



Chemical Composition of Xylem Sap from *Citrus sinensis* L. Osbeck (Sweet Orange)

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Currently, Huanglongbing is the major disease in Florida citrus. The disease is caused by the phloem-restricted, uncultivable bacterium, *Candidatus Liberibacter asiaticus* and transmitted by the Asian citrus psyllid (ACP), *Diaphorina citri*. Many of the phloem sap-feeders occasionally feed on the xylem sap. It has been shown that ACP also spends time on xylem activities and a proportion of psyllids feeds from xylem indicating that xylem sap contains the essential nutrients needed for psyllid. We studied the chemical composition of citrus xylem sap and carried out a comparison with citrus phloem sap. Xylem sap was collected by centrifugation. The collected sap was derivatized with trimethylsilyl (TMS) and analyzed with gas chromatography-mass spectrometry (GC-MS). Five amino acids (proline, glycine, threonine, GABA, and serine), six sugars (sucrose, glucose, fructose, and three inositol isomers), and six organic acids (succinic acid, fumaric acid, malic acid, citric acid, quinic acid, and threonic acid), were detected in the xylem sap. Mannose and galactose were found in trace amounts. The profile of the xylem sap was similar to that of the phloem sap. However, the xylem sap was less concentrated than the phloem sap. Because the xylem sap contains most of the components found in the phloem sap, it could support the survival and the growth of ACP. This information expands our knowledge about the nutrition requirements for citrus phloem-sap feeders, such as ACP, and can help define a suitable minimal artificial diet. The diet will greatly help in laboratory studies such as testing the efficiency of RNAi and antimicrobial peptides.

Plant phloem sap is rich in nutrients (sugars and amino acids) and it is the main diet for many phloem-feeding insects. Due to the high concentration of sugars in the phloem sap, its osmotic pressure is 2–5 times greater than the osmotic pressure of insect's hemolymph (Douglas, 2006). Consequently, phloem sap feeding insects must overcome the high osmotic potential of the phloem sap (Douglas, 2006). In animals, osmoregulation can be achieved by eliminating of the excess solutes or by getting more water (Albers and Bradley, 2004). Aphids reduce the osmotic potential of their guts by two strategies: 1) by cleaving sucrose and polymerization of one of its moiety (glucose) into oligosaccharides which is then excreted through honeydew; and 2) by transferring water from the hind gut to the stomach (Douglas, 2003; Douglas, 2006; Shakesby et al., 2009). Additionally, Pompon et al. (2001) also showed that aphids can reduce their osmotic stress by increasing their consumption of the xylem sap. The percentage of time spent by alate aphids feeding on the xylem sap increased when fed on: (1) sucrose (0.4 M); (2) a non-metabolized compound (0.01 M inulin); and (3) when aphids were deprived of their primary symbionts which are responsible for synthesis of oligosaccharides (Pompon et al., 2001). In addition, it has been found that dehydrated aphids increase their consumption of xylem sap to maintain their water balance (Spiller et al., 1990).

Phloem sap-sucking insects transmit many plant pathogens while feeding on phloem sap. Many of the economically important citrus diseases, such as huanglongbing (HLB), citrus tristeza, and citrus stubborn disease are transmitted by piercing-sucking

insects (Bové and Garnier, 2002). *Candidatus Liberibacter asiaticus* (CLAs), which is associated with huanglongbing (HLB), is a phloem-restricted Gram-negative bacterium that is transmitted by the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Jagoueix et al., 1994). The ACP transmits the CLAs bacterium during its feeding on the phloem sap of citrus plants.

Many phloem sap feeding insects such as aphids, whiteflies, plant-hoppers, leafhoppers, and psyllids occasionally feed on xylem sap (Pompon et al., 2001). The ACP mainly feeds on the phloem sap. However, it sometimes feeds on the xylem sap of citrus plants (Cen et al., 2012; Bonani et al., 2010). Cen et al. (2012) found that the percentage of time spent by ACP feeding on the phloem sap was significantly reduced in severely symptomatic CLAs-infected plants. On the other hand, the percentage of psyllids feeding from xylem and the time spent on the xylem activities increased as symptom expression increased. The increase in salivation time (E1) and the decrease in ingestion time of phloem sap (E2) indicated that the phloem sap of CLAs-infected plants either was not suitable for ACP or the ACP was not able to feed on the distorted, crushed, and necrotic phloem cells (Cen et al., 2012). Cen et al. (2012) proposed that psyllids feeding on CLAs-infected plants visits the xylem more frequently and spend more time feeding on its sap to maintain water balance.

In contrast to the phloem sap, xylem sap is poor in nutrients (amino acids and sugars) and it exists under negative tension (Novotny and Wilson, 1997). Consequently, a xylem-sucking insect must be able to overcome this negative tension and low nutrient concentration (Novotny and Wilson, 1997). To overcome the low nutrient concentration in the xylem sap, xylem-sucking insects must consume the xylem at very high rate (100–800 mg/day) (Novotny and Wilson, 1997). Recently, we found that the

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phloem sap of citrus plants was rich in sugars (sucrose, glucose, fructose, and inositol), amino acids, and organic acids including succinic, malic, benzoic, and citric acids (Hijaz and Killiny, 2014). However, no data are available about the composition of the citrus xylem sap. The objectives of this study were to determine the xylem sap composition and compare it with the phloem sap in order to explain why the ACP feeds on the xylem sap of healthy and CLAs-infected citrus plants. The finding may also lead to the design of a minimal diet solution that can be used to evaluate the efficiency of certain compounds against ACP without the influence of citrus plants.

Material and Methods

PLANT MATERIALS. We used one-year old 'Pineapple' sweet orange [*Citrus sinensis* (L.) Osbeck] plants (0.75–1 m tall). Plants were kept in a greenhouse with the temperature controlled at 28 °C to 32 °C. Plants were regularly watered twice a week and fertilized once a week using 20–10–20 fertilizer.

XYLEM SAP COLLECTION. Stems of 10–20 cm length (0.3–0.5 cm diameter) were collected from the greenhouse grown plants. The bark was stripped into two pieces and was removed from the twig. The peeled stem was rinsed with deionized water and dried on Kimwipes to exclude any contamination from the phloem sap. Then, the peeled stems were cut into about 1-cm pieces using a sterile razor blade. To collect the xylem sap, the peeled stem pieces were centrifuged at 14,000 rpm for 15 min at 4 °C. The collected xylem sap was stored at –80 °C until analysis. Xylem sap was collected from three twig pieces from each plant and was pooled together and considered as one biological replicate. Five biological replicates were collected from five plants and were used for the analysis by gas chromatography-mass spectrometry (GC-MS).

TRIMETHYLSILYL-DERIVATIZATION OF XYLEM SAP. Aliquots of 15 μ L of xylem sap were dried under nitrogen stream in 2-mL micro-reaction vessel tubes. Then, the dried samples were mixed with 30 μ L of methoxyamine hydrochloride solution (MOX) in pyridine (2%) and allowed to react for 17 h at room temperature (Gullberg et al. 2004). At the end of the incubation time, 80 μ L of N-methyl-(N-trimethylsilyl) trifluoroacetamide (MSTFA) were added and left for 2 h at room temperature. Finally, 0.5 μ L of derivatized sample was injected into the GC-MS running in full scan mode. Twenty μ L volumes of 500, 1000, and 2000 ppm standard mixes (sucrose, glucose, fructose, inositol, malic acid, quinic acid, succinic, citric acid, proline, alanine, serine, GABA, glycine, threonine, and glutamic acid) were prepared as described above and these standards were used to quantify the concentration of xylem sap components.

GC-MS ANALYSES. Derivatized samples and standards were analyzed using a Clarus 500 GC-MS system (Perkin Elmer, Waltham, MA, USA) fitted with a ZB-5MS column (cross-linked 5% Phenyl 95% Dimethylpolysiloxane, 30 m, 0.25 mm, and 0.25 μ m film thickness). The flow rate for the hydrogen carrier gas was 1 mL/min. The GC temperature program was as follows: initial temperature was held at 70 °C for 1 min, and then increased to 220 °C at a rate of 7 °C/min, held for 2 min, increased further to 280 °C at 7 °C/min, held for 1 min, increased to 300 °C, and finally held for 5 min. The injector and the detector temperatures were set at 250 °C and 180 °C, respectively.

MS PEAK IDENTIFICATION. GC-MS chromatograms were analyzed using TurboMass software version 5.4.2 (Perkin Elmer,

Waltham, MA). Peaks were first identified by comparing their mass spectra with library entries [NIST mass spectra library (National Institute of Standards and Technology, Gaithersburg, MA), Wiley 9th edition (John Wiley and Sons, Inc., Hoboken, NJ)]. Identification of detected compounds was further confirmed by comparing their retention time and mass spectra with that of authentic standards. A schematic representation of the methodology used in this study is illustrated in Fig. 1.

Results and Discussion

Trimethylsilyl (TMS) derivatization method was used because TMS reacts with a broad-spectrum of compounds including sugars, organic acids, and amino acids. Nineteen compounds were detected in the xylem sap of citrus plants by GC-MS after TMS derivatization (Fig. 1, Fig. 2). These compounds contained 5 amino acids, 5 sugars, 3 sugar alcohols, and 6 organic acids. The percentages of the detected compounds are shown in Fig. 1.

Four protein amino acids and one non-protein amino acid (GABA) were detected in xylem sap of 'Pineapple' sweet orange. The total concentration of these amino acids in the xylem sap was about 33 mM and they composed 37.6 % of the total detected compounds. Proline (24.8 ± 19.3 mM) was the main amino acid followed by GABA (5.8 ± 2.0 mM), serine (1.5 ± 0.8 mM), glycine (0.91 ± 0.4 mM), and threonine (0.2 ± 0.1 mM) (Fig. 1, Fig. 2).

One disaccharide (sucrose), two monosaccharides (fructose and glucose), and three sugar alcohols (inositol, and two unknown sugar alcohols) were detected in the xylem sap. In addition, mannose and galactose were detected in trace amounts. The total concentration of the detected sugars was about 27.5 mM and they composed about 30% of the detected compounds. Sucrose was the major sugar (8.6 ± 2.6 mM) followed by fructose (7.0 ± 2.5 mM) and glucose (6.0 ± 2.1 mM). The total concentration of sugar alcohols was 5.8 mM and they composed 21.2% of the total sugars.

Six organic acids were detected in xylem sap: succinic acid, fumaric acid, malic acid, citric acid, quinic acid, and threonic acid. The total concentration of these organic acids in xylem sap was 27.8 mM and they composed 31.4% of the detected compounds. Malic acid was the most predominant organic acid (27.8 ± 8.0 mM) followed by quinic acid (3.7 ± 1.5 mM) and citric acid (2.1 ± 1.7 mM). The concentration of the rest of the organic acids was less than 1 mM each.

All of the compounds detected in xylem sap were also found in the phloem sap of citrus (Hijaz and Killiny, 2014). In addition, the profile of the xylem sap components was similar to that of the phloem sap. For example, proline was the most abundant amino acid found in the phloem (Hijaz and Killiny, 2014) and the xylem sap. In addition, sucrose, glucose, and fructose were the main sugars in both the xylem and the phloem. Furthermore, malic acid was the major organic acid in both of the xylem and the phloem sap (Hijaz and Killiny, 2014).

The concentration of most of the xylem components was lower than that of the phloem sap (Hijaz and Killiny, 2014). For example, the level of sucrose and glucose in the phloem sap (Hijaz and Killiny, 2014) were 7.6 and 3.4 times higher than that in the xylem. The concentrations of citric acid and threonic acid in the phloem sap (Hijaz and Killiny, 2014) was 10 times higher than found in the xylem sap. The level of amino acids in the phloem sap (Hijaz and Killiny, 2014) was also higher than those of the xylem sap (Fig. 2).

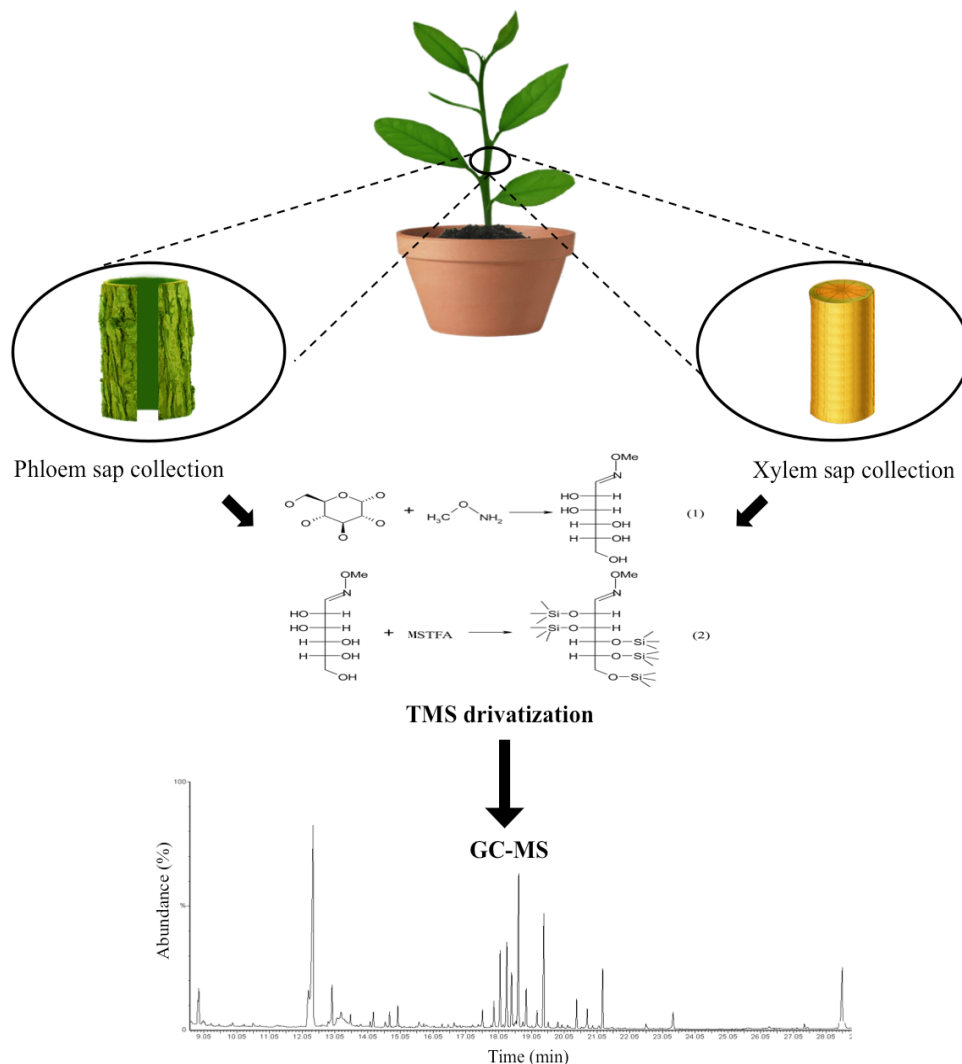


Fig. 1. A schematic representation of methods used to analyze phloem and xylem saps collected from 'Pineapple' sweet orange (*Citrus sinensis*). The figure illustrates the different steps from sap collection and derivatization to gas chromatographic (GC) analysis.

Most of the compounds detected in the 'Pineapple' sweet orange xylem sap were reported in the xylem of other plants. Andersen et al. (1992) detected 19 amino acids, 6 organic acids (oxalic acid, citric acid, tartaric acid, malic acid, succinic acid, and fumaric acid), and three sugars (sucrose, glucose, and fructose) in the xylem sap of *Lagerstroemia indica* L. The total concentration of the amino acids, organic acids, and sugars were 1.2, 1.4, and 0.1 mM, respectively. Sucrose was the main components of the xylem sap of maple (*Acer platanoides*) and its concentration ranged between 10–25 mM during winter and it reached its minimum during the period of bud burst (Schill et al., 1996). Brodbeck et al. (1990) reported the presence of seventeen amino acids in the xylem sap of the host of four common species of the xylem leafhopper, *Homalodisca vitripennis* (Homoptera: Cicadellidae). The total concentration of these amino acids in the four host plants (*Lagerstroemia indica*, *Baccharis halimifolia*, *Prunus persica*, and *Prunus salicina*) ranged from 1.4–13.1 mM (Brodbeck et al., 1990). Furukawa et al. (2011) detected 20 amino acids, two organic acids (citrate and malate), and two sugars (sucrose and glucose) in the xylem sap of *Populus nigra*. The amino acid concentrations ranged from 1–623 μ M. Citrate

concentration ranged from 11–58 μ M and malate from 224–1186 μ M. Glucose concentration ranged from 2 to 17 μ M and sucrose from 1–314 μ M. Although the previous studies showed that the xylem sap was a dilute solution, the sucrose concentration in the xylem sap of willow (*Salix*) plants may reach up to 5% (w/v) (Stanislawek et al., 1987, Sauter, 1980).

Infection with CLAs may induce changes the phloem sap composition of infected plants. Fan et al. (2010) showed a significant increase in sucrose and fructose in both midribs and lobes of symptomless CLAs-infected leaves and an increase in glucose level in the midribs of symptomless leaves (Fan et al., 2010). In addition, the level of starch increased significantly in the leaves from CLAs-infected plants (Fan et al., 2010). The previous results indicated that sugars and starch might accumulate in the phloem sap of the CLAs-infected plants. The accumulation of sugars and starch in the phloem sap increases the osmotic tension of phloem sap and makes it less suitable (higher osmotic tension) for ACP. Consequently, ACP feeding on the phloem sap of CLAs-infected plants suffering from osmotic stress would increase its consumption of the xylem sap to reduce the osmotic stress of its gut. Although it is possible that the plugged and distorted

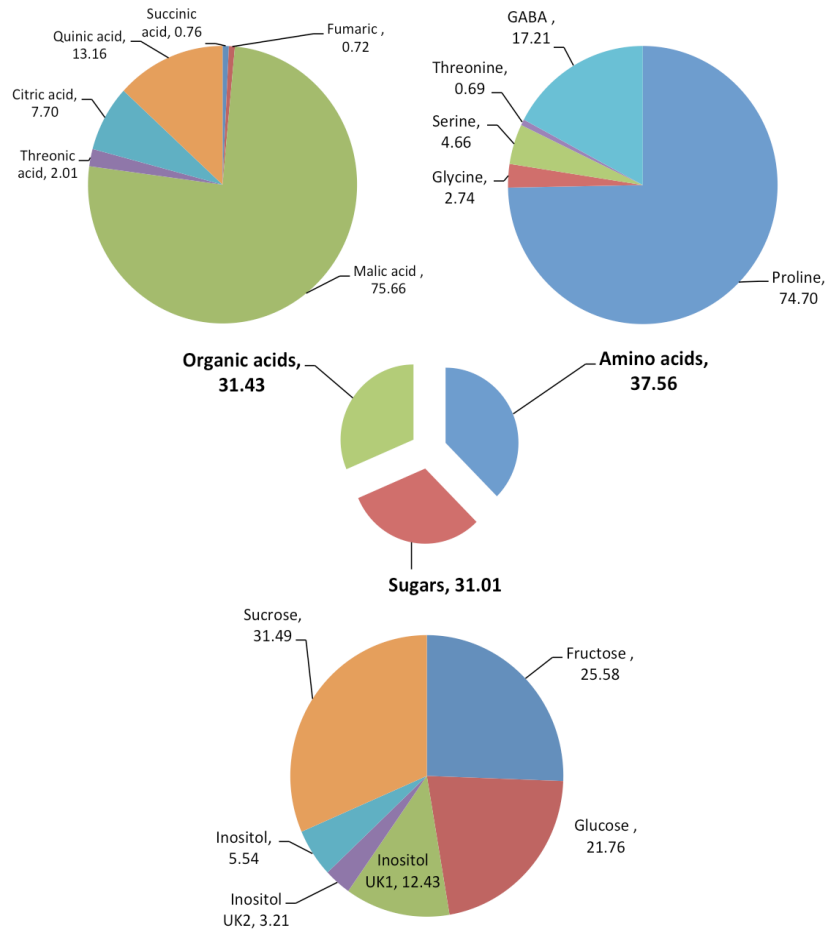


Fig. 2. Percentage composition of different compounds in the xylem sap from 'Pineapple' sweet orange (*Citrus sinensis*).

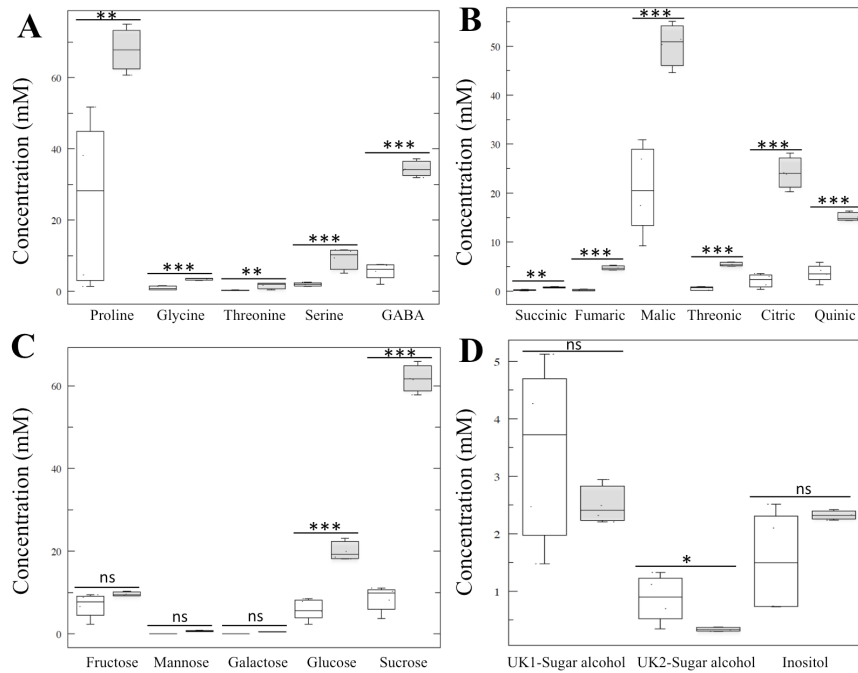


Fig. 3. Concentration in milli-molarity (mM) of compounds in xylem and phloem saps collected from 'Pineapple' sweet orange (*Citrus sinensis*). White boxes represent xylem sap while gray boxes represent phloem sap. Horizontal thick lines indicate the median values; boxes show the interquartile ranges including 50% of the values; whiskers reflect the highest and the lowest number of visits; and asterisks indicate statistically significantly differences. * indicates $P < 0.05$, ** indicate $P < 0.005$, and *** indicate $P < 0.0001$.

phloem cells prevent ACP from feeding (Cen et al., 2012), our results together with the results of Pompon et al. (2001) support the osmoregulation hypothesis.

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