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Secondary Metabolite Composition in Citrus × *Poncirus trifoliata* Hybrids

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***Poncirus trifoliata* L. Raf. is used as a parent in citrus rootstock breeding because it confers desirable characteristics, such as disease resistance and cold hardiness. However, fruit of *P. trifoliata* hybrids typically have unpleasant flavor. The objective of this study was to determine the chemical composition of juice from *P. trifoliata* hybrids for comparison with the fruit of *P. trifoliata*. Six hybrids were studied: the female parent (5-14-96, 1/8 *P. trifoliata*), the male parent (1-11-7, no *P. trifoliata*), and four siblings (6-49-96, 6-49-116, 6-49-148, and 6-49-163, all 1/16 *P. trifoliata*). Juice from these hybrids was analyzed for volatiles, flavonoids, limonoids, sugars, and acids. Juice of *P. trifoliata* was also analyzed. The volatile profile of juice from the female parent showed the most similarity to *P. trifoliata*, with many sesquiterpene hydrocarbons and esters. The hybrid 6-49-163 presented a similar pattern regarding volatile composition. However, another hybrid, 6-49-116, also presented the high content of limonoids and flavonoids measured in the female parent and in *P. trifoliata*. For these preliminary results, we observed differences among siblings with the same parents based on their secondary metabolite composition. Complex differences in volatiles, limonoid and flavonoid compounds among *P. trifoliata* hybrids were revealed in this study. When eventually correlated with sensory data, our results could be used to evaluate the chemical basis of juice quality and thus select *P. trifoliata* hybrids for consumption.**

Orange (*Citrus sinensis* L. Osb.), mandarin (*C. reticulata* Blanco), and grapefruit (*C. paradisi* Macf.) are among the largest fruit commodities in the US citrus market. Brazil and USA are the largest producers of orange juice concentrate with 1.3 and 0.7 million tons at 65 °Brix, respectively (USDA, 2012–2013). Therefore, maintaining a steady supply of good quality orange juice from these regions is crucial for the juice industry. In the recent decade, citrus diseases have threatened citrus production. The most serious disease, huanglongbing (HLB), significantly reduces fruit production (Gottwald et al., 2007), is detrimental to juice quality (Baldwin et al., 2010; Bassanezi et al., 2009; Plotto et al., 2010), and could potentially disrupt citrus juice supply. Hybridization is one of the techniques used to create disease-resistant citrus varieties (Hearn, 1987). Because *P. trifoliata* is more tolerant to HLB (Albrecht and Bowman, 2011), it might be possible to breed citrus scions with resistance to HLB.

Poncirus trifoliata L. Raf. (*P. trifoliata*) is one of the genera most used in breeding citrus due to its valuable characteristics, absent in some commercial citrus varieties, such as cold tolerance, multiple stress tolerance, and disease resistance (Gurtskaya, 1981; Hearn, 1987; Kapanadze, 1979). Therefore, as reviewed by Hearn (1987), *P. trifoliata* and *P. trifoliata* hybrids have been the subject of numerous studies.

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Even though Citrus–*P. trifoliata* hybrids and *P. trifoliata* have the advantage of being highly disease-resistant compared to pure citrus, their fruit are considered inedible for fresh consumption (Gershtein, 1976; Gurtskaya, 1981; Kapanadze, 1973, 1979). It was suggested that this unpleasant flavor might be related to the high content of secondary metabolites in the essential oil in the juice sacs (Gershtein, 1972; Gurtskaya, 1981; Ogawa et al., 2000; Ueno et al., 1985). However, specific crosses could produce fruit with no oil at all or no bitter oil, such as hybrids involving *Fortunella* and *P. trifoliata*. Apparently the effect of genes responsible for the production of bitter essential oil in *P. trifoliata* are suppressed by the genes from *Fortunella* (Kapanadze, 1977; Kokaya, 1981).

The objective of our study was to determine the secondary metabolite compounds including terpenoids, limonoids and flavonoids (Buckingham, 2007) composition of *P. trifoliata* hybrid juices to correlate this information with their *P. trifoliata* background. Six hybrids were studied: the female parent (5-14-96, 1/8 *P. trifoliata*), the male parent (a citrus hybrid: 1-11-7, no *P. trifoliata*), and four siblings (6-49-96, 6-49-116, 6-49-148, and 6-49-163, all 1/16 *P. trifoliata*). Juice was analyzed for secondary metabolites and also general volatiles, sugars and acids. Finally, based on secondary metabolite composition, hierarchical cluster analyses were performed to observe similarities between hybrids and *P. trifoliata*.

Materials and Methods

Plant material

Citrus hybrids with and without *P. trifoliata* in their pedigrees (Fig. 1), and pure *P. trifoliata* fruit were grown at the USDA, ARS research farm in Fort Pierce, FL. Fruit were harvested in Nov. 2012

(*P. trifoliata*) and in Jan. 2013 (all six hybrids). Optimal harvest dates resulting in acceptable eating quality are not known for these new hybrids and they were harvested based on their availability. Once collected, fruit were washed and sanitized before juicing.

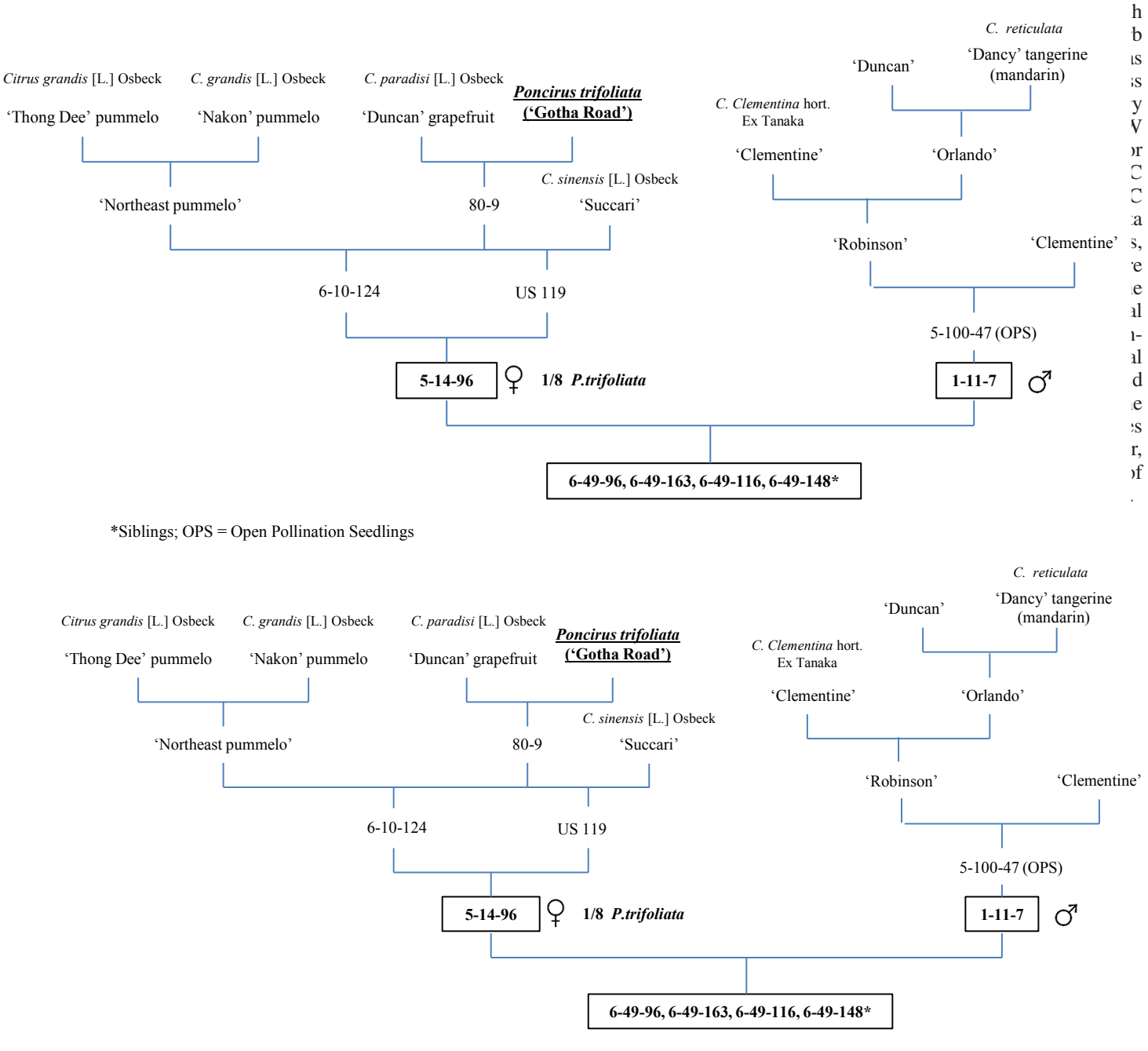
Sample preparation

Juice was extracted using a manual juicer (Citrus Juicer Model 318, 20 oz, from Oster distributed by Sunbean Products Inc., Delray Beach, FL) by gently squeezing halved fruit to minimize introducing peel oil and albedo into the juice.

Volatiles. A mixture of 10 mL juice, 10 mL NaCl saturated solution and 20 µL 3-hexanone at 1228 mL·L^{-1} (internal standard, Supernatant was filtered (Whatman #4 filter paper, Batavia, IL) by vacuum. The filtered solution was brought to 100 mL with 80% ethanol. A total of 10 mL of the filtered solution was then filtered through a C-18 Sep-Pak (Waters/Millipore), followed by a 0.45-µm Millipore (Siemens-Millipore, Shrewbury, MA) filter.

Identification of volatile compounds

Samples were equilibrated for 30 min at 40 °C in a Gerstel MPS2 autosampler (Gerstel Inc.). A 2-cm solid-phase micro-extraction (SPME) fiber (50/30 µm DVB/Carboxen/PDMS; Supelco, Bellefonte, PA) was then exposed to the headspace for 60 min at 40 °C without stirring or shaking. After exposure, the



Citrus grandis [L.] Osbeck
'Thong Dee' pummelo
C. grandis [L.] Osbeck
'Nakon' pummelo
'Northeast pummelo'
C. paradisi [L.] Osbeck
'Duncan' grapefruit
Poncirus trifoliata ('Gotha Road')
C. sinensis [L.] Osbeck
80-9
'Succari'
6-10-124
US 119
5-14-96
1/8 P.trifoliata
C. reticulata
'Duncan'
'Dancy' tangerine (mandarin)
C. Clementina hort. Ex Tanaka
'Clementine'
'Orlando'
'Robinson'
'Clementine'
5-100-47 (OPS)
1-11-7
6-49-96, 6-49-163, 6-49-116, 6-49-148*
*Siblings; OPS = Open Pollination Seedlings

Fig. 1. Pedigrees of hybrids considered in this study. Hybrid fruits studied are in bold and framed.

Quantification of limonoids and flavonoids

Juice samples were analyzed by HPLC-MS, using a Waters 2695 Alliance HPLC (Waters, Medford, MA) instrument connected in parallel with a Waters 996 photodiode array detector and a Waters/Micromass ZQ single quadrupole mass spectrometer equipped with an electrospray ionization source. Compound separation was achieved with a Waters XBridge C8 column (5 µm, 4.6 × 150 mm). Elution conditions included two-solvent gradients composed initially of acetonitrile/0.5% formic acid (10/90, v/v) and increased with linear gradients to 20/80 (v/v) over 10 min, then to 25/85 (v/v) by 15 min, then to 40/60 (v/v) by 23 min, to 70/30 (v/v) by 45 min, and finally to 10/90 (v/v) by 53 min, at a flow rate of 0.75 mL·min^{-1}. Data recording and processing was done with MassLynx software version 4.1 (Micromass, Division of Waters Corp., Beverly, MA). The internal standard was used to normalize the mass spectrometer instrument response during sequential runs. For quantification, stock solutions of flavonoids (naringin, neohesperidin, poncirin, narirutin, isosakuranetin rutinoside and hesperidin) and limonoids (limonin, nomilin, deacetyl nomilinic acid glucoside, limonin gluoside and nomilinic acid glucoside) were prepared in dimethyl sulfoxide. Five to seven dilutions of the stock solutions were injected to obtain calibration curves. To normalize the peak area data, correction factors were calculated from the ratio of the peak areas with that of the internal standard, then the peak area of each compound was divided by the correction factor. Quantitative results are expressed as µg·g^{-1} of juice.

Quantification of sugars and acids

Titratable acidity (TA) was determined using a titrator (Mettler Toledo DL15, Columbus, OH) and soluble solids content (SSC) using a refractometer (Atago PR-101α, Tokyo, Japan). Sugars were analyzed by HPLC equipped with a refractive index detector (Agilent 1100 Series). 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 mobile phase was 0.001 M CaEdta with a flow rate of 0.3 mL·min^{-1} at 90 °C. The injection volume was 60 µL using a Perkin-Elmer Series 200 autosampler and pump (Perkin-Elmer, Waltham, MA). Quantification of sugars was based on the external standard method (EZChrom Elite software, Version 3.3.2. SP2, Santa Clara, CA) using standards of sucrose, glucose and fructose. All results are expressed as g per 100 mL of juice.

Organic acids were analyzed by HPLC equipped with a Spectra System UV 6000 LP photo diode array detector (Thermo Fisher Scientific, Waltham, MA). The column used was an AltechOA1000 Prevail organic acid column (9 µm, 300 mm × 6.5 mm) (Grave Davison Discovery Sciences, Deerfield, IL) with a flow rate of 0.2 mL·min^{-1} at 35 °C and a mobile phase of 0.01 N H_2SO_4. The injection volume was 60 µL using a Perkin-Elmer Series 200 autosampler (Perkin-Elmer) and a Spectra System P4000 pump (Thermo Fisher Scientific). Quantification of acids was based on the calibration curves of citric and malic acids, expressed as g per 100 mL of juice.

Statistical analysis

Cluster analysis, taking into account the presence/absence of secondary metabolites, was performed using XLSTAT v 2012.6.02 (Addinsoft, Paris, France). The purpose was to have a general view of the distribution of these compounds among the six Citrus–*Poncirus* hybrids and *P. trifoliata*. The unweighted pair-group average agglomeration method was used. The Kulzinski coefficient was employed to measure similarities between samples.

In the same way cluster analysis based on volatile peak area and limonoid/flavonoid measurements was performed using XLSTAT. Thus, cluster was made according to the average distance between all the samples. The unweighted pair-group average agglomeration method was used. The Euclidean distance measured dissimilarities between samples.

Results and Discussion

Secondary metabolite composition

The juice of *P. trifoliata* was characterized by having a large amount of esters and sesquiterpene hydrocarbons (Table 1). Twenty esters and 32 sesquiterpene hydrocarbons were identified in *P. trifoliata*, in addition to alcohols, monoterpenes, and aldehydes. Our results are in agreement with the volatile composition of *P. trifoliata* described by Heinrich et al. (1979) and Scora et al. (1966), where a majority of sesquiterpene hydrocarbons (β-caryophyllene,64.2%) was detected in juice vesicles or pulp.

Similar to the juice of *P. trifoliata*, the juice of the female parent 5-14-96 (1/8 *P. trifoliata*) and one of the siblings, 6-49-163 (1/16 *P. trifoliata*) were also rich in esters (8 and 10 compounds, respectively) and sesquiterpene hydrocarbons (34 compounds in both samples). A few compounds were unidentified (5 volatiles for 5-14-96 and 10 volatiles for 6-49-163). In contrast to these hybrids, the male parent 1-11-7 (no *P. trifoliata*) presented only two esters and no sesquiterpene hydrocarbons, and the other three siblings (1/16 *P. trifoliata*) presented one to three esters and zero to four sesquiterpene hydrocarbons (Table 1). One to four unknown compounds were detected but not identified in these four juice samples. Except for ethyl acetate, which was present in all samples, ethyl esters (ethyl butanoate, 2-ethyl butenoate, ethyl hexanoate and ethyl octanoate) were present in *P. trifoliata*, the female parent and in the 6-49-163 progeny but not in the male parent, suggesting an overexpression of alcohol acyl transferase with preferential ethanol substrate (Sanz et al., 1997). Further a large number of sesquiterpene hydrocarbons (e.g., δ-elemene, α-cubenene, α-ylangene, α-copaene and β-elemene) were detected in *P. trifoliata*, in the female parent and in 6-49-163. This observation can be explained by the overexpression of farnesyl diphosphate synthase in the mevalonate pathway, which leads to the formation of farnesyl diphosphate, precursor to all the sesquiterpene hydrocarbons (Robinson, 1991). Besides these volatiles, two terpene alcohols (terpinen-4-ol and α-terpineol) and esters of acetic acid (octyl acetate and citronellyl acetate) were detected only in *P. trifoliata* and in 6-49-163. Only a few aldehydes ((Z)-3-hexenal and heptanal) and two ketones (2-hydroxy-2-butanone and 2-methyl-3-pentanone) were produced by the male and/or female parent that were not present in the pure *P. trifoliata* fruit. The diversity of volatiles produced by *P. trifoliata*, and the fact that only a sub set of these volatiles were found in Citrus × *P. trifoliata* hybrids suggest that *P. trifoliata* characteristics might be transmitted at different levels through subsequent generations.

Among the non-volatile compounds, the bitter compounds are limonin, nomilin, naringin, neohesperidin and poncirin (Horowitz and Gentili, 1963; Nagy and Attaway, 1980). Limonin and nomilin were detected at 10.9 µg·g^{-1} and 1.3 µg·g^{-1} of juice, respectively, in *P. trifoliata* (Table 2). This limonin concentration was higher than its taste thresholds in sucrose-acid solution (6.2 µg·g^{-1}) or in orange juice (3.4 µg·g^{-1}) (Dea et al., 2013). Limonin and nomilin were also present in the female parent at 1.0 and 0.8 µg·g^{-1}, respectively, and in sibling 6-49-116 at 2.2 and 0.7 µg·g^{-1}, respectively (Table 2). The male parent (no *P. trifoliata* in the background)

Table 1. Volatile composition of one citrus (male parent), five *Citrus–P. trifoliata* hybrids and *P. trifoliata* (*Poncirus*). Values are the peak area of each compound divided by the peak area of the internal standard.

Family	I^T DB5	Volatile	Identification	1-11-7 (male parent)	5-14-96 (female parent)	6-49-116*	6-49-148*	6-49-163*	6-49-96*	**Poncirus**
Total alcohol	509	Ethanol	Standard, MS, I^T	0.12	0.28	0.05	0.47	0.62	0.20	0.56
	1128	Linalool	Standard, MS, I^T							0.33
	1225	Terpinen-4-ol	Standard, MS, I^T					**0.05**		**0.12**
	1236	α-Terpineol	Standard, MS, I^T					**0.07**		**0.06**
	1254	Citronellol	Standard, MS, I^T							0.02
	1263	*(E)*-Carveol	Standard, MS, I^T					0.14		
	1541	*(E)*-Nerolidol	MS, I^T					0.20		
		Total		**0.12**	**0.28**	**0.05**	**0.47**	**1.09**	**0.20**	**1.08**
		%		**4.65**	**7.42**	**1.35**	**28.04**	**2.48**	**2.95**	**2.92**
Esters	598	Ethyl acetate	Standard, MS, I^T	0.05	0.03	0.01	0.04	0.22	0.04	0.69
	702	Methyl butanoate	Standard, MS, I^T		**<0.01**					**0.01**
	789	Ethyl butanoate	Standard, MS, I^T		**0.25**			**0.06**		**0.93**
	841	2-Ethyl butenoate	MS, I^T		**<0.01**			**0.01**		**0.10**
	847	2-Methylethyl butanoate	MS, I^T							0.03
	851	3-Methylethyl butanoate	MS, I^T							0.08
	877	3-Methylbutyl acetate	MS, I^T							0.04
	879	2-Methylbutyl acetate	MS, I^T							0.01
	901	Propyl butanoate	MS, I^T							0.04
	931	Methyl hexanoate	Standard, MS, I^T	<0.01	**<0.01**					**<0.01**
	967	Ester 967								0.01
	1012	Butyl butanoate	MS, I^T							1.49
	1012	Ethyl hexanoate	Standard, MS, I^T		**0.11**	**0.01**		**0.18**	**0.05**	**1.76**
	1028	Hexyl acetate	MS, I^T							0.12
	1080	2-Methylbutyl butanoate	MS, I^T							0.13
	1156	3-Hydroxyethyl hexanoate	Standard, MS, I^T		0.01					
	1213	Butyl hexanoate	MS, I^T							0.18
	1216	Ethyl octanoate	Standard, MS, I^T		**0.01**			**0.12**	**<0.01**	**0.68**
	1229	Octyl acetate	Standard, MS, I^T					**0.05**		0.08
	1352	*(E)*-Carveol acetate	MS, I^T					0.03		
	1358	Citronellyl acetate	MS, I^T					**0.40**		0.06
	1384	Geranyl acetate	Standard, MS, I^T					0.09		
	1397	Ethyl decanoate	MS, I^T							0.02
	1431	Esters 1431						0.04		
		Total		**0.05**	**0.42**	**0.01**	**0.04**	**1.20**	**0.09**	**6.46**
		%		**2.05**	**11.01**	**0.41**	**2.56**	**2.73**	**1.31**	**17.38**
Aldehydes	640	3-Methylbutanal	MS, I^T							0.01
	679	Pentanal	Standard, MS, I^T		0.00					
	743	*(E)*-2-Penten-1-al	MS, I^T		0.00					
	790	*(Z)*-3-Hexenal	MS, I^T	0.01		0.16	0.03	0.07	0.16	
	793	Hexanal	Standard, MS, I^T	0.02	0.34	0.18	0.03	0.05	0.28	0.03
	856	*(E)*-2-Hexenal	Standard, MS, I^T	0.01	0.21	0.10	0.02	0.06	0.08	0.07
	910	Heptanal	Standard, MS, I^T	0.00	0.01	0.01	0.00	0.00	0.01	
	925	*(E,E)*-2,4-Hexadienal	Standard, MS, I^T		0.01	0.01				

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Table 1. Continued.

Family	I^T DB5	Volatile	Identification	1-11-7 (male parent)	5-14-96 (female parent)	6-49-116*	6-49-148*	6-49-163*	6-49-96*	**Poncirus**
Aldehydes	1086	*(E)*-2-Octenal	Standard, MS, I^T		0.00					
	1233	Decanal	Standard, MS, I^T							0.03
	1318	Perillaldehyde	Standard, MS, I^T					0.01	0.01	
		Total		**0.04**	**0.58**	**0.46**	**0.08**	**0.19**	**0.54**	**0.14**
		%		**1.64**	**15.18**	**13.29**	**4.95**	**0.43**	**7.86**	**0.38**
Furans	681	2-Ethylfuran	MS, I^T		**0.01**	**0.02**				
		%			**0.34**	**0.71**				
Ketones	693	3-Hydroxy-2-butanone	MS, I^T		<0.01					
	735	2-Methyl-3-pentanone	MS, I^T	<0.01	<0.01					
	874	4-Heptanone	MS, I^T	0.01	0.01	0.01	0.01	0.01	0.01	0.01
	1284	Carvone	Standard, MS, I^T				0.01	0.02		
		Total		**0.01**	**0.01**	**0.01**	**0.02**	**0.03**	**0.01**	**0.01**
		%		**0.30**	**0.31**	**0.22**	**1.13**	**0.07**	**0.13**	**0.02**
Monoterpene hydrocarbons	945	*α*-Thujene	MS, I^T					0.02	0.01	0.04
	959	*α*-Pinene	Standard, MS, I^T	0.02	<0.01	0.03	0.01	0.56	0.09	0.31
	983	Camphene	Standard, MS, I^T							0.02
	1001	Sabinene	Standard, MS, I^T					0.03	0.01	0.07
	1008	*β*-Myrcene	Standard, MS, I^T	0.06	<0.01	0.08	0.02	2.65	0.29	3.01
	1036	Monoterpene 1036		<0.01		0.01		0.15	0.02	0.09
	1038	*α*-Phellandrene	Standard, MS, I^T	<0.01		<0.01		0.13	0.01	0.81
	1049	*α*-Terpinene	Standard, MS, I^T	<0.01		0.01		0.14	0.01	0.15
	1057	Monoterpene 1057		0.01	0.01	0.02	0.01	0.05	0.04	
	1060	Monoterpene 1060		<0.01		0.01		0.18	0.02	0.90
	1063	Limonene	Standard, MS, I^T	2.13	0.17	2.59	0.98	23.22	5.21	5.41
	1070	*(Z)*-*β*-Ocimene	MS, I^T	0.08	0.01	0.07	0.02		0.20	
	1071	*(E)*-*β*-Ocimene	MS, I^T							2.27
	1073	*β*-Phellandrene	Standard, MS, I^T					0.70		3.93
	1091	*γ*-Terpinene	Standard, MS, I^T	<0.01		<0.01		0.09	0.01	0.15
	1107	Monoterpene 1107								0.09
	1122	Terpinolene	Standard, MS, I^T	0.01	0.01	0.02	<0.01	0.38	0.04	0.10
	1125	Monoterpene 1125						0.03	0.05	
	1127	Monoterpene 1127		0.01	<0.01	0.02	0.01	0.18		
	1152	*(E,E)*-2,6-Dimethyl-1,3,5,7-octatetraene	MS, I^T							0.10
	1163	Monoterpene 1163								0.37
	1171	Monoterpene 1171								0.29
		Total		**2.33**	**0.19**	**2.83**	**1.04**	**28.50**	**5.98**	**18.09**
		%		**91.36**	**5.09**	**82.60**	**62.71**	**65.19**	**87.74**	**48.70**
Sesquiterpene hydrocarbons	1363	Sesquiterpene 1363			**0.01**			**0.14**		**0.03**
	1366	*δ*-Elemene	MS, I^T		**0.09**			**1.55**		**0.33**
	1378	*α*-Cubebene	Standard, MS, I^T		**<0.01**			**0.06**		**0.03**
	1403	*α*-Ylangene	MS, I^T		**<0.01**			**0.05**		**0.02**
	1409	*α*-Copaene	MS, I^T		**0.01**			**0.19**		**0.11**
	1415	*β*-Elemene	MS, I^T		**0.06**			**0.48**		**0.27**
	1421	Sesquiterpene 1421						**0.03**		**0.01**
	1423	Sesquiterpene 1423								0.01

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Table 1. Continued.

Family	I^T DB5	Volatile	Identification	1-11-7 (male parent)	5-14-96 (female parent)	6-49-116*	6-49-148*	6-49-163*	6-49-96*	**Poncirus**
Sesquiterpene hydrocarbons	1437	*(Z)*-Caryophyllene	MS, I^T		**0.07**			**0.20**		**0.29**
	1448	*γ-E*lemene	MS, I^T		**0.03**			**0.66**		**0.35**
	1452	*(Z)-β*-Farnesene	MS, I^T							0.31
	1453	*(E)*-Caryophyllene	Standard, MS, I^T		0.68		<0.01	4.04		6.36
	1458	Sesquiterpene 1458			**0.02**			**0.15**		**0.27**
	1462	6,9-Guaiadiene	MS, I^T		0.01			0.10		
	1467	(+)-Epi-bicyclosesquiphellandrene	MS, I^T		<0.01			0.04		
	1471	Spirolepechinene	MS, I^T		**0.03**					**0.06**
	1473	Alloaromadendrene	Standard, MS, I^T		**0.02**			**0.12**		
	1474	Sesquiterpene 1474								**0.20**
	1480	*(E)*-Selina-1(6)-4-diene	MS, I^T		<0.01			0.05		
	1483	*α*-Humulene	Standard, MS, I^T		**0.08**			**0.85**		**1.24**
	1484	Sesquiterpene 1484			<0.01			0.05		
	1488	Sesquiterpene 1488			<0.01			0.12		
	1490	*γ*-Muurolene	MS, I^T		**0.02**			**0.32**		**0.14**
	1493	*α*-Farnesene	MS, I^T		**0.01**			**0.12**		**0.14**
	1495	Sesquiterpene 1495			0.01			0.45		
	1497	Sesquiterpene 1497			0.02					
	1500	*δ*-Selinene + epizonarene	MS, I^T		0.01			0.49		
	1503	Sesquiterpene 1503			0.09	<0.01		0.16		0.17
	1506	Sesquiterpene 1506								0.06
	1507	*α*-Muurolene	MS, I^T		**<0.01**			**0.12**		**0.05**
	1510	Valencene	Standard, MS, I^T		0.77	0.05		0.24		0.10
	1514	*α*-Selinene	MS, I^T		0.09	<0.01		0.28		0.09
	1518	*β*-Selinene	MS, I^T		0.05			0.04		
	1521	*δ*-Cadinene	MS, I^T		**0.04**			**0.85**		**0.25**
	1527	*(Z)-C*alamenene	MS, I^T		**<0.01**			**0.05**		**0.10**
	1530	*(E)*-Calamenene	MS, I^T		**0.02**			**0.11**		**0.05**
	1535	7-Epi-*α*-selinene	MS, I^T		0.08	<0.01		0.03		0.02
	1536	*(E)*-Cadina,1,4-diene	MS, I^T					0.07		0.03
	1539	*α*-Cadinene	MS, I^T		**<0.01**			**0.11**		**0.05**
	1546	Sibirene	MS		**0.01**			**0.17**		**0.07**
	1550	Selina-3,7(11)-diene	MS, I^T		**0.01**			**0.16**		**0.08**
	1605	Sesquiterpene 1605						0.06		0.08
		Total			**2.31**	**0.05**	**<0.01**	**12.72**		**11.37**
		%			**60.76**	**1.60**	**<0.01**	**29.09**		**30.60**
Oxides	1589	Caryophyllene oxide	Standard, MS, I^T							0.11
		Total								**0.11**
		%								**0.31**
		TOTAL		**2.55**	**3.81**	**3.42**	**1.66**	**43.73**	**6.82**	**37.14**

*Siblings.

Standard: Identification based on co-injection with authentic standards by GC-MS;

MS: Identification based on mass spectra matching;

I^T: Identification based on I^T matching with DB-5 column;

Table 2. Limonoid and flavonoid content (μg·g^{-1} of juice) in one citrus (male parent), five Citrus–*P. trifoliata* hybrids and *P. trifoliata*.

	Component	1-11-7 (male parent)	5-14-96 (female parent)	6-49-116*	6-49-148*	6-49-163*	6-49-96*	***P. trifoliata***
Limonoids	Limonin	0.5	**1.0**	**2.2**				**10.9**
	Nomilin		**0.8**	**0.7**				**1.3**
	deacetyl nomilinic acid glucoside		**19.6**					**16.1**
	limonin glucoside	17.9	37.6	73.0	54.1	22.2	99.4	17.4
	nomilinic acid glucoside	156.1	335.1	59.9	388.4	226.4	35.4	1.4
Flavonoids	Naringin		**133.0**	**41.5**				**115.9**
	neohesperidin			38.0				
	poncirin							50.2
	narirutin	16.9	58.5	24.8	227.1	95.1	80.1	22.4
	isosakuranetin rutinoside	21.0					7.4	38.8
	hesperidin	**219.6**		42.0	**166.5**	82.3	**149.5**	**17.0**

*Siblings.

contained no nomilin and some limonin (0.5 μg·g^{-1}) but this level would probably not induce bitterness. Limonin content of the two parents and 6-49-116 were at levels typically found in orange juice (Baldwin et al., 2010), whereas *P. trifoliata* had a higher content. Likewise, the content of nomilin in *P. trifoliata* was higher than reported values for orange juice (Baldwin et al., 2010). Deactyl nomilinic acid glucoside, a tasteless limonoid, was present in *P. trifoliata* (16.1 μg·g^{-1}) and in the female parent (19.6 μg·g^{-1}).

Limonin glucoside and nomilinic acid glucoside were present in all samples analyzed. Limonin glucoside concentrations were high in the siblings 6-49-116 and 6-49-96 (73.0 and 99.4 μg·g^{-1}, respectively) and nomilinic acid glucoside content was high in the female parent (335.1 μg·g^{-1}) and in the juice of two of the siblings (6-49-148 and 6-49-163) (226–388 μg·g^{-1}), as well as in the male parent (156.1 μg·g^{-1}). However, this limonoid occurred at lower concentrations in the juice of two other siblings (around 35-60 μg·g^{-1} for 6-49-116 and 6-49-96) and was very low in the juice of *P. trifoliata* (1.4 μg·g^{-1}).

Among the bitter flavonoids, high levels of naringin were measured in the juice of *P. trifoliata* (115.9 μg·g^{-1}), in the female parent (133.0 μg·g^{-1}) and in the progeny 6-49-116 (41.5 μg·g^{-1}), while poncirin was only found in *P. trifoliata* (50.2 μg·g^{-1}) (Table 2). These two bitter flavonoid s are typically found in *P. trifoliata*; for example a naringin level of 937 μg·g^{-1} fresh weight (Nogata et al., 2006) and poncirin levels of 13,400 μg·g^{-1} fresh weight (Nogata et al., 2006) and 2,890 μg·g^{-1} dry weight (Kawaii et al., 1999) were reported. In this study, naringin and poncirin concentrations were equal or higher than their recognition taste thresholds in water: 33–119 μg·g^{-1} for naringin (Schiffman et al., 1994) and <30 μg·g^{-1} for poncirin (Horowitz, 1964). Neohesperidin, another bitter flavonoid (Horowitz and Gentili, 1963), was only found in the sibling 6-49-116 (38.0 μg·g^{-1}).

Narirutin was found in all the samples, with contents in the siblings (80.1 to 227.1 μg·g^{-1}) greater than in either parent (16.9 and 58.5 μg·g^{-1}) or than in *P. trifoliata* (22.4 μg·g^{-1}). Isosakuranetin rutinoside was found in the male parent, the sibling 6-49-96, and in *P. trifoliata*. It is worth noting that hesperidin (non-bitter flavonoid), is typically present at 25.6–392.6 μg·g^{-1} in fresh sweet orange fruit or juice,and is reported at 43.1–470.8 μg·g^{-1} in fresh tangerine fruit or juice (Peterson et al., 2006). In this study, hesperidin exhibited the highest occurring flavonoid in the male parent with no *P. trifoliata* in the background while the levels were about half that in 6-49-148 and 6-49-96 (149.5–166.5 μg·g^{-1}) and lower in 6-49-163 (82.3 μg·g^{-1}). The lowest hesperidin content was detected in *P. trifoliata* and in 6-49-116, and no hesperidin was detected in the female parent.

Overall, the female parent with ¼ *P. trifoliata* in its background had a limonoid and flavonoid composition similar to *P. trifoliata*, followed by the progeny 6-49-116. This was different than the progeny 6-49-163, which had a volatile profile more similar to *P. trifoliat*a than the other siblings. Similar to the situation with volatiles, limonoids and flavonoids that characterize the *P. trifoliata* species might be transmitted differently through subsequent generations. Moreover, our results suggest different inheritance patterns for limonoids and flavonoids than for volatiles.

Poncirus trifoliata inheritance expression

Cluster analyses were performed based on the "quality" of compounds (presence/absence of volatile and non-volatile compounds) (Fig. 2A) and on "quantity" of compounds (volatile corrected peak areas and limonoid and flavonoid concentrations) (Fig. 2B) in each hybrid and *P. trifoliata* samples. These analyses grouped similar hybrids and their parents in clusters. For the "quality" analysis, *P. trifoliata* had its own cluster, indicating that it was clearly distinguished from the hybrid samples regarding the composition of volatiles and secondary metabolites (Fig. 2A). With the background of 1/4 of *P. trifoliata*, the female parent, 5-14-96, was included in the same cluster as its progeny 6-49-163 (1/8 of *P. trifoliata*). The male parent (no *P. trifoliata* background) was grouped with the remaining hybrids (Fig. 2A). In the "quantity" analysis, *P. trifoliata* had its own cluster, as did 6-49-163 and the female parent (5-14-96) (Fig. 2B). All the other

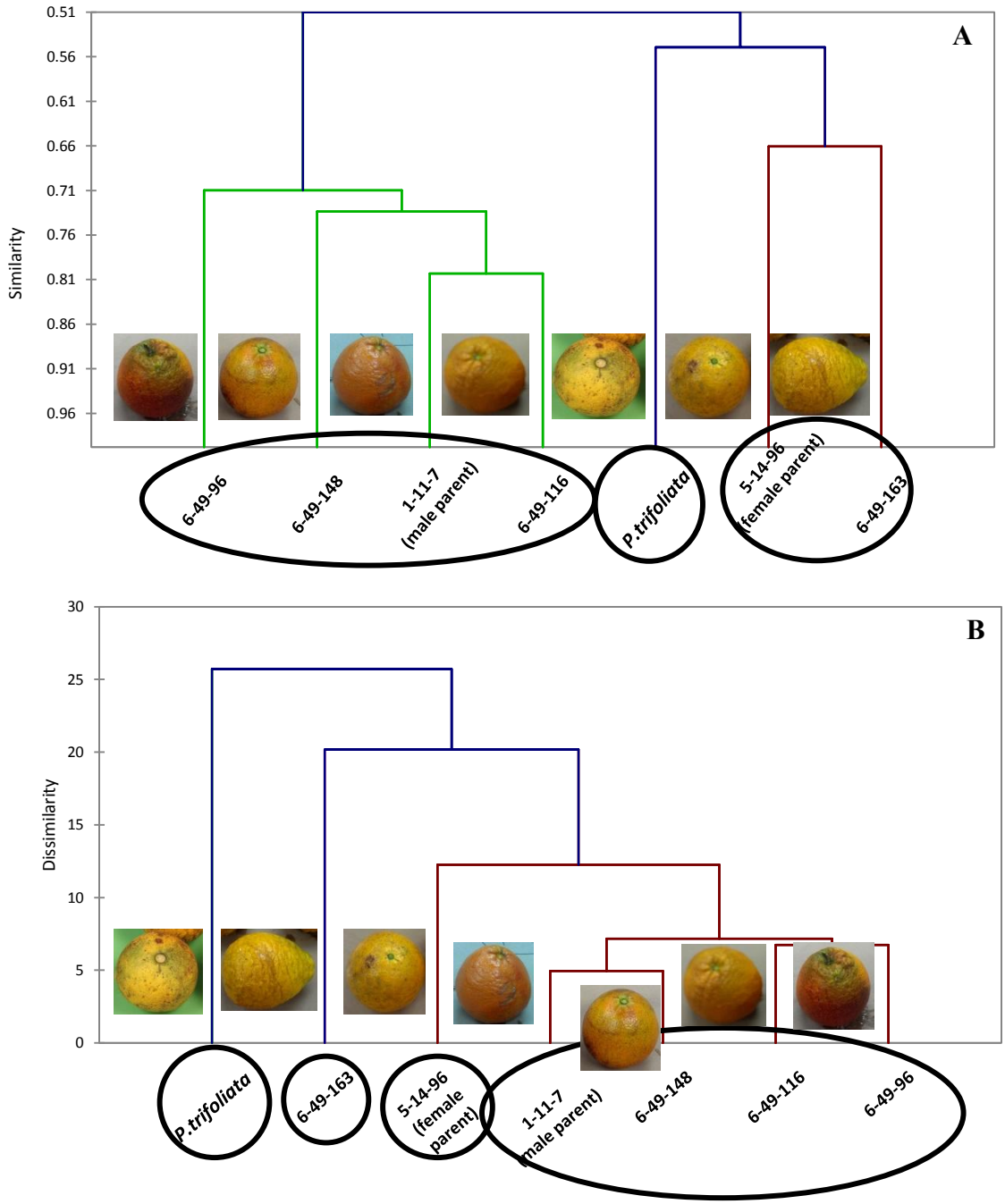

Fig. 2. Cluster analysis using the presence/absence of secondary metabolites (**A**) and using the volatile corrected peak area and limonoids and flavonoids contents (**B**) per sample.

hybrids, 1-11-7 (male parent, no *P. trifoliata* in its background), 6-49-148, 6-49-116 and 6-49-96 (1/16 of *P. trifoliata*), were clustered in one group. In both cluster analyses, it appears that grouping was determined by the composition of volatile profile more than by the non-volatile compounds. Indeed, even with a composition similar to *P. trifoliata* for limonoids and flavonoids, 6-49-116 was one of the least similar to *P. trifoliata* in volatiles, and in both cluster analyses, it was grouped with the male parent. More investigation on 6-49-116 hybrid would be interesting to pursue to estimate if other *Poncirus* characteristics, such as disease resistance, was transmitted and expressed, and to evaluate its eating quality.

Fruit Maturity

Sugars and acids are recognized indicators of fruit maturity and juice quality. Table 3 reports the sugar and acid contents of the six Citrus and Citrus–*Poncirus* hybrids harvested on the same day. It appears that the female parent and all siblings had high TA (0.8 to 1.3 g/100 mL) and SSC (9.3 to 14.1 g/100 mL) in comparison with the male parent (TA: 0.2 g/100 mL, SSC:

Table 3. Sugar and acid content (g/100 mL) in the juice of one citrus (male parent) and five *Citrus–P. trifoliata* hybrids.

Measurement	1-11-7 (male parent)	5-14-96 (female parent)	6-49-116*	6-49-148*	6-49-163*	6-49-96*
Titratable Acidity (TA)	0.2	0.9	1.0	1.3	1.3	0.8
Citric	0.4	1.5	1.5	2.0	2.0	1.5
Malic	0.2	0.1	0.1	0.1	0.1	0.1
Total acids**	**0.6**	**1.5**	**1.6**	**2.2**	**2.1**	**1.6**
Soluble Solids Content (SSC)	8.3	14.1	11.0	10.3	13.4	9.3
SSC/TA	**34.0**	**15.8**	**11.6**	**8.0**	**10.3**	**12.2**
Sucrose	4.1	4.4	3.3	2.3	4.3	3.7
Fructose	1.9	2.1	3.2	3.2	3.4	2.3
Glucose	1.9	2.2	3.0	3.0	2.8	2.0
Total sugars**	**7.9**	**8.7**	**9.5**	**8.4**	**10.5**	**8.1**

*Siblings

**Total acids and sugars are the sum of citric and malic acids and sucrose, glucose and fructose contents respectively

8.3). However, SSC/TA was the highest in the male parent (34.0), mostly due to a low TA. SSC/TA ratio gives an idea of which juice sample can be perceived as more sweet than sour. Comparing the SSC and SCC/TA values with those from the packing house maturity chart from the Florida Department of Agriculture & Consumer Services (USDA, 2009), it appears that all hybrids had a commercial maturity within an acceptable range according to the orange, tangerine and grapefruit standards. Only 6-49-148 hybrids did not correspond to the orange standard values: for a range of SSC values from 8.0 to 11.0, SCC/TA ratio has to be from 10.5 to 9.

Among individual acids, citric acid content was very high (1.5 to 2.0 g/100 mL) in all the hybrids with *P. trifoliata* in their background. The male parent had a citric acid content (0.4 g/100 mL) lower than most orange cultivars (Kelebek and Selli, 2011; Manley, 1983). Total sugar content was the highest in the sibling 6-49-163 (10.5 g/100 mL) and the lowest in the male parent (7.9 g/100 mL). These values may indicate that the siblings were less ripe than the male parent.

Sugars and acids, together with secondary metabolites (volatiles, limonoids, flavonoids) determine fruit juice aroma and flavor. Thus, their detection or/and quantification have to be considered to evaluate the juice quality. In a future study, sensory analysis evaluating flavor/taste of the juice from these hybrids will be conducted to determine which hybrid(s) have acceptable commercial juice quality.

Conclusion

In this study we identified volatiles and quantified limonoids and flavonoids in the juice from fruit of six Citrus and Citrus–*P. trifoliata* hybrids, including two parents and four siblings, and compared them to the secondary metabolite composition of *P. trifoliata* juice. Interestingly, only two siblings, 6-49-163 and 6-49-116, were similar to *P. trifoliata* regarding their secondary metabolite composition. Overall, 6-49-163 was the closest sibling to the female parent and expressed more its *P. trifoliata* background, mostly for volatiles. In contrast, 6-49-116 was closer to the female parent for its limonoid/flavonoid composition, but closer to the male parent for its volatile composition. These preliminary results reveal a complex inheritance pattern for volatile, limonoid and flavonoid compounds. From our results, further investigations could be conducted, i) to evaluate the juice quality and select *P. trifoliata* hybrids for consumption by correlating our chemical data with sensory data and ii) to determine the disease resistance or tolerance, especially to citrus greening or Huanglongbing (HLB), for the *P. trifoliata* hybrids which have acceptable eating quality.

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