

## ***Metabolic Control of Hass Avocado Fruit Growth***

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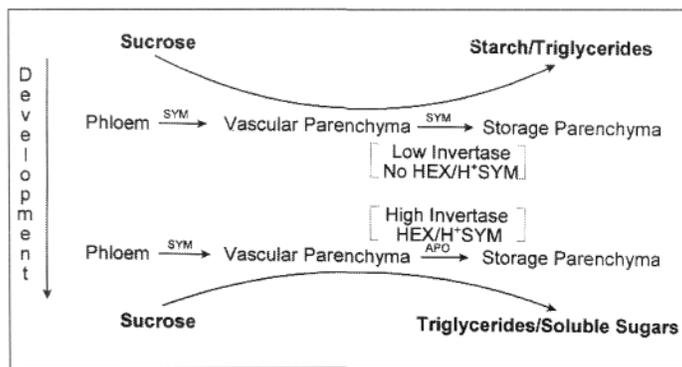
### **ABSTRACT**

To address the physiology of the Hass small fruit problem, the following question was asked: Why are small Hass fruit small? Some obvious possibilities included impaired seed development and the state of health of the seed coat. However, in depth studies confirmed that differences in fruit sizes were a consequence of cell number and therefore, cell division. Thus: Is the Hass small fruit problem symptomatic of a loss of cell division activity or, does loss of cell division arise due to appearance of the small fruit phenotype? A detailed biochemical/physiological study has revealed the importance of isoprenoid metabolism and cytokinin-abscisic acid interaction in the control of avocado fruit growth. Seemingly, cytokinin exerts control over abscisic acid by affecting synthesis of molybdenum-containing cofactors which impacts on carbohydrate concentration and composition via sugar metabolizing enzymes to suppress isoprenoid synthesis and cell division cycle activity. The consequence: elevated fruit abscisic acid turnover and loss of sink strength which contribute to nucellar-seed coat senescence and a slowing or cessation of fruit growth (ethylene-induced fruit drop, in the early stages). It is concluded that regulation of fruit growth is the result of altered purine metabolism which is determined by seed cytokinin homeostasis.

### **INTRODUCTION**

Fruit size is a consequence of cell division and cell expansion, processes that depend upon the importation of carbohydrates synthesized elsewhere in the plant. Recent studies have revealed that the major transportable carbohydrate, sucrose, enters the fruit along vascular traces that permeate the mesocarp and coalesce distally at the funiculus from where sucrose is transported to the seed coat- the major zone of phloem unloading in developing avocado fruit (Moore-Gordon, 1997). It is from within the seed coat that solute (i.e. sucrose and potassium) is distributed to the growing seed and/or mesocarp and the fluxes between each of these terminal sinks is determined by differences in hydrostatic pressure of source and sink tissue, sustained by metabolism and compartmentalisation. A schematic illustrating the currently accepted model of solute flow into developing terminal sinks is shown in figure 1. During the early stages of development, solute flow is symplastic and imported sucrose is stored as starch (triglycerides in avocado). Towards the conclusion of fruit development transport becomes apoplastic and the shift from symplastic to apoplastic transport is associated

with increased extracellular acid invertase activity and accumulation of imported carbohydrate as soluble sugar (Patrick, 1997). Symplastic transport occurs via plasmodesmata and is therefore driven by diffusion along gradients of changing osmotic or solute potential. By comparison, apoplastic transport is an energy requiring process that relies upon activation of a plasma membrane localised hexose/H<sup>+</sup> symporter. Quite clearly these biochemical and physiological events that occur within the major phloem unloading region viz. the seed coat, can and do exercise control over fruit growth and development. For example, breakdown of the integuments (seed coat) is a primary event in the production of seedless avocado fruits (Tomer *et al.*, 1980; Steyn *et al.*, 1993). However, where normal embryo development takes place, a senescent seed coat has been associated with abscission of Fuerte fruit (Blumenfield and Gazit, 1974) and detailed studies have revealed that senescence of the seed coat is responsible for ethylene production by the nucellus-seed coat and that these processes are a prerequisite to avocado fruitlet abscission i.e. fruit drop (Davenport and Manners, 1982). Thus, ethylene is considered by these authors to be the result rather than the cause of nucellar-seed coat senescence. We too have attributed changes in avocado fruit growth to alterations in the physiological state of the seed coat (Cowan *et al.*, 1997a). In fact we have argued that seed coat health is directly related to fruit size and that senescence of the seed coat, at any stage in fruit development, signals cessation of growth and appearance of small or undersized fruit. Thus, we have used seed coat senescence as the basis for a definition of the Hass "small fruit syndrome" (Cowan *et al.*, 1997a). Unfortunately the trigger mechanism responsible for induction of seed coat senescence and hence appearance of small fruit remains to be identified. In an effort to resolve this issue we continue to ask why are small Hass fruit small?



SYM, Symplastic; APO, apoplastic; HEX/H, hexose/proton symporter

Figure 1. Current model describing solute movement into developing fruit illustrating the transition from starch accumulation to accumulation of soluble sugars,

## CELL DIVISION AND HMGR ACTIVITY: SIMULATING THE SMALL FRUIT SYNDROME

Preliminary studies on the kinetics of Hass avocado fruit growth revealed that final fruit size was limited by cell number and not cell size (Moore-Gordon, 1997; Cowan *et al.*,

1997b). The implications of this, and an earlier observation (Schroeder, 1953), were that loss of cell division cycle activity had flattened or eliminated the solute potential gradient between source and sink tissue so that much less or no net movement of sugar from seed coat to mesocarp/seed was occurring (i.e. loss of sink strength). The resultant change in tissue sugar content and composition would be expected to influence the rate of sucrose removal from the seed coat thus lessening sink strength and causing growth of the fruit to slow.

Assuming the developing embryo (seed) is the major sink; a reduction in sink strength might be expected to influence cytokinin levels particularly as this group of plant hormone has been linked to cell division and development of sink strength. Unfortunately, there is insufficient evidence to suggest that a change in cytokinin content and composition is a major contributing factor in the appearance of small Hass fruit (Cutting, 1993). An alternative explanation might therefore be that changes in hormone status are manifest in increased synthesis and/or metabolism of inhibitors such as abscisic acid. The results presented in table 1 clearly show that abscisic acid metabolism in small Hass fruit is substantially greater than in tissues from similarly aged control fruit. Interestingly, no accumulation of abscisic acid was evident in mesocarp tissue of small fruit whereas in seed tissue abscisic acid concentration was increased by >100%. This result might suggest that increased seed abscisic acid is responsible for reducing sink strength and fruit growth. If so, by what mechanism?

Table 1. Metabolism of abscisic acid in phenotypically small and normal Hass avocado fruit.

Tissue	DPA <sup>a</sup>	ABA-GE <sup>a</sup>	PA <sup>a</sup>	ABA-GS <sup>a</sup>	ABA <sup>a</sup>
<i>µg g<sup>-1</sup> Dry Weight</i>					
<b>Mesocarp</b>					
<i>Control</i>	27.10 ± 8.76	4.86 ± 1.17	0.79 ± 0.43	2.39 ± 1.20	21.85 ± 0.45
<i>Small</i>	133.39 ± 15.72	25.33 ± 2.06	ND <sup>b</sup>	3.36 ± 0.34	20.05 ± 3.47
<b>Seed</b>					
<i>Control</i>	1.36 ± 0.04	199.84 ± 23.34	ND	84.35 ± 2.07	14.03 ± 0.79
<i>Small</i>	220.85 ± 26.73	5120.41 ± 539.71	20.50 ± 3.41	1646.45 ± 210.14	29.04 ± 5.91

DPA, dihydrophaseic acid; ABA-GE, abscisic acid glucose ester; PA, phaseic acid; ABA-GS, abscisic acid glucoside; ABA, abscisic acid. ND<sup>b</sup>, not detected;

In an attempt to simulate onset of the small fruit syndrome we injected developing Hass avocado fruit via the pedicel with mevastatin, a competitive inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR). These enzyme catalyses the first step in isoprenoid biosynthesis (i.e. reduction of HMG-CoA to mevalonate) in a pathway that supplies plant hormones (e.g. gibberellins, abscisic acid, brassinolides, cytokinins), pigments (e.g. carotenoids, phytyl side-chain of chlorophyll) and a variety of other essential metabolites required for growth and development. HMGR is also

particularly important for cell division cycle activity. Inhibition of growth by mevastatin, or any other inhibitor of HMGR, can be relieved by co-treatment with mevalonate, the product of the reaction catalysed by HMGR. With regard to Hass avocado fruit growth, this is adequately demonstrated in figure 2. Thus, simulation of the small fruit phenotype can be achieved through inhibition of HMGR. In addition, mevastatin caused seed coat senescence and increased abscisic acid concentration. Also, sugar content and composition of mevastatin-treated fruit resembled closely that of small fruit particularly the decline in sucrose and concomitant rise in glucose content of the seed (figure 3). In all cases the deleterious effects of mevastatin were negated by co-treatment with either mevalonate or the cytokinin, isopentenyl adenine including the mevastatin-induced rise in abscisic acid content of the fruit (Cowan *et al*, 1997b; Moore-Gordon, 1997). Abscisic acid treatment of fruit also reduced growth and induced seed coat senescence and these effects were likewise negated by co-treatment with isopentenyl adenine (Moore-Gordon, 1997). This suggests that the interaction of phytohormones, in particular abscisic acid and cytokinin, is crucial to continuation of fruit growth and development.

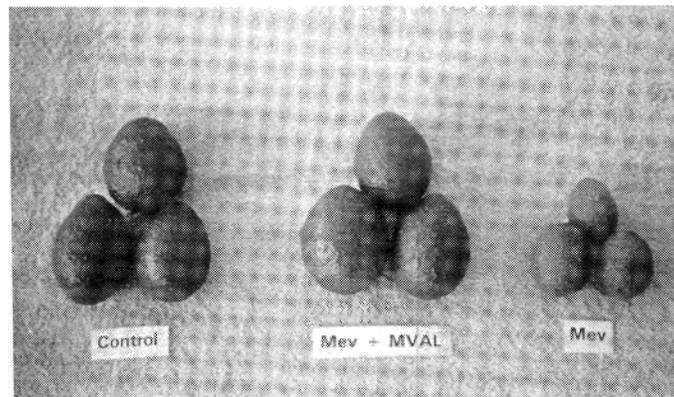


Figure 2. Effect of mevastatin (Mev), injected via the pedicel, on Hass avocado fruit growth, and reversal of mevastatin-induced inhibition of growth by co-treatment with mevalonate (MVAL) the product of the reaction inhibited by mevastatin

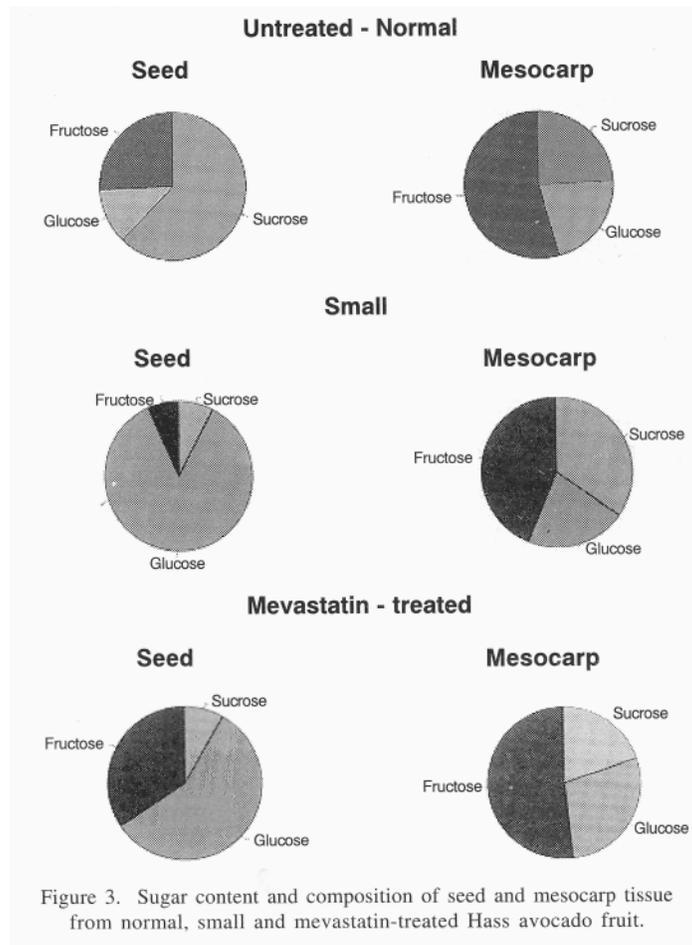


Figure 3. Sugar content and composition of seed and mesocarp tissue from normal, small and mevastatin-treated Hass avocado fruit.

## CARBOHYDRATE TRANSPORT/DISTRIBUTION: HORMONAL VERSUS SUGAR MEDIATION

Detailed analyses of [ $^{14}\text{C}$ ] sucrose movement into developing Hass avocado fruit revealed preferential distribution to the seed coat within 24h in both normal and small fruit (figure 4). Thereafter, radioactivity from sucrose accumulated in the mesocarp of small fruit (figure 4A) whereas in normal fruit, the seed continued to exercise priority for sugar (figure 4B). These observations confirmed that sink strength in avocado is in all probability controlled by the seed and that utilization of sugar within the seed serves to maintain the solute potential gradient between source (seed coat) and sink tissue. Thus, abolishment of sink strength results in the mesocarp assuming sink dominance. Interestingly, the pattern of distribution of radioactivity from [ $^{14}\text{C}$ ] sucrose was identical for both small fruit and abscisic acid-treated fruit, and co-treatment of fruit with isopentenyl adenine plus abscisic acid negated the abscisic acid-induced effect (Moore-Gordon, 1997).

Sugar transport into developing sinks is either symplastic or apoplastic or both. To examine this aspect of avocado fruit growth in more detail both micro-iontophoretic and electron microscope studies were carried out. In brief, the results of this investigation revealed a high degree of symplastic connectivity in untreated Hass fruit tissues,

abscisic acid-induced gating of plasmodesmata and diminution of cell-cell chemical communication, and abolishment of the electrochemical potential gradient between source and sink tissue by abscisic acid (Moore-Gordon, 1997). Similar observations were recorded for the small fruit.

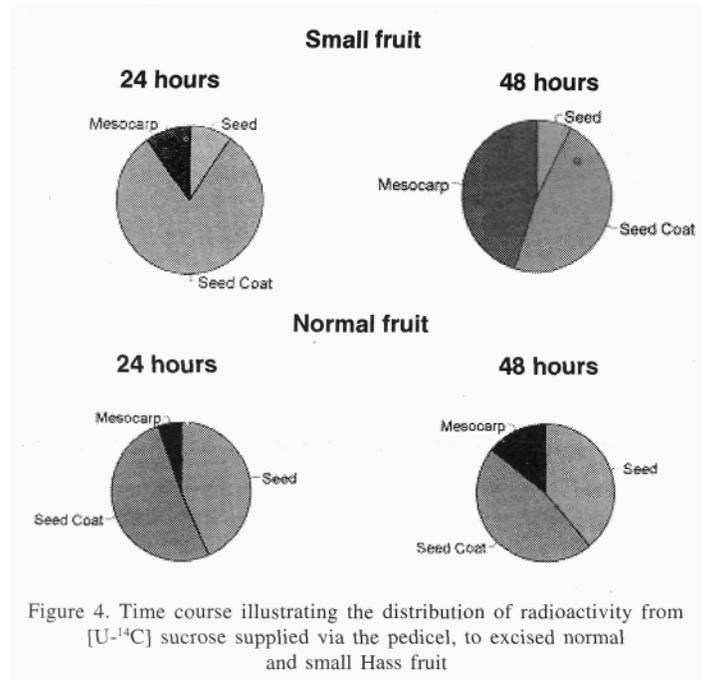


Figure 4. Time course illustrating the distribution of radioactivity from [U-<sup>14</sup>C] sucrose supplied via the pedicel, to excised normal and small Hass fruit

The deleterious effects of abscisic acid treatment were negated in the presence of equimolar isopentenyl adenine indicative of cytokinins-abscisic acid antagonism. Cessation of symplastic solute transport in small and abscisic acid-treated fruit must therefore effect, or be the result of, changes in sugar content and composition.

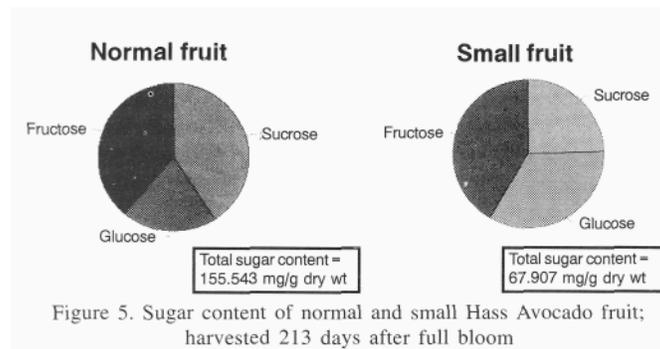


Figure 5. Sugar content of normal and small Hass Avocado fruit; harvested 213 days after full bloom

Bulk sugar content and composition of small and normal Hass fruit is illustrated in figure 5. Total sugar content of small fruit was less than 50% that of the control. While fructose, expressed as percentage of total sugar, was similar for both small and normal

fruit, glucose content of small fruit increased at the expense of sucrose. Analysis of the activity of sugar metabolising enzymes in small and normal 'Hass' fruit revealed a fourfold increase in total activity (i.e. invertase + sucrose synthase + sucrose phosphate synthase) in small fruit and as shown in figure 6, the bulk of this activity was in the form of sucrose synthase (cleavage). By comparison, total invertase and sucrose synthase activities were similar in normal fruit.

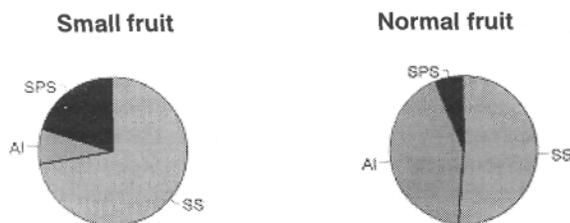


Figure 6. Sucrose synthase (SS), sucrose phosphate synthase (SPS) and acid invertase (AI) activity, expressed as a percentage of total sugar metabolizing enzyme activity, of small and normal Hass fruit.

Both sucrose synthase and invertase catalyse the hydrolysis of sucrose and activity of these enzymes has been correlated with sink functions such as cell division and sugar import and storage (Sung *et al.*, 1994). Similarly, sucrose phosphate synthase plays an important role in the accumulation of sucrose by fruit and does so by converting glucose to sucrose in order to maintain hexose import and thus the solute potential gradient (Miron and Schaffer, 1991). However, high hexose concentrations decrease invertase activity (Weber *et al.*, 1995) and can therefore contribute to reduced sink strength. In fact glucose comprised 85% of the sugars of the seed of small Hass fruit (see figure 3) suggesting glucose suppression of growth. Glucose repression of a gibberellin-dependent signaling pathway in barley embryos has recently been established (Perata *et al.*, 1997) supporting the idea that sugars act as signals to affect plant metabolism and development. Not surprisingly, analysis of HMGR activity revealed a 50% reduction in activity in extracts prepared from seed of small fruit. This, coupled with the measured increase in glucose content of seed from mevastatin-treated fruit (see figure 3) strongly suggests that glucose accumulation and activity of HMGR are interrelated processes. Thus a model linking activity of HMGR, mediated by sugar content and composition, and biosynthesis of cytokinin, formed from mevalonate the product of the reaction catalysed by HMGR was proposed (Cowan, 1997). Briefly, this model suggested that activity of HMGR was mediated by the enzyme HMGR kinase and that the latter was activated or deactivated in accordance with changes in glucose concentration. Since fruit growth and development would be sensitive to changes in either of these processes, the question arises: What is the trigger mechanism responsible for cessation of cell division and appearance of the Hass small fruit variant?

## CYTOKININ-MEDIATED REGULATION OF ABSCISIC ACID

The preceding information and presented data strongly suggests that arrested

isoprenoid biosynthesis, coupled with the subsequent increased level and/or turnover of abscisic acid (and ethylene), plays a significant role in contributing to the appearance of small Hass fruit.

In terms of fruit development, cell division cycle activity and sink strength are synonymous and both require cytokinins and sugars to drive growth. Cytokinin homeostasis is believed to be the result of cytokinin oxidase (specifically isopentenyl adenine oxidase) activity, an enzyme catalysing the oxidation of the isoprenoid side chain at the N<sup>6</sup>-position (Jones and Schrieber, 1997). The product of this reaction sequence is the *nor*-cytokinin, adenine. All studies published to date indicate that unlike cytokinin, adenine either has no effect or stimulates, albeit slightly, abscisic acid biosynthesis/turnover in plants. We have recently demonstrated, contrary to published results, that cytokinin stimulates abscisic acid metabolism (A. K. Cowan & A. L. P. Cairns, unpublished data). The order of efficacy appears to be zeatin > isopentenyl adenine > adenine, i.e. opposite to the proposed sequence of cytokinin formation.

Abscisic acid is produced during increased respiratory activity particularly evident in ripening of avocado fruit and is formed from the C-15 intermediate xanthoxal in a reaction catalysed by an aldehyde oxidase that requires a molybdenum-containing cofactor for full catalytic activity. This type of enzyme (reaction) is negatively affected by adenine and the degree of N<sup>6</sup> substitution of adenine is proportional to the severity of inhibition of aldehyde oxidation. Thus, in theory, cytokinin overproduction should induce symptoms similar to abscisic acid deficiency and this is precisely what has been observed for some species (Deikman, 1997). Cytokinin overproduction is a likely consequence of reduced cytokinin oxidase activity. Alternatively, cytokinin may induce its own formation (Kamínek *et al*, 1997). These authors therefore propose that in response to xylem/phloem-derived cytokinin, cells exhibiting strong positive feedback or weak induction of cytokinin oxidase may develop cytokinin autonomy and/or become meristematic. If positive feedback is based on auto-induction of cytokinin biosynthesis, then such cells would become sites of cytokinin biosynthesis and cytokinin overproduction would ensue. Thus, the half-life of free abscisic acid within the developing fruitlet would be substantially reduced preventing this hormone from fulfilling its normal physiological function. We have recently confirmed that cytokinin does indeed stimulate abscisic acid turnover by influencing the conversion of xanthoxal to abscisic acid and oxidation of the latter to pascic acid and dihydrophaseic acid (A. K. Cowan, A. L. P. Cairns & B. Rahm, unpublished results) so rendering it inactive.

## CONCLUSION

The data summarised in this paper strongly supports the hypothesis that isoprenoid synthesis and sugar metabolism (i.e. changes in sugar content and composition) contribute to the metabolic control of avocado fruit growth. This interrelationship seems to reside at the level of cell division cycle activity, the inhibition of which results in glucose accumulation, preferential suppression of seed HMGR presumably through sugar-induced activation of HMGR kinase and a decline in growth-promoting isoprenoids, particularly cytokinins. Cytokinin homeostasis, which is responsible for sustaining cell proliferation, may be adversely affected and result in either cytokinin

withdrawal and mitotic arrest or cytokinin overproduction and increased abscisic acid turnover which prevents this hormone from fulfilling its normal physiological function.

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## REFERENCES

- BLUMENFELD, A. & GAZIT, S. 1974. Development of seeded and seedless avocado fruits. *Journal of the American Society of Horticultural Science* 99: 442 - 448.
- COWAN, A. K. 1997. Why are Hass fruit small? *South African Avocado Growers' Association Yearbook* 20: 52 - 54.
- COWAN, A. K., MOORE-GORDON, C. S. & WOLSTENHOLME, B.N. 1997a. Defining the Hass small fruit syndrome. *South African Avocado Growers' Association Yearbook* 20: 50 - 51.
- COWAN, A. K., MOORE-GORDON, C. S., BERTLING, I. & WOLSTENHOLME, B. N. 1997b. Metabolic control of avocado fruit growth: isoprenoid growth regulators and the reaction catalysed by 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Plant Physiology* 114: 511 - 518.
- CUTTING, J. G. 1993. The cytokinin complex as related to small fruit in Hass avocados. *Acta Horticulturae* 329: 147 - 149.
- DAVENPORT, T. L. & MANNERS, M. M. 1982. Nucellar senescence and ethylene production as they relate to avocado fruitlet abscission. *Journal of Experimental Botany* 33: 815 - 825.
- DEIKMAN, J. 1997. Elucidating cytokinin response mechanisms using mutants. *Plant Growth Regulation* 23: 33 - 40.
- JONES, R. J. & SCHRIEBER, B. M. N. 1997. Role and function of cytokinin oxidase in plants. *Plant Growth Regulation* 23: 123 - 134.
- KAMÍNEK, M., MOTYKA, V. & VA KOVÁ, R. 1997. Regulation of cytokinin content in plant cells. *Physiologia Plantarum* 101: 689 - 700.
- MIRON, D. & SCHAFFER A. 1991. Sucrose phosphate synthase, sucrose synthase, and invertase activities in developing fruit of *Lycopersicon esculentum* Mill, and sucrose accumulating *Lycopersicon hirsutum* Humb. and Bonpl. *Plant Physiology* 95: 623 - 627.
- MOORE-GORDON, C. S. 1997. Aspects of avocado fruit growth and development: towards an understanding of the small fruit syndrome. Ph. D. Thesis, University of Natal.

- PATRICK, J. W. 1997. Phloem unloading: sieve element unloading and post-sieve element transport. *Annual Review of Plant Physiology and Plant Molecular Biology* 348: 191 - 222.
- PERATA, P., MATSUKURA, C., VERNIERI, P. & YAMAGUCHI, J. 1997. Sugar repression of a gibberellin-dependent signaling pathway in barley embryos. *The Plant Cell* 9: 2197 - 2208.
- SCHROEDER, C. A. 1953. Growth and development of the Fuerte avocado fruit. *Proceedings of the American Society of Horticultural Science* 68: 253 - 258.
- STEYN, E. M. A., ROBERTSE, P. J. & SMITH, D. 1993. An anatomical study of ovary-to-cuke development in considerably low producing trees of Fuerte avocado (*Persea Americana* Mill.) with special reference to seed abortion. *Sexual Plant Reproduction* 6: 87 - 97.
- SUNG, S., SHEIH, W. & BLACK, C. 1994. Growth, sucrose synthase and invertase activities of developing *Phaseolus vulgaris* L. fruits. *Plant Cell and Environment* 17: 419 - 426.
- TOMER, E., GAZIT, S. & EISENSTEIN, D. 1980. Seedless fruit of Fuerte and Ettinger avocado. *Journal of the American Society of Horticultural Science* 105: 341 - 346.
- WEBER, H., BORISJUK, L., HEIM, U., BUCHNER, P. & WOBUS, U. 1995. Seed coat-associated invertases of fava bean control both unloading and storage functions: cloning of cDNA's and cell type-specific expression. *The Plant Cell* 1: 1835 - 1846.