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Developmental differences in antioxidant compounds and systems in normal and small-phenotype fruit of 'Hass' avocado (*Persea americana* Mill.)

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ABSTRACT

Antioxidant compounds and reactive oxygen-scavenging systems were investigated in 'Hass' avocado fruit set by determinate (DFS) and indeterminate floral shoots (IFS). During early development, DFS produced the largest fruit, IFS the smallest. Normal-phenotype (large) fruit (NPF) from DFS and IFS were faster growing, greater in length, diameter and weight and contained more protein than small-phenotype fruit (SPF) from DFS and IFS, resulting in SPF having greater ascorbic acid and total phenol concentrations and antioxidant capacity per g fresh weight. In contrast, NPF had greater catalase, ascorbate peroxidase, and glutathione reductase activities per g fresh weight than SPF on a greater number of sample dates; SPF had greater superoxide dismutase activity. Per mg protein, ascorbic acid and phenol concentrations, antioxidant capacity, peroxidase, polyphenol oxidase, catalase and ascorbate peroxidase activities were greater in SPF than NPF on more dates and at maturity. Thus, SPF would be a healthier food choice due to their greater antioxidant compounds and capacity compared to NPF.

1. Introduction

The 'Hass' avocado (*Persea americana* Mill.) dominates the avocado industry worldwide (Garner et al., 2011). 'Hass' avocado fruit are attractive to consumers not only for their aroma, taste, color and texture, but also for their nutritional value and health benefits. Thus, the chemical composition (mineral, protein, vitamin, fatty acid, and antioxidant content) of the fruit is important. Avocados are nutrient dense. They contain more protein and potassium than most fruits and many compounds with antioxidant activity, such as phenolics, ascorbic acid, vitamin E and carotenoids (Bertling et al., 2007; Dreher and Davenport, 2013; Silva et al., 2002; Wang et al., 2012) that quench free radicals and thus, prevent oxidative damage detrimental to human health (Sun et al., 2002). In recent years, more attention has been paid to the antioxidants contained in fruit because epidemiological studies have documented that antioxidants are associated with the reduced incidence of cardiovascular diseases, diabetes and some types of cancer (Davì et al., 2010; Toor et al., 2006). Specifically, research has provided evidence that phytochemicals found in avocado fruit inhibit the growth of cancerous tumors, mitigate obesity and diabetes, and reduce cardiovascular disease (Devalaraja et al., 2011; Ding et al., 2007; Dreher and Davenport, 2013). Thus, developing strategies to enhance avocado fruit antioxidant composition has become a goal of avocado industries globally as a means to promote avocado consumption.

To this end, studies have been conducted to determine the impact of maturity, ripening and post-harvest storage on the biochemistry of various antioxidants in avocado fruit (Lu et al., 2009; Villa-Rodríguez et al., 2011; Wang et al., 2012). When fruit were harvested at early maturity, they retained greater concentrations of antioxidants, even after being stored for an extended period (35 days compared to 21 days), than mature fruit harvested later in the season (Wang et al., 2012). Producing avocado fruit with greater antioxidant concentrations pre-harvest and harvesting early would translate to greater antioxidant concentrations post-harvest and improve the associated health benefits of avocado fruit for the consumer. Despite this, factors influencing antioxidant synthesis and accumulation during avocado fruit development remain largely unknown. This lack of knowledge precludes our ability to manipulate the antioxidant composition of developing avocado fruit to increase the health benefits. Keeping in mind that the role of antioxidants is to protect the developing fruit from reactive

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oxygen species (ROS) generated during normal oxidative metabolism, and at dramatically increased rates during stress (drought, high light intensity, abnormally high and low temperatures, ultra-violet radiation and pathogens) (Mittler, 2002), this lack of knowledge also compromises our ability to increase fruit growth and size, especially when adverse climatic conditions prevail.

'Hass' avocado trees produce two populations of fruit, large normal-phenotype fruit (NPF) and phenotypically small fruit (SPF) (Richings et al., 2000). The small fruit variant is limited by cell number, not by cell size, with determinants of mitosis and cell division playing key roles in final fruit size (Dahan et al., 2010). Of importance to the present study, ROS serve critical functions in plant signaling that control growth, including mitosis and cell division, developmental events, and responses to both abiotic and biotic stresses (Livanos et al., 2012a,b). Common ROS are scavenged by enzymes, e.g., superoxide radical by superoxide dismutase (SOD), hydrogen peroxide by peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) via the ascorbate-glutathione cycle, requiring glutathione reductase, and non-enzymatically, e.g., hydroxyl radicals and singlet oxygen by metabolites such as ascorbate, carotenoids and phenols (Bertling et al., 2007; Huang et al., 2007). In addition, polyphenol oxidase (PPO), though not considered part of antioxidant systems, plays an important role in oxidative processes, especially during stress (Polovnikova and Voskresenskaya, 2008). During stress, ROS homeostasis is disrupted by the increased production of ROS (Mittler, 2002) that damage proteins, DNA, RNA, and lipids and negatively impact mitosis and cell division (Livanos et al., 2012a,b).

Thus, the objectives of this research were to determine the following: (*i*) whether differences existed in the antioxidant concentrations and/or activity of reactive oxygen-scavenging systems between NPF and SPF of the 'Hass' avocado from early development to early maturity; (*ii*) whether these differences provided evidence of a compromised capacity of the small fruit variant to scavenge ROS or that the small size of the fruit had a concentrating effect on antioxidant accumulation, capacity and enzyme activity; and (*iii*) whether fruit growth rate and size were related to the type of floral shoot (determinate or indeterminate) on which the fruit developed, providing evidence consistent with the vegetative shoot apex of the indeterminate floral shoot serving as a source of metabolites promoting fruit development or a competing sink that reduced fruit growth. The overall goal of the research presented herein was to identify developmental factors and phenological events influencing antioxidant concentrations of 'Hass' avocado fruit pre-harvest in order to subsequently be able to harvest fruit with more antioxidants and greater health benefits for consumers.

2. Material and methods

2.1. Plant material

Avocado fruit (*Persea americana* Mill., cv. Hass) were collected from bearing trees on Mexican seedling rootstocks in a commercial orchard located in Irvine, California, USA (Latitude: 33°40′10″ N; Longitude: 117°49′23″ W; Elevation: 22 m above sea level). The study was carried out over 2 years. Due to the low percent fruit set of the 'Hass' avocado, 100 determinate (DFS) and 100 indeterminate floral shoots (IFS), randomly selected at a height of 1.2–1.5 m above ground around the tree, were tagged on three separate sets of 40 trees (three replications, 120 trees total) in a completely randomized design. Fruit collection began 45 days after full bloom (DAFB; full bloom occurred in April of each year) through 300 DAFB (early fruit maturity; 20.8% dry matter content) (Dixon, 2013). Note that mature avocado fruit do not undergo ripening while on the tree. A minimum of 20 fruit of similar age was collected for each phenotype from each floral shoot type on each sampling date from the three separate sets of trees (three replications), with a minimum of 20 trees represented in each replication. At early collection dates, when fruit were small, more than 20 fruit were collected to have sufficient biomass for all the analyses. Collected fruit were placed in plastic bags, containing moist paper toweling, and the plastic bags were placed in a cooler box for transport from the orchard to the lab at the University of California-Riverside for immediate processing.

2.2. Fruit biometrics

For 20 fruit in each of three replicate samples, fresh weight (FW) was determined, length and transverse diameter were measured with an electronic caliper, and dry weight (DW) was determined after each fruit was dried to a constant weight using a microwave oven (Lee et al., 1983). Fruit growth rate was calculated based on the change in FW from one sample date to the next. Soluble protein concentration was determined in 1 g FW of avocado mesocarp tissue (the edible portion of the fruit) homogenized in distilled water (or the buffer specified below) using the Bradford method (Bradford, 1976).

2.3. Analysis of fruit antioxidant compounds

Ascorbic acid concentration was determined by the method of Kyaw (1978). To prepare the color reagent, a solution of sulfuric acid (5 ml) and water (15 ml) was poured slowly into a 30 ml aqueous solution containing sodium tungstate (20 g) and disodium hydrogen phosphate (10 g), and boiled gently for 2 h. Avocado mesocarp tissue (1 g FW) was homogenized in 4 ml of distilled water and then centrifuged at 10,000*g* for 20 min (4 °C), and 2 ml of the supernatant was added to 2 ml of color reagent and mixed thoroughly. The solution was left at room temperature for 30 min and then centrifuged at 10,000*g* for 5 min, the absorbance at 700 nm was determined relative to a standard ascorbic acid concentration curve and the results were expressed as mg 100 g^{-1} FW or mg^{-1} protein.

Total phenol concentration was measured using the Folin-Ciocalteu method (Spanos and Wrolstad, 1990). Avocado mesocarp tissue (1 g FW) was homogenized in 4 ml of distilled water and then centrifuged at 10,000*g* for 20 min (4 °C), and 0.02 ml of the supernatant was combined with 1.58 ml of distilled water and then 0.1 ml of Folin-Ciocalteau's reagent. The reaction was neutralized by adding 0.3 ml of 20% (w/v) sodium carbonate and incubated at 75 °C for 10 min, at which time the absorbance at 760 nm was determined. Gallic acid was used for the standard concentration curve, and results were expressed as mg gallic acid equivalents (GAE) 100 g^{-1} FW or mg^{-1} protein.

The ferric reductive ability of plasma (FRAP) assay was used to quantify the capacity of the mesocarp to reduce Fe^{3+} to Fe^{2+}, which is a direct measure of the concentration of electron-donating antioxidants (antioxidant capacity, AOC) of a tissue (Halvorsen et al., 2002). The FRAP assay was carried out according to the modifications of Thaipong et al. (2006). To prepare the FRAP reagent, a solution of 0.3 mM sodium acetate (pH 3.6), 10 mM 2,4,6-tripyridyl-2-triazine (TPTZ) and 20 mM ferric chloride (10:1:1, v/v/v) was made. An aliquot of 0.06 ml of avocado mesocarp extract (0.25 g ml^{-1} distilled water) was added to 1.8 ml of FRAP reagent and mixed thoroughly. After the reaction had been left at 37 °C for 10 min, the absorbance at 593 nm was determined. Calibration was based on concentrations of ferrous ion from 25 to 1600 μM using freshly prepared ammonium ferrous sulfate. Results for AOC were reported as μmol Fe^{2+} g^{-1} FW or mg^{-1} protein.

Table 1
Effect of determinate (DFS) and indeterminate floral shoot (IFS) type on the diameter (mm) and length (mm) of normal-phenotype fruit (NPF) versus small-phenotype fruit (SPF) of the 'Hass' avocado from 45 to 300 days after full bloom (DAFB).

Floral shoot and fruit type	Days after full bloom							
	45		100		160		300	
	Diam.	Len.	Diam.	Len.	Diam.	Len.	Diam.	Len.
DFS NPF	11.9 a[z]	16.3 a	43.6 a	62.5 a	55.8 a	74.1 a	64.6 a	89.8 a
IFS NPF	10.7 b	13.4 b	42.0 a	56.8 b	54.8 a	77.2 a	64.7 a	91.4 a
DFS SPF	5.6 c	7.4 c	29.7 b	39.8 c	38.1 b	54.4 b	49.4 b	69.2 b
IFS SPF	5.1 d	6.4 d	25.0 c	35.5 d	39.4 b	54.7 b	48.1 b	66.4 b
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[z] Mean values for 20 fruit of each phenotype for each floral shoot type collected from three separate sets of trees (three replications) within a vertical column followed by different letters are significantly different at specified *P* values by Fisher's Protected LSD Test.

2.4. Analysis of reactive oxygen-scavenging systems

Avocado mesocarp tissue (2 g FW) was homogenized in 4 ml of cold 100 mM sodium phosphate buffer (pH 7.5), containing 2% (w/v) polyvinylpolypyrolidone (PVPP). The homogenate was centrifuged at 10,000*g* at 4 °C for 20 min and the supernatant was collected and used in the analyses described below.

Peroxidase (POD) (EC 1. 11. 1. 7) activity was analyzed according to the method of Lurie et al. (1997). The reaction solution contained 50 mM phosphate buffer (pH 7.8) (2 ml), 25 mM guaiacol (500 μl), 500 mM H_2O_2 (200 μl) and mesocarp extract (100 μl). The reagent blank used 50 mM phosphate buffer (pH 7.8) (100 μl) in place of the mesocarp extract. Activity of POD was expressed as an increase in absorbance at 470 nm g^{-1} FW min^{-1} or mg^{-1} protein min^{-1}.

Polyphenoloxidase (PPO) (EC 1. 14. 18. 1) activity was analyzed according to the method of Lurie et al. (1997). The reaction solution contained 50 mM phosphate buffer (pH 7.8) (2.5 ml), 50 mM *o*-dihydroxybenzene (0.5 ml) and avocado mesocarp extract (100 μl). In the blank, 50 mM phosphate buffer (pH 7.8) (100 μl) replaced the mesocarp extract. One unit of PPO activity was defined as a change in absorbance at 420 nm g^{-1} FW min^{-1} or mg^{-1} protein min^{-1}.

Superoxide dismutase (SOD) (EC 1. 15. 1. 1) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT), according to the method of Larrigaudiere et al. (2004). The reaction solution (3 ml) contained 50 mM phosphate buffer (pH 7.8) (1.7 ml), 130 mM methionine (MET, 0.3 ml), 750 μM NBT (0.3 ml), 100 μM ethylene diaminetetracetic acid disodium salt dihydrate (EDTA-Na_2) (0.3 ml), 20 μM riboflavin (0.3 ml) and avocado mesocarp extract (100 μl). The blank utilized 50 mM phosphate buffer (pH 7.8) (100 μl) in place of mesocarp extract. The reaction solutions and one blank were placed in the light and a second blank was placed in the dark, each for 15 min, at which time absorbance at 560 nm was determined. One unit (U) of SOD was considered to be the amount of enzyme corresponding to 50% inhibition of the reaction. Results were expressed as units 100 g^{-1} FW min^{-1} or mg^{-1} protein min^{-1}.

Catalase (CAT) (EC 1. 11. 1. 6) activity was measured according to Larrigaudiere et al. (2004). The reaction solution contained 20 mM hydrogen peroxide (H_2O_2) (2.9 ml) in 100 mM phosphate buffer (pH 7.5) and avocado mesocarp extract (100 μl). The activity of CAT was expressed as a decrease in absorbance at 240 nm g^{-1} FW min^{-1} or mg^{-1} protein min^{-1}.

Ascorbate peroxidase (APX) (EC 1. 11. 1. 11) activity was determined spectrophotometrically according to the method of Nakano and Asada (1981). The reaction solution contained 100 mM phosphate buffer (pH 7.5) (2.5 ml), 5 mM ascorbic acid (50 μl), 2 mM H_2O_2 (300 μl) and mesocarp extract (100 μl). In the blank, 100 mM phosphate buffer (pH 7.5) (100 μl) replaced the avocado mesocarp extract. The reaction was started with the addition of H_2O_2. The APX activity was expressed as the decrease in absorbance at 290 nm g^{-1} FW min^{-1} or mg^{-1} protein min^{-1}.

Glutathione reductase (GR) (EC 1. 6. 4. 2) was determined spectrophotometrically according to the method of Halliwell and Foyer (1978). The reaction solution contained 100 mM phosphate buffer (pH 7.5) (2.7 ml), 5 mM glutathione oxidized (GSSG, 0.1 ml), 3 mM nicotinamide hypoxanthine dinucleotide phosphate (NADPH) (50 μl) and avocado mesocarp extract (200 μl). In the blank, 100 mM phosphate buffer (pH 7.5) (200 μl) replaced the mesocarp extract. The reaction was initiated with the addition of NADPH. One unit of GR was reported as the decrease in absorbance at 340 nm g^{-1} FW min^{-1} or mg^{-1} protein min^{-1}.

2.5. Statistical analysis

On each collection date, samples of a minimum of 20 fruit were collected for each shoot type from three separate sets of trees (three replicate samples) and all biochemical assays were run at least twice with similar results. Mean comparisons were conducted using analysis of variance (ANOVA). When ANOVA testing indicated significant differences, post-hoc comparisons were run utilizing Fisher's Least Significant Difference (LSD) procedure with a family error rate of $\alpha \leq 0.05$. Relationships that had a significant Pearson's correlation coefficient were subsequently subjected to regression analysis using the least squares method of the general linear model. All analyses were conducted using SAS (version 9.2, SAS Institute Inc., Cary, NC).

3. Results

3.1. Relationship between inflorescence type and fruit size

The relationship between floral shoot type and fruit size changed over time. At 45 DAFB, both the diameter and length of the large NPF set by DFS were significantly greater than NPF set by IFS and greater than SPF set by both DFS and IFS ($P < 0.0001$) (Table 1). In addition, the diameter and length of SPF set by DFS were significantly greater than SPF set by IFS, but both had smaller diameters and length than NPF set on either DFS or IFS ($P < 0.0001$). Thus, the diameter and length of SPF collected from IFS were significantly smaller than those of all other fruit ($P < 0.0001$). These relationships, with limited variation, persisted through 120 DAFB, with the exception that the diameters of NPF collected from DFS and IFS were no longer significantly different by this date (Table 1). At 160 DAFB through the end of the research at 300 DAFB (early maturity), NPF borne on DFS were no longer larger (diameter or length) than NPF produced by IFS (Table 1). Also the diameter and length of SPF borne on DSF and IFS were no longer different at 160 DAFB. However, on all sample dates, the diameter and length of NPF from both DFS and IFS were always significantly greater than SPF produced by either DFS or IFS ($P < 0.0001$) (Table 1).

Based on the results above, for subsequent biometric and biochemical analyses, NPF fruit from DFS and IFS were pooled and

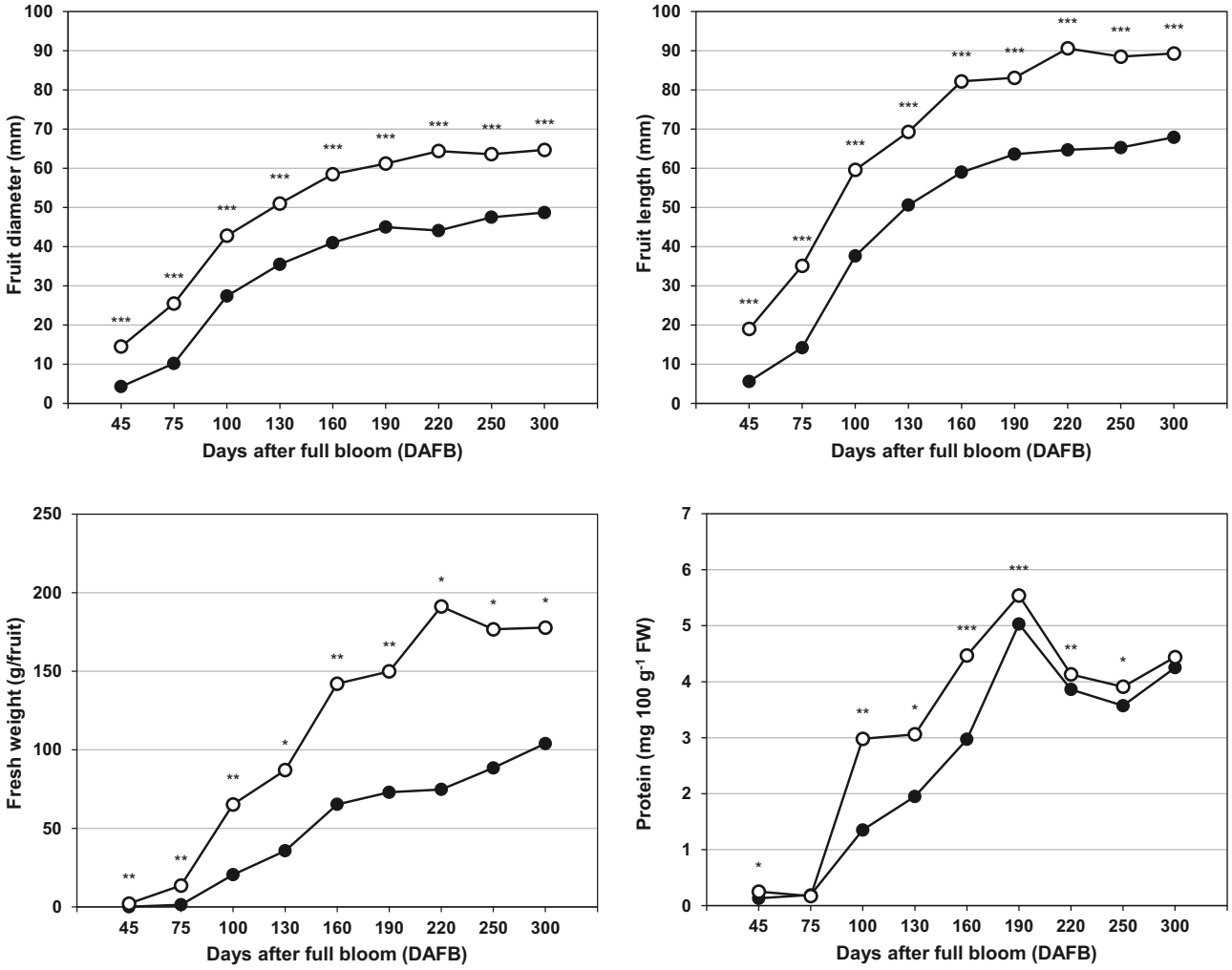

Fig. 1. Diameter (upper left panel), length (upper right panel), fresh weight (lower left panel) and mesocarp protein concentration (lower right panel) of normal-phenotype (-○-) and small-phenotype (-●-) fruit of the 'Hass' avocado from 45 through 300 days after full bloom. Paired mean values for 20 fruit for each phenotype collected from three separate sets of trees (three replications) for a given date marked with *, **, or *** are significantly different at $P \leq 0.05$, 0.01, or 0.001, respectively, by Fisher's Protected LSD Test.

compared with a pooled sample of SPF collected from both DFS and IFS. In each case, the sample was comprised of an equal number of fruit from each floral shoot type.

3.2. Fruit biometrics

Full bloom for 'Hass' avocado trees in this study was in April of each year. For the period 45 through 300 DAFB, NPF had significantly greater diameters, length and FW than SPF on all sample dates (Fig. 1). Based on FW, the average growth rate of NPF (1.15 g/day) was significantly greater than SPF (0.46 g/day) ($P = 0.0137$). The mesocarp protein concentration of NPF was greater than SPF on all sample dates, except 75 DAFB (Fig. 1). Mesocarp protein content was positively correlated with fruit FW ($r = 0.86$; $P < 0.0001$).

3.3. Fruit antioxidant concentrations

Mesocarp ascorbic acid concentrations decreased with time in both NPF and SPF over the period from 45 to 160 DAFB ($P < 0.0001$) (Fig. 2a). Ascorbic acid concentrations increased again in both NPF and SPF starting 190 DAFB. The concentration of ascorbic acid in SPF mesocarp was significantly greater than NPF from 45 to 130 DAFB, equal to that of NPF from 190 through 250 DAFB and significantly greater than NPF again at 300 DAFB (Fig. 2a). The mesocarp total phenol concentration of SPF was also greater than that of NPF for the period 45–130 DAFB (Fig. 2c). By 100 DAFB, the total phenol concentration of avocado mesocarp had decreased dramatically in both NPF and SPF and remained at this new low through 300 DAFB. During this period the concentration of phenols was greater in NPF at 190 and 250 DAFB and greater in SPF at 220 DAFB. The AOC of SPF mesocarp (determined by the FRAP assay) was greater than NPF from 75 to 130 DAFB and remained constant over this period in SPF and NPF, respectively (Fig. 2e). The AOC of both types of fruit was significantly reduced at 160 DAFB and then increased to a new high for each fruit type at 220 through 300 DAFB; at 220 DAFB AOC was again greater in SPF than NPF. Taken together, the results are consistent with the small size of the SPF having a concentrating effect on the antioxidant compounds present in the mesocarp tissue (per g FW) of 'Hass' avocado fruit during early development.

Avocado mesocarp ascorbic acid and total phenol concentrations and AOC (per mg protein) of were all significantly greater in SPF compared to NPF at 45 DAFB (Fig. 2b, d and f). By 100 DAFB, the concentrations of all three had decreased dramatically to a low level that persisted through 300 DAFB in both types of fruit. However, during this period, SPF had greater concentrations of the following antioxidants than NPF on the sample dates specified: (*i*) ascorbic acid at 100, 130, 220, 250 and 300 DAFB; (*ii*) total phenols

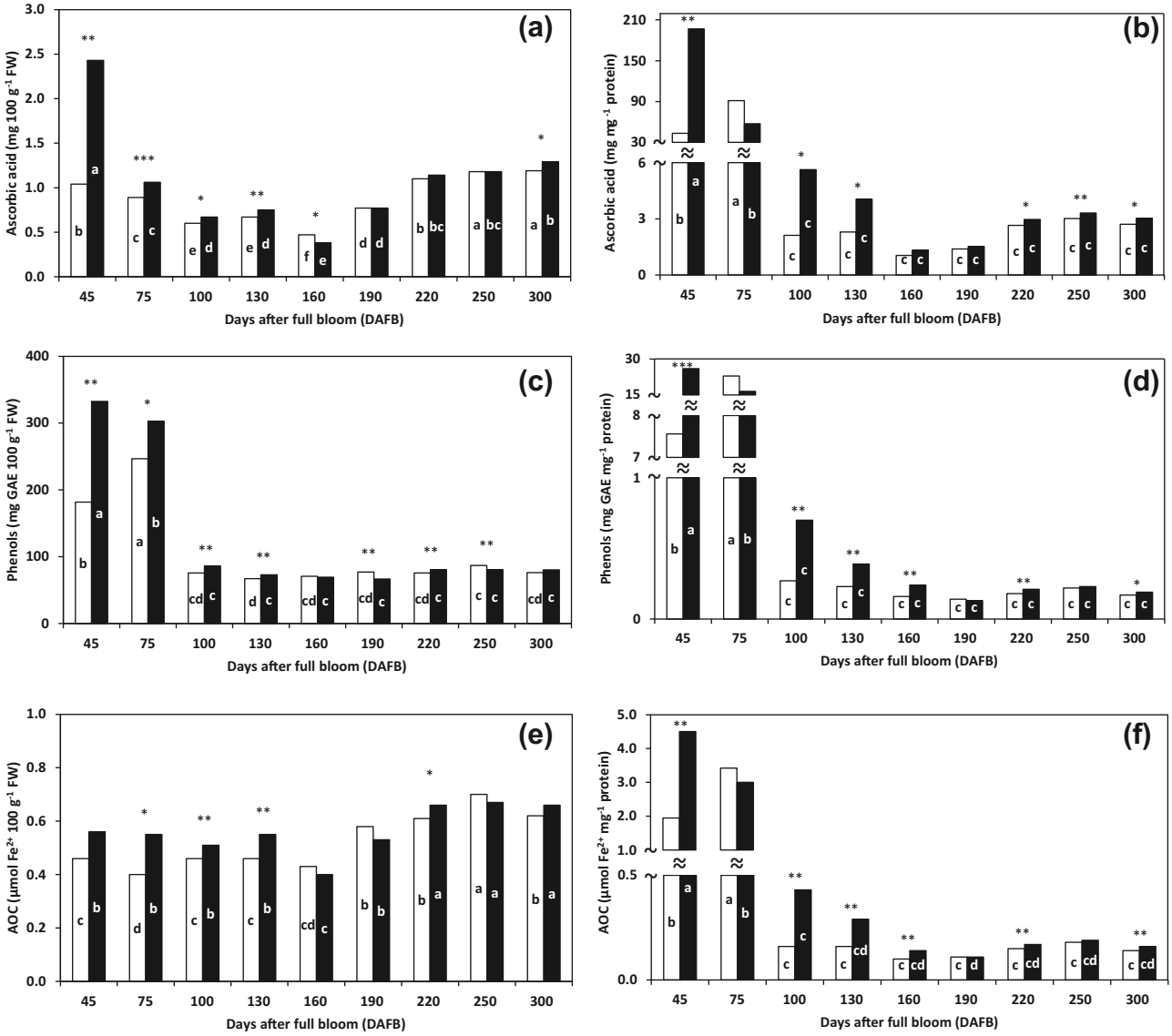

Fig. 2. Ascorbic acid and phenol concentrations and antioxidant capacity per gram fresh weight (a, c and e, respectively) and per mg protein (b, d and f, respectively) in the mesocarp of normal-phenotype (□) and small-phenotype (■) fruit of the 'Hass' avocado from 45 through 300 days after full bloom. Paired mean values for 20 fruit for each phenotype collected from three separate sets of trees (three replications) for a given sample date marked with *, ** or *** are significantly different at $P \leq 0.05$, 0.01, or 0.001, respectively, by Fisher's Protected LSD Test. Bars of the same color with different letters are significantly different across time at $P \leq 0.05$ by Fisher's Protected LSD Test.

at 100, 130, 160, 220 and 300 DAFB; and (*iii*) AOC at 100, 130, 160, 220, and 300 DAFB. However, only the concentration of total phenols was significantly correlated with the protein concentration of avocado mesocarp and this relationship was negative ($r = -0.75$; $P < 0.0001$). In contrast, in the mesocarp of NPF and SPF, significant positive correlations were found between the concentrations of (*i*) total phenols and AOC ($r = 0.98$; $P < 0.0001$) and (*ii*) ascorbic acid and AOC ($r = 0.91$; $P < 0.0001$). Based on the results of stepwise linear regression analyses, mesocarp total phenol and ascorbic acid concentrations together explained 97% of the variation in the antioxidant capacity of 'Hass' avocado mesocarp tissue during fruit development with phenols explaining a greater proportion of the variation ($P < 0.0001$).

3.4. Antioxidant enzyme activity

At 45 and 75 DAFB, POD activity (per g FW) in the mesocarp of NPF and SPF was low, but POD activity increased steadily from 100 through 160 DAFB, remaining relatively stable thereafter (Fig. 3a). During fruit development, the mesocarp of NPF had greater POD activity than SPF at 45, 130 and 160 DAFB, whereas SPF had greater POD activity than NPF during later development at 200 and 300 DAFB. The activities of PPO, CAT and APX (per g FW) in the mesocarp of NPF and SPF all had a pattern similar to POD, low at 45 and 75 DAFB, increasing over the period from 100 to 130 DAFB (PPO) (Fig. 3c) or 100 to 190 DAFB (CAT and APX) (Fig. 3g and i) and remaining relatively stable thereafter. Activities of POD and PPO were similar in that both were significantly greater in the mesocarp of NPF during early development, in this case at 45, 130 and 160 DAFB and significantly greater in SPF later in development at 220 and 300 DAFB for POD (Fig. 3a) and 200 DAFB for PPO (Fig. 3c). In contrast, CAT and APX activities were significantly greater in the mesocarp of NPF than SPF at 100,130, 160, and 220 DAFB (Fig. 3g) and at 45, 75, 100, 130, 160, and 250 DAFB (Fig. 3i), respectively. The activity patterns of SOD (Fig. 3e) and GR (Fig. 3k) (g FW) were different from the other enzymes and each other. The activity of SOD in the mesocarp of NPF decreased from 45 to 100 DAFB, whereas it continued to decrease in SPF through 130 DAFB (Fig. 3e). For NPF,

SOD activity increased from 130 through 250 DAFB, before reaching the level of activity expressed at 45 DAFB, only to drop below that rate at 300 DAFB. In contrast, for SPF fruit, SOD activity increased from 160 to 190 DAFB when it reached the same activity as 45 DAFB, which was maintained through 300 DAFB. The activity of SOD was greater in the mesocarp of than NPF only at 100 and 300 DAFB. The activity of GR was lower during early development (45–130 DAFB) for both NPF and SPF and greater during later development from 160 to 300 DAFB for NPF and from 190 to 300 DAFB for SPF (Fig. 3k). The activity of GR was greater in the mesocarp of NPF fruit than SPF mid-way through fruit development at 100, 130, 160 and 190 DAFB.

The results above left open the possibility that the greater activity of the antioxidant enzymes on a FW basis in NPF mesocarp compared to SPF mesocarp might be due to the greater protein concentrations in the mesocarp of NPF compared to SPF. Consistent with this possibility, the activities of all the antioxidant enzymes were significantly positively correlated with mesocarp protein concentration, with the exception of SOD activity: CAT ($r = 0.94$; $P < 0.0001$); APX ($r = 0.87$; $P < 0.0001$); POD ($r = 0.79$; $P < 0.0001$); GR ($r = 0.76$; $P < 0.0001$); PPO ($r = 0.50$; $P < 0.0001$); and SOD ($r = 0.10$; $P = 0.3045$). In further support of this interpretation, when the activities of the antioxidant enzymes were expressed per mg protein, SPF had greater activities than NPF on a greater number of sample dates. Thus, POD activity was greater in the mesocarp of NPF only at 45 DAFB, whereas it was greater in SPF at 100, 160, 190 220 and 300 DAFB (Fig. 3b). Similarly, PPO was greater in NPF only at 45 DAFB, whereas it was greater in SPF at 100, 130, and 200 DAFB (Fig. 3d). For SOD, activity was greater in SPF than NPF at 45, 130, 160, 190, 220 and 250 DAFB (Fig. 3f). For CAT, there were no differences in activity between NPF and SPF during early fruit development, but CAT activity was greater in SPF than NPF at 160, 190, 250 and 300 DAFB (Fig. 3h). Similarly, APX activity was greater in the mesocarp of SPF than NPF at 100, 130, 160, 190 and 250 DAFB (Fig. 3j). In contrast, GR activity was greater in the mesocarp of NPF than SPF at 100, 130, 160, 190 DAFB (Fig. 3l). The similarities and differences in the activity patterns of the antioxidant enzymes (per mg protein) during fruit development identified above were further documented by calculating a Pearson's correlation coefficient for

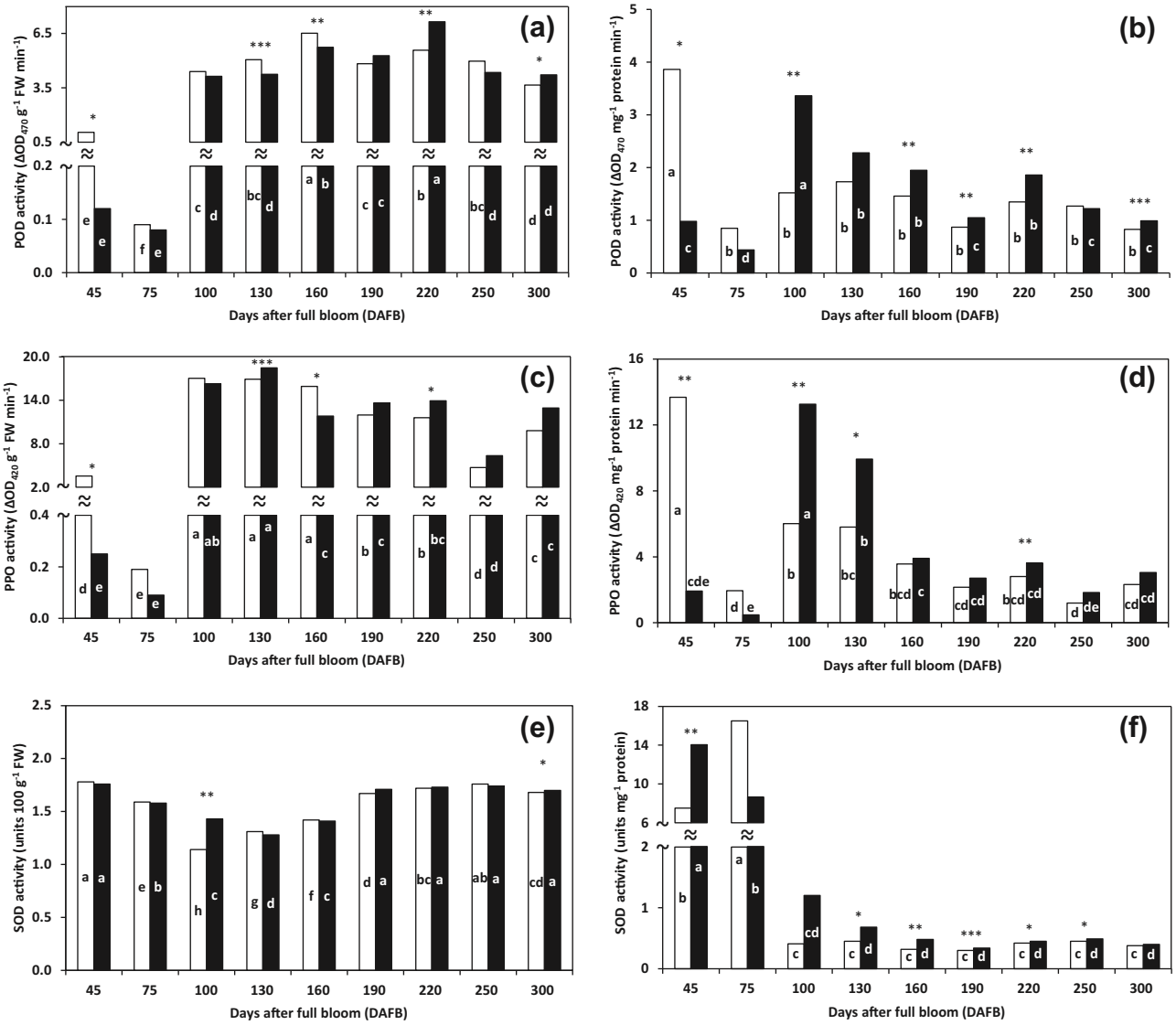

Fig. 3. Activities of peroxidase (POD), polyphenoloxidase (PPO), superoxide distmutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) per gram fresh weight (a, c, e, g, i and k, respectively) and per mg protein (b, d, f, h, j and l, respectively) in the mesocarp of normal-phenotype (□) and small-phenotype (■) fruit of the 'Hass' avocado from 45 through 300 days after full bloom. Paired mean values for 20 fruit for each phenotype collected from three separate sets of trees (three replications) for a given sample date marked with *, ** or *** are significantly different at $P \leq 0.05$, 0.01, or 0.001, respectively, by Fisher's Protected LSD Test. Bars of the same color with different letters are significantly different across time at $P \leq 0.05$ by Fisher's Protected LSD Test.

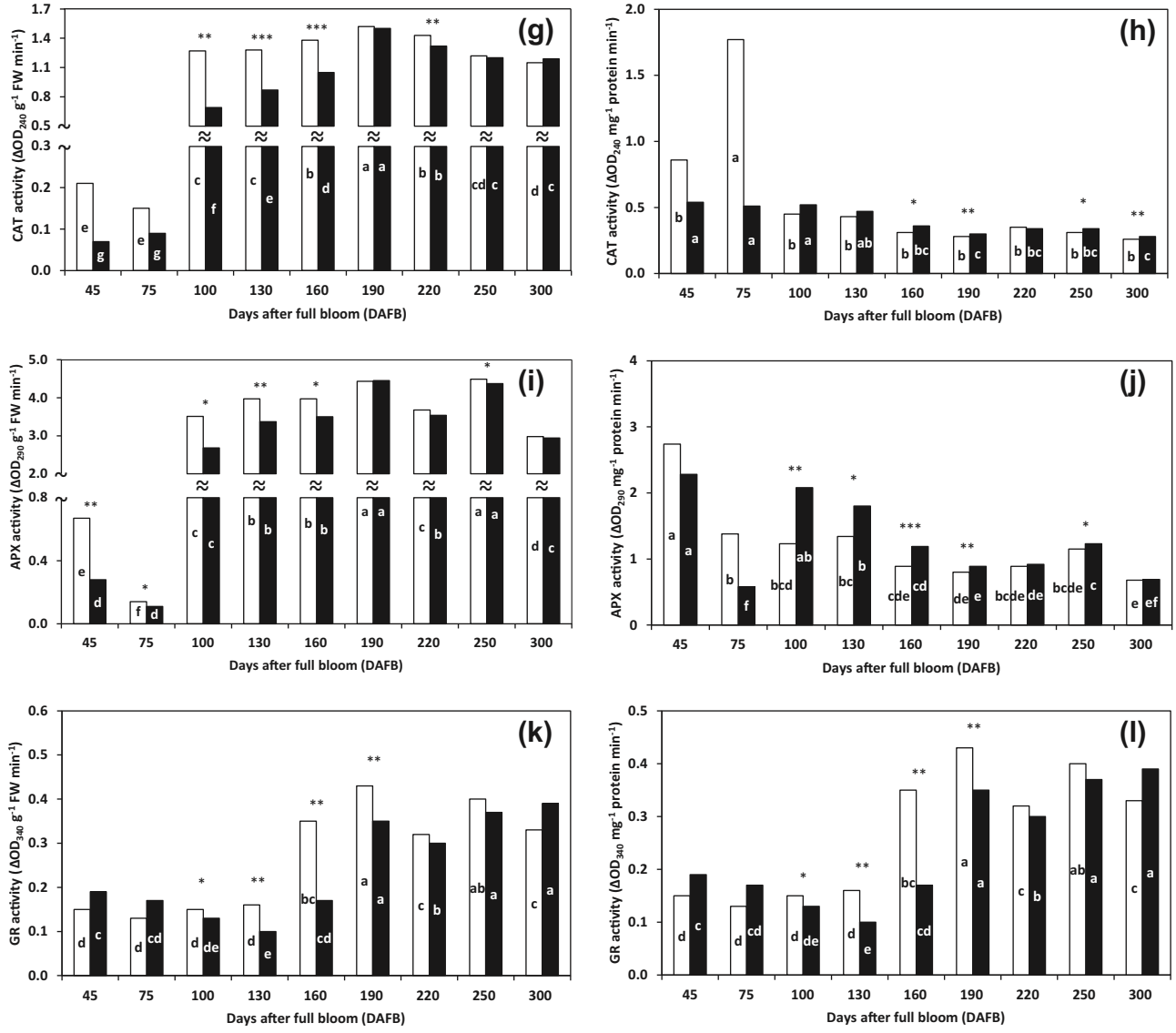

Fig. 3. (*Continued*)

each relationship. The activity of POD was significantly ($P < 0.0001$) positively related to the activities of PPO ($r = 0.73$), CAT ($r = 0.88$), and APX ($r = 0.88$), respectively, but the relationship between CAT and APX activity was stronger ($r = 0.94$; $P < 0.0001$). Interestingly, GR activity more closely paralleled the activity of CAT than APX ($r = 0.63$ versus $r = 0.55$, respectively; $P < 0.0001$ for each). The activity of SOD was negatively correlated with all antioxidant enzyme activities except GR ($P < 0.0001$). Taken together, these results provide strong evidence that the reactive oxygen-scavenging systems of the small fruit variant of the 'Hass' avocado were not compromised and likely not a factor contributing to the reduced growth rate that characterized these fruit.

4. Discussion

The results of this research provided clear evidence that DFS produced significantly larger fruit (diameter and length) than IFS during fruit set and early development. Both NPF and SPF borne on DFS were larger at 45 through 120 DAFB than NPF and SPF developing on IFS, respectively. Shoot extension and leaf expansion of the vegetative shoot apex of IFS are initiated at approximately full bloom (Salazar-García et al., 1998) and would have occurred concurrently with early growth of fruit borne on IFS. The results reported herein support the interpretation that the developing vegetative shoot apex of IFS was a competing sink that negatively impacted early fruit growth, including exponential fruit growth (diameter and length) (approximately 45-160 DAFB) (Lahav and Kalmer, 1977). Prior to the end of exponential fruit growth, the majority of leaves of the vegetative shoot apex of IFS would likely have been fully mature and no longer competing sinks but instead sources of photosynthate and metabolites for the developing fruit. Consistent with this interpretation, the results also demonstrated that at 160 DAFB (end of exponential fruit growth) through the end of the sampling period at 300 DAFB (early maturity), the sizes of NPF and SPF, respectively, were no longer related to the type of inflorescence on which they were developing. These results are consistent with demonstrations that inhibiting the growth of the vegetative shoot apex of IFS with foliar-applied plant growth inhibitors or removing it with pruning eliminates competition and increases fruit set and fruit size if implemented sufficiently early during fruit development (Bower and Cutting, 1992; Cutting and Bower, 1990; Salazar-García and Lovatt, 1998; Whiley, 1990). The results of this

study suggested that such treatments should be imposed prior to 45 DAFB, by which time SPF are already 50% smaller in diameter and length and lower in FW than NPF.

The results of this research also established that commercially bearing 'Hass' avocado trees in California produced distinct pools of normal and small-phenotype fruit consistent with 'Hass' avocado fruit production in South Africa (Richings et al., 2000) and Israel (Dahan et al., 2010). On all sampling dates, NPF were significantly greater in diameter, length and FW than SPF. Fresh weight accumulation stopped in NPF at 220 DAFB, but continued for SPF with an upward trajectory in FW evident at the end of the research at 300 DAFB, consistent with delaying harvest to increase fruit packing carton size, which is based on FW, and increase the commercial value of the fruit. Normal fruit were equal to or greater than 178 g FW per fruit (≥ packing carton size 60) at harvest and had attained this approximate FW at early maturity (300 DAFB; mid-February). The small fruit variant were only 100 g FW per fruit at this time, but were typically between 99 to 134 g FW per fruit (packing carton size 84) at harvest. In California, mature fruit undergo a second period of exponential fruit growth from May to July, with peak harvest between March and July.

The results of this research provided clear evidence that the small size of the 'Hass' avocado small fruit variant had a concentrating effect on the accumulation of antioxidant compounds. The SPF had significantly greater ascorbic acid and total phenol concentrations and AOC per gram FW than NPF on more sample dates. Specifically, ascorbic acid concentration was significantly greater in SPF on five sample dates versus only one date for which ascorbic acid was greater in NPF; total phenol concentration was greater in SPF on five dates versus only two dates for NPF; and AOC was significantly greater in SPF on four sampling dates, with NPF never having a greater AOC than SPF. For both fruit types, mesocarp AOC (g FW) was low and stable during early fruit development (45-130 DAFB) as first reported by Bertling et al. (2007).

Significant ($P < 0.0001$) positive relationships between total phenol and ascorbic acid concentrations and AOC determined by FRAP in avocado mesocarp with $r = 0.98$ and $r = 0.91$, respectively, were very similar to those reported for eight horticultural crops, for which the relationship between total phenols and AOC determined by FRAP was $r = 0.92$ ($P < 0.01$) and for ascorbic acid and AOC (FRAP) was $r = 0.85$ ($P < 0.01$) (Corral-Aguayo et al., 2008). Also the finding that total phenol and ascorbic acid concentrations together explained 97% of the variation in the antioxidant capacity of 'Hass' avocado mesocarp tissue during fruit development, with phenols explaining majority of the variation ($P < 0.0001$), is consistent with ascorbic acid generally being a lesser component of AOC compared to the total phenol concentrations present in fruit (Corral-Aguayo et al., 2008; Thaipong et al., 2006; Vinson et al., 2001).

In contrast to the concentrating effect of small fruit size on the concentrations of antioxidant compounds in SPF on a FW basis, the results of this research provided evidence that the majority of the antioxidant enzymes exhibited greater activity in NPF than SPF when expressed on a FW basis. Specifically, when significant differences occurred in the activities of CAT, APX and GR, they were always significantly greater in NPF than SPF. The activities of POD and PPO were significantly greater in NPF than SPF on one and two additional sampling dates, respectively, compared to the number of dates that SPF had greater activities of these enzymes than NPF. When the activity of POD and PPO were greater in SPF than NPR, it occurred later in fruit development at 220 DAFB or later. In contrast, the activity of SOD was never greater in NPF relative SPF but SOD activity was only greater in SPF than NPF on two dates. Thus, the activity of the majority of reactive oxygen-scavenging enzymes was not concentrated in the small fruit variant.

In addition, the mesocarp protein concentration of developing 'Hass' avocado fruit was not concentrated in the small fruit variant but instead significantly positively correlated with avocado fruit FW. Thus, SPF consistently had lower protein concentrations than NPF throughout development, with the exception of 75 DAFB. In addition, the activities of all the antioxidant enzymes were strongly significantly and positively correlated with the protein concentration of the mesocarp tissue, with the exception of SOD activity. These results support the conclusion that the greater activity of the majority of reactive oxygen-scavenging enzymes on a FW basis in NPF than SPF on a greater number of sample dates was due to the low protein concentration of SPF. This interpretation was corroborated by the fact that when antioxidant enzyme activity was expressed per mg protein, on any sampling date for which there was a significant difference in enzyme activity due to fruit type, the activity was always significantly greater in SPF than NPF. Specifically, on every date that SOD, CAT and APX activities (per mg protein) were significantly different, they were significantly greater in SPF than NPF. For POD and PPO, NPF had significantly greater activity only at 45 DAFB, whereas SPF had significantly greater POD and PPO activity on numerous sampling dates during later fruit development. Only GR activity was greater in NPF than SPF on all dates where significant differences occurred. Thus, the results of this research suggest that the activities of the reactive oxygen-scavenging systems on a fresh weight basis were not compromised in the 'Hass' avocado small fruit variant.

The results of this research demonstrated that a significant change in cell metabolism occurred in both types of fruit between 75 and 100 DAFB that was not directly correlated with the rapid increase in fruit FW or mesocarp protein concentration. For example, mesocarp total phenol concentrations (per g FW and mg protein), ascorbic acid and AOC (per mg protein) and SOD and CAT activity (per mg protein) decreased from 75 to 100 DAFB and remained low through early fruit maturity (300 DAFB). In contrast, the activities of POD and PPO (per g FW and mg protein) and CAT and APX (per g FW) increased from 75 to 100 DAFB. The differences likely represent the variation in the roles played by these different antioxidant compounds and enzyme systems in scavenging ROS, which progressively increase during fruit development (López et al., 2010).

Taken together, the results of this research did not provide any evidence that differences in antioxidant concentrations, capacity or enzyme activities between NPF and SPF were factors contributing to the reduced growth rate of the 'Hass' small fruit variant, but instead suggested that these differences were the consequence of smaller fruit size. Thus, due to the concentrating effect of small fruit size on antioxidant concentrations and capacity, per unit of mespocarp consumed, small 'Hass' avocado fruit should be a healthier food choice than large fruit.

5. Conclusions

From 45 DAFB through exponential fruit growth (120 DAFB), NPF and SPF developing on DFS were significantly larger than NPF and SPF developing on IFS, respectively, with smallest fruit borne on IFS. However, by 160 DAFB, fruit size was no longer influenced by the floral shoot type on which the developing fruit were set. The results supported the interpretation that early fruit growth was reduced due to competition with the developing vegetative shoot apex of indeterminate floral shoots and identified that corrective treatments would need to be implemented earlier than 45 DAFB. In addition, the results established that 'Hass' avocado trees in California produced distinct pools of normal (large) size fruit and a small fruit variant. From 45 to 300 DAFB (early fruit maturity), NPF were significantly larger (diameter and length), weighed more and had a greater concentration of protein in the edible portion of the fruit (mesocarp) than SPF. However, the smaller size of SPF had a concentrating effect, resulting in the mesocarp of SPF having greater

concentrations of total phenols and ascorbic acid and a greater antioxidant capacity than the mesocarp of NPF. Further, despite SPF having lower mesocarp protein concentrations per g FW than NPF throughout development, the activities of all antioxidant enzymes expressed per mg protein, except GR, were more frequently greater in SPF than NPF, especially on sample dates closer to early fruit maturity, suggesting that the ability of the 'Hass' avocado small fruit variant to scavenge ROS was not likely compromised. Moreover, based on these results, small avocado fruit would appear to provide a healthier food choice than larger 'Hass' avocado fruit due to their greater concentrations of antioxidant compounds and greater antioxidant capacity per unit weight of edible mesocarp. Presently SPF (99–134 g/fruit) are not as commercially valuable as normal fruit (178–325 g/fruit). However, the significantly greater concentrations of antioxidant compounds and greater total antioxidant capacity might change consumer preference in favor of smaller 'Hass' avocado fruit due to their potential to provide greater health benefits.

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