). Since pedicel ring-neck is associated with plant water stress (Whiley *et al.*, 1986), one could surmise that mulching reduced the impact of adverse environmental pressure. It is worth noting here that the degree of this disorder is less advanced in the mesic KwaZulu-Natal midlands climate than in the more stressful environment of the Northern Province and Mpumalanga.

Table 2
Summary of the effect of mulching on the incidence of pedicel ring-neck. Values were calculated from a total of 100 fruits per tree

Year	*Incidence of pedicel ring-neck (%)*	
	Control	*Mulch*
1993/1994	17,5 ± 2,2	7,5 ± 2,4
1994/1995	13,3 ± 2,7	7,2 ± 1,9

Foliage temperature

The surface temperature of a leaf is the tangible manifestation of its energy balance and therefore is affected by abiotic and biotic factors. The most prominent of the latter are the stomates which, in closing, limit the amount of energy that can be dissipated by transpiration, and consequently cause the leaf temperature to increase (Raschke, 1960). These facts led Tanner (1963) to postulate that the surface temperature of the leaf may be used to assess the water status of the canopy, i.e. the degree of water stress. Since leaf canopy temperatures of control trees are generally higher than that on the mulch treatment ($T_{control} - T_{mulch}$ is approximately 0,5 °C on average) (figure 6), this implies that mulching has reduced overall plant water stress.

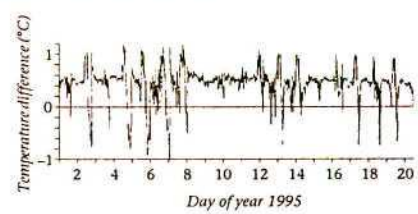

Figure 6
Foliage canopy temperature differences between the control and mulch treatments ($T_{control} - T_{mulch}$)

CONCLUSIONS

A thick, composted pine bark mulch applied in February 1993, supplemented by the natural avocado leaf mulch, was compared to no mulch (regular removal of fallen leaves). Mulched trees showed more prolonged and more extensive root growth, especially in the summer/autumn root flush but also throughout the year, including the critical fruit set period. Fruit growth on the mulch treatment was significantly increased, in spite of increased numbers of fruit per tree. Resultant fruit mass at harvest was 11,8 % greater in both years, and total yield per tree 18,5 % and 42,2 % greater, in the first and second seasons respectively. These results lend support to the hypothesis that a healthy and vigorous root environment, ameliorated by reinforced mulching, can lead to larger average fruit size and mass.

A probable explanation why mulching has a positive effect on fruit size is that this practice might reduce overall plant stress, thus creating favourable physiological conditions for fruit growth. Mulching considerably reduced the incidence of premature seed coat degeneration and pedicel ring-neck, both of which are associated with tree water stress. Mulching also reduced leaf canopy temperatures by approximately 0,5 °C during the ecological dry period, providing further evidence that mulching reduced overall plant stress. In addition, the role of improved mineral uptake must be mentioned. In leached acid soils in Queensland, Australia, Smith *et al.* (1995) found that soil boron applications improved Hass fruit size by 11–15 % Improved boron nutrition may be another beneficial effect of mulching.

Properly regulated mulches thus provide a practical solution to the grower to increase average fruit size, presumably by ameliorating plant stress at critical periods. The Everdon climate can be classified as only moderately stressful, being cool and mesic with high rainfall and humidity, and with good orchard management. Benefits of mulching are therefore likely to be greater in more stressful environments, provided that crop load is not excessive. Canopy management to ensure sufficient leaf area per fruit is therefore vital, particularly for Hass in warmer areas where initial fruit set can be excessive.

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Some Pros and Cons of Mulching Avocado Orchards

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ABSTRACT

Mulching is a powerful manipulatory tool to ameliorate the root environment and reduce tree stress. It has been shown to improve both fruit size and yield in Hass in the cool, mesic KwaZulu-Natal midlands, and could be expected to be even more beneficial in more stressful areas. Pros and cons of mulching are discussed. Choice is affected by C : N ratio of the mulch, availability, expense and speed of decomposition. Timing is important, and tree nutrition and soil moisture must be monitored. Overall, in most situations the advantages of mulching, including water conservation, outweigh the disadvantages.

INTRODUCTION

Mulching is the application of any layer of plant material or other suitable material to the surface of the soil, without incorporation into the soil. It is an ancient technique and has three main benefits, viz. improved soil physical properties, improved water conservation and reduction of weed growth. Overriding these benefits are the effects on root growth and root health, i.e. amelioration of the root environment to improve conditions for the vitally important 'hidden half' of the plant. Wolstenholme (1981) stressed the importance of the roots in avocado tree performance.

A wide range of materials are used for mulching, e.g. manure, sludge, sawdust, wood-chips, straw, shredded prunings, plant foliage, paper, plastic, sand and gravel (Turney & Menge, 1994). These authors reviewed the use of mulching to control root disease in avocado and citrus trees. Recent research by Moore-Gordon *et al.* (1995, 1996) has highlighted the benefits of a composted pine bark mulch in increasing Hass fruit size, through the partial alleviation of stress associated with improved root growth. This ongoing research, surprisingly one of the few detailed studies of avocado orchard mulching, has raised questions in the minds of growers, more particularly the practical and economic implications of the promising research results.

This paper gives a broad overview of the pros and cons of organic mulching, with particular reference to the South African situation. Readers wishing for more general detail are referred to the excellent book on growing media by Handreck & Black (1994). The principles of composting are, however, not discussed in this overview.

BENEFITS OF MULCHING

Water conservation

Mulching conserves water by reducing evaporation from the soil; decreasing water run-off, soil puddling, compaction and erosion; increasing soil permeability; and increasing soil water holding capacity (Turney & Menge, 1994). More water is therefore available during stress and drought periods. This is of cardinal importance for maintenance of tree function during prolonged droughts such as we have experienced for the past four or five years, and during critical periods such as fruit set and early fruit growth. Furthermore, substantial savings in irrigation water can be effected — a scarce resource which will become far more expensive in future. These water savings need to be quantified in avocado orchards.

Improved root growth and reduced physiological stress

Good mulches allow more root growth, both in the litter layer and in the more fertile topsoil. Addition of organic matter improves soil structure, porosity and aeration and therefore also allows deeper root growth. Avocado roots have a high oxygen requirement (Stolzy *et al.*, 1971). Moore-Gordon *et al.* (1995; 1996) showed substantially more root growth, and for longer periods during the two main root growth flushes (Whiley *et al.*, 1988) as a result of mulching under the drip with composted pine bark.

More root growth means greater uptake of water and minerals, and probably also greater synthesis and translocation of growth-promoting hormones such as cytokinins and gibberellins in and from the roots. This is accompanied by reduced levels of the growth inhibitor abscisic acid in aerial parts. The net result is reduced stress, resulting in more cell division in flowers and fruits, better fruit set, larger fruits, and higher yields. Moore-Gordon *et al.* (1995, 1996) have shown that anatomical (less ring-neck of fruit stalks) and physiological (reduced incidence of premature seed coat abortion) indicators of stress are ameliorated.

More mesic soil environment

It is well known that mulched soils experience less temperature fluctuation, mainly because of improved moisture status (Gregoriou & Rajkumar, 1985; Lanini *et al.*, 1988). This also improves root growth and reduces plant stress. Optimum temperatures for root growth of Duke 7 and Velvick avocado rootstocks lay between 18 and 28 °C (Whiley *et al.*, 1990).

Suppressive soils for root disease reduction

The use of mulches and gypsum to help create more suppressive soils to combat *Phytophthora cinnamomi* root rot in avocado orchards was pioneered in Australia (Broadbent & Baker, 1974; Pegg *et al.*, 1982). In the 1970s, before chemical control of *Phytophthora* was available, the so-called 'Pegg Wheel' concept was widely promoted in South Africa (Wolstenholme, 1977). However, Trochoulias *et al.* (1986) showed in eastern Australia that organic amendments plus gypsum were unable to prevent tree decline on shallow and/or poorly drained soils, especially during high rainfall episodes. This has also been the South African experience, where Wolstenholme & Le Roux (1974) recommended unimpeded drainage to at least 1,5–2,0 m to ensure reasonable long-term success in the fight against root rot. The return of heavy rains during the 1995/96 season has resulted in rapid decline of many trees on poorly drained or shallow parts of orchards.

The mechanisms of root disease and nematode control by mulching are fully discussed by Turney & Menge (1994). They include increased soil populations of microbial antagonists; production of inhibitory volatiles such as ammonia and nitrite, and toxins such as saponins and organic acids; encystment of *Phytophthora* zoospores by organic matter; increased host resistance (phytoalexins); and improved aeration and drainage in mulch and soil. Lower soil temperatures also favour the tree over *Phytophthora*.

The advent of effective chemical control, and especially phosphonate fungicides (Darvas *et al.*, 1984, Pegg *et al.*, 1985), has

shifted the emphasis from biological to integrated control. Nevertheless, the principles of multi-faceted control still apply, and mulches are an important component.

Mineral nutrition

Although the primary aims of mulching are not specifically related to organic fertilization, all organic mulches decompose to release mineral elements for root uptake. Humus, the end-product of decomposition, substantially increases the cation exchange capacity of the soil. Nitrogen is initially released as ammonium, which can be taken up by plant roots or adsorbed to clay and humus particles. Ammonium can also be nitrified to nitrate, which is more subject to leaching and may pollute groundwater. However, the fact that nitrogen from organic matter is released slowly, reduces this risk, and if the mulch has a low C : N ratio (see later) the need for inorganic N fertilization will be reduced (Maynard, 1989).

Three mineral elements are regarded as especially important for healthy and prolific root growth, viz. phosphorus (P), calcium (Ca) and boron (B). All three have been observed to increase under a mulch (Stephenson & Schuster, 1945). Whiley *et al.* (1996) regard better root boron uptake in deficient soils as one reason for the increased Hass fruit size in mulched trees found by Moore-Gordon *et al.* (1995; 1996). Composted pine bark mulches are good sources of, *inter alia*, potassium and boron. In fact, in acid leached soils most of the boron will be in the organic matter (and mulch), from where it is slowly released by the action of micro-organisms (Gupta, 1979).

Weed control

Mulches usually reduce weed problems by reducing weed seed germination or reducing light levels. However, the opposite may apply with uncomposted mulches infested with weed seed.

MULCHING PROBLEMS

Cost

One of the main reasons cited for not using mulches is their cost. This applies more to mulching materials not available on site. Costs of transport are high due to the bulky nature of mulches, and application costs must be considered. These costs must be balanced against the increased fruit size (Hass) and yield, and the water and fertilizer savings achieved. This is a difficult exercise in view of lack of data. However, where the very existence of trees and yield was seriously compromized, as in the recent prolonged drought where irrigation water often ran out, surely some form of mulching was obligatory! The senior author was amazed to see avocado trees and bare orchard soils in extreme water stress baking in the sun, while all around was grass and other litter which could have relieved their plight. A partial costing for a commercial composted pine bark mulch is presented later.

Danger of nitrogen 'draw-down' (negative period)

Mulches with a high carbon to nitrogen (C : N) ratio have insufficient nitrogen for the increased populations of soil micro-organisms which help to decompose them. This nitrogen must also be supplied by the soil. The result is a N 'draw-down' or 'negative period', when the tree roots cannot obtain sufficient N. This can be overcome, at some expense by extra N fertilization (Handreck & Black, 1994; Turney & Menge, 1994),

Table 1 gives typical N contents and C : N ratios for a range of materials which have been used as mulches. C : N ratios above 100 are very high, so that material such as sawdusts (containing mostly cellulose-rich wood) and uncomposted barks are not good mulching materials. On the other hand, humus has a C : N ratio of 10 : 1. Proper composting usually reduces the C : N ratio of bark to about 30, and a ratio of 10 : 1 is hardly ever achieved. Maggs (1985) and Wright (1987) found that uncomposted South African pine bark had ratio of more than 100, up to 450, the latter especially when the wood (cellulose) content is high.

Table 1

Carbon : nitrogen ratios, and percentage nitrogen content of a range of mulching materials (modified from Handreck & Black, 1994)

Material	*%N in D.M.*	*C : N Ratio*
Pinus radiata sawdust	0,09	550
Cardboard	—	500
Pinus radiata bark	0,1	500
Eucalyptus sawdust	0,1	500
Eucalyptus bark	0,2	250
Paper	0,2	170
Bagasse	0,4	120
Woody prunings	—	100
Composted eucalyptus sawdust	0,45	100
Composted *P. radiata* bark	0,4	100
Wheat or oats, straw	0,4	100
Mature leaves	0,7	60
Composted pine bark[1]	1,1	30–40
Maize stalks	1,2	33
Peat	1,5	30
Grasses	1,8	22
Mixed weeds	2,0	19
Cow manure	2,6	15
Lucerne hay	3,1	13
Peanut shells	4,4	12
Poultry litter	2,4	10–11
Poultry droppings	5,5	7
Pig manure	—	5
Urine	—	2

[1]The Gromed Organics composted pine bark used in the mulching trial at Everdon had a nitrogen content of 1,1 % and a C : N ratio of about 37.

Availability of mulch

Availability is determined by the nature of nearby farming operations. Most avocado growing areas in South Africa are adjacent to exotic forestry plantations of eucalyptus or pines. Waste materials, especially barks (composted and/or thoroughly aged) should be utilized, but sawdusts make poor mulches. In KwaZulu-Natal, aged sugarcane bagasse can be used, although its C : N ratio is rather high. Poultry droppings and broiler/pullet and breeder deep litter, as well as kraal manure can be considered, but have a very low C : N ratio and a high N content. Van Ryssen *et al.* (1977) also noted their high copper content.

Stubbles of various kinds (wheat, oats, barley etc.) have been widely used in subtropical high rainfall Australia, usually together with gypsum ($CaSO_4$). They make excellent mulches. Similarly, stalky grasses with a high fibre content can be used. Remains of weeds, grasses and cover crops in the orchard can and should be used to reinforce the natural leaf mulch under avocado trees. However, fresh blady grass clippings (e.g. kikuyu) are relatively high in N, low in fibre, compact easily, get slimy, and are poor mulches. In Australia, peanut shell mulches are discouraged because they increase *Verticillium* root rot.

Increased frost hazard

In the U.S.A., mulches extending beyond the tree drip have increased the frost hazard in orchards where frost is a danger. They do this by reducing soil heating and storage of heat (from the sun) in the soil, and by raising the coldest air by the height of the mulch (Leyden & Rohrbaugh, 1963). In South Africa this is unlikely to be a problem, since frost hazard is lower and most mulches are placed only under the drip of the tree.

Increased fire hazard in winter

Dry winters increase the possibility of runaway fires gaining access to orchards, especially if dry vegetation is present. Mulches may then act as 'funeral pyres' for trees, and the result can be devastating. Sensible precautions will reduce this risk.

Incorrect use of mulches

Mulches are a powerful management tool if correctly used. Problems arise where the wrong mulch is chosen, or applied at the wrong time. These issues are discussed further below.

Upset nutrient balance

Again, mulches (especially those with a low C : N ratio and which decompose rapidly) can supply significant amounts of nutrients. The danger then exists of upsetting the vegetative-reproductive balance of the tree (Wolstenholme & Whiley, 1990). It stands to reason that leaf and soil analysis becomes even more important in the correct management of mulches in avocado orchards. Farm-yard and chicken manures applied to heavy soils in Israel reduced avocado yields, possibly through reducing soil hydraulic conductivity and through nutrient imbalances (Lahav, 1984).

IDEAL MULCHES

Ideal avocado orchard mulches include those with the following properties:

- C : N ratio of more than 25 : 1, but less than 100 : 1
- Fibrous, stalky, strawy materials with a moderate rate of breakdown
- Composted, chunky pine barks

As previously noted, sawdust is a poor mulch material. It was widely used in avocado orchards in the drought years of the 1960s, and did help to save drought-stricken trees. However, its disadvantages became apparent when the rains returned — poor physical properties; very low N content; excessive wetness; and toxic residues (pine sawdust) if not composted or aged. Similarly, paper and waste cardboard make poor mulches — they must be forked to allow water infiltration, are untidy, also have a high C : N ratio, and soon become a soggy mess (Handreck & Black, 1994).

Rapidity of mulch decomposition

The speed of decomposition (mineralization) of a mulch depends on its nature, and on environmental conditions. An organic mulch should not break down quickly — its prime function is soil cover rather than organic fertilizer. To reduce decomposition, it is never worked into the soil.

- *Quick:* Mulches derived from young plant materials with low C : N ratios and little fibre break down very quickly, e.g. young leaves, weeds, green manure crops and most animal manures (Handreck & Black, 1994). More stalky materials such as hay and straw do not last much longer, as they are usually well chopped up.
- *More slowly:* Bulkier, fibrous materials such as mealie cobs and stubble, and wood chips break down fairly slowly.
- *Very slowly:* Composted pine barks with medium particle size, and bark products as used for landscaping decompose very slowly. The same applies to large wood prunings.

Gromed avocado mulch (composted pine bark)

The mulch chosen for the Hass small-fruit trial at Everdon Estate, Howick was initially a commercial composted pine bark. At the time, it was selected because of its good physical properties, ready availability, known composition, and to establish a principle. Economic viability was not an issue. This Gromed mulch, available from Gromed Organics at Cramond, KwaZulu-Natal, is widely used in the nursery industry and has the following characteristics:

- thoroughly composted pine bark;
- particle size (graded) 16–24 mm, i.e. cannot compact;
- half-life of 5 years, i.e. very slow decomposition;
- high levels of potassium, calcium and boron.

The mulching trial at Everdon has now run for over 3 years, with no addition to the original mulch and little evidence of decomposition. Originally, 1,5 m^3 was applied under the drip of the seven-year-old trees, in a layer approximately 15 cm thick, to simulate (with the natural leaf mulch) the deep litter layer of an ideal indigenous avocado rainforest habitat. Due to this longevity of the mulch, it is conceivable that when orchards are thinned, the mulch under thinned trees could be transferred to the remaining trees.

An exercise in economic viability of this mulch showed that initial costs per hectare were very high, but that these could be amortized over the life of the mulch, and offset against the gains. Some salient figures are:

Cost of mulch @ 1,5 m_3/tree, 200 trees/ha, delivered from Cramond to Everdon was R26 035/ha in 1994 and R31 265/ha in 1996. Equivalent costs for Cramond to Tzaneen would be R45 673 and R55 340.

However, based on 1994 FOB less export costs supplied by Avodata, Tzaneen, the increased fruit size (ca. 12 %) and greater yield (ca. 42 %) over 2 years resulting from mulching, greatly increased the value of the crop. In 1993/94, extrapolating from the mulching trial, control trees would have grossed R42 377/ha, compared with R78 834 for mulched trees. Figures for 1994/95, a lower crop season, were R15 390 and R38 426. These figures do not take all costs into consideration, nor all benefits such as reduced fertilizer bill and reduced irrigation. Nor is it certain whether these mulching benefits will be as great in a good rainfall season. Nevertheless, they indicate that the 'pay-back' time for Everdon would have been about 11 months, and for Tzaneen 19 months. So even expensive mulches, applied under the tree drip, are not quite as expensive as perceived if their benefits and longevity are taken into account.

IMPORTANT DO'S FOR MULCHING

- Choose a suitable, economic mulch.
- The main aim is to *reinforce* the natural leaf and litter mulch under the tree. Avocado trees typically produce spring and summer/autumn growth flushes, but the leaves are short-lived (9–10 months, Whiley & Schaffer, 1994). Therefore a healthy avocado tree will have a good, thick natural leaf mulch, with most leaves falling in late spring (or before flowering in stressed trees). The excellent leaf mulch under avocado trees in Israel was a feature of field tours during the recent World Avocado Congress III. In contrast, weak and unhealthy trees will have virtually no natural mulch.
- The best time to apply the mulch, under our climatic conditions, is in autumn — after the summer rains, and in time to tide the trees over the dry, stressful winter and spring. The mulch will also have broken down significantly before the onset of the heavy summer rains (this does not of course apply to chunky composted pine bark, or to large tree prunings which take years to decompose).
- Do not mulch excessively wet soils, or areas of the orchard that become wet after heavy rains — e.g. the lower slopes where drainage water reaches the surface.
- Monitor tree nutrient status (annual leaf and soil analysis) as well as moisture status/irrigation need (tensiometers), both of which are affected by mulches.
- Never remove vegetative material from orchards. Cut up larger limbs and trunks of thinned trees, and place them under the

drip — although they have a high C : N ratio, they decompose very slowly so their adverse N draw-down effect is diluted over time. Use a brush-cutter to break down smaller prunings.
- Adjust mulch type, thickness and timing to suit your particular orchard situation. A thickness of 7,5 cm may be quite sufficient in some situations; 10–15 cm in others.

SOME DON'TS FOR MULCHING

- Do not use materials that pack into a water-shedding layer (Handreck & Black, 1994), e.g. green lawn clippings, sawdust. Green grass with little fibre soon turns slimy, due to poor aeration and encouragement of anaerobic organisms. Anaerobic organisms produce organic acids that are toxic to plants (the pH can drop as low as 2), and nitrogen loss can be high (Handreck & Black, 1994).
- Avoid materials with very high C : N ratios (> 100 : 1, unless they decompose very slowly and have good physical properties), or very low C : N ratios (< 20 : 1). The latter supply considerable nutrients as they decompose, and must be considered as organic fertilizers rather than mulches.
- Don't mulch already wet areas (lower slopes) or during very wet periods.
- If tree barks are used, they should be composted or aged, especially pine bark which contains resins.
- Do not apply thick, poorly aerated mulches just before the summer rains.
- Do not use wet, unleached composts or manures from, for example, kraals or piggeries — they may have a high salt content if they have not been thoroughly leached.
- Do not place mulches right up against the tree trunk — they can encourage *Phytophthora* collar rot.

CONCLUSIONS

Under South African and Australian conditions, with warm to cool climates and summer rainfall, mulching under and sometimes slightly beyond the drip of the tree has proven highly beneficial on well-drained soils. It simulates the rainforest floor conditions of the indigenous habitat, and benefits the rather shallow feeder root system of this 'litter feeder'. Root growth and root health are promoted, reducing the tree stress syndrome, especially at critical periods. This has led to larger fruit size and increased yield in Hass. Suppressive soils are also encouraged.

However, the choice of mulch must take into account factors such as C : N ratio, cost and availability, and speed of breakdown. Mulches must be applied correctly, and avoided where soil wetness is a problem. Composted pine bark has proved highly beneficial, and its initial expense is offset by a long life and greatly improved tree performance. The do's and don'ts of mulching are discussed. Overall, advantages outweigh disadvantages, and growers are strongly advised to use suitable mulches to their advantage, especially where water conservation is important. Moreover, mulching is environmentally friendly in a world increasingly conscious of 'clean and green' issues.

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Mulching of Avocado Orchards to Increase Hass Yield and Fruit Size and Boost Financial Rewards – a Three Season Summary of Research Findings

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ABSTRACT

Avocado yields are low when compared to other fruit tree crops, and the Hass cultivar, specifically, possesses an inherent tendency to produce a high percentage of small fruit. The application of a thick composted pinebark mulch was investigated as a strategy to increase yield and to alleviate the small fruit problem in this cultivar. Mulched trees showed more prolonged and extensive root growth throughout the duration of the trial. Over three seasons, mulching elevated average fruit yields by 22,6%, and increased mean fruit mass by 6,6%. The number of fruit that were considered highly suitable, and acceptable for export, were increased by 45% and 20% respectively. Initial costs of the pinebark were off-set within two seasons, thus providing growers with a practical means of boosting financial returns, especially since pinebark is considered to have a half life of approximately five years.

INTRODUCTION

Avocado yields are low when compared to other fruit crops (Wolstenholme, 1986). In the Hass cultivar, problems of low fruit productivity are intensified by its inherent tendency to produce large numbers of small fruit (Kremer-Köhne & Köhne, 1995). There is poor consumer acceptance for small fruit on the overseas market, and since the South African avocado industry is predominantly export orientated, yearly financial losses by the industry are considerable. There is thus a need to find solutions to these problems, as potential financial rewards to producers could be substantial.

The long-term approach is to breed or select a new high-yielding, large-fruited and black-skinned cultivar. Unfortunately, breeding programmes are time consuming and thus there is a need for an interim solution. Mulching with composted pinebark was investigated as a strategy to at least partly alleviate the extent of the problem. This strategy is based on the avocado's rainforest origin and adaptation to soils with a litter layer and a high humic content. Reinforced mulching (in addition to natural litter fall) simulates rainforest floor conditions, thus providing roots with improved and more natural edaphic growing conditions. It also alleviates several aspects of environmental stress.

The benefits derived from mulching include increased water and nutrient availability (Gregoriou & Rajkumar, 1984), improved soil structure and porosity (Gallardo-Laro & Nogales, 1987) and a narrowing in the diurnal soil temperature range (Gregoriou & Rajkumar, 1984). In addition, mulching creates a suppressive environment for *Phytophthora cinnamomi* thus reducing the impact of this phytopathogen (Turney & Menge, 1994). All of these benefits of mulching, together with an adequate assimilate/nutrient supply, sustain fruit growth and development and reduce the incidence of small fruit by reducing the confounding effects of either stress-induced abscisic acid (ABA) accumulation and/or feedback regulation of photosynthesis. Mulching would be expected to increase the proportion of growth promoting hormones (especially cytokinins) relative to inhibitors (ABA), and seed coat viability would therefore be maintained and prolonged. This is known to be critical in permitting the fruit to continue rapid growth, through maintaining anatomical and physiological connections between fruit flesh and the seed (Blumenfeld & Gazit, 1974). Likewise, the incidence of pedicel 'ring neck' which may occur due to elevated xylem ABA levels (Adato & Gazit, 1976), would be reduced in healthy, non-waterstressed trees (Whiley *et al.*, 1986).

The objective of this research was to investigate whether mulching could be a practical cultural method of increasing mean Hass fruit size and overall yield through improved root activity, and to assess whether this practice was a commercially viable option available to growers. The authors have reported on the first two seasons results (Moore-Gordon *et al.*, 1996). This paper summarizes results for a full three seasons.

MATERIALS AND METHODS

The study was conducted using six-year-old (in 1993) Hass trees on clonal Duke 7 rootstocks at a spacing of 7m x 7m. A total of 1,5m³ per tree of coarse composted pinebark (Gromed® coarse potting mix) was applied in February 1993 under the canopy of six trees to a depth of approximately 15cm, and these trees were compared to six adjacent unmulched trees (figure 1). No additions were made to this mulch during the three year duration of the trial.

At harvest, fruit size distributions were determined for each tree and classified according to the number of fruit per standard 4 kg export carton. Total tree yields were calculated by adding the product of the number of fruit per count size and the class centre of all the count sizes.

To determine the effect of pinebark mulching on export potential, fruit was classified into three broad categories:

Highly suitable for export:	Counts 14 – 18
Acceptable for export:	Counts 10 – 12; 20 – 22
Not suitable for export:	Counts ≥ 24

For the breakdown of costs and financial rewards of the pinebark mulch, the following assumptions were made:

- 65% of fruit in the count size range of 10 – 22 were exported.
- Yearly mean on farm returns per hectare were used for the calculations.
- Labour costs of application were not taken into account.
- Potential savings on water and fertilizer bills were not taken into account.

It is important to remember that the cost of transport will obviously vary with distance from source.

RESULTS AND DISCUSSION

Control (unmulched) trees show a typical fruit size distribution

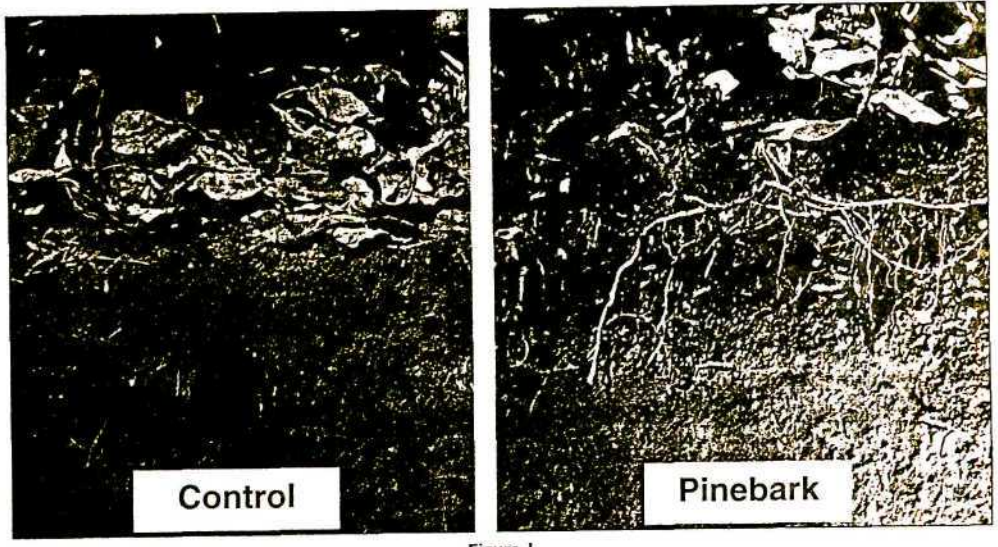

Figure 1
The composted pinebark mulch and control treatment

of the Hass cultivar with many fruit in the count size range of 22 to 26, and a high proportion of factory grade avocados (figure 2). Mulching with pinebark had the effect of shifting the overall count size distribution in favour of large fruit (figure 2). Overall fruit productivity was significantly ($P \leq 0,01$) increased by mulching with composted pinebark, and this positive response was achieved in three successive seasons (table 1). Over the three year duration of the trial, mulched trees produced an average of 22,0 ± 1,2 kg more than control trees, representing a 22,6 % increase in yield. Harvest results also confirmed the biennial bearing nature of cropping in avocado trees. A heavy crop in 1993/1994 was followed by a relatively light crop in 1994/1995, with high yields for the following season (table 1). Assuming that assimilate supply to growing fruit in a season of low yield is limiting, any improvement in resource accumulation and distribution to developing fruit as a result of mulching should considerably enhance fruit productivity.

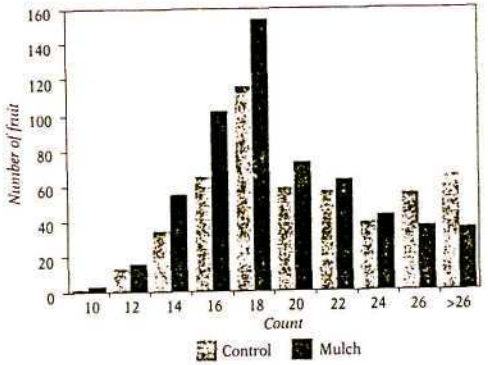

Figure 2
Mean Hass fruit size distribution at harvest. Values are expressed as a mean for each tree averaged over three seasons

Table 1
Summary of the effects of pinebark mulching on Hass avocado fruit quality and quantity

	Control	*Mulch*	*Percentage increase*
1993/1994			
Mean fruit mass (g)	198,0	221,3	11,8**
Yield (t ha⁻¹)	21,2	23,8	18,5**
1994/1995			
Mean fruit mass (g)	178,2	199,2	11,8**
Yield (t ha⁻¹)	9,4	13,4	42,2**
1995/1996			
Mean fruit mass (g)	216,1	220,4	2.0
Yield (t ha-1)	31,7	35,8	18,9**
Overall			
Mean fruit mass (g)	203,1	216,5	6,6**
Yield (t ha⁻¹)	20,0	24,4	22,6**

Data are means of six trees.
**denotes a significant ($p \leq 0,01$) increase in response to mulching

Mulching also resulted in fruit size being significantly ($P \leq 0,01$) increased by an average 13,4 ± 1,2g, and this was achieved in spite of the increase in the number of fruit per tree. This response is particularly significant since problems of fruit size principally arise in trees with heavy crops (Lahav & Kalmer, 1977), as resources available for fruit growth have to be allocated to more sinks. A 12% increase in fruit mass in the first season was achieved despite a yield of over 20 t ha⁻¹ in control trees and nearly 24 t ha⁻¹ in mulched trees. A similar 12% increase in fruit size was obtained in the second season of low yield (9,4 t ha⁻¹ and 13,4 t ha⁻¹ in control and mulched trees respectively). Only in the third season of a very high yield (30,2 t ha⁻¹ and 35,8 t ha⁻¹

respectively), was fruit size not significantly increased. To have maintained an excellent mean fruit size in this season, despite the high yield, was nevertheless remarkable.

Since the South African avocado industry is predominantly export orientated, it would be extremely beneficial to increase the proportion of export quality fruit. The increase in mean fruit mass coupled with elevated yields in response to mulching, resulted in an increase in the number of fruit that meet export requirements for fruit size (table 2). Over the three season duration of the trial, mulching increased the number of fruit that are considered highly suitable for export (counts 14 – 18) by 45%, and in addition the number of fruit that are acceptable for export (counts 10 – 12; 20– 22) by 20%. During the same period the number of fruit that are deemed unsuitable for export was reduced by 29% in the mulch treatment (table 2).

Table2

Summary of the effects of pinebark mulching on export potential related to fruit size

	Control	*Mulch*	*Percentage increase*
1993/1994			
Suitable	200	344	+ 72,0
Acceptable	152	145	- 4,6
Not suitable	157	51	- 67,5
1994/1995			
Suitable	53	117	+ 120,8
Acceptable	71	114	+ 60,6
Not suitable	138	102	- 26,1
1995/1996			
Suitable	374	447	+ 19,5
Acceptable	152	190	+ 25,0
Not suitable	172	177	+ 2,9
Overall			
Suitable	209	303	+ 45.0
Acceptable	125	150	+ 20.0
Not suitable	155	110	- 29.0

Counts 14 – 18 were considered to be highly suitable for export; counts 10 – 12 and 20 – 22 were considered to be acceptable for export; and counts ≥ 24 were considered not to be suitable for export. Figures are mean numbers of fruit per category per tree. Figures preceded by a positive sign indicate an increase by mulching, and figures preceded by a negative sign indicate a decrease by mulching.

The increased yield and mean fruit size coupled with improved export potential as a result of mulching, means that financial rewards to avocado producers could be considerably boosted, although costs of the mulch would have to be off-set. Considering that the half-life of composted pinebark is regarded as five years (Wolstenholme *et al.*, 1996), and that the initial cost of the pinebark were off-set within two seasons (table 3), the application of pinebark or similar mulches provides avocado growers with another option of increasing profitability.

Table 3

Breakdown of costs of and extra revenue generated by the application of a pinebark mulch

1993/94 Cost of pinebark + transport = R26 300 / ha (-R26 300)

Return / ha (On farm)

	Control	Mulch	Extra revenue	
1993/94	R34 700	R47 800	R13 100	(-R13 200)
1994/95	R16 300	R30 100	R13 800	(+R600)
1995/96	R70 500	R85 400	R14 900	(+R15 500)

CONCLUSIONS

Although yield and fruit size are under the control of many interacting factors, and crop failures can be caused by climatic extremes and poor flowering, *inter alia*, this study has shown that mulching, through creating a more mesic root environment and reduced environmental stress, has the potential to substantially increase avocado yield and Hass fruit size. The practice of mulching thus presents avocado producers with an option to increase financial rewards, although cost of the mulch would have to be off-set. Choice of mulch must take into account factors such as cost, availability, C:N ratios and speed of breakdown. The initial expense of the coarse composted mulch used in this trial was off-set by a long life (the half-life is approximately five years), and greatly improved tree performance. It should be noted that these results were obtained in a relatively low stress (more mesic) environment and in a well managed orchard. Benefits of mulching might be greater under more stressful growing conditions.

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