Effect of Vector Control and Foliar Nutrition on Quality of Orange Juice Affected by Huanglongbing: Chemical Analysis

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Abstract. ‘Valencia’ orange trees from groves with 90% infection by *Candidatus liberibacter asiaticus* (CLAs), the presumed pathogen for citrus greening or huanglongbing (HLB) disease, were treated with insecticide (I), a nutritional spray (N), and insecticide plus nutritional spray (I + N). Controls (C) were not treated. Fruit were harvested in March to April, 2013, 2014, and 2015, juiced, and the juice was frozen for later chemical analyses. Titratable acidity (TA), soluble solids content (SSC), SSC/TA ratio, many volatiles, flavonoids, and limonoids showed differences because of season, whereas SSC, several volatiles (ethanol, *cis*-3 hexenol, α -terpinene, ethyl acetate, and acetone), flavonoids (narirutin, vicenin-2, diosmin, nobiletin, heptamethoxy flavone), and limonoids (nomilin and nomilinic acid glucoside) showed differences because of treatment. However, consistent patterns for chemical differences among seasons were not detected. TA tended to be higher in N and C the first two seasons and SSC/TA higher in I and I + N for all seasons (not significant for 2014). Bitter limonoids tended to be higher in I, N or I + N over the seasons. Principal Component Analysis showed that there was a good separation by season overall and for treatment in 2013. In 2014 and 2015, the insecticide treatments (either I or I + N) had the highest sugar and SSC/TA levels and lowest TA levels, although not always significant, as well as higher juice CLAs cycle threshold (Ct) levels, indicating lower levels of the pathogen.

Orange juice is the most popular fruit juice because of its flavor and more notably for nutritional benefits (De Ancos et al., 2002). Florida and California are the largest orange producing states in the U.S., with Florida oranges being mostly processed and California mostly consumed as fresh fruit. However, the future of orange fruit production

and subsequently the processing industry is highly jeopardized by citrus greening disease, or HLB, first discovered in Florida in 2005 (Bové, 2006; Gottwald, 2010; Lin et al., 2008; Shokrollah et al., 2011). Despite major research efforts, there is no cure for the disease and Florida orange production has fallen from around 244 million boxes in the 1997–98 season to 81.5 million for this past 2015–16 season (USDA-NASS, 2016). Processing plants are, therefore, not running at full capacity and face closures because of lack of fruit volume. The disease is putatively incited by the bacterium CLAs and is spread by the Asian citrus psyllid (ACP) vector (*Diaphorina citri*) which causes severe tree decline (Bové, 2006). HLB fruit that look normal (asymptomatic) generally taste like healthy fruit, but fruit that are symptomatic for HLB (small, green and misshapen) tend to be off-flavored and drop prematurely, thus, contributing to yield reductions. HLB symptomatic fruit that impart off-flavor to the juice have lower sugars, sometimes higher acids, higher levels of bitter limonoids and astringent flavonoids as well as an altered volatile

profile (Baldwin et al., 2010; Dagulo et al., 2010; Plotto et al., 2010). In general, because HLB symptomatic fruit have significantly lower SSC and often higher acidity (often measured as TAs) compared with healthy fruit, they exhibit a lower soluble solids/acid ratio (SSC/TA) (Baldwin et al., 2010; Dagulo et al., 2010). The SSC/TA is often used to evaluate fruit maturity, with a lower ratio indicating immature fruit (Bassanezi et al., 2009). Based on this ratio, the quality of symptomatic fruits is similar to that of unripe fruit and hence the reported bitter and sour taste (Dea et al., 2013; Plotto et al., 2010). However, the SSC/TA tends to increase with later harvest dates, and as such, variability due to variety and harvest date were found to be higher than that due to disease (Baldwin et al., 2010). Early season fruit juice tends to be less sweet and more bitter, sour, and astringent (Dea et al., 2013; Plotto et al., 2010). Bitterness in orange fruit and juice is caused mainly by two limonoids: limonin and nomilin (Hasegawa et al., 1989). Higher concentrations of limonin and nomilin were found in the juice from asymptomatic and even more so from HLB symptomatic fruit compared with healthy fruit (Baldwin et al., 2010).

Many growers are coping with HLB by controlling the psyllid vector (Stansly et al., 2010, 2014; Tansey et al., 2016) with insecticides and managing tree disease symptoms with foliar nutrient sprays (Giles, 2011; Tansey et al., 2016). Foliar applications deliver micronutrients to counteract HLB-induced deficiencies (Masuoka et al., 2011), salts of phosphorus acid to aid in nutrient assimilation and compounds such as salicylic acid, that are thought to activate systemic acquired resistance pathways (SAR) in the tree. Insect vector control and foliar nutrient/SAR treatments increased yields in the sampled blocks, and thus were purported to lessen disease expression in these same trees (Stansly et al., 2014; Tansey et al., 2016). The objective of this study was to investigate whether the preharvest vector control (insecticide) and foliar nutrient sprays would also mitigate HLB-induced off-flavor symptoms in the fruit and juice, as exhibited by flavor and aroma chemical profiles.

Materials and Methods

Field treatments. Experiments were carried out on a 5.2-ha grove located in Collier Co., Florida, planted in 2001 with *Citrus sinensis* (L.) Osbeck cv. Valencia, on Swingle citrumelo, *C. paradisi* Macf. × *Poncirus trifoliata* L., rootstock. Planting density was 373 trees·ha⁻¹ (151 trees·ac⁻¹) at 7.3 m between rows and 3.7 m within rows (Stansly et al., 2014; Tansey et al., 2016). Trees were irrigated with undertree, microsprinklers, and standard weed control and fertilization practices were followed (Davies and Jackson, 2009). The grove was over 90% infected with HLB within 18 months of commencing treatments in 2008 as ascertained by sampling every fifth tree previously for qPCR

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detection by method of Li et al. (2006). The grove was divided into 16 plots in a randomized complete block (RCB) design with two factors: insecticide and foliar nutrients, each at two levels (with and without) (Stansly et al., 2014). Treatments included insecticide applications (*I*) as needed for ACP control, 2–3 applications of foliar nutrition (*N*), a combination of insecticide plus foliar nutrition (*I* + *N*), and an untreated control (*C*). Each treatment was replicated four times (Stansly et al., 2014).

Insecticide treatments were applied twice during the dormant seasons and during the growing seasons based on a threshold of 0.2 adults per tap sample in 2012 and 0.1 per tap in 2013–15 (Tansey et al., 2016). Insecticide treatments were grouped by growing season from the end of harvest through the beginning of harvest the next year. The two dormant spray applications of broad-spectrum insecticides were made to the entire study site in the winter of 2012–13 and to *I* and *I* + *N* treatment trees only during growing seasons and the winters of 2013–14 and 2014–15 (Table 1). Foliar nutrition was applied during major flush periods (spring, summer, and fall) when leaves were fully expanded but not yet hardened (Stansly et al., 2014; Tansey et al., 2016), with slight differences in the nutrition program in the 2012 to Sept. 2013 than for the rest of the experimental period (included *Bacillus subtilis* and boron) (Table 2).

Fruit sampling. All mature ‘Valencia’ fruit were harvested and weighed by plot mid-March to late-April of 2013, 2014, and 2015 (Tansey et al., 2016). For 2013, a subsample of four bags of fruit subsamples (replicates per treatment were 151–283 fruit/bag-replicate) were delivered to the USDA-ARS Horticultural Research Laboratory and stored at 10 °C for 1–2 weeks until washed and juiced using a JBT extractor (JBT FoodTech, Lakeland, FL) then pasteurized (Model 25DH; MicroThermics Inc., Raleigh, NC) at 92 °C for 18 s and the resulting juice frozen until chemical analyses. Before juicing, a bag of control fruit was used

to prime the juicer. The situation was similar for 2014 and 2015, but the juicing was done at the USDA laboratory using a JBT juicer and MicroThermics pasteurizer. After washing, fruit were juiced using a fresh juicer (Fresh’n Squeeze® Point-of-Sale Juicer; JBT FoodTech), and pasteurized using a pilot pasteurizer (UHT/HTST Laboratory 25EHV Hybrid; Microthermics Inc.) at 90 °C for 10 s.

Quantification of sugars and acids. For quality determination, SSC and TA of the control and blends were determined before individual sugar and acid analyses. SSC, determined by refractive index, was measured with a digital ATAGO PR-101 refractometer (Atago Co, Tokyo, Japan), and TA and pH were calculated from titration of 10 mL of juice with 0.1 mol·L⁻¹ NaOH to a pH 8.1 endpoint using an 808 Titrando (Metrohm, Riverview, FL).

Individual sugars were analyzed with a high-performance liquid chromatography (HPLC) system following an optimized extraction of the juice samples (Baldwin et al., 2012). Twenty grams of juice samples was centrifuged (Avanti J-E centrifuge; Beckman-Coulter, Brea, CA) at 11,952 g_n for 20 min at 10 °C. A total of 10 mL of the supernatant was passed through a C-18 Sep-Pak (Waters/Millipore), and the eluate was filtered with a 0.45 µm Millipore (Siemens-Millipore, Shrewbury, MA) filter before analysis using HPLC. The column used was a Sugar-Pak™ I (10 µm, 6.5 mm × 300 mm) (Waters, Milford, MA) operated at 90 °C in a CH-30 column heater and a TC-50 controller (FIATron, Milwaukee, WI). The samples were analyzed by injecting 60 µL of the filtered juice using a Perkin-Elmer Series 200 autosampler and pump (Perkin-Elmer, Waltham, MA) and running through an isocratic system of 0.001 mol·L⁻¹ CaEDTA mobile phase with a flow rate of 0.3 mL·min⁻¹. Detection of peaks was done with an Agilent 1100 series refractive index detector (Agilent Technologies, Santa Clara, CA). Quantification was based on the external standard method (Version 3.3.2. SP2; EZChrom Elite

software, Santa Clara, CA) using standards for sucrose, glucose, and fructose. All results are expressed as g/100 mL of juice. The sugars sucrose, glucose, and fructose were added to give total sugars, and the glucose and fructose values were multiplied by 0.74 and 1.73, respectively, to normalize to sucrose for sweetness and then added to sucrose to give sucrose equivalents (Koehler and Kays, 1991).

Organic acids were also analyzed using HPLC of the same extracts that were prepared for the individual sugars. Chromatographic separation was done with an AltechOA1000 Prevail organic acid column (9 µm, 300 mm × 6.5 mm) (Grave Davison Discovery Sciences, Deerfield, IL). The samples were introduced to the HPLC system by injecting 60 µL at a flow rate of 0.2 mL·min⁻¹ at 35 °C and a mobile phase of 0.005 mol·L⁻¹ H₂SO₄. The analytes of interest (citric and malic acids) were detected with a Spectra System ultraviolet 6000 LP photo diode array detector (Thermo Fisher Scientific, Waltham, MA). Quantification was based on the calibration curves for standards of citric and malic acids, expressed as g/100 mL of juice.

Total ascorbic acid was quantified by mixing 2 g of homogenate with 20 mL metaphosphoric acid mixture (6% HPO₃ containing 2N Acetic acid). The samples were then filtered (0.22 µm) before HPLC analysis. Ascorbic acid analysis was conducted using a Hitachi LaChromUltra UHPLC system with a diode array detector and a LaChromUltra C18 2 µm column (2 mm × 50 mm) (Hitachi Ltd., Tokyo, Japan). The analysis was performed under isocratic mode at a flow rate of 0.5 mL·min⁻¹ with absorbance measured at 254 nm. Sample injection volume was 5 µL, each with duplicate HPLC injections. Mobile phase was buffered potassium phosphate monobasic (KH₂PO₄, 0.5%, w/v) at pH 2.5 with metaphosphoric acid (HPO₃, 0.1%, w/v). The retention time of the ascorbic acid peak was 2.5 min. After comparison of retention time with the ascorbic acid standard, the peak was identified. The

Table 1. Insecticides applied to *I* (insecticide-treated) and *I* + *N* (insecticide + nutrition-treated) trees from 2012 to 2015.^z

Date	Brand name	Active ingredient	Rate/ha	HMO ^y (%)	Company
1 May 2012	Movento® MPC ^x	Spirotetramat	1.17 L	2	Bayer CropScience LP
15 June 2012	Imidan® 70-W	Phosmet	1.12 kg	1	Gowan Company
16 Aug. 2012	Dimethoate 4EC ^x	Dimethoate	1.75 L	2	Helena Chemical
8 Nov. 2012	Delegate® WG ^x	Spinetoram	0.37 kg	1	Dow AgroSciences LLC
5 Dec. 2012	Danitol® 2.4 EC ^w	Fenpropathrin	1.17 L	—	Valent
24 Jan. 2013	Movento® MPC ^w	Spirotetramat	1.17 L	2	Bayer CropScience LP
10 Apr. 2013	VoliamFlexi® ^x	Thiamethoxam + Chlorantraniliprole	0.51 kg	1	Syngenta
31 Oct. 2013	Closer™ SC Insecticide ^x	Sulfoxaflor	0.37 L	3	Dow AgroSciences LLC
19 Dec. 2013	Imidan® 70-W ^w	Phosmet	1.12 kg	1	Gowan Company
22 Jan. 2014	Danitol® 2.4 EC ^w	Fenpropathrin	1.17 L	—	Valent
22 Mar. 2014	Mustang® ^x	Zeta-Cypermethrin	0.31 L	—	FMC Corporation
7 July 2014	Exirel® ^x	Cyantraniliprole	1.46 L	—	Du Pont
19 Dec. 2014	Lorsban® Advanced ^w	Chlorpyrifos	5.85 L	1	Dow AgroSciences LLC
14 Jan. 2015	Baythroid® XL ^w	Fenpropathrin	0.44 L	—	Bayer CropScience LP
1 May 2015	Agri-Flex® ^x	Thiamethoxam + Abamectin	0.62 L	1	Bayer CropScience LP
27 July 2015	Apta® ^x	Tolfenpyrad	1.82 L	—	Ninchino America Inc

^zTansey et al. (2016).

^yHMO = horticultural mineral oil.

^xGrowing season sprays.

^wDormant spray sprays.

Table 2. Components of foliar nutrition applications in 2012–15.^z

	Product	Function	Rate/ha	Company
2012 to Sept. 2013	Serenade Max WP (<i>Bacillus subtilis</i> 26.2%)	SAR inducer	2.52 kg	AgraQuest, Inc.
	Saver(Potassium salicylate)	SAR inducer	2.34 L	Plant Food Systems
	3–18–20 w/K-Phite® (KH ₂ PO ₃ + K ₂ HPO ₃)	Macronutrient/Fungicide	74.83 L	Plant Food Systems
	13–0–44 fertilizer (KNO ₃)	Macronutrient	9.53 kg	Diamond R Fertilizer
	Techmangan (MnSO ₄)	Micronutrient	9.53 kg	Diamond R Fertilizer
	Zinc Sulfate	Micronutrient	3.14 kg	Diamond R Fertilizer
	Sodium Molybdate	Micronutrient	0.06 kg	Diamond R Fertilizer
	Epsom Salts (MgSO ₄)	Micronutrient	9.53 kg	Diamond R Fertilizer
	Purespray Green® (435 Oil)	Adjuvant	46.77 L	Petro-Canada Lubricants, Inc.
	Saver™ (Potassium salicylate)	SAR inducer	9.35 L	Plant Food Systems
Oct. 2013 to Sept. 2015	K-Phite® (KH ₂ PO ₃ + K ₂ HPO ₃)	Fungicide	4.68 L	Plant Food Systems
	13–0–44 fertilizer (KNO ₃)	Macronutrient	9.53 kg	Diamond R Fertilizer
	Techmangan (MnSO ₄)	Micronutrient	9.53 kg	Diamond R Fertilizer
	Zinc Sulfate	Micronutrient	3.14 kg	Diamond R Fertilizer
	Sodium Molybdate	Micronutrient	0.06 kg	Diamond R Fertilizer
	Epsom Salts (MgSO ₄)	Micronutrient	9.53 kg	Diamond R Fertilizer
	Purespray Green® (435 Oil)	Adjuvant	46.77 L	Petro-Canada Lubricants, Inc.
	Beau-Ron® D (Boron)	Micronutrient	1.68 kg	Drexel Chemical Co.

^zTansey et al. (2016).

amount of total ascorbic acid content in orange juice was quantified using calibration curves obtained from different concentrations (10, 20, 30, 50, 100, 150, 200, and 300 µg·mL⁻¹) of ascorbic acid standards.

Quantification of limonoids and flavonoids.

Concentrations of limonoids and flavonoids in orange juice were determined using high-performance liquid chromatography – mass spectrometry (HPLC–MS) following a previous method (Baldwin et al., 2010). Each juice sample (10 mL) was added to 30 mL of methanol and 70 µL of 1.8 mg·mL⁻¹ mangerifin (internal standard). After manually shaking 60 times, the mixture was incubated at 55 °C for 15 min in a shaking incubator (130 rpm), and then exposed to a –20 °C freezer for 5 min. The cooled mixture was centrifuged at 15,000 g_n for 15 min at 5 °C, and the supernatant was collected. The pellets were extracted again with 10 mL of DI water and 30 mL of methanol by repeating the above shaking, incubation, and centrifuging regimen. The supernatants were merged, and concentrated using a rotary evaporator to yield 2.5 mL extract. The concentrated sample was then passed through a 0.45 µm PTFE filter for HPLC–MS analysis. A Waters 2695 Alliance HPLC (Waters, Medford, MA) connected in parallel with a Waters 996 PDA detector and a Waters/Micromass ZQ single quadrupole mass spectrometer equipped with an electrospray ionization source was used for the analysis. Compound separations were achieved with a Waters Atlantis dC18 column (2.1 mm × 100 mm), using solvent gradient conditions as reported previously (Baldwin et al., 2010). Elution conditions included a binary solvent gradient composed initially of 0.1 mL formic acid/100 mL water, and acetonitrile (90/10 v/v) and increased with linear gradients to 85/15 (v/v) over 10 min, then to 75/25 (v/v), 60/40 (v/v), and 30/70 (v/v) over 15, 23, and 40 min, respectively, and finally equilibrating to the initial condition of 90/10 (v/v) over 60 min, at a flow rate of 0.75 mL·min⁻¹. Postcolumn split to the PDA and mass ZQ detector was 10:1. MS parameters were as

follows: ionization mode, ES⁺; capillary voltage 3.0 kV, extractor voltage 5 V; source temperature 100 °C; desolvation temperature 225 °C; desolvation N₂ flow 465 L·h⁻¹; cone N₂ flow 70 L·h⁻¹. Protonated ions [M + H]⁺ were monitored in scan mode. Quantification was based on the calibration curves for authentic standards of each flavonoid and limonoid compounds analyzed and expressed as g/100 mL of juice.

Quantification of aroma volatiles. Three milliliters of juice was transferred to a 10-mL crimp-capped vial at the pilot plant, transported on ice to the laboratory, and then stored at –80 °C. Frozen samples were thawed under running tap water and injected onto an Agilent 6890 (Agilent Technologies) gas chromatography (GC) using a Gerstel multipurpose autosampler equipped with Stabilwax and HP-5 low bleed columns. The flow rate was split equally to the two columns at 17 mL·min⁻¹ at 40 °C with an increase in temperature at 6 °C·min⁻¹ up to 180 °C, where the temperature was held constant for an additional 5.8 min. The GC peaks for the aroma volatile compounds were quantified using standard curves as determined by enrichment of deodorized orange juice by known concentrations of authentic volatile compound standards (Baldwin et al., 2010). Volatile compound identification was confirmed using a headspace and solid phase microextraction (SPME) fibers along with mass spectroscopy (MS) following the methods described by Bai et al. (2014). Briefly, the juice samples were incubated for 30 min at 40 °C. A 2-cm SPME fiber (50/30 µm DVB/Carboxen/PDMS; Supelco, Bellefonte, PA) was then exposed to the headspace for 30 min at 40 °C. After exposure, the SPME fiber was inserted into the injector of a GC–MS (Model 6890; Agilent) to desorb the extract for 15 min at 250 °C. The GC–MS equipment and settings were as follows: DB-5 (60 m length, 0.25 mm i.d., 1.00 µm film thickness; J&W Scientific, Folsom, CA) columns, coupled with a MS detector (5973 N; Agilent). Mass units were monitored from 30 to 250 m/z and ionized

Fig. 1. Principle component analysis plot of all four preharvest treatments of ‘Valencia’ orange trees including untreated control (C), foliar nutritional sprays (N), insecticide sprays (I), I + N (M) for all 3 years (February–March harvests in 2013, 2014 and 2015) for 55 attributes that were measured postharvest in fruit juice and included 12 sugar and acid measurements, 26 aroma volatiles, and 17 secondary metabolites (flavonoids or limonoids).

at 70 eV. Data were collected using a data system (ChemStation G1701 AA; Hewlett-Packard, Palo Alto, CA). A mixture of C-5 to C-18 n-alkanes was run at the beginning of each day to calculate retention indices.

DNA extraction and qPCR detection of CLAs from juice. DNA was extracted from 500 µL of orange juice using a modified CTAB method as described in a patent publication (Zhao et al., 2015). DNA quality (260/280 and 260/230 ratio) and quantity were assessed using spectrophotometry (NanoDrop; Thermo Scientific). CLAs detection was accomplished by qPCR. Specific primers targeting CLAs 16S rRNA gene (Li primers) (Li et al., 2006) or CLAs hyv1 (LJ primers) (Morgan et al., 2012) were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). PCR mixtures with a total volume of 15 µL contained 7.5 µL of TaqMan

PCR master mix (Applied Biosystems) for Li primers, or SYBR Green PCR Master Mix (Applied Biosystems) for LJ primers, 250 nM each primer, 150 nM probe (for Li primers) and 100 ng of template DNA. Real-time PCR amplifications were performed in a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The qPCR parameters were as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min, with fluorescence signal capture at each stage of 60 °C. For SYBR® Green Real-Time PCR (with LJ primers), the default Melt Curve (disassociation) stage is continued after the 40 cycles of PCR. Ct values were analyzed using ABI 7500 Software version 2.0.6 (Applied Biosystems, Inc., Carlsbad, CA) with a manually set threshold at 0.02 and automated baseline settings.

Statistics. Principle component analysis (PCA), which is a method for projecting the 55 attributes space onto a few dimension space, was conducted for each season and across all seasons using JMP (version 11.2.0; SAS Institute, Cary, NC). SAS (version 9.4; SAS Institute) was used for all other analysis.

Analysis of variance (ANOVA) for each measurement was done separately for each year and for the 3 years combined. For the analyses of each individual year, an RCB design with three replicates was used. Mean separations were accomplished using Tukey's test ($P < 0.05$). For the analyses of the 3-year combined data, the design was a 2 (I) × 2 (N) factorial, with a split plot over years.

Results and Discussion

Overall, there were differences by season, regardless of treatment, as can be expected because of environmental conditions and cumulative effects of both treatment and disease. In a previous publication involving the same treatments, the yields were highest on $I + N$ trees followed by I trees, followed by N trees and finally C trees (Tansey et al., 2016). A PCA plot using all the chemical data measured in the orange juice samples showed groupings by year (Fig. 1), with the first two principal components explaining 55.7% of the data, for all the treatments combined.

Component three explained an additional 11.6% of the variation (data not shown).

Overall, only the year 2013 showed separation for chemical compounds by treatment. This is seen in a PCA (Fig. 2), explaining 53.2% of the variation in the first two components, with component three explaining an additional 14% of the variation (data not shown). The first two components show C and N separating from each other and both from I and $I + N$. The N treatment was correlated with pH, TA, citric and malic acids, aroma volatiles γ -terpinene, α -terpineol, 2-methyl propanol, and limonene as well as with many flavonoids. The untreated C was correlated with SSC, TA, SSC/TA, and many aroma volatiles. Treatments I and $I + N$ correlated with the volatiles hexanol, methyl butanoate, methanol, α -terpineol, linalool, and somewhat with SSC/TA.

For 2013, there were differences for all sugar and acid measurements except for pH and malic acid (Table 3). Treatment N was highest in TA, SSC, sucrose, glucose, fructose, total sugars, sucrose equivalents, and citric acid. Treatment N compound levels were not always significantly higher than I or $I + N$ but were all significantly higher than untreated C , whereas $I + N$ was highest in SSC/TA among the treatments. SSC, glucose, fructose, sucrose, total sugars, and sucrose equivalents (where the sweetening power of glucose and fructose are normalized to sucrose) indicate or impart sweetness, whereas TA, citric, malic, and to some extent ascorbate, impart sourness to orange juice. For 2014, there again were no differences for pH and treatment N was again highest in TA (along with C) and SSC (although the differences were very small between C , I , and N), there was no difference in SSC/TA, whereas I was highest in sucrose, glucose, fructose, total sugars, and sucrose equivalents (in contrast to 2013 where N was highest and now is lowest in these for 2014) as well as citric and malic acids (along with C). For 2015, there were no differences in pH or malic acid, as for 2013 and 2014, nor for SSC or fructose. Treatment $I + N$ was lowest in

Fig. 2. Principle component analysis (PCA) for year 2013 for four preharvest treatments of ‘Valencia’ orange trees including untreated control (C), foliar nutritional sprays (N), insecticide sprays (I) and $I + N$ (M) (a, PCA scores plot) for 55 attributes that were measured postharvest in the fruit juice and included 12 sugar and acid measurements, 26 aroma volatiles, and 17 secondary metabolites (flavonoids and limonoids) (b, PCA loading plot).

Table 3. Effect of insecticide, nutritional spray, or both during growth season on contents of sugar and acid measurements in ‘Valencia’ orange juice over three harvest seasons.

Attribute	2013				2014				2015			
	Control	Insecticide (I)	Nutrition (N)	$I + N$	Control	Insecticide (I)	Nutrition (N)	$I + N$	Control	Insecticide (I)	Nutrition (N)	$I + N$
pH	4.1 ²	4.1	4.2	4.1	4.1	4.1	4.1	4.1	4.2	4.3	4.2	4.3
Titrateable acidity (TA, %)	0.68 b	0.65 b	0.73 a	0.66 b	0.77 a	0.71 b	0.78 a	0.73 b	0.71 a	0.66 a	0.69 a	0.63 b
Soluble solids content (SSC, %)	11.0 b	10.0 c	11.4 a	10.9 b	10.4 ab	10.2 ab	10.7 a	9.9 b	9.8	10.3	10.4	9.5
SSC/TA	16.2 b	15.4 c	15.6 c	16.5 a	13.6	14.2	13.7	13.5	13.8 b	15.7 a	15.1 b	15.1 b
Sucrose (%)	3.9 b	4.9 ab	5.4 a	4.6 b	3.8 b	4.3 a	3.2 c	3.6 b	3.7 b	4.2 ab	4.3 a	4.4 a
Glucose (%)	1.4 b	1.8 ab	1.9 a	1.6 ab	1.9 ab	2.0 a	1.5 c	1.7 b	1.9 b	2.1 ab	2.0 ab	2.1 a
Fructose (%)	1.9 b	2.3 ab	2.6 a	2.1 ab	2.2 ab	2.3 a	1.8 c	2.0 b	2.2	2.4	2.3	2.4
Total sugars (%)	7.2 b	9.0 ab	9.9 a	8.4 ab	7.9 ab	8.6 a	6.5 c	7.3 b	7.8 b	8.7 ab	8.7 ab	9.0 a
Sucrose equivalence (SE)	8.2 b	10.3 ab	11.3 a	9.5 ab	8.9 ab	9.7 a	7.3 c	8.3 b	8.9 b	9.9 ab	9.9 ab	10.2 a
Citric acid (%)	0.42 b	0.41 b	0.87 a	0.48 b	0.80 a	0.80 a	0.50 b	0.66 b	0.67 ab	0.61 b	0.73 a	0.70 ab
Malic acid (%)	0.13	0.11	0.17	0.14	0.18 a	0.18 a	0.15 b	0.16 b	0.25	0.24	0.24	0.23

²Mean values ($n = 4$) followed by different letters in the same attributes and same year (row) are significantly different at $P \leq 0.05$ using Tukey's test.

TA, treatment *I* was highest in SSC/TA, and either *N* or *I + N* highest in sucrose, glucose, total sugars, or sucrose equivalents with *N* being highest in citric acid. Overall, this is somewhat similar to 2013. There were no significant differences for total ascorbic acid, which was analyzed in 2014 and 2015 and ranged from 14.5 to 32.7 mg/100 g, with no significant differences because of treatment (data not shown).

For aroma volatiles in 2013, there were no differences for acetaldehyde, octanal, or 2-methyl propanol (Table 4). Treatment *I* was highest in methanol along with *I + N*, octanol, linalool, and α -terpineol. Treatment *N* was highest in hexanal, decanal, ethanol (along

with *C*), terpinene-4-ol, α -pinene, sabinene, myrcene (along with *C*), limonene (along with *C*), γ -terpinene, ethyl butanoate, ethyl hexanoate, and acetone (the last three along with *C* and acetone along with *I + N*). Other than already mentioned, treatment *I + N* was highest in hexanol and methyl butanoate. In 2014, there was little relationship to 2013 aroma volatile levels, except again, there were no differences for acetaldehyde or 2-methyl propanol and unlike in 2013, there were differences for octanal. In addition to octanal, there were also differences for hexanal, methanol, ethanol, hexanol, *cis*-3-hexanol, α -pinene, limonene, γ -terpinene, valencene, ethyl acetate, methyl butanoate, and ethyl

butanoate as in 2013; however, different treatments showed elevated levels as compared with 2013. For 2015, aroma volatiles, decanal, octanol, linalool, terpinene-4-ol, α -pinene, sabinene, myrcene, limonene, γ -terpinene, valencene ethyl acetate, methyl butanoate, ethyl hydroxyhexanoate, and acetone showed no differences among treatments, which was completely different from 2013 and 2014. Acetaldehyde, which showed differences unlike 2013 and 2014, hexanal was highest in treatment *I* as was hexanal (as in 2014), and octanal was highest in *N* and *I + N*, methanol was lowest in *I + N*, and ethanol and 2-methyl propanol were highest in *I* with *cis*-3-hexanol showing high levels in *C*, as in 2013 and 2014,

Table 4. Effects of insecticide, nutritional spray, or both during growth season on contents ($\mu\text{L}\cdot\text{L}^{-1}$) of aroma volatile compounds in 'Valencia' orange juice over three harvest seasons.

Attribute	2013				2014				2015			
	Control	Insecticide (<i>I</i>)	Nutrition (<i>N</i>)	<i>I + N</i>	Control	Insecticide (<i>I</i>)	Nutrition (<i>N</i>)	<i>I + N</i>	Control	Insecticide (<i>I</i>)	Nutrition (<i>N</i>)	<i>I + N</i>
Acetaldehyde	14.8 ^z	14.7	14.9	14.8	11.2	10.5	10.6	10.2	8.8 ab	10.5 a	8.9 ab	7.8 b
Hexanal	0.32 b	0.26 c	0.38 a	0.31 b	0.05 ab	0.07 a	0.03 b	0.03 b	0.20 ab	0.24 a	0.17 b	0.15 b
Octanal	1.2	1.1	1.2	1.2	1.6 a	1.3 b	1.4 ab	1.4 ab	0.13 a	0.09 b	0.12 a	0.14 a
Decanal	0.86 ab	0.89 ab	0.91 a	0.74 b	0.66	0.58	0.59	0.69	0.39	0.32	0.32	0.32
Methanol	204 b	249 a	190 c	246 a	116 a	87 b	100 b	90 b	61.2 a	59.6 a	61.5 a	50.5 b
Ethanol	948 a	840 b	898 a	810 b	813 a	606 b	614 b	542 c	715 ab	689 ab	749 a	594 b
2-Methylpropanol	0.020	0.022	0.025	0.017	0.01	0.01	0.01	0.01	0.17 ab	0.16 ab	0.20 a	0.13 b
Hexanol	0.24 c	0.30 ab	0.27 b	0.33 a	0.08 a	0.06 b	0.07 ab	0.04 b	0.15 ab	0.18 a	0.13 b	0.13 b
<i>cis</i> -3-Hexanol	0.44 a	0.40 b	0.41 ab	0.39 b	0.38 a	0.34 b	0.32 b	0.34 b	0.30 a	0.28 a	0.23 b	0.25 b
Octanol	1.7 b	1.8 a	1.7 b	1.5 c	1.6	1.7	1.8	1.9	1.3	1.3	1.4	1.4
Linalool	0.92 b	1.15 a	1.00 ab	0.95 b	0.64	0.59	0.59	0.60	0.66	0.65	0.65	0.54
Terpinen-4-ol	0.39 b	0.42 ab	0.45 a	0.36 b	0.20	0.17	0.19	0.20	0.27	0.27	0.25	0.23
α -Terpineol	0.44 b	0.62 a	0.40 b	0.44 b	0.48	0.47	0.44	0.49	0.60	0.73	0.63	0.60
α -Pinene	1.9 b	1.7 c	2.1 a	1.7 c	1.4 b	1.5 a	1.4 b	1.4 b	1.5	1.4	1.5	1.3
Sabinene	1.6 b	1.2 c	2.1 a	1.2 c	2.1	1.9	1.9	1.6*	0.94	0.95	0.73	0.80
Myrcene	5.5 a	5.2 ab	5.7 a	5.1 b	4.5	4.8	4.5	4.6	4.1	4.0	4.1	3.9
Limonene	220 a	212 b	223 a	207 b	195.8 b	205.1 a	197.3 b	202.2 b	183	181	180	179
γ -Terpinene	0.061 b	0.062 ab	0.064 a	0.063 ab	0.25 a	0.23 a	0.20 b	0.20 b	0.12	0.11	0.11	0.10
Valencene	6.43 a	5.81 b	5.59 c	5.93 b	3.9 a	3.9 a	3.3 b	3.4 b	4.6	5.4	4.9	4.9
Ethyl acetate	0.19 a	0.17 b	0.16 b	0.14 b	0.16 a	0.12 b	0.10 bc	0.08 c	0.10	0.08	0.11	0.08
Methyl butanoate	0.011 d	0.016 b	0.013 c	0.027 a	0.023 a	0.015 b	0.016 b	0.018 b	0.009	0.016	0.016	0.012
Ethyl butanoate	0.42 a	0.37 b	0.43 a	0.35 b	0.31 a	0.19 b	0.19 b	0.21 ab	0.19 ab	0.22 a	0.22 a	0.17 b
Ethyl hexanoate	0.044 a	0.034 b	0.044 a	0.033 b	0.012	0.013	0.014	0.016	0.014 a	0.013 a	0.008 b	0.011 b
Ethyl 3-hydroxyhexanoate	82.3 a	75.1 b	72.7 b	73.7 b	53.7	53.8	47.6	51.1	71.1	86.1	81.6	83.2
Acetone	0.29 a	0.16 b	0.26 a	0.28 a	0.17	0.13	0.13	0.08	0.11	0.09	0.09	0.10

^zMean values ($n = 4$) followed by different letters in the same attributes and same year (row) are significantly different at $P \leq 0.05$ using Tukey's test.

Table 5. Effects of insecticide, nutritional spray, or both during growth season on contents ($\text{mg}\cdot\text{L}^{-1}$) of flavonoid and limonoid compounds in 'Valencia' orange juice over three harvest seasons.

Attribute	2013				2014				2015			
	Control	Insecticide (<i>I</i>)	Nutrition (<i>N</i>)	<i>I + N</i>	Control	Insecticide (<i>I</i>)	Nutrition (<i>N</i>)	<i>I + N</i>	Control	Insecticide (<i>I</i>)	Nutrition (<i>N</i>)	<i>I + N</i>
Hesperidin	96 ^z	104	109	92	194	213	218	175	92	101	100	97
Narirutin	17 b	18 b	21 a	17 b	20	22	23	20	20	23	22	22
Vicenin-2	25 ab	22 ab	26 a	21 b	38 a	39 a	38 a	33 b	26	27	26	26
Isosakuranetin rutinoside	9.5 b	8.8 b	12.3 a	8.6 b	7.8 b	9.8 a	10.0 a	8.5 b	8.6	9.9	10.5	10.9
Diosmin	1.1 b	1.1 b	1.2 a	1.0 b	1.1 ab	1.4 a	1.1 ab	0.7 b	1.1	1.2	1.1	0.9
Sinensetin	1.8	1.9	2.1	1.6	2.1	2.4	2.6	2.1	1.8 ab	1.9 a	2.1 a	1.6 b
Tangeretin	0.76	0.68	0.91	0.74	0.83 ab	0.44 b	1.18 a	0.40 b	0.76 b	0.68 b	0.91 a	0.74 b
Nobiletin	2.7 b	2.6 b	3.2 a	2.5 b	4.3 b	3.5 bc	5.6 a	2.5 c	2.7	2.6	3.2	2.5
Heptamethoxyflavone	2.1 b	2.2 b	2.7 a	2.0 b	2.1 b	2.4 ab	3.2 a	2.0 b	2.1 b	2.2 b	2.7 a	2.0 b
Limonin	1.6 c	3.7 a	3.9 a	2.2 b	1.8 ab	1.5 b	2.4 a	2.1 ab	2.2 ab	2.2 ab	2.0 b	2.4 a
Limonin glucoside	63 ab	65 ab	67 a	60 b	163 a	151 ab	163 a	140 b	66	67	69	70
Nomilin	0.10 bc	0.26 a	0.16 b	0.08 c	0.06 c	0.18 a	0.16 a	0.10 b	0.13	0.12	0.10	0.19
Nomilinic acid glucoside	183 ab	181 ab	232 a	161 b	291 a	286 b	293 a	292 ab	162 b	187 a	167 b	186 a
Deacetyl nomilinic acid glucoside	4.4 b	5.5 a	6.1 a	5.2 b	3.2 ab	3.5 a	3.1 ab	2.7 b	4.7	5.2	5.6	5.2

^zMean values ($n = 3$) followed by different letters in the same attributes and same year (row) are significantly different at $P \leq 0.05$ using Tukey's test.

Table 6. Attributes which were significantly affected by insecticide and nutritional sprays over three seasons.

Attribute	Mean value		ANOVA (<i>F</i> value and significance)			
	Insecticide (<i>I</i>)		Nutrition (<i>N</i>)		<i>I</i>	<i>N</i>
	No	Yes	No	Yes		
Titrateable acidity (TA, %)	0.73	0.67			11.4***	
Soluble solids content (SSC, %)	10.6	10.1			6.3*	
Ethanol	789.6	680.3			6.2*	
α -Terpineol	0.50	0.56			4.2*	
Sabinene	1.57	1.26			4.1*	
Vicenin-2	29.8	27.9			4.1*	
Ethyl acetate			0.14	0.11		4.3*
Diosmin			1.16	1.02		5.2*
Hesperidin	134.9	130.4	133.4	132.0		14.2***
Narirutin	20.6	20.1	20.0	20.7		5.4*
Heptamethoxyflavone	2.48	2.13	2.18	2.43		4.1*
Limonin	2.31	2.37	2.18	2.51		4.7*
Nomilin	0.120	0.154	0.141	0.133		7.7**

*, **, ***Significant at $P \leq 0.05$, 0.01, and 0.001.

Fig. 3. Cycle threshold (Ct) values from qPCR of orange juice from fruit harvested from 'Valencia' orange trees, untreated (controls) or treated preharvest with foliar nutritional sprays (*N*), insecticide sprays (*I*) or *I* + *N*. Different letters in the same year (**A** and **C**) indicate significant differences between treatments, and different letters (**B** and **D**) indicate significant differences between treatments according to Tukey's test at $P < 0.05$. (**A** and **B**) are Ct values using Li primers, and (**C** and **D**) are Ct values using LJ primer.

along with *I*. Ethyl butanoate was highest in *I* and *N*, whereas ethyl hexanoate showed high levels in *C*, as in 2013, and in *I*. Otherwise, there was no consistency in aroma values across the years. The aroma volatiles analyzed in this study are reported to contribute to orange juice flavor, although there is no one odor-active compound. Aldehydes, and esters particularly, contribute giving green, citrus, and fruity aromas along with terpenoid hydrocarbons (Perez-Cacho and Rouseff, 2008).

For flavonoids in 2013, there were no differences for hesperidin, narirutin, vicenin-2, diosmin, sinensetin, tangeretin, and nobiletin. Treatment *N* was highest in isokuraretin rutinoside, heptamethoxyflavone, limonin (along with *I*), limonin glucoside, nomilinic acid glucoside, and deacetyl nomilinic

acid glucoside (along with *I*) (Table 5). Other than already mentioned, *I* was highest in nomilin. Treatment *I* + *N* was intermediate in most secondary metabolites, but lowest in nomilin, whereas untreated *C* was lowest in limonin. Limonin and nomilin impart bitterness to orange juice, whereas the flavonoids impart mostly astringency (Horowitz and Gentili, 1969; Manners, 2007). The 2014 flavonoid and limonoid measurements had more compounds showing differences among treatments including vicenin-2, diosmin, tangeretin, and nobiletin, which did not show differences in 2013. For these compounds and the other compounds that showed differences in 2013, either *I*, *N*, or both showed the highest values, although not always significantly different from *C* or *I* + *N*. For 2015, only sinensetin,

tangeretin, heptamethoxyflavone, limonin, and nomilinic acid glucoside showed differences among treatments. For sinensetin, tangeretin, and heptamethoxyflavone, *I* or *N* were highest; for limonin and nomilinic acid glucoside, *I* or *I* + *N* were highest.

Regarding the main effects of *I* or *N*, the sugar and acid measurements TA and SSC were lower with *I*, along with the volatiles ethanol, α -terpineol, sabinene, and vicenin-2, which were lower with *I* except for α -terpineol (Table 6). Meanwhile, the volatile ethyl acetate and flavonoid diosmin were both lower for *N*. The flavonoids hesperidin (lower), narirutin (higher), heptamethoxyflavone (lower) as well as the limonoids, limonin (higher), and nomilin (lower) for *N* were all also significant for *I* + *N*.

Measurement of CLAs levels using qPCR of the juice, expressed as Ct values, generally showed a significant effect of insecticide treatments for both primers used (Fig. 3). Although initially leaves of every fifth tree had been tested to determine that the grove sites were more than 90% infected, the sectors of the tree can differ in CLAs levels, and the effect of insecticides preventing subsequent infections also can affect subsequent CLAs levels. Therefore, we tested the fruit juice to see how infected the fruit were. Because there is much less nucleic acid material in juice than in leaves, the LJ primer (Fig. 3C and D) (Morgan et al., 2012) was used in addition to the Li primer (Fig. 3A and B) (Li et al., 2006), as it is more sensitive because of the higher copy number of target genes (a prophage embedded numerous times in the CLAs genome). Figure 3A and C show that insecticide treatments (*I* and *I* + *N*) were generally lower in CLAs infection indicated by higher Ct values. Treatments *I* and *I* + *N* were always significantly different from controls, with *I* being significantly different from *N* as well. Over all 3 years (Fig. 3B and D), both insecticide treatments (*I* and *I* + *N*) had higher Ct values (lower CLAs levels) than *C* or *N*.

In conclusion, this study investigated whether foliar nutritional, insecticide, or both treatments of orange trees affected by HLB could improve flavor as determined by measuring flavor-related chemicals. The *I*, *N*, and *I* + *N* preharvest treatments of orange trees

did not have a consistent postharvest effect on flavor chemicals in the subsequent fruit juice over the 3 years of the study. Across the years, total sugars, TA, SSC/TA, and the bitter limonoids (limonin and nomilin), the most important taste predictors (sweetness, sourness, and bitterness) affected by HLB (Raithore et al., 2015; Plotto et al., 2010), were similar in 2013 and 2015, dipping slightly (sugars and bitter limonoids) or increasing slightly (TA) in 2014 (Tables 3 and 5), thus showing no trend. Therefore, it is not likely that these treatments consistently impacted flavor by raising sugar levels or reducing concentrations of acids, bitter limonoids, or both. Neither were there any significant trends in aroma compounds. However, in 2014 and 2015, either *I* or *I* + *N* had the highest sugar and SSC/TA levels and lowest TA levels, although not always significantly different from *C* (TA) or *N* (SSC/TA), suggesting a cumulative effect of the treatments over the years. The chemical results are reflected in sensory results (Plotto et al., 2017) of the same samples, which were not consistent but showed that *I* or *I* + *N* were associated with positive orange juice characteristics (orange, fruit flavor, and sweetness), especially in 2015. Thus, use of insecticides (with or without nutritional treatment) seemed to improve flavor. In addition, the insecticide treatments (*I* and *I* + *N*) did seem to have a consistent effect on Ct values indicating lower CLas levels for those treatments (Fig. 3). There is evidence that CLas titer is correlated to flavor quality (Zhao et al., 2015). These results are consistent with previously reported beneficial effects on yield (Stansly et al., 2014; Tansey et al., 2016).

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