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The Hass small fruit syndrome: solving a 50 million rand per season problem

R.F. Cripps, E.W. Richings, N..J Taylor & A.K. Cowan
Horticultural Science, School of Agricultural Sciences & Agribusiness, University of Natal Pietermaritzburg, Private Bag X01, Scottsville 3209

ABSTRACT

This paper describes the results of studies carried out to determine the underlying biochemical and physiological processes that contribute to Hass avocado fruit growth. To achieve this, we have concentrated our efforts on the following; sugar metabolism, activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and the role of isoprenoids, purine metabolism, and plant hormone homeostasis. An interactive scheme is described to explain the relationship between these processes in the metabolic control of avocado fruit growth. Our findings point to a role for cytokinin overproduction as a causative factor in the initiation of the appearance of small Hass fruit. It suggested that cytokinin overproduction results in an increase in aldehyde oxidase activity with a concomitant increase in reactive oxygen species which together, down regulate HMGR activity and cause a decline in cell division and fruit growth.

INTRODUCTION

It has been estimated that the Hass small-fruit problem costs the South African avocado industry well over R50 million per season in lost revenue. In attempting to address this problem we posed the question: Why are small-fruit small? To answer this question we initially defined the small-fruit syndrome and then sought to examine the underlying mechanisms responsible for the metabolic control of avocado fruit growth in an effort to explain the Hass small-fruit syndrome. The small-fruit syndrome was defined as a physiological phenomenon occurring along the continuum: abortion of embryo growth → seed → coat senescence → cessation of mesocarp cell division → slowing of fruit growth → small fruit, a process that can be initiated at any stage in the Hass fruit development programme. (Cowan *et al.,* 1997b). Cell division cycle activity was confirmed as the major limiting process in the growth of the small-fruit variant (Cowan *et al*., 1997a). Secondly, stimulation of the small-fruit phenotype was achieved by injecting fruit in the linear phase of rapid growth with mevastatin, an inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) responsible for catalysing the committed step in the isoprenoid biosynthetic pathway. A consequent reduction in fruit growth and *in vivo* HMGR activity confirmed the importance of this enzyme and hence, isoprenoid production in the metabolic control of avocado fruit growth (Cowan, 1997;

Cowan *et al.,* 1997a). Detailed hormone analysis revealed higher rates of abscisic acid (ABA) metabolism in the small-fruit variant and in fruit treated with mevastatin. Furthermore, an increase in fruit size was positively correlated with endogenous cytokinin (CK) concentration but negatively correlated with internal ABA content - indicating the possibility that ABA-CK antagonism was responsible, at least in part, for the induction of the appearance of phenotypically small fruit. Confirmation of this antagonism was obtained following analysis of symplastic solute transport in developing fruit which showed that ABA-induced retardation of solute transport was negated in the presence of equimolar CK (Moore- Gordon *et al,* 1998). In more recent work, changes in solute transport were correlated with alterations in sugar metabolism in the small-fruit variant (Richings, Cripps & Cowan, unpublished data). Seed tissue displayed preferential sink strength over the mesocarp, increased insoluble acid invertase activity, sucrose depletion, and apparent accumulation of glucose relative to total soluble sugars. Similar observations were recorded for seed tissue from ABA- and mevastatin-treated fruit suggesting a relationship between fruit growth (size), and seed HMGR activity and sugar metabolism.

The question therefore arises: Are the aforementioned biochemical changes causative in the appearance of the small-fruit or, do they arise as a result of expression of the small-fruit phenotype? To address this issue we are currently investigating the following aspects as they relate to avocado fruit growth and development:

1. sucrose metabolism,
2. the relationship between sugar and hormone signaling,
3. the contribution of purine metabolism,
4. the biochemical basis for ABA-CK antagonism.

In pursuing these lines of investigation we have been able to elucidate a sequence of metabolic events that, once initiated, would seem to culminate in a slowing of Hass avocado fruit growth resulting in the appearance of the so-called small-fruit variant. In addition, we have utilized these biochemical processes to evaluate the suitability of a novel plant growth substance (comprising a mixture of molybdenum and allopurinol) for use in avocado cultivation - in particular for the stimulation of flowering and fruit set. Results indicate that a single application of the aforesaid plant growth regulator increases flowering and fruit set, and that it has the potential to improve fruit quality including size.

SUCROSE METABOLISM IN SMALL AND NORMAL HASS AVOCADO FRUIT

Sucrose is generally regarded as the primary sugar transported in most flowering plants (Sung *et al.*, 1998). Thus, it is assumed that the metabolism of this sugar in developing fruits influences the sink strength of these organs. Three enzymes are commonly associated with sucrose metabolism and the control of sink strength, viz. invertase, sucrose synthase and sucrose phosphate synthase. Invertase hydrolyses sucrose to glucose and fructose, and has been associated with carbohydrate influx (Estruch and Beltrán, 1991), sugar accumulation (Sonnewald *et al.,* 1991) and growth (Miller and Chourey, 1992). Sucrose synthase catalyses the interconversion of sucrose and UDP-

glucose and fructose, and plays a role in determining the rate of carbohydrate flow into fruit (Ho, 1996), starch accumulation (Déjardin *et al*., 1997) and dry matter accumulation (Deminitz-King *et al*, 1997). Sucrose phosphate synthase catalyses the synthesis of sucrose from UDP-glucose and fructose-6-phosphate, and allows for the maintenance of a sugar gradient from the outside to the inside of sink cells (Miron and Schaffer, l991), permitting transport, compartmentalization, and sugar accumulation (Klann *et al*., 1993).

Our research has shown that sugar metabolizing enzyme activity is greater in tissues of normal Hass fruit. Small and ABA-treated fruit appear to show either a reduction in, or a change in distribution of, enzyme activity. Acid invertase is the most active sucrose metabolising enzyme in avocado, with the insoluble form predominating. In the small-fruit variant, seed tissue has significantly higher acid invertase activity which contrasts with normal fruit wherein the mesocarp has highest acid invertase activity. Treatment with ABA appears to mimic the small-fruit in that soluble acid invertase activity of mesocarp and seed coat tissue is greater than in untreated fruit. Other studies have shown that the breakdown products of sucrose metabolism (i.e. glucose and fructose), like plant hormones, have the ability to modulate gene expression (Zhou *et al*., 1998) and change many sink related processes including, carbohydrate storage, sugar utilization, and fruit growth (Koch, 1996). Based on this assumption it is possible that changes in sugar metabolizing enzyme activity in Haas avocado fruit tissue impacts on fruit growth through altered hexose sugar content and composition. Thus, appearance of the small-fruit phenotype might be expected to occur concomitant with changes in sucrose metabolism which may or may not parallel changes in hormone content.

Table 1. ***Sugar content (mg g dry weight $^{-1}$) and composition (% of total) of seed and mesocarp tissue from developing normal and small Hass avocade fruit.***

Type	Tissue	Sugar content (mg g DW $^{-1}$)			
		Sucrose	Glucose	Fructose	Total
Normal	Seed	32 783 (79)	2.241 (5)	6.574 (16)	41.598 (100)
	Mesocarp	14 090 (54)	4.827 (19)	6.904 (27)	25.821 (100)
Small	Seed	0 240 (1)	12.419 (77)	3.454 (22)	16.113 (100)
	Mesocarp	10.818 (51)	4.139 (19)	6.389 (30)	21.346 (100)

A detailed analysis of soluble sugar content and composition in seed and mesocarp tissue of normal and small Haas fruit showed that differences were most pronounced in the seed tissue (Table 1). The total sugar content was substantially reduced in the seed of the small-fruit variant and glucose accounted for 77% of the total soluble sugars

compared to 5% in the seed of normal fruit. The seed of the small-fruit variant also showed a marked reduction in sucrose content and sucrose only accounted for 1% of the total soluble sugars, compared to 79% in the seed of normal fruit. These results closely resembled those obtained in the 1996/1997 season i.e. glucose as a proportion of the total soluble sugar in the seed of small fruit was increased substantially, presumably due to sucrose depletion (Cowan *et al*., 1998).

SUGARS AND HORMONES: SIGNALS IN FRUIT GROWTH AND DEVELOPMENT

As mentioned above, both HMGR activity and isoprenoid synthesis are essential for normal fruit development. HMGR kinase is the enzyme thought to modulate activity of HMGR and is a member of the SNF1-related protein kinase family, a group of enzymes that is apparently activated or deactivated in response to changes in glucose concentration (Barker *et al*., 1996). Thus, it was hypothesized that reduced HMGR activity might be expected to occur concomitant with changes in glucose concentration. Since sugars and hormones have been implicated in the control of plant growth and development (De Wald *et al.,* 1994; Dijkwe *et al*., 1997; Mason *et al.,* 1992; Mita *et al.,* 1997; Perata *et al.,* 1997), an interaction between sugars, hormone metabolism and HMGR activity in the control of avocado fruit growth seemed likely.

Table 2. ***Sugar content (mg g dry weight $^{-1}$) and composition (% of total) of seed and mesocarp tissue from developing Hass avocado fruit treated with and without mevastatin.***

Type	Tissue	Sugar content (mg g DW $^{-1}$)			
		Sucrose	Glucose	Fructose	Total
Control	Seed	29.639 (83)	1.622 (4)	4.491 (13)	35.752 (100)
	Mesocarp	3.277 (13)	10.002 (410	11.395 (46)	24.674 (100)
Mevastatin	Seed	20.983 (42)	8.730 (17)	20.346 (41)	50.059 (100)
	Mesocarp	5.899 (27)	7.588 (35)	8.287 (38)	21.774 (100)

Treatment of Haas avocado fruit with mevastatin (a competitive inhibitor of HMGR activity) has been shown to reduce fruit growth, decrease HMGR activity and increase ABA content (Cowan ef a/., 1 997b). Thus, mevastatin-treatment results in characteristics associated with the Haas small- fruit phenotype. As shown in Table 2, mevastatin treatment also induced major changes in the content and composition of soluble sugars in the seed tissue. Glucose concentration was substantially increased in the seed of mevastatin-treated fruit and in- creased as a percentage of total sugars from 4 to 1 7%, while the sucrose content and percentage of sucrose, relative to the total sugar content of the seed, was reduced. These results suggested that an interaction

between increased glucose (relative to total soluble sugars) and/ or sucrose depletion (Table 1), increased ABA metabolism (Cowan *et al.,* 1 998) and a reduction in HMGR activity (Cowan ef a/., 1 997b) were important factors in the control of Haas avocado fruit growth. To further investigate the potential hormone- sugar interaction, the effect of glucose on HMGR activity, CK content and ABA metabolism was investigated. The data presented in Figure 1, shows that within 6 hours of glucose application, HMGR activity of seed and mesocarp tissue had declined by 52 and 38% respectively. By 24 hours, HMGR activity of glucose-treated seed tissue was still approximately 20% below that of the control, while by 48 hours HMGR activity was restored. Thus, a change in sugar content and composition caused a transitory decline in HMGR activity which seems to suggest that increased glucose content of seed of the small-fruit (Table 1) is linked to reduced HMGR activity. The results in Table 3 show that as the concentration of applied glucose was increased, ABA and dihydrophaseic acid (DPA) levels declined while CK levels increased in the mesocarp of ripening Hass avocado fruit. Since DPA is the major acidic catabolite of ABA in avocado (Hirai and Koshimizu, 1 983), glucose application appeared to enhance ABA metabolism which was thus negatively correlated to the effect of glucose on CK content. Taken together, and in view of the discussion outlined below, further evidence in support of an antagonistic relationship between CK and ABA was thus proposed. However, this relationship seems to be indirect and may be due to both ABA and CK sharing, at least in part, a common biosynthetic origin. ABA and auxin production is also thought to be linked as the enzyme(s) controlling the final step in the biosynthesis of these plant hormones is related (Sagi *et* a/., 1998). To what extent this interrelationship involves the gibberellins (GA) is currently unknown. Nevertheless, one potential indirect effect of changes in glucose content in developing avocado fruit might include alternations in plant hormone (CK, ABA and auxin) homeostasis and the subsequent appearance of phenotypically small fruit.

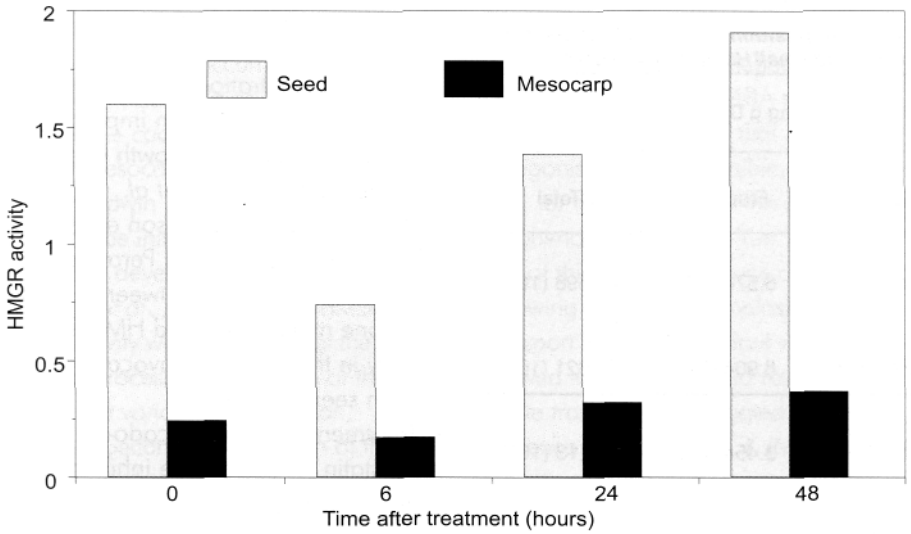

Figure 1: Effect of glucose on seed and mesocarp HMGR activity in Hass avocado fruit

PURINE METABOLISM AND HASS FRUIT SIZE

In order to investigate the relationship between CK, ABA and auxin in avocado fruit, the activity of key enzymes involved in the synthesis and metabolism of these hormones

was determined. Firstly, cytokinin oxidase (CKOX) activity was measured. This enzyme is responsible for the irreversible breakdown of CK and thus plays an important role in regulating CK homeostasis in plant tissue (Motyka ef a/., 1 996). Secondly the enzyme xanthine dehydrogenase (XDH) involved in purine metabolism, was measured. This enzyme catalyzes the conversion of hypoxanthine and xanthine to uric acid (Perez-Vicente ef a/., 1 988). Thirdly, activity of aldehyde oxidase (AO) was determined. This enzyme catalyzes the last step in the biosynthesis of auxin and ABA (Sagi et a/., 1998). Both AO and XDH require a molybenum cofactor (MoCo) for full catalytic function. MoCo is derived from the purine metabolic pathway (Rajagopalan, 1 997) and thus any change in purine metabolism will impact an AO activity and auxin and ABA production. Changing environmental conditions can also impact on the relative allocation of the MoCo to these two enzymes (Lips, 1 997). Lastly, the activity of auxin oxidase (IAAOX) was determined. This enzyme is thought to regulate the level of indole-3- acetic acid (IAA) in tissues (Milrad de Forchetti and Tigier, 1983) which is important as both IAA and CK have been implicated in the control of plant cell division - the limiting factor responsible for appearance of the small-fruit variant.

Figure 2 shows that CKOX, XDH and AO activity are greater in the small-fruit. In contrast, IAAOX activity was greatest in normal fruit. These results suggest that there is increased turnover of CK in small fruit, together with an increased rate of purine metabolism and ABA production. IAA content is presumably reduced in normal fruit. The increase in CK and purine metabolism and ABA content, combined with decreased levels of IAA would seem to suggest that changes in phytohormone homeostasis do occur coincident with alterations in cell division cycle activity in avocado fruit and that an imbalance in hormone content can contribute to the appearance of the small-fruit. Thus, maintenance of CK and IAA homeostasis combined with increased ABA turn- over, leading to ABA breakdown, might be expected to sustain cell division cycle activity and fruit growth. This might best be achieved by manipulating some of the key enzymes thought to be involved in the control of phytohormone (CK, ABA, IAA and GA) homeostasis.

ABSCISIC ACID-CYTOKININ INTERACTION

The information outlined above suggested ABA-CK antagonism as a mitigating factor in the appearance of the Hass small-fruit variant. However, the underlying biochemical mechanism was unknown. In studies on the control of dormancy and germination in cereals we had previously demonstrated a role for molybdenum in the alleviation of pre-harvest sprouting, implying involvement of a MoCo- containing AO enzyme in this process. This investigation was extended to include allopurinol, an inhibitor of XDH activity, which appeared to enhance the effect of molybdenum presumably by impacting on ABA metabolism. We then turned our attention to avocado primarily because of its well known ability to accumulate ABA during ripening - in order to investigate in more detail the biochemistry of the proposed ABA-CK interaction. The results of his study (Cowan ef a/., 1 999) are summarized in Figure 3. In brief, the three major proteins that incorporate molybdenum as the MoCo are illustrated in relation to CKOX. The scheme proposes that elevated CKOX activity increases the adenine content of tissue leading to inhibition of XDH. As a consequence, there is a build up of CK by feedback. Also,

increased amounts of MoCo become available for incorporation into the AO for ABA (and IAA) biosynthesis. Since ABA induces its own breakdown there is enhanced ABA turnover but sustained or increased levels of CK and IAA.

Table 3. Effect of glucose on CK, ABA and DPA content of mesocarp of ripening Hass avocado fruit.

	CK	ABA	DPA
Treatment	ng (g dry weight)$^{-1}$	µg (g dry weight)$^{-1}$	
Control	16.480	7.810	5.430
+ 0.5 µmol glucose	23.100	5.290	1.670
+ 1.0 µmol glucose	25.740	4.250	1.800
1.5 µmol glucose	41.190	0.070	1.070

Results are the mean of two experiments. Data for ABA and DPA are expressed as net increase relative to values obtained from mesocarp at t_0 (i.e. $t_{24} - t_0$)

A byproduct of AO-catalyzed reactions is reactive oxygen (i.e. free radical oxygen) which interferes with HMGR resulting in reduced activity and a slowing of cell division while increasing the possibility of cell death. Our recent results demonstrate that a range of free radical generating agents reduces HMGR activity *in vivo* and in vitro (Richings and Cowan, unpublished results). Thus, reactive oxygen species have the potential to arrest cell division and fruit growth.

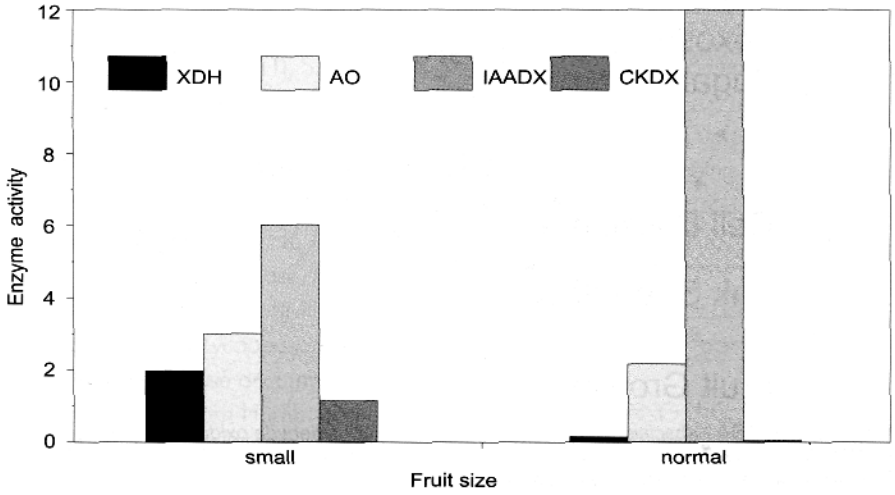

Figure 2: Activity of xanthine dehydrogenase (XDH), aldehyde oxidase (AO) auxin oxidase (IAAOX) and cytokinin oxidase (CKOX) in normal and small Hass avocado fruit

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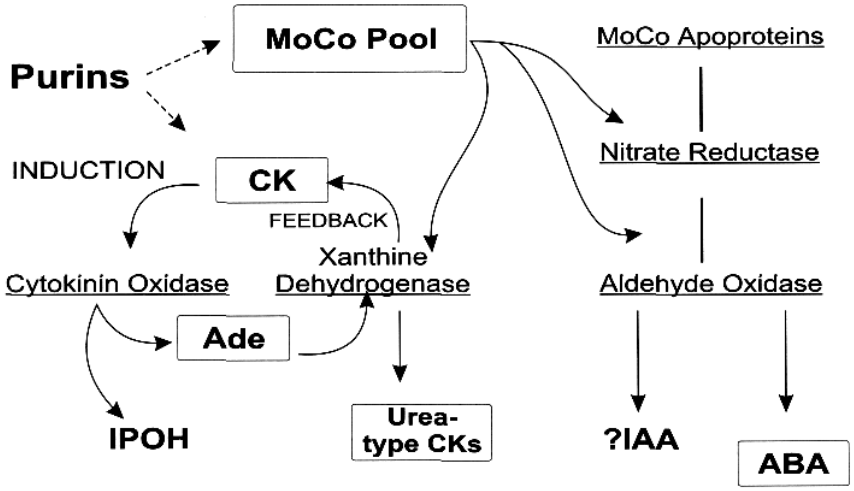
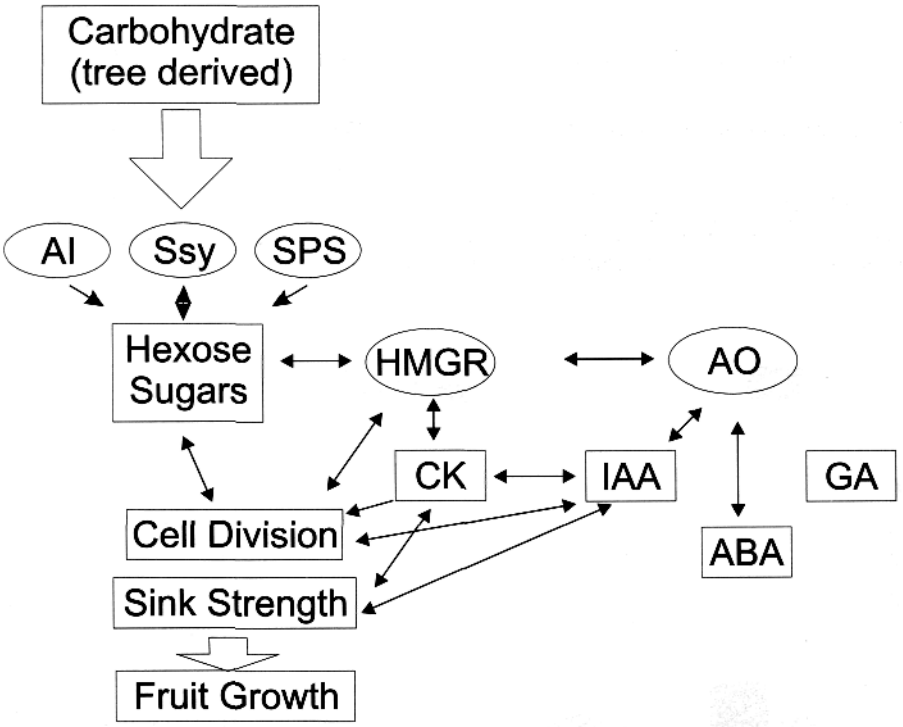
CONCLUSION

Taken together, the results of our studies have enabled us to propose an integrated scheme to explain the metabolic control of Haas avocado fruit growth (Figure 4). This scheme illustrates the major physiological processes in relation to potential regulatory molecules considered to be important in the control of fruit growth. Thus, hexose sugars and plant hormones are assigned major roles in this developmental programme and are linked via the activity of sugar metabolizing enzymes. HMGR and AO. Based on our observations, activity of HMGR is crucial in the metabolic control of fruit growth. It is possible that the following sequence of events once initiated, results in the appearance of the small-fruit: induction of CKOX which, via increased formation of adenine, regulates activity of AO by increasing the amount of MoCo for sulfur/lotion. Increased AO activity stimulates ABA turnover and reactive oxygen species production which adversely affects HMGR. Down regulation of HMGR results in reduced cell division, slowing of growth and cell death (particularly evident in the embryo and seed coat of phenotypically small fruit). It would thus appear that induction of CKOX activity could be the major causative factor of the Hass small fruit syndrome. Although CKOX is CK-induced, implying that CK overproduction is responsible for the small-fruit syndrome, the origin of this CK is currently unknown. Perhaps the excess CK is produced by microbial symbionts of Hass avocado - in particular, organisms such as the *pink-pigmented* facultative meffiyltropbs or PPFMs (Holland, 1997; Holland and Polacco, 1994).



Abbreviation: ABA - Abscisic acid; ADe - adenine; CK - cytokinin; IAA - auxin; MoCo - molybdenum co-factor

Figure 3: Proposed scheme illustrating the biochemical interaction between HBA and Cytokinin metabolism in Hass avocado fruit.



Abbreviations: ABA - abscisic acid; AI - acid invertase; AO - aldehyde oxidase; GA - gibberellin; HMGR - 3-hydroxy, 3-methylglutaryl coenzyme A reductase; IAA - auxin; CK - Cytokinnin; SPS - sucrose phosphate synthase; Ssy - sucrose synthase

Figure 4: ***Scheme illustrating the interaction between the major regulatory components in the metabolic control of Hass avocado fruit growth.***

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