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, c-terpinene, ethyl acetate, and acetone), flavonoids (narinrutin, vicenin-2, nobiletin, heptamethoxy flavone), and limonoids (nomilin and nomilinic acid glucoside) showed differences because of treatment. However, consistent patterns for chemical differences among seasons were found in 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 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 astrin gent (Dea et al., 2013; Plotto et al., 2010). Bitterness in orange fruit and juice is caused mainly by two limonoids: nomilin and nomilinic acid (Hasegawa et al., 1989). Higher concentrations of nomilin and nomilnic acid 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 plastic 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.
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 spray applications of broad-spectrum insecticide treatments 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).

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 Titrand (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 were centrifuged (Avanti J-E centrifuge; Beckman-Coulter, Brea, CA) at 11,952 g 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 AlttechOA1000 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) and metaphosphoric acid mixture (6% HPO₃, 0.3%, 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.

Table 1. Insecticides applied to I (insecticide-treated) and I + N (insecticide + nutrition-treated) trees from 2012 to 2015.¹

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

¹Tansey et al. (2016).

²HMO = horticultural mineral oil.

³Growing season sprays.

⁴Dormant season sprays.
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 manganin (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 for 5 min. The cooled supernatant was collected. The concentrated sample was then passed repeating the above shaking, incubation, centrifuging regimen. The supernatant was merged, and concentrated using the supernatant was collected. The pellets were extracte d again with 10 mL of DI water and 30 mL of methanol by re-
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Quantification of aroma volatiles. Three milliliters of juice was transferred to a 10-mL crimp-capped vial at the pilot plant, transport ed 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 Sta-
bilwax 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: ionization mode, ES +; capillary voltage 3.0 kV, extractor voltage 5 V; source temperature 100 °C; desorption 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.

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 rDNA 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

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

| Product | Function | Rate/ha | Company |
|---------|----------|---------|---------|
| Serenade Max WP (Pseudobacte rium putida) 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® (KH2PO4 + K2HPO4) | Macronutrient/Fungicide | 74.83 L | Plant Food Systems |
| 13–0–44 fertilizer (KNO3) | Macronutrient | 9.53 kg | Diamond R Fertilizer |
| Techmangam (MnSO4) | 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 (MgSO4) | Micronutrient | 9.53 kg | Diamond R Fertilizer |
| Purespray Green® (435 Oil) | Adjuvant | 46.77 L | Petro-Canada Lubricants, Inc. |
| Oct. 2013 to Sept. 2015 | | | |
| Saver™ (Potassium salicylate) | SAR inducer | 9.35 L | Plant Food Systems |
| K-Phite® (KH2PO4 + K2HPO4) | Fungicide | 4.68 L | Plant Food Systems |
| 13–0–44 fertilizer (KNO3) | Macronutrient | 9.53 kg | Diamond R Fertilizer |
| Techmangam (MnS04) | 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 (MgSO4) | 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. |

*Tansey et al. (2016).
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 replications 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 $2 (I) \times 2 (N)$ factorial, with a split plot over years.

### Results and Discussion

Overall, there were differences by season, regardless 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 $\gamma$-terpineine, $\alpha$-terpinol, 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, $\alpha$-terpinol, 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 and $I + N$ 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)](image)

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±    | 4.1           | 4.2           | 4.1     | 4.1      | 4.1           | 4.1           | 4.1     | 4.2     | 4.3           | 4.2           | 4.3    |
| Titratable 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 a | 10.4 | 9.5 a |
| SSC/TA          | 16.2 b | 15.4 c | 15.6 c | 16.5 a | 13.6       | 14.2       | 13.7       | 13.5 a | 13.8 b | 15.7 a | 15.1 b | 15.1 b |
| Sugar (%)       | 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 a | 1.9 a | 1.6 b | 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 a | 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 (%)     | 8.2 b   | 10.3 ab | 11.3 a | 9.5 b | 8.9 ab | 9.7 a | 7.3 c | 8.3 b | 8.9 b | 9.9 ab | 9.9 ab | 10.2 a |
| Equivalence (SE) | 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 |
| Citric 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 a | 0.24 a | 0.23 a |

*Mean 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-pinen, sabine, myrcene (along with C), limonene (along with C), γ-terpine, 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-pinen, limonene, γ-terpine, 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, terpine-4-ol, α-pinen, sabine, myrcene, limonene, γ-terpine, 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 (µg·L⁻¹) of aroma volatile compounds in 'Valencia' orange juice over three harvest seasons.

| Attribute         | Control | Insecticide | Nutrition | I + N |
|-------------------|---------|-------------|-----------|-------|
| Acetaldehyde      | 14.8±  | 14.7        | 14.9      | 14.8  |
| Hexanal           | 0.32 b  | 0.26 c      | 0.38 a    | 0.31 b |
| Octanal           | 1.2 ±   | 1.1 ±       | 1.2 ±     | 1.2 ±  |
| Decanal           | 0.86 ±  | 0.89 ±      | 0.91 ±    | 0.74 ± |
| Methanol          | 204 b   | 249 a       | 190 c     | 246 a  |
| Ethanol           | 948 a   | 840 b       | 898 a     | 810 b  |
| 2-Methy propanol  | 0.20 ±  | 0.022       | 0.025     | 0.017 |
| Hexanol           | 0.24 c  | 0.30 ab     | 0.27 b    | 0.33 a |
| cis-3-Hexenal     | 0.44 a  | 0.40 b      | 0.41 ab   | 0.39 b |
| Octanol           | 1.7 b   | 1.8 a       | 1.7 b     | 1.5 c  |
| Linalool          | 0.92 b  | 1.15 a      | 1.00 ab   | 0.95 b |
| Terpine-4-ol      | 0.39 c  | 0.42 a      | 0.45 a    | 0.36 b |
| α-Terpine         | 0.44 b  | 0.62 a      | 0.40 b    | 0.44 b |
| α-Pine            | 1.9 b   | 1.7 c       | 2.1 a     | 1.7 c  |
| Sabine            | 1.6 b   | 1.2 c       | 2.1 a     | 1.2 c  |
| Myrcene           | 5.5 ± a | 5.2 ab      | 5.7 ± a   | 5.1 ± b |
| Limonene          | 220 a   | 212 b       | 223 a     | 207 b  |
| γ-Terpine         | 0.061 b | 0.062 ab    | 0.064 a   | 0.063 ab |
| Valencene         | 6.43 a  | 5.81 b      | 5.99 c    | 5.93 b |
| Ethyl acetate     | 0.19 a  | 0.17 b      | 0.16 b    | 0.14 b |
| Methyl butanoate  | 0.011 d | 0.016 b     | 0.013 c   | 0.027 a |
| Ethyl butanoate   | 0.42 a  | 0.37 b      | 0.43 a    | 0.35 b |
| Ethyl hexanoate   | 0.044 a | 0.034 b     | 0.044 a   | 0.033 b |
| Ethyl             | 82.3 ±  | 75.1 b      | 72.7 b    | 73.7 b |
| 3-Hydroxyhexanoate| 0.29 a  | 0.16 b      | 0.26 a    | 0.28 a |

Table 5. Effects of insecticide, nutritional spray, or both during growth season on contents (mg·L⁻¹) of flavonoid and limonoid compounds in 'Valencia' orange juice over three harvest seasons.

| Attribute         | Control | Insecticide | Nutrition | I + N |
|-------------------|---------|-------------|-----------|-------|
| Hesperidin        | 96±     | 104         | 109       | 92    |
| Naringenin        | 17 b    | 18 b        | 21 a      | 17 b  |
| Vicenin-2         | 25 ab   | 22 ab       | 26 a      | 21 b  |
| Isosakuranetin rutinoside| 9.5 b | 8.8 b | 12.3 a | 8.6 b |
| Diosmin           | 1.1 b   | 1.1 b       | 1.2 a     | 1.0 b  |
| Sinensetin        | 1.8 ± a | 1.9 ± b     | 2.1 ± a   | 1.6 ± b |
| Tangeretin        | 0.76 ± a| 0.68 ± b    | 0.91 ± q  | 0.74 ± |
| Nobiletin         | 2.7 b   | 2.6 b       | 3.2 a     | 2.5 b  |
| Heptamethoxyflavone| 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  |
| Limonin glucoside | 63 ab   | 65 ab       | 67 a      | 60 b  |
| Nomilin           | 0.10 bc | 0.26 a      | 0.16 b    | 0.08 c |
| Nomilinic acid glucoside| 8.3 ab | 11.1 ab | 13.2 a | 10.8 b |
| Deacetyl nomilinic acid glucoside| 4.4 b | 5.5 a | 6.1 a | 5.2 b |

3Mean values (n = 3) followed by different letters in the same attributes and same year (row) are significantly different at P ≤ 0.05 using Tukey’s test.

3Mean values (n = 4) followed by different letters in the same attributes and same year (row) are significantly different at P = 0.05 using Tukey’s test.
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 Roseff, 2008).

For flavonoids in 2013, there were no differences for hesperidin, narirutin, vicenin-2, diosmin, sinetetin, tangeretin, and nobiletin. Treatment N was highest in isokuranetin 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 nomilinic acid 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 sinetetin, tangeretin, heptamethoxyflavone, limonin, and nomilinic acid glucoside showed differences among treatments. For sinetetin, 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, sabine, 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 (with or without nutritional treatment) seemed 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 Cl 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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