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Phytohormone Changes and Carbohydrate Status in Sweet Orange Fruit from Huanglongbing-infected Trees

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Abstract Huanglongbing (HLB) infection alters citrus fruit growth and development, resulting in small, misshapen, and poorly colored fruit containing aborted or partially developed seeds. Typically, symptomatic fruit have delayed maturation and abscise prematurely. We studied carbohydrate and phytohormone changes in HLB-affected fruit to explain symptom development because (1) carbohydrate shortage has been linked to fruit growth arrest and eventually abscission and (2) hormonal signals regulate, at least partially, fruit set and development. Symptomatic fruit (S), asymptomatic fruit (AS) from symptomatic trees, and healthy fruit (H) from asymptomatic trees were harvested from 'Valencia' sweet orange trees [*Citrus sinensis* (L.) Osbeck] infected with the HLB pathogen or not, as verified by PCR. Mature S weighed less, had lower °Brix, were smaller, had more aborted seeds, and were greener than AS or H. Starch and sucrose contents were lower in mature S flavedo compared with that of H and AS. S and AS harvested 7 and 12 months after full bloom produced significantly less ethylene than H. Indole-3-acetic acid (IAA) and abscisic acid (ABA) contents in flavedo removed from the stylar end, middle section, or stem end of fruit generally were higher in S flavedo than in AS and H. ABA content was fourfold higher in flavedo from the middle section of S than in AS and H. Flavedo excised from the large shoulder of misshapen S had significantly higher IAA content when compared with the normal-sized area of the same fruit on the opposite side. This increase corresponded to an increase in hypodermal cell area in S flavedo. Overall, these data reveal an imbalance of carbohydrate and phytohormone status in fruit from HLB-infected trees and suggest a role of such changes in fruit symptom development.

Keywords Citrus · Greening disease · Carbohydrates · Phytohormones

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Introduction

Citrus huanglongbing (HLB or citrus greening), caused by the phloem-limited proteobacterium '*Candidatus* Liberibacter spp.,' is a significant worldwide threat to sustainable citrus production. Economic losses due to HLB have been documented in Asia and Africa (reviewed by da Graça and Korsten 2004), and predictions for Brazil and Florida estimate productivity reductions between 13 and 31% by 2020, depending on the percentage incidence of HLB (Spreen and others 2006). Typical symptoms of HLB-infected trees include reduced plant height, yellowing of leaves, blotchy mottle, and/or chlorotic patterns of leaves resembling those induced by zinc and iron deficiencies, followed by leaf drop and twig dieback at later stages (Bové 2006). Fruit produced by infected trees are smaller, lopsided, poorly colored with coloration beginning in the stem end (color inversion), have a high rate of seed abortion, and abscise prematurely (da Graça 1991). Juice from symptomatic fruit has higher acidity, lower sugars, and lower Brix/acid ratio, resembling juice from less mature fruit (Dagulo and others 2010). Recent research has focused on the Asian citrus psyllid vector of HLB (Setamou and others 2008; Qureshi and others 2009; Boina and others 2010), the bacterial causal agent (Li and others

2006; Tatineni and others 2008; Duan and others 2009; Magomere and others 2009; Sechler and others 2009; Tyler and others 2009), and changes in leaf physiology and morphology (Manthey 2008; Cevallos-Cevallos and others 2009; Etxeberria and others 2009; Kim and others 2009; Sagaram and Burns 2009; Achor and others 2010). In contrast, literature on HLB infection effects on citrus fruit growth and development is limited. Studies in fruit have been largely restricted to HLB effects on juice quality and physical fruit characteristics (Bassanezi and others 2009; Baldwin and others 2010; Dagulo and others 2010).

Fruit growth and development are complex processes involving a number of physiological and biochemical changes under genetic, nutritional, hormonal, and environmental control (Giovannoni 2004). In citrus, fruit growth arrest was associated with sucrose deficiency (Gomez-Cardenas and others 2000). In sweet oranges infected with the phloem-limited HLB bacterium, plugging of sieve cells via callose deposition in and around sieve pores has been reported (Schneider 1968; Etxeberria and others 2009; Kim and others 2009), presumably resulting in disruption of carbohydrate transport and massive accumulation of starch in plastids of symptomatic leaves. As duration of HLB infection increases, nutrient deficiencies and carbohydrate imbalance in leaves and roots (Etxeberria and others 2009) may develop, arresting plant growth and development and eventually leading to tree mortality.

Phytohormones influence citrus fruit set and productivity and plant response to plant pathogen attack. Auxins promote cell enlargement, affect fruit abscission, and stimulate citrus fruit growth (Talon and others 1997; Josan and others 1999; Iglesias and others 2007). Furthermore, indole-3-acetic acid (IAA) induces parthenocarpy in a number of species (Ozga and Reinecke 2003). Nonpollinated parthenocarpic fruit have aborted seeds (Heuvelink and Körner 2001) and are commonly smaller and misshapen compared to pollinated fruit. Abscisic acid (ABA) plays a crucial role in plant adaptation to different environmental stresses and in several physiological processes such as seed maturation and fruit development (Zeevaart and Creelman 1988). In citrus, ABA has been associated with water and salinity stress (Agusti and others 2007). ABA transiently accumulates in drought-stressed roots and leaves and recovers after rewatering (Mehouachi and others 2005). Ethylene (ET) participates in regulating defense responses in plants: contributing to hypersensitive responses against necrotrophic pathogens (Bari and Jones 2009), regulating abscission (Goren 1993; Kazokas and Burns 1998; Yuan and others 2001; Agusti and others 2007), and participating in fruit maturation processes, including coloration and carotenoid synthesis (Goldschmidt and others 1993). The fruit aroma compound valencene accumulated during citrus fruit maturation and was responsive to ethylene (Sharon-Asa and others 2003).

We analyzed changes in IAA, ABA, ET, starch, and sucrose contents in fruit flavedo of HLB-infected and healthy 'Valencia' oranges [*Citrus sinensis* (L.) Osbeck] in an attempt to understand the physiological control of HLB symptom development in sweet orange fruit, such as misshape, seed abortion, and fruit development arrest. Based on physiological changes reported for HLB-affected trees and anatomical changes observed for fruit, we hypothesized that HLB-affected fruit had altered carbohydrate and phytohormone contents. Analysis of the spatial distribution of IAA and ABA in the fruit could help to explain color inversion and development of misshapen fruit areas in symptomatic fruit. Due to the role of IAA in cell enlargement, we analyzed the area of flavedo parenchyma cells located three to five cell layers under the epidermis (hypodermis); larger cells in a localized position of symptomatic fruit could explain the formation of abnormal growing areas and consequently misshapen fruit. Such changes could explain development of symptoms and perhaps lead to disease-mitigating treatments. The aim of this work was to determine changes in IAA, ABA, ET, starch, and sucrose contents in fruit flavedo of HLB-infected 'Valencia' orange trees. We confirm that the flavedo from HLB-infected trees has lower carbohydrate content and demonstrate that HLB-infected fruit have an altered phytohormone balance.

Materials and Methods

Plant Material

Quantitative PCR (Q-PCR) (Li and others 2006) confirmed the presence of the HLB bacterium in HLB symptomatic trees. Healthy fruit (H) were harvested from healthy (PCR-negative) trees, and asymptomatic and symptomatic fruit (AS and S, respectively) were harvested from HLB-infected (PCR-positive) 'Valencia' sweet orange trees [*Citrus sinensis* (L.) Osbeck grafted on Swingle rootstock] located in Lake Placid, FL, USA, and at the Citrus Research and Education Center, Lake Alfred, FL, USA. Trees were between 15 and 18 years of age. S and AS were harvested from the same PCR-positive trees, but AS were located on branches or sectors of trees not showing symptoms. Fruit were harvested 7 months (immature) or 12 months (mature) after full bloom. Fruit were harvested from four replicate trees; at least eight fruit were harvested from each tree replicate. Measurements were averaged to give a single value for each replicate.

Fruit Physical and Biochemical Characteristics

Samples (at least 5 fruit/replicate) were hand-juiced using a juicer extractor (Sunkist Growers, Inc., Ontario, CA) and juice was immediately used for analysis. Soluble solids content (°Bx) was measured using a handheld Brix refractometer (Fisher Scientific, Pittsburgh, PA), and titratable acidity (% citric acid) was measured by titrating juice samples with 0.1 N NaOH using phenolphthalein as an indicator (Boland 1990). Peel color at three equidistant locations along the fruit equator was measured using a Chroma meter CR-400 (Konica Minolta Sensing, Inc., Osaka, Japan) and expressed as a*/b* ratio (a/b ratio). The three measurements were averaged for each fruit. Seeds were removed from H, AS, and S and separated into normal (filled and light in color), intermediate (reduced in size or incompletely developed), and aborted (small seed with collapsed endosperm, Fig. 1) categories. The number of aborted seeds was expressed as percent of the total seeds in each fruit.

Light Microscopy, Cell Area Measurements, and Cell Counting

Due to the role of IAA in cell enlargement, we analyzed the area of flavedo parenchyma cells located three to five cell layers under the epidermis (hypodermis). Flavedo samples were isolated with a razor blade from H, AS, and S and from the misshapen section of S. Misshapen areas were typically located on the fruit shoulder in the stem-end region, with one shoulder visibly larger than the opposite shoulder of the same fruit. Flavedo sections (1–2 mm) were fixed in 3% glutaraldehyde in 0.1 M potassium phosphate buffer at pH 7.2 and kept overnight at 4°C. Sections were then washed in the same buffer and postfixed 4 h at room temperature in 2% osmium tetroxide in the above buffer. Samples were dehydrated in an acetone series and embedded in Spurr's resin (Spurr 1969). For light microscopy, 1-μm sections were cut with glass knives, stained with methylene blue/azure A, and poststained in basic fuchsin (Schneider 1981). Light micrographs were taken on a Leitz Laborlux S compound microscope (Wetzlar, Germany) with a Canon Powershot S31S digital camera (Tokyo, Japan). At least 15 cells in three different slide fields in the hypodermis region were measured in a minimum of four slides per fruit replicate; data are the means of the average cell areas of each slide. The fields were 0.01 mm^2. We considered each cell as an ellipse and calculated cell area using the formula $A = \pi \cdot r^1 \cdot r^2$, where r^1 and r^2 are the length of the semimajor and semiminor axes, respectively (half of the longest and shortest diameters of the ellipse, respectively). To determine the cell number in the flavedo for H, AS, S, and the misshapen area of S, parenchyma cells from the hypodermis region of each sample were counted in the same 0.01-mm^2 slide fields used for cell area measurements.

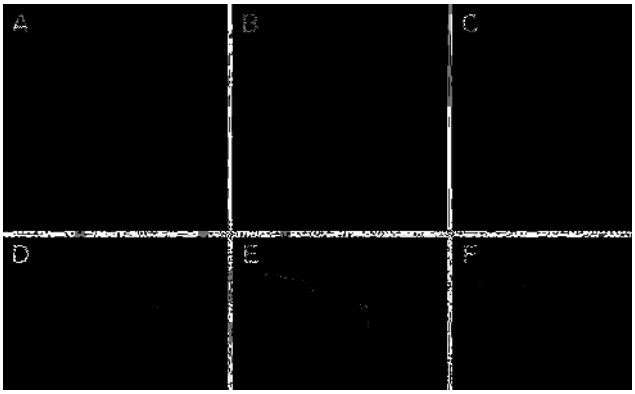

Fig. 1 **a** Healthy fruit from PCR-negative trees (healthy), **b** asymptomatic and **c** symptomatic fruit from PCR-positive (HLB-infected) 'Valencia' orange trees 12 months after full bloom. **d** Filled, healthy seed (*left*) and aborted seed (*right*) from healthy fruit. **e** Filled, healthy seed (*left*) and aborted seed (*right*) from asymptomatic fruit. **f** Partially developed (*left* and *middle*) and aborted (*right*) seeds from symptomatic fruit

Phytohormone Determination

For determination of spatial distribution of phytohormones in fruit flavedo, H, AS, and S tissues were isolated from the stem end, equatorial region (middle section) and stylar end of mature fruit. The flavedo was isolated (8 fruit/replicate) using an apple peeler. For determination of phytohormone content of misshapen areas, the flavedo was removed from this region (generally restricted to the shoulder in the stem end and in some cases extending closer to the equator of symptomatic fruit) and the region opposite the misshapen area exactly from the same fruit position. Care was taken to remove only the flavedo and minimize contamination with albedo. Flavedo samples were frozen in liquid nitrogen and stored at −80°C until used.

ABA was determined according to Serrano and others (1995) in dim light. Briefly, 1 g of ground flavedo was extracted in 80% acetone (1:20 w:v) containing 100 mg/L butylated hydroxytoluene (BHT) and 0.5 g/L citric acid for 16 h at 4°C. The extracts were centrifuged at 2700×*g* for 10 min. The supernatant was recovered, diluted 1:20 with TBS buffer (50 mM Tris, 1 mM $MgCl_2$, 150 mM NaCl) pH 7.2, and quantified by enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody against ABA (Agdia Inc., Elkhart, IN). ELISA procedures were conducted following the manufacturer's instructions. At least two extractions were made for each replicate and extracts were quantified in duplicate. ABA content was estimated from the standard curve prepared for each plate.

IAA was quantified from the flavedo obtained as described above. IAA was extracted, partially purified, and quantified according to Yuan and others (2001) with some modification. The procedure was performed in dim light. Briefly, 3 g of ground flavedo was extracted in 80% methanol (1:25 w:v) containing 50 mg/L BHT overnight at 4°C. The crude extract was filtered through Whatman No. 2 filter paper. Filtrates were evaporated to the aqueous phase in vacuum at 35°C, then adjusted to pH 8 with 0.1 N NaOH and purified with 1:1 v/v ethyl acetate and saturated NaCl. The aqueous/organic mixture was vortexed and the organic phase discarded. The aqueous phase was adjusted to pH 2.5 with 1 N HCl and partitioned at least twice with one volume of ethyl acetate. The organic phases were combined and dried over anhydrous $NaSO_4$, then evaporated to dryness under vacuum at 35°C. Samples were resuspended in 1.5 ml of ethyl acetate:methanol (2:1 v/v) and evaporated to dryness. Samples were dissolved in 500 µl of 100% methanol and 25-µl aliquots were separated over a 30-min period by high-performance liquid chromatography (HPLC) (1200 Series, Agilent Technologies, Santa Clara, CA), using a reverse phase, semipreparative, C18 column (Agilent Eclipse, XDB-C18, 5 µm, 4.6 × 150 mm), at a flow rate of 1 ml min^{-1} with a methanol/acetic acid water gradient. The constant gradient went from 15% methanol in 1% acetic acid to 80% methanol in 1% acetic acid. Absorbance of the effluent was measured at 220 and 282 nm. Fractions (3 ml) were collected according to IAA standard's retention time and reduced to dryness with vacuum. The residue was dissolved in 700 µl of ethyl acetate:methanol (5:2 v/v) and methylated with 100 µl of 2 M trimethylsilyldiazomethane in n-hexane. After 1 h in the dark, samples were dried under N_2 and redissolved with 500 µl of TBS buffer pH 7.2 for ELISA using a monoclonal antibody against IAA (Agdia Inc.) following the manufacturer's instructions. At least two extractions were made for each replicate and extracts were quantified in triplicate. IAA content was estimated from the standard curve prepared for each plate in the same way as the samples (extraction, separation by HPLC, methylation, and ELISA quantification).

For ethylene measurements, fruit weight and volume were measured and fruit were sealed in a 750-ml (H and AS) or 390-ml (S) Rubbermaid® container for periods up to 8 h. Ethylene production was measured by injecting 1 ml of gas sample into a 5890 series II gas chromatograph (Agilent Technologies) equipped with a flame ionization detector as described (Yuan and others 2001).

Starch and Sucrose Determination

Starch quantification was performed according to the method of Etxeberria and Gonzalez (unpublished protocol). At least two extractions were made for each replicate. Juice vesicles were isolated from fruit segments, and seeds and segment membrane were discarded. Flavedo and juice vesicle tissue were frozen in liquid N_2 and stored at −80°C until used. When needed, tissues were removed from storage and dried in a 60°C oven overnight. Dried tissues (50 mg) were ground to a powder, suspended in 700 µl of distilled water, and homogenized in a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Recovered samples and a standard were boiled in water for 10 min and cooled by submersion in cold water for 2 min. Samples were vortexed and centrifuged for 2 min at $2000\times g$. Supernatant aliquots were transferred into a new tube for starch and sucrose determination. For starch determination, 900 µl of 100% ethanol were added to 300 µl of supernatant. The mixture was vortexed and centrifuged for 10 min at $10{,}000\times g$. The supernatant was discarded and 1 ml of distilled water was added to dissolve the pellet. Fifty microliters of $KI:I_2$ (8 mM:50 mM) were added. Quantification of starch was accomplished by monitoring color change in a spectrophotometer at 594 nm. Rice starch (Sigma Aldrich, St. Louis, MO) was used as a standard. For sucrose determination, the supernatant (25 and 10 µl for flavedo and juice vesicles, respectively) was diluted to 100 µl with H_2O and 100 µl of 30% KOH were added to each tube. Standards and samples were boiled for 10 min and then transferred to cold water for 2 min. Tubes were vortexed and 3 ml of anthrone reagent (0.14% anthrone in 25.8 N H_2SO_4) was added to each one and vortexed. The mixture was incubated at 40°C for 35 min. Quantification was accomplished by spectrophotometry at 620 nm using a sucrose (Fisher Chemical, Pittsburgh, PA) standard curve.

Statistical Analysis

Data were analyzed as a completely randomized design using ANOVA and means were separated by Duncan's multiple-range test using Statistical Analysis Systems for PC (SAS Institute Inc., Cary, NC). Regression analysis was performed using R: A Language and Environment for Statistical Computing 2009 (Foundation for statistical computing, Vienna, Austria).

Results

In HLB-infected 'Valencia' orange trees (PCR-positive), two fruit phenotypes could be distinguished: asymptomatic (AS) and symptomatic (S). AS was similar to healthy (H) fruit in weight, diameter, volume, and peel color (Table 1; Fig. 1a, b). In contrast, S fruit were misshapen

Table 1 Physical and juice quality characteristics of healthy (H), asymptomatic (AS), and symptomatic (S) 'Valencia' orange fruit harvested at 12 months after full bloom from PCR-negative (healthy) and PCR-positive (HLB-infected) trees

	H	AS	S
Fruit weight (g)	202a	190a	96b
Fruit diameter (cm)	7.1a	6.9a	5.5b
Fruit volume (cm^3)	190a	173a	88b
Healthy seeds/fruit (No.)	8.1a	3.8b	0.4c
Partially developed seeds/fruit (No.)	0b	0b	1.9a
Aborted seeds/fruit (No.)	1.1b	0.8b	2.4a
% Abortion	17.5b	20b	52a
Peel color (a/b ratio)	0.29a	0.23a	−0.13b
°Bx	12.9a	12.6a	9.7b
Acid (% citric acid)	1.07b	0.94c	1.29a
°Bx/acid ratio	12.1b	13.3a	7.5c

Different letters within each row indicate that means are statistically different (Duncan test, $p < 0.05$)

and had significantly lower weight, diameter, volume and peel color (Fig. 1c). S fruit had significantly greater numbers of partially developed and aborted seeds compared with AS or H fruit, and consequently percent abortion was significantly greater. When present, aborted seeds from all fruit were flat and shrunken (Fig. 1d–f). Seeds removed from S fruit were brown in color. As reported previously (Dagulo and others 2010), juice from S fruit had a 60% lower Brix/acid ratio due to both lower sugar content and higher acidity (Table 1).

Starch and Sucrose Contents are Altered by HLB Infection

In the immature flavedo of H, starch (Fig. 2a) and sucrose (Fig. 2c) contents were numerically higher than that of AS and S, but not significantly different. Mature fruit flavedo starch (Fig. 2b) and sucrose (Fig. 2d) contents were significantly lower in S compared with AS and H. Sucrose content in juice vesicle tissue was threefold lower in S compared with H and AS (Table 2). However, starch content in juice vesicle tissue was similar in S, AS, and H.

ABA and IAA Distributions in the Flavedo are Altered by HLB Infection

To determine the relationship between ABA and IAA contents and HLB symptom development, measurements were made in the flavedo isolated from three fruit positions. Total ABA content increased about threefold in the flavedo of S compared with H and AS (Table 3). However, ABA was not equally distributed in the flavedo longitudinally from the stem end to the stylar end of the fruit. Higher ABA content was measured in the middle section of fruit irrespective of infection. The flavedo from the middle section of S fruit had a greater than a fourfold increase in ABA content when compared with AS and H. Little change occurred in the stem- or stylar-end positions, although ABA was lower in AS flavedo from the stem end. Total IAA content was higher in flavedo of S than AS or H. IAA

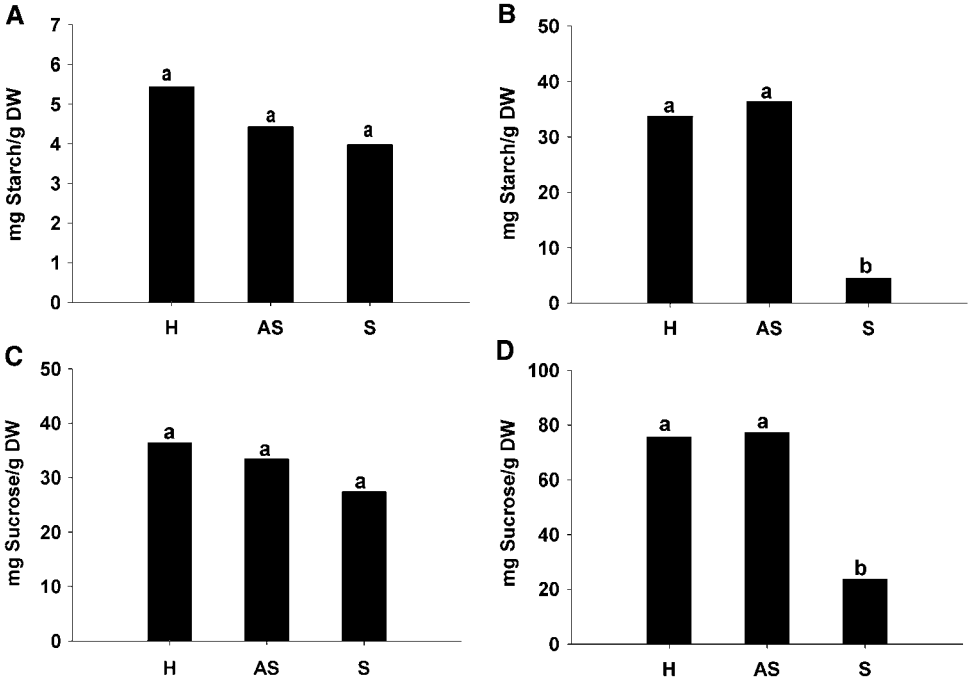

Fig. 2 Starch content in flavedo from **a** immature and **b** mature 'Valencia' orange fruit. Sucrose content in flavedo from **c** immature and **d** mature 'Valencia' orange fruit. *H* healthy fruit flavedo from PCR-negative trees, *AS* asymptomatic fruit flavedo from PCR-positive HLB-infected trees, *S* symptomatic fruit flavedo from PCR-positive HLB-infected trees. Values are the means of four replicates. *Different letters on bars* indicate means are statistically different using Duncan's test; $p < 0.05$

Table 2 Starch and sucrose content in juice vesicle tissue of healthy, asymptomatic, and symptomatic mature 'Valencia' orange fruit harvested from PCR-negative (healthy) and PCR-positive (HLB-infected) trees

	Starch (mg/g DW)	Sucrose (mg/g DW)
Healthy	4.5a	232a
Asymptomatic	4.9a	223a
Symptomatic	5.5a	70b

Different letters within each column indicate that means are statistically different (Duncan test, $p < 0.05$)

Table 3 ABA and IAA content at three positions of flavedo from healthy and HLB-affected mature 'Valencia' oranges

	ABA nmol/g DW			IAA pmol/g DW		
	H	AS	S	H	AS	S
Stem end	1.5a	0.9b	1.7a	538a	122b	107b
Middle section	5.4b	4b	24a	1054b	967b	1857a
Stylar end	1.3a	1.1a	0.7a	191a	262a	394a
Total	9.1b	7.2b	27a	1782ab	1303b	2359a

Flavedo was removed from healthy (H), asymptomatic (AS), and symptomatic (S) fruit. Different letters within each row and individual treatment indicate that means are statistically different (Duncan test, $p < 0.05$)

content was significantly greater in S middle-section flavedo than H or AS. However, IAA was four- to fivefold lower in AS and S stem end than in H, and no significant differences occurred in the stylar end, although numerically IAA was higher in S fruit at that location. To investigate the lateral distribution of ABA and IAA in the flavedo of misshapen fruit areas of HLB-infected fruit, the content of both phytohormones was measured. There were no differences in ABA content (data not shown); however, the IAA content in misshapen regions of S fruit flavedo was twofold higher than that of stem-end flavedo taken from the opposite, or normal-shaped, side of the fruit (Fig. 3).

Cell Area of Flavedo Hypodermis

Stem-end hypodermal cells from S fruit were significantly larger than those from H and AS (Fig. 4a). Cells from the misshapen stem-end area of S fruit were 26% larger than cells from the flavedo of the normal area of the same fruit on the opposite fruit side, and 57 and 77% larger than cells from H and AS fruit, respectively. As hypodermal cell area increased, IAA content increased (Fig. 4b). As cell area increased, the number of cells per unit area decreased (Fig. 4b, inset).

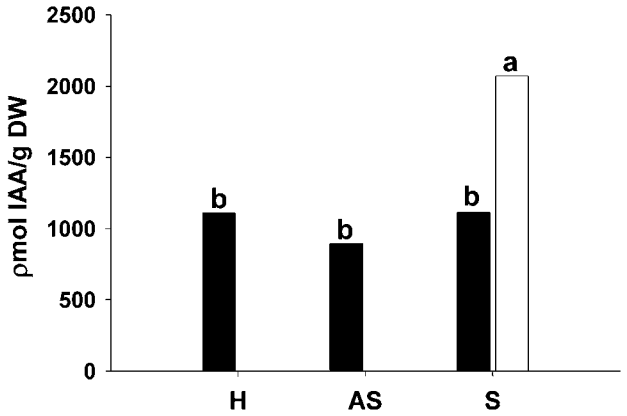

Fig. 3 IAA content in flavedo from H, AS, and S mature 'Valencia' orange fruit. *Black bars* depict IAA content in flavedo from the stem end of healthy (H), asymptomatic (AS), and symptomatic (S) fruit on the side with visually normal size and appearance. The *white bar* shows the IAA content in flavedo from the misshapen section of S fruit located on the opposite side of the fruit depicted by the *black bar*. Misshapen areas were visually identified as large exaggerated fruit shoulders on the stem end. Values are the means of four replicates. *Different letters on bars* indicate means are statistically different using Duncan's test; $p < 0.05$

Ethylene Production in HLB-affected and Healthy Fruit

S immature and mature fruit produced significantly less ET than did H fruit (Fig. 5a, b). Mature and immature H 'Valencia' orange fruit produced over twofold more ET than S. ET production in AS was intermediate between H and S, but not significantly different.

Discussion

Symptomatic 'Valencia' mature fruit from HLB-infected trees generally have a juice profile characteristic of immature fruit in that percent acid is higher and °Bx lower (Plotto and others 2008; Bassanezi and others 2009; Baldwin and others 2010; Dagulo and others 2010). S fruit are smaller, poorly colored, and have partially or completely aborted seeds and a reduced number of total seeds per fruit (Albrecht and Bowman 2009). Our results agree with previous reports.

In citrus, carbohydrate supply is a critical factor governing fruit development. Sucrose content correlates positively with fruit growth (Mehouachi and others 1995). Girdling and fruit thinning, two techniques that make more photosynthate available to fruit, increase fruit size (Goldschmidt 1999). In HLB-affected trees, carbohydrate flow is disrupted due to the blockage of phloem sieve elements, resulting in an accumulation of starch in symptomatic leaves (Schneider 1968; Kim and others 2009), the aerial stem, and bark tissue (Etxeberria and others 2009),

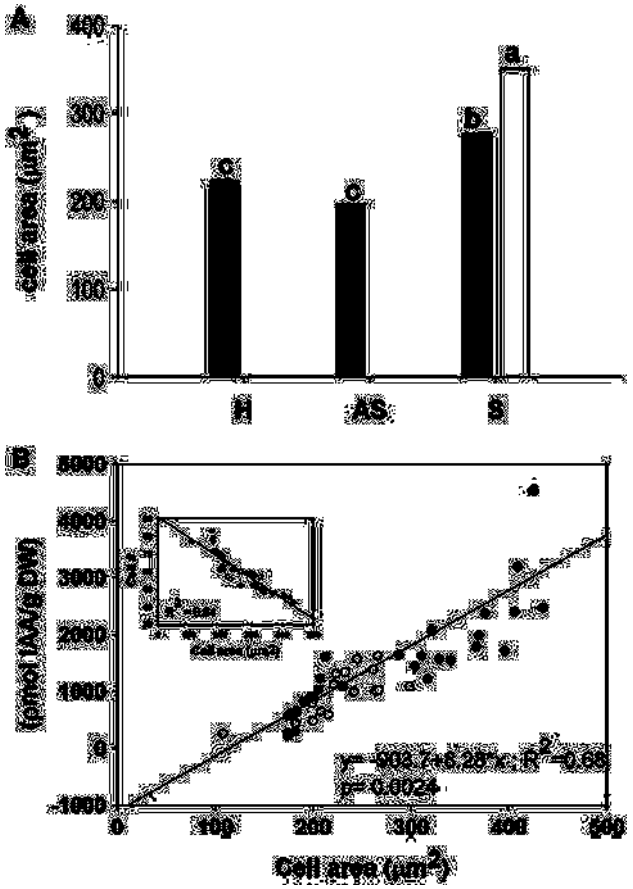

Fig. 4 **a** Hypodermal cell area from flavedo of mature 'Valencia' orange fruit harvested from healthy (H) PCR-negative and HLB-infected PCR-positive trees (*AS* asymptomatic, *S* symptomatic). *Black bars* depict the hypodermal cell area in flavedo from the stem end of H, AS, and S fruit on the side with visually normal size and appearance. The *white bar* shows the hypodermal cell area in flavedo from the misshapen section of S fruit located on the opposite side of the fruit depicted by the *black bar*. Misshapen areas were visually identified as large exaggerated fruit shoulders on the stem end. Values are the means of four replicates. *Different letters on bars* indicate means are statistically different using Duncan's test; $p < 0.05$. **b** Correlation between IAA content and hypodermal cell area in flavedo from H (*white circles*), S (*black circles*), and AS (*gray circles*) fruit. *Inset* shows the correlation between hypodermal cell area and number of cells in the measurement field

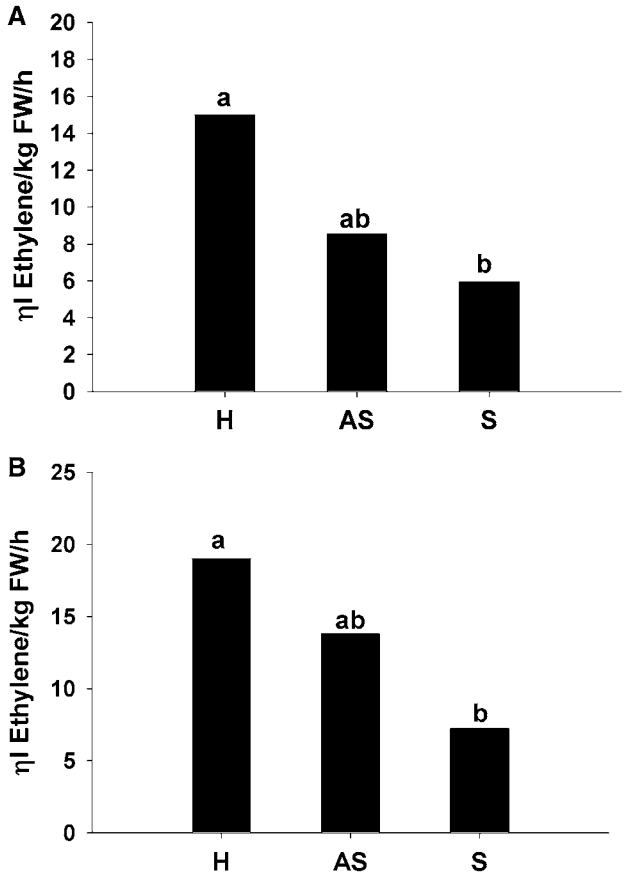

Fig. 5 Ethylene production in healthy (H), asymptomatic (AS), and symptomatic (S) 'Valencia' orange fruit. **a** Immature fruit (7 months of age). **b** Mature fruit (12 months of age). Values are the means of four replicates. *Different letters on bars* indicate means are statistically different using Duncan's test; $p < 0.05$

and in a decrease in roots (Etxeberria and others 2009). Thus, phloem blockage results in starch accumulation in source organs but depletion in sink or storage organs. We demonstrated that mature S has lower starch content in the flavedo and lower sucrose content in the flavedo and juice vesicle. Sucrose is one of the main but not the only carbohydrate affecting Brix in orange juice. Fructose and glucose are also major sugars in orange juice and could explain why in mature fruit sucrose content is threefold lower in S compared with H, while Brix is only 40% lower. This carbohydrate deficiency can influence fruit growth and development and could explain, in part, the size reduction in S.

The spatial distribution of phytohormones in fruit within a cluster has been studied in grapes (Nito and Kuraishi 1979) and tomatoes (Bohner and Bangerth 1988) in relation to fruit set and abscission. Little information exists in citrus fruit with respect to spatial distribution of IAA and ABA content. In this work, ABA and IAA in the flavedo of H and HLB-affected (S and AS) fruit were asymmetrically distributed, with high content measured in flavedo isolated from the fruit middle section. More importantly, IAA and ABA contents were significantly higher in the middle sections of S when compared to AS and H, even though fruit size was smaller. Increased IAA content has been associated with cell enlargement and fruit expansion (Talon and others 1997). Cell measurements in fruit hypodermis indicated that S had higher IAA content and larger cells but a lower number of cells, suggesting that IAA played a role in continued cell expansion in S but that overall fruit size remained smaller because there were

fewer cells. IAA levels were significantly higher in flavedo isolated from misshapen sections of S. Such elevated and localized IAA content could be affecting cell enlargement and result in the development of misshapen fruit areas. Other phytohormones such as gibberellin or cytokinin may influence hypodermal cell size and number in HLB-affected fruit. Future work should focus on quantifying these phytohormones and determining their impact on fruit symptom development.

ABA regulates several developmental processes such as seed and fruit maturation (Zeevaart and Creelman 1988; Zacarias and others 1998). ABA content also accumulates as stress is applied, thus functioning as a mediating signal (Gomez-Cardenas and others 2000). Increased ABA levels detected in S flavedo are similar to that found in fruit under stress (Rodrigo and others 2006; Agusti and others 2007) and could be a response to HLB infection.

Ethylene production was lower in S than in H, and such differences were independent of fruit maturity. Plant pathogen infections can either increase or have no effect on ethylene production, depending on the host–pathogen interaction (Lawton and others 1994; Lahey and others 2004; Van Loon and others 2006). Lower levels of ethylene produced in S could have an implication in the retention of green color and fruit aroma. *Citrus sinensis terpene synthase1*, a gene that encodes an enzyme involved in synthesis of the important sesquiterpene flavor components valencene and nootkatone in grapefruit and sweet orange flavedo and juice oils, was regulated developmentally and enhanced by ethylene (Sharon-Asa and others 2003). Moreover, Dagulo and others (2010) found lower concentrations of valencene in juices from HLB S Valencia fruit compared with H and AS fruit, which suggests that together with lower ethylene production, ethylene could play a role in differential flavor and aroma profiles in S, AS, and H. Finally, fruit from HLB-infected trees abscise prematurely. In citrus, carbohydrate availability has been connected to abscission in developing fruitlets (Iglesias and others 2007), although the primary role of carbohydrate availability in mature fruit abscission is less clear. Although ethylene accelerates abscission, the ethylene–IAA balance plays a regulating role in controlling the process (Hall 1952; Sexton and Roberts 1982). The fact that IAA content is fourfold lower in the stem end of S may promote abscission, even though ethylene production in the whole fruit is lower.

In conclusion, we measured starch and sucrose deficiency in S that could lead to reduced fruit size. S had lower ethylene production that may impact fruit aroma. Total ABA and IAA content in flavedo from S was higher. Compared with normal-growing areas of S, IAA was higher in the misshapen region of those fruits, and, together with increased hypodermal cell size, this suggests a role for IAA in the development of misshapen fruit areas. Further experiments are needed to investigate interactions between carbohydrate shortage and plant hormonal imbalance in HLB infection. Overall, the results show that HLB infection alters the phytohormone and carbohydrate balance in 'Valencia' orange fruit and suggests that such perturbations affect fruit symptom development.

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