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SHORT COMMUNICATION

Occurrence of free fatty acids in the phloem sap of different citrus varieties

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ABSTRACT
Candidatus Liberibacter asiaticus is a phloem restricted bacterium that causes citrus greening disease or huanglongbing (HLB), a major treat to commercial citrus production in Florida. It is transmitted by the Asian citrus psyllid, a phloem sap-feeding insect. Studies conducted on the composition of citrus phloem sap revealed the presence amino acids, organic acids and sugars and of low amounts of free fatty acids. In the present study, the phloem sap of 12 citrus varieties with different degrees of tolerance to HLB were extracted with ethyl acetate and analyzed by GC-MS after derivatization with boron trifluoride, a fatty acid-specific reagent. Nine free fatty acids were detected in all varieties. Of the 9 fatty acids detected, only capric acid was significantly different among varieties.

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Huanglongbing (HLB) or citrus greening disease is the most serious threat to commercial citrus production in Florida since its identification in 2005. It also affects other major citrus production areas like Brazil and China.[1] The symptoms of HLB include the characteristic "blotchy mottle" in leaves, severe root loss, reduced fruit yield, and small, misshapen, bitter fruit unsuitable for the market. The disease is transmitted by the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama, a phloem sap-sucking insect. The causal agent of the disease is the *Candidatus* liberibacter asiaticus (*C*Las) bacterium in Florida. Other bacteria associated with the disease are *Candidatus* liberibacter americanus (reported in Brazil) and *Candidatus* liberibacter africanus (South Africa). This Gram-negative bacterium is restricted to the phloem sieve tubes of plants.[2] The citrus phloem supports the development and multiplication of *C*Las and ACP and it is believed to contain all the nutrients required by them.

The phloem has an important role in transporting photoassimilates throughout the plant vascular system, but in recent years, its function has expanded to include both intracellular and long-distance plant lipid signaling.[3] In plants, lipids are most often bound to proteins or phosphate groups in the cell membranes of the sieve elements.[3] Plant responses to abiotic stresses are thought to induce lipases or other cell-wall degrading enzymes which release lipids from the membranes into the phloem.[3] Although lipid compounds are present in the phloem, their role as signaling or long distance transport have not been fully elucidated.[3] Lipid analysis of canola phloem sap revealed the presence of phloem-specific fatty acids[4] and phloem exudates of *Arabidopsis thaliana*[5] showed the presence of unusual fatty acids that also appear to be specific to the phloem. Even though their function is unknown, it was suggested that they are involved in pathogen response or signaling.[3] Fatty acids are precursors for jasmonic acid which is released in response to wounding and herbivore attack.[6] Qualitative and quantitative differences in these compounds may help to evaluate their possible role in explaining the differences in tolerance to *C*Las.

Different citrus genotypes present differences in HLB disease symptoms and tolerance to *C*Las, the causal agent of the disease, under field conditions and in greenhouse studies.[7,8,9] The study of the phloem composition is important to provide clues to the mechanisms involved in this different degree of sensitivity and tolerance to citrus greening disease.

Killiny & Hijaz (2016)[10] studied the phloem sap composition of 14 citrus cultivars classified as tolerant, moderately tolerant and sensitive to *C*Las and ACP as the phloem sap in their main source of nutrients. In addition to amino acids and organic acids they found low amounts of 5 fatty acids, even though the derivatization method used was not specific for the analysis of fatty acids. Currently, our objective is to focus on the analysis of the fatty acid composition of the phloem sap of 12 citrus varieties. Valencia and Hamlin sweet oranges, the most important varieties used commercially in Florida, are very susceptible to HLB disease,[7] while a new mandarin hybrid, Sugar Belle, has shown tolerance to HLB.[9] Table 1 shows all of the citrus varieties used for this study and their different tolerances toward HLB.

Young citrus trees (6 months old; 5 biological replicates for each variety) were maintained under greenhouse conditions (16 h:8 h L:D photoperiod, 27 °C, 65% RH), with watering 3 times weekly and weekly fertilization with water soluble fertilizer (24–8–16 NPK). Phloem sap was collected according to Hijaz and Killiny (2014).[11] Briefly, stems were cut from the trees and were diced into 1 cm sections. The bark tissue was scored with a razor blade and peeled from the sections with forceps. The tissue was packed into nested centrifuge tubes for centrifugation. About 20 μL of phloem sap was obtained from each tube and samples were pooled together for each biologic replicate. Samples were kept frozen at −20 °C until analysis. Fatty acids were extracted from 100 μL of phloem sap with

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Table 1. Citrus varieties used in this study and their tolerance to citrus greening disease (HLB).

Variety	Abbreviation	CLas response
C. sinensis 'Valencia' sweet orange[a]	VA	Sensitive
C. sinensis 'Hamlin' sweet orange[a]	HA	Sensitive
C. paradisi 'Duncan' grapefruit[a]	DU	Sensitive
C. reticulata 'Clementine' mandarin[b]	CL	Sensitive
Tangelo "Minneola"[b] Duncan grapefruit (*C. paradisi*) x Dancy tangerine (*C. reticulata*)	MN	Sensitive
C. aurantifolia 'Mexican' lime[a]	ML	Moderately tolerant
C. aurantium 'Sour' orange[a]	SO	Moderately tolerant
C. volkamericana "Volkamer" lemon[a]	VL	Moderately tolerant
C. macrophylla[a]	MP	Moderately tolerant
Carrizo citrange[a]	CA	Tolerant
Sugar Belle[c] Clementine mandarin (*C. reticulata*) x Minneola tangelo, Duncan grapefruit (*C. paradisi*) x Dancy tangerine (*C. reticulata*)	SB	Tolerant
C. latipes[a]	LA	Tolerant

[a]Folimonova et al (2009)
[b]McCollum et al (2016)
[c]Stover et al (2016)

ethyl acetate[12] and were derivatized using boron trifluoride–methanol solution 10.0% (14:86, w/w) from Sigma-Aldrich (St Louis, Missouri). Heptadecanoic acid (C17:0) was added as the internal standard, as it is rarely found in plant tissues (for details, see ref 13). Hexane extracts from methylated samples were analyzed using GC-MS (PerkinElmer Autosystem XL with Turbomass software, Waltham, MA) operated in electron impact mode at 70 eV with selected ion monitoring (SIM) mode. For optimal GC separation, a 60 m Stabilwax column (Carbowax 60 m, 0.25 mm i.d., 0.50 μm film thickness, Restek, Bellefonte, PA) was used with ultrapure hydrogen as the carrier gas. The GC oven program was at 70 °C, held for 5 min, ramped to 180 °C at a rate of 10 °C min^{-1} and finally to 240 °C at 3 °C min^{-1}, and held at the final temperature for 10 min. Compounds were identified by comparison of the mass spectra and retention times obtained from authentic standards (Supelco, St. Louis, MO). Peak areas were normalized to the internal standard and converted to μM or nM concentrations using standard curves generated from fatty acid standards derivatized and injected under the same conditions as phloem sap samples.

In the present study we used both a specific method to extract the fatty acids from the phloem sap and a more specific derivatization reagent for fatty acids analysis.[12,13] This procedure allowed the detection and quantification of 9 fatty acids in all citrus varieties in concentrations ranging from 17.47 nM to 54.50 μM (Table 2). Palmitic and stearic acids were the major fatty acids present in all varieties. Other fatty acids detected were: capric, caprylic, lauric, myristic, myristoleic, oleic and linoleic acids. As found in this study, there were no significant differences among the number and the amount of fatty acids in the different varieties, except for capric acid. Capric acid was highest in Sugar Belle (SB) and Carrizo citrange (CA) (> 1.0 μM) which are both considered tolerant. However, Minneola tangelo (MN), which is susceptible to HLB also had a about the same concentration. The lowest levels of capric acid were found in 'Hamlin' orange (susceptible) and *Citrus latipes* (LA) which is considered tolerant. Most likely, these differences were varietal and not related to tolerance to HLB.

Previous work on phloem sap composition of 14 citrus varieties using a trimethylsilyl (TMS) procedure found trace amounts of fatty acids including myristic, palmitic and stearic acids.[14] The principal component analysis did not reveal any clustering among the varieties studied according to their fatty acids concentration.[14] Myristic, palmitic and stearic acids were also detected in orange jasmine (*Murraya paniculata* (L.) Jack), an ornamental plant that is a close relative to citrus and a host for ACP.[15]

Killiny & Hijaz (2016)[10] used methyl chloroformate to derivatized the phloem sap of 13 citrus varieties. Even though the focus of their work was to evaluate the amino acids implicated in plant defense against *C*Las, low amounts of 5 fatty acids (myristic, palmitic, stearic, oleic and linoleic) were reported, all of which were also detected in the present study. The authors used simple linear regression and stepwise forward regression to investigate the relationship between the phloem sap composition and citrus tolerance to *C*Las. Fatty acids were not implicated in citrus tolerance to *C*Las (p-value > 0.05) but stepwise forward regression showed that linoleic acid and stearic acid were necessary in the model.

Most citrus varieties are susceptible to HLB but field surveys have shown that some cultivars have some tolerance and some decline faster than others. Genomic analysis of *C*Las revealed that this phloem-restricted bacterium is not capable of growing on fatty acids.[16] Nevertheless, a vigorous production of

Table 2. Free fatty acids in phloem sap of different citrus varieties.

	Caprylic acid [C8:0] (μM)	Capric acid [C10:0] (μM)	Lauric acid [C12:0] (μM)	Myristic acid [C14:0] (μM)	Myristoleic acid [C14:1] (nM)	Palmitic acid [C16:0] (μM)	Stearic acid [C18:0] (μM)	Oleic acid [C18:1] (nM)	Linoleic acid [C18:2] (μM)
VA	0.62 ± 0.11	0.94 ± 0.18[ab]	3.46 ± 1.28	3.15 ± 1.64	38.67 ± 11.59	21.70 ± 4.82	41.42 ± 29.46	20.64 ± 2.00	7.74 ± 4.17
HA	0.54 ± 0.04	0.48 ± 0.30[c]	4.68 ± 2.50	2.38 ± 0.55	34.34 ± 11.48	35.49 ± 20.56	45.01 ± 19.90	17.47 ± 5.76	17.87 ± 6.98
DU	0.54 ± 0.02	0.94 ± 0.15[ab]	4.04 ± 0.48	2.73 ± 0.62	30.09 ± 2.12	24.47 ± 4.80	42.65 ± 28.47	20.74 ± 1.96	10.17 ± 7.87
CL	0.56 ± 0.05	0.78 ± 0.13[abc]	2.86 ± 0.30	1.96 ± 0.42	28.51 ± 0.74	26.98 ± 13.16	37.73 ± 20.62	22.70 ± 3.27	6.57 ± 2.97
MN	0.58 ± 0.05	1.01 ± 0.08[a]	4.16 ± 1.49	2.50 ± 1.18	29.17 ± 1.34	32.34 ± 21.27	40.52 ± 22.21	21.84 ± 0.69	6.05 ± 5.57
ML	0.67 ± 0.24	0.88 ± 0.14[ab]	4.61 ± 3.38	6.78 ± 10.40	33.74 ± 8.66	39.86 ± 13.18	43.42 ± 9.18	20.18 ± 3.34	10.76 ± 14.31
SO	0.57 ± 0.06	0.84 ± 0.10[abc]	3.19 ± 0.57	3.81 ± 2.61	28.57 ± 0.60	33.43 ± 9.01	54.50 ± 49.74	18.45 ± 4.08	7.71 ± 9.06
VL	0.61 ± 0.03	0.89 ± 0.18[ab]	5.06 ± 3.80	3.11 ± 1.23	28.85 ± 0.83	23.21 ± 2.15	35.76 ± 15.18	19.36 ± 4.47	6.79 ± 6.26
MP	0.67 ± 0.20	0.92 ± 0.10[ab]	3.62 ± 1.25	5.05 ± 5.50	30.78 ± 2.36	27.89 ± 15.35	38.85 ± 26.50	19.11 ± 4.24	8.35 ± 9.17
CA	0.90 ± 0.71	1.06 ± 0.15[a]	4.08 ± 1.55	2.37 ± 0.82	30.24 ± 1.60	28.22 ± 9.44	40.07 ± 28.51	19.20 ± 4.35	12.17 ± 10.41
SB	0.77 ± 0.22	1.04 ± 0.14[a]	5.51 ± 1.99	6.49 ± 6.98	29.04 ± 1.61	36.13 ± 20.36	49.37 ± 27.26	20.80 ± 1.96	5.90 ± 1.42
LA	0.57 ± 0.06	0.64 ± 0.27[bc]	3.05 ± 0.46	2.17 ± 0.52	28.36 ± 0.86	22.42 ± 7.58	41.88 ± 29.94	19.36 ± 4.37	2.71 ± 1.70

Values represent means ±SD (n = 10). Different superscript letters on values indicate significant difference using Tukey honestly significant different tests. Fatty acids without superscript letters are not significantly different.

oxygenated fatty acids (oxylipins) is a characteristic response to pathogenesis and herbivory.[17] Therefore, these fatty acids may be essential for bacterial pathogenicity and their function should be further investigated.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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