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Leaf Volatile Compounds of Six Citrus Somatic Allotetraploid Hybrids Originating from Various Combinations of Lime, Lemon, Citron, Sweet Orange, and Grapefruit

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Volatile compounds were extracted by a pentane/ether (1:1) mixture from the leaves of six citrus somatic allotetraploid hybrids resulting from various combinations of lime, lemon, citron, sweet orange, and grapefruit. Extracts were examined by gas chromatography−mass spectrometry (GC-MS) and compared with those of their respective parents. All hybrids having an acid citrus parent exhibit the same relative contents in hydrocarbons and oxygenated compounds as the acid citrus, while the (grapefruit + orange) hybrid behaves similarly to its two parents. When volatile compound contents (μg g^{-1}) are examined in detail, several behaviors are encountered in hybrids and seem to depend on the presence/absence of the considered parental compound and on the corresponding hybrid combination. Meanwhile, the sesquiterpene hydrocarbons are present in all hybrids at concentrations systematically lower than those of the highest parental producers. Statistical analyses show that hybrids exhibit hardly discriminable aromatic profiles, meaning that no strong dominance of one or the other parent was observed in hybrids with regards to the leaf volatile compound production.

KEYWORDS: ***Citrus aurantifolia*; *Citrus limon*; *Citrus medica*; *Citrus sinensis*; *Citrus paradisi*; Rutaceae; allotetraploid somatic hybrids; leaf volatile compounds; statistical analyses**

INTRODUCTION

Citrus, as first in the world's fruit production, are of a considerable economic importance; thus, improvement of cultivars and rootstocks is a permanent major goal (*1*). Sexual hybridization, although largely used, is limited by several constraints such as polyembryony and interspecific and intergeneric incompatibilities. So somatic hybridization has appeared as an appropriate tool for varietal creation and has been successfully applied to the *Citrus* genus to generate new allotetraploid hybrids by fusion of diploid parental protoplasts (*1*). The main application of protoplast fusion is the production of rootstocks with improved resistances to pathogens and an increased tolerance to different stresses (*2*−*5*). Another objective is to create cultivars able to produce fruits with original qualities: tetraploid hybrids can be, for example, crossed with diploids resulting in the production of seedless triploid cultivars (*6*−*8*). Others factors, such as morphology, color, acidity, sugar content, and aroma compounds, are under consideration. These latter factors are major determinants of the sensory characteristics of not only fresh fruit but also derived products, such as juices or essential oils extracted from the peel, flowers, and leaves.

Depending on the considered citrus species, the leaf volatile compounds show different relative distributions in hydrocarbons and oxygenated compounds. Sweet orange (*9*, *10*) and grapefruit (*11*) leaves contain high proportions of hydrocarbons (typically > 70%); sabinene, a monoterpene hydrocarbon, is dominant (*10*, *12*). The leaves of lemon (*9*), lime (*11*), and citron (*13*), three acid citrus fruits (*14*), have high relative contents in oxygenated compounds (~50%) with predominant levels of monoterpene aldehydes (geranial, neral). The relative similarities of aroma profiles of, on one hand orange and grapefruit, and on the other hand lemon, lime, and citron, agree with their genetic origins: Grapefruit is an hybrid of sweet orange and pummelo while lime and lemon are hybrids sharing citron as their common parent (*15*).

To our knowledge, only four studies concerning the composition of leaf essential oils from citrus somatic hybrids [(sweet

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orange + "Femminello" lemon) (*16*), ("Milam" lemon + "Femminello" lemon) (*17*), (lime + grapefruit) (*18*), and (seven mandarin-derived hybrids) (*12*)] have been recently published. These studies showed that somatic hybridization does not result in a simple addition of parental traits with regards to the biosynthesis of aroma compounds: for instance, in our previous study presenting leaf volatile compounds of seven allotetraploid hybrids sharing willow leaf mandarin as their common parent (*12*), whatever the second parent fused to the mandarin one, hybrids exhibited an aromatic profile close to their mandarin parent. One of the questions raised by this study was to know if other hybrids sharing other nonmandarin parents behave the same way, in other words, if one of the parents used in the somatic fusion is dominant for the production of aroma compounds in the hybrids.

Tetraploid hybrids are bred at the Station de Recherches Agronomiques INRA-CIRAD (San Ghjulianu, Corsica, France). With the aim of establishing common inheritance rules, we analyzed leaf volatile compounds from six somatic allotetraploid hybrids obtained by fusion of various combinations of lime [*Citrus aurantifolia* (Christm.) Swing.], lemon [*Citrus limon* (L.) Burm.], citron [*Citrus medica* (L.)], sweet orange [*Citrus sinensis* (L.) Osb.], and grapefruit (*Citrus paradisi* Macfayden). Leaves from the five parents were also analyzed, and the results are presented hereafter.

MATERIALS AND METHODS

Plant Materials. The 1 year old parents, all grafted onto volkameriana rootstock (*Citrus limonia* Osb.) and growing in the same field of the Station de Recherches Agronomiques (INRA-CIRAD) of San Ghjulianu, were of the following species: lime (cv. Mexican lime; hereafter designated ML in tables and figures), lemon (cv. lac = lemon apireno Cantinella, LAC), citron (cv. Corsican, CDC), sweet orange (cv. Shamouti, SO), and grapefruit (cv. Star Ruby, SRG). We also analyzed 1 year old somatic tetraploid hybrids, obtained by the fusion of protoplasts from (i) the nucellar callus line of grapefruit (common parent) and, respectively, callus-derived protoplasts of lime [hybrid (SRG + ML)], lac lemon [hybrid (SRG + LAC)], and sweet orange [hybrid (SRG + SO)], and leaf-derived protoplasts of citron [hybrid (SRG + CDC)]; also analyzed were 1 year old somatic tetraploid hybrids resulting from the fusion of (ii) callus-derived protoplasts of sweet orange (common parent) with callus-derived protoplasts of lime [hybrid (SO + ML)] and lac lemon [hybrid (SO + LAC)]. These hybrids were all grafted onto volkameriana rootstock and planted the same week in the same field as their parents. Batches of leaves were randomly hand-picked, revolving around the shrubs on the same day (April 2002), and immediately air-freighted to our laboratory. Three individual shrubs were sampled for each parent and hybrid, and each batch of leaves was analyzed separately as follows. Leaves (50 g) were cut in half with scissors after removal of the central rib and then ball-milled in liquid N_2 with a Dangoumill 300 grinder for 2 min. Finely pulverized leaf powder was then stored under argon at −80 °C before extraction and analysis of volatile compounds the day after.

Solvents and Chemicals. The solvents (*n*-pentane and ether) were of analytical grade. Reference compounds and *n*-alkane (C_5−C_{22}) standards were from Aldrich Chimie (Saint Quentin Fallavier, France).

Extraction of Volatile Compounds. The internal standard (30 μg of *n*-hexanol) was added to leaf powder (500 mg), which was then homogenized using a Potter Elvejhem homogenizer with 20 mL of pentane/ether (1:1) for 5 min. The slurry was then filtered on a glass crucible (porosity 4) filled with anhydrous sodium sulfate. The extract was finally concentrated at 42 °C to a volume of 2 mL with a 25 cm Vigreux distillation column.

Gas Chromatography (GC) and GC-Mass Spectrometry (MS) Analysis. Solvent extracts were analyzed by GC-flame ionization detection (FID) using two fused silica capillary columns of DB-Wax (column A, J&W Scientific, Folsom, CA) (60 m × 0.32 mm i.d. × 0.25 μm film) and DB-1 (column B, J&W Scientific) fused silica capillary column (30 m × 0.32 mm i.d. × 0.25 μm film). The oven temperature was increased from 40 °C at a rate of 1.5 °C min^{-1} (DB-Wax) or at a rate of 3 °C min^{-1} (DB-1) up to 245 °C where it was held for 20 min. The on-column injector was heated from 20 to 245 °C at 180 °C min^{-1}. The detector temperature was 245 °C. Hydrogen was used as the carrier gas at a flow rate of 2 mL min^{-1}. Injected volumes were 2 μL of concentrated extract.

Solvent extracts were also analyzed by GC-MS using a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5973 quadrupole mass spectrometer with electron ionization mode (EI) generated at 70 eV. The ion source and quadrupole temperatures were 230 and 150 °C, respectively, and the filament emission current was 1 mA. Volatile compounds were separated on a DB-Wax (column A, J&W Scientific) fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film) and on a DB-1 (column B, J&W Scientific) fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film). The oven temperature was increased from 40 °C at a rate of 3 °C min^{-1} up to 250 °C where it was held for 20 min. The on-column injector was heated from 20 to 245 °C at 180 °C min^{-1}. The detector temperature was 245 °C. Helium was used as carrier gas at a flow rate of 1.1 mL min^{-1}. Electron impact mass spectra were recorded in the 40−600 amu range at 1 s^{-1} intervals. Injected volumes were 1 μL of the concentrated extract. Compounds were identified on the basis of linear retention indices on both columns (DB-Wax and DB-1), and EI mass spectra (Wiley 275.L library) were from the literature or from authentic standard compounds.

Quantitative data were obtained from the GC-FID analyses. Integration was performed on compounds eluted from the DB-Wax column between 3 and 110 min. Response factors of 10 reference compounds from different classes (monoterpenes, sesquiterpenes, monoterpene alcohols and aldehydes, and esters) were determined and found to range from 0.85 to 1.2 vs *n*-hexanol, averaging 1.0. Response factors were therefore taken as 1.0 for all compounds with reference to the internal standard. It was also confirmed that the internal standard was fully recovered after extraction and concentration from a leaf powder, by the separate injection of 2 μL of a standard solution of *n*-hexanol (15 μg mL^{-1}) in pentane/ether (1:1). Amounts were expressed as μg *n*-hexanol equivalent g^{-1} of dry weight. Linear retention indices were calculated with reference to *n*-alkanes (C_5−C_{22}). Concentrations (see **Table 1**) are given as the average of data from three individual shrubs. The total content in volatile compounds of the leaves from hybrids and their parents was calculated by summing concentrations of all volatile compounds eluted from the DB-Wax column between 3 and 110 min and expressed as percent dry weight.

Statistical Analyses. All statistical analyses were performed with the XLSTAT 6.0 software (Addinsoft, Paris, France). A dendrogram based on the absence/presence of each compound for all of the parents and hybrids has been obtained using the Sokal and Michener dissimilarity coefficient matrix and the UPGMA method (**Figure 1**). For each hybrid combination, Euclidian distances were calculated between both parents, between the grapefruit or the orange parents and the hybrid, and between the hybrid and the other parent (**Figure 2**). Calculations were based on the average concentrations of each volatile compound (see **Table 1**) from leaves of three individual shrubs. Principal component analysis was conducted with the different classes of volatile compounds (μg g^{-1} dry weight) as variables (see **Table 1**). **Figure 3A** was obtained from the correlation matrix calculated with the standardized matrix. Parents were used as active units for the calculation of the distribution of variables, whereas the somatic hybrids were considered as supplementary individuals and projected on the factorial planes with the aim to show the positioning of these hybrids with regards to the parents (**Figure 3B**).

RESULTS AND DISCUSSION

Our major objective was to qualitatively and quantitatively analyze volatile compounds extracted from leaves of young citrus somatic hybrids produced by the fusion of protoplasts from (i) the nucellar callus line of grapefruit (common parent) with callus-derived protoplasts of lime, lac lemon, and sweet

Table 1. Compounds of Leaves (μg g^{-1} Dry Weight and Relative Percent) from Parents and Their Tetraploid Hybrids

no.	compound	RI DB-Wax	RI DB-1	ML[a]	LAC[b]	CDC[c]	SO[d]	SRG[e]	SRG + ML	SRG + LAC	SRG + CDC	SRG + SO	SO + ML	SO + LAC	reliability of identification[f]
							monoterpene hydrocarbons								
1	α-pinene	1017	927	20	20	22	60	50	28	32	29	49	43	32	1
2	α-thujene	1019	921	–[g]	2	1	10	7	2	4	3	11	9	6	2
3	*β*-pinene	1097	964	22	40	4	58	61	41	69	31	64	20	48	1
4	sabinene	1112	963	20	44	47	1257	1091	400	242	352	1040	430	310	1
5	*δ*-3-carene	1140	998	4	298	4	394		3	321	3	201	557	462	1
6	*β*-myrcene	1157	984	123	134	109	188	94	108	175	105	183	253	180	1
7	α-phellandrene	1158	991				15			6		12	25	9	1
8	α-terpinene	1167	1002	1		1			1	2		2	3	2	1
9	limonene	1191	1020	3579	2068	3120	655	220	2065	2947	2726	2483	3678	2079	1
10	*β*-phellandrene	1195	1014	12	12	11	34	13	12	34	15	46	69	51	1
11	(*Z*)-*β*-ocimene	1227	1031	53	17	24	10	10	14	10	18	11	25	19	1
12	*γ*-terpinene	1235	1047	4	1	5	3			4	1	1	4	2	1
13	(*E*)-*β*-ocimene	1244	1041	267	82	36	326	192	258	139	147	370	346	160	1
14	*p*-cymene	1254	1006		5	2	11			4	1	2	8	8	1
15	α-terpinolene	1271	1075	5	18	3	41		4	17	2	26	71	50	1
	total			4110	2741	3389	3062	1738	2936	4006	3433	4501	5541	3418	
	relative % (%)			30.7	39.0	36.0	56.0	55.0	46.2	50.6	52.8	68.4	42.7	49.9	
							monoterpene aldehydes								
16	citronellal	1464	1131	61	442	125	257	384	475	346	450	612	1206	691	1
17	neral	1663	1214	2072	549	1744	147	12	662	992	805	228	1318	545	1
18	geranial	1719	1246	3420	1184	2639	208	21	1010	1267	1155	273	2080	827	1
	total			5553	2175	4508	612	417	2147	2605	2410	1113	4604	2063	
	relative % (%)			41.5	31.0	47.8	11.2	13.2	33.7	32.8	37.0	16.9	35.5	30.1	
							monoterpene alcohols								
19	linalool	1539	1087	61	49	64	341	162	58	64	80	189	313	140	1
20	α-terpineol	1682	1168	13	8	11	16	5	10		9	22	21	12	1
21	citronellol	1757	1214		102	6	24	43	55	91	18	106	170	109	1
22	nerol	1786	1214	51	151	37	15	8	48	94	16	43	123	98	1
23	geraniol	1895	1246	119	239	65	30	17	80	188	27	48	168	99	1
	total			244	549	183	426	235	251	437	150	408	795	458	
	relative % (%)			1.8	7.8	1.9	7.8	7.4	3.9	5.5	2.3	6.2	6.1	6.7	
							monoterpene esters								
24	citronellyl acetate	1658	1333	2	235	18	33	81	40	54	17	39	182	74	1
25	methyl geranate	1678	1298		37		13		7			18	34	16	2
26	neryl acetate	1717	1340	37		255	28	30	95	377	36	28	233	150	1
27	geranyl acetate	1744	1358	176	555	514	54	67	110	180	62	16	309	62	1
	total			215	827	787	128	178	252	611	115	101	758	302	
	relative % (%)			1.6	11.8	8.3	2.3	5.6	4.0	7.7	1.8	1.5	5.9	4.4	
							sesquiterpene hydrocarbons								
28	*δ*-elemene	1460	1320	4											2
29	α-ylangene	1470	1351								2				2
30	α-copaene	1478	1355				4	6							1
31	*β*-bourbonene	1502	1362	19											2
32	*β*-cubebene	1527	1367				5	6							2
33	*trans*-α-bergamotene	1575	1414	141	65	41			43	15	28		68	21	2
34	*β*-elemene	1575	1370				37	28	6			4	24	8	2
35	(*E*)-*β*-caryophyllene	1580	1391	924	433	251	229	137	104	60	89	90	400	170	1
36	α-humulene	1650	1423	101	32	15	76	37	21	3	13	24	56	22	1
37	(*E*)-*β*-farnesene	1660	1438	29			79	47	135	24	33	15	48	16	1
38	*γ*-selinene	1672	-	11											2
39	germacrene D	1690	1457	170		8	4	4	9		5	4	17		2
40	*β*-selinene	1698	1458	40											2
41	α-selinene	1703	1467	43											2
42	bicyclogermacrene	1719	1468	33	17	5	19	22	28	9	15	18	5	3	2
43	sesquiterpene [h]	1719	1492	223											
44	α-bisabolene	1720	1493	54	109	62			102	33	25		82	43	2
45	(*E*,*E*)-α-farnesene	1740	1490	334					44						2
46	germacrene A	1741	1476	313			313	118	58	8	45	69	135	105	2
47	germacrene C	1754	1493	127					15						2
48	germacrene B	1805	1528	415					14				34		2
	total			2981	656	382	766	405	579	152	255	224	869	388	
	relative % (%)			22.3	9.3	4.0	14.0	12.8	9.1	1.9	3.9	3.4	6.7	5.7	
							sesquiterpene aldehydes								
49	*β*-sinensal	2200	1664				168	81	73	36	33	131	233	67	2
50	α-sinensal	2268	1716				31		26			5	36	10	2
	total			0	0	0	199	81	99	36	33	136	269	77	
	relative % (%)			0.0	0.0	0.0	3.6	2.6	1.6	0.5	0.5	2.1	2.1	1.1	
							aliphatic aldehydes								
51	hexanal	1072	771	6			53	22	3	4	2	8	5	17	1
52	(*E*)-2-hexenal	1200	827	16		1	35	21	4	5	5	9	18	26	1
53	octanal	1277	984	17			1								1

Table 1 (Continued)

no.	compound	RI DB-Wax	RI DB-1	ML[a]	LAC[b]	CDC[c]	SO[d]	SRG[e]	SRG + ML	SRG + LAC	SRG + CDC	SRG + SO	SO + ML	SO + LAC	reliability of identification[f]
	aliphatic aldehydes														
54	nonanal	1380	1083	6		11	1								1
55	decanal	1485	1184	54	4		3	1	4			5			1
	total			99	4	12	93	44	11	9	7	22	23	43	
	relative % (%)			0.7	0.1	0.1	1.7	1.4	0.2	0.1	0.1	0.3	0.2	0.6	
	others														
56	1-penten-3-ol	1151							1	2			2	3	2
57	1,8-cineole	1198	1021	15	7	5	27				5				1
58	*cis*-2-penten-1-ol	1310		3			15	9	2	2		3	1	3	2
59	6-methyl-5-hepten-2-one	1323	969	2				1							1
60	*cis*-3-hexen-1-ol	1373		5			5	2	3	6		7		1	2
61	2-hexen-1-ol	1394		4				1	2	3		2	1		1
62	*cis*-limonene oxide	1426	1116	2	5	17			7	8	12	16	9	13	1
63	acetic acid	1433		5	2				4	4				3	1
64	*trans*-limonene oxide	1439	1121	2	5	9			5	7	7	11	9	9	1
65	epoxyterpinolene	1447			3				1	3	2		4	5	2
66	*trans*-sabinene hydrate	1456	1050	2	7	1	25	13	8	10	9	21	19	14	2
67	*cis*-caryophyllene oxide	1955	-	10		10	8	13	10	2	22	3	10	12	1
68	*trans*-caryophyllene oxide	1962	1580			31	27	7	26	13	16	6	18	24	1
69	(*E*)-nerolidol	2026	1544		13	21	5		7	11			21	10	1
	total			50	42	94	112	46	76	71	73	69	94	97	
	relative % (%)			0.4	0.6	1.0	2.0	1.5	1.2	0.8	1.1	1.0	0.7	1.4	
	total hydrocarbons			7091	3397	3771	3828	2143	3515	4158	3688	4725	6410	3806	
	relative % (%)			53.0	48.3	40.0	70.0	67.8	55.3	52.5	56.7	71.8	49.4	55.6	
	total oxygenated compounds[i]			6111	3555	5490	1458	955	2760	3698	2715	1780	6449	2943	
	relative % (%)			45.7	51.0	58.2	26.2	30.2	43.4	46.6	41.7	27.0	49.9	43.0	

[a] Lime. [b] Lac lemon. [c] Citron. [d] Sweet orange. [e] Grapefruit. [f] Key for reliability of identification: 1, identified by linear retention index and mass spectrum of reference compounds; 2, tentatively identified by linear retention index and mass spectrum similar to mass libraries. [g] Not detected. [h] MW = 204. [i] Others excluded.

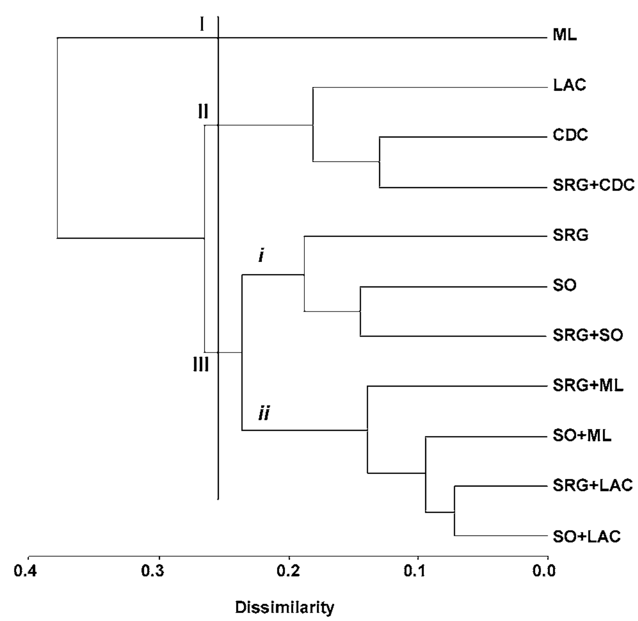

Figure 1. Dendrogram built from the Sokal and Michener dissimilarity index using the UPGMA method. The vertical line separates the main clusters. ML = lime; LAC = lac lemon; CDC = citron; SO = sweet orange; SRG = grapefruit; SRG + ML = hybrid (grapefruit + lime); SRG + LAC = hybrid (grapefruit + lac lemon); SRG + CDC = hybrid (grapefruit + citron); SRG + SO = hybrid (grapefruit + sweet orange); SO + ML = hybrid (sweet orange + lime); and SO + LAC = hybrid (sweet orange + lac lemon).

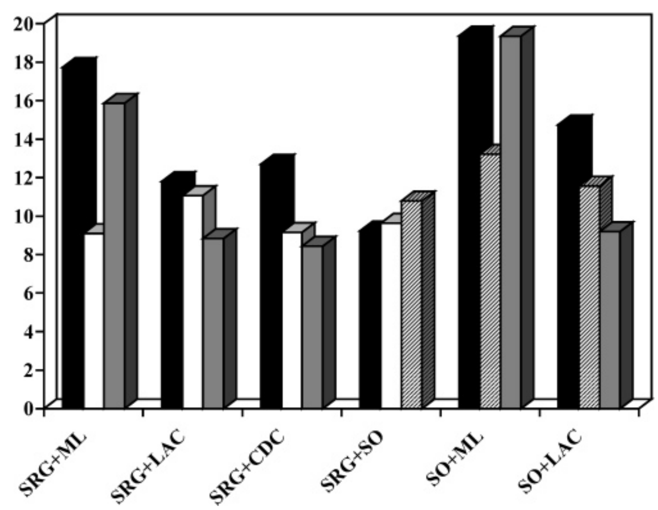

Figure 2. Euclidean distances between both parents (black bars), between the grapefruit parent and the hybrid (white bars), between the orange parent and the hybrid (hatched bars), and between the hybrid and the other parent (gray bars). ML = lime; LAC = lac lemon; CDC = citron; SO = sweet orange; SRG = grapefruit; SRG + ML = hybrid (grapefruit + lime); SRG + LAC = hybrid (grapefruit + lac lemon); SRG + CDC = hybrid (grapefruit + citron); SRG + SO = hybrid (grapefruit + sweet orange); SO + ML = hybrid (sweet orange + lime); and SO + LAC = hybrid (sweet orange + lac lemon).

orange and leaf derived-protoplasts of citron and from (ii) the nucellar callus line of sweet orange (common parent) with callus-derived protoplasts of lime and lac lemon. The six hybrids were shown to be allotetraploid ($2n = 4x = 36$) hybrids by flow cytometry and isozyme analysis (*4*). Volatile compounds of leaves from the five parents (ML, LAC, CDC, SO, and SRG) were also analyzed.

Total Content in Volatile Compounds. The total contents in volatile compounds of leaves (percent dry weight) from the

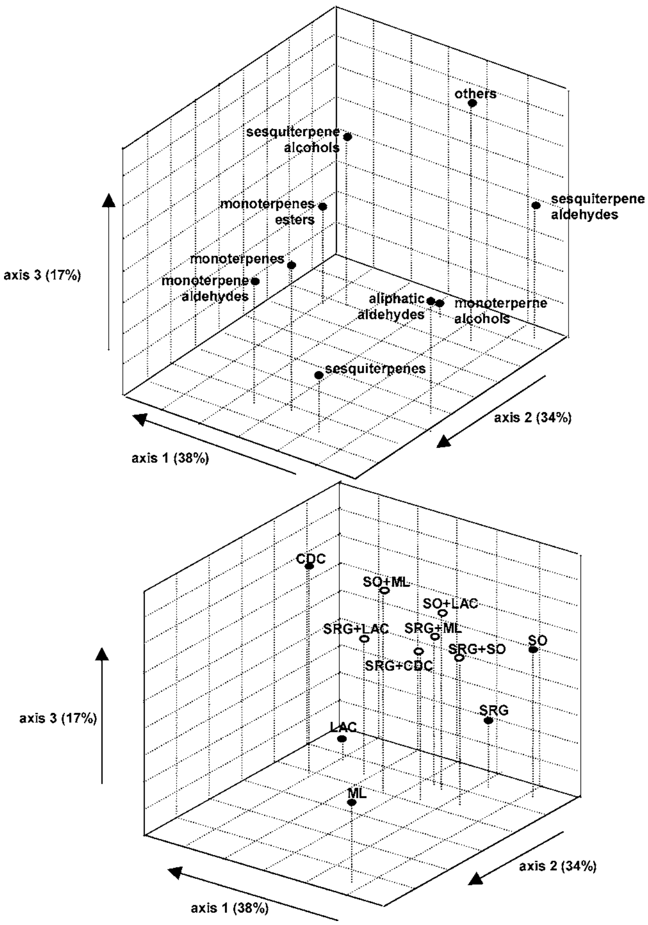

Figure 3. Results from PCA analysis. (A) Distribution of variables; (B) distribution of individuals. ML = lime; LAC = lac lemon; CDC = citron; SO = sweet orange; SRG = grapefruit; SRG + ML = hybrid (grapefruit + lime); SRG + LAC = hybrid (grapefruit + lac lemon); SRG + CDC = hybrid (grapefruit + citron); SRG + SO = hybrid (grapefruit + sweet orange); SO + ML = hybrid (sweet orange + lime); and SO + LAC = hybrid (sweet orange + lac lemon).

parents were as follows: lime, 1.33; lac lemon, 0.70; citron, 0.94; sweet orange, 0.54; and grapefruit, 0.31. The leaf volatile contents of hybrids were as follows: SRG + ML, 0.64; SRG + LAC, 0.79; SRG + CDC, 0.65; SRG + SO, 0.66; SO + ML, 1.30; and SO + LAC, 0.68.

Leaf Volatile Compounds from Parents. The composition of leaf extracts from the parents is given in **Table 1**. Hydrocarbons, among volatile compounds, were relatively more represented in sweet orange and grapefruit than in the three acid citrus fruits (lime, lemon, and citron) (~70 vs ~40−50%, respectively). They were present in lime at a higher concentration than in the other parents. Leaves of orange and grapefruit have higher sabinene (monoterpene hydrocarbon, compound 4) contents than the other parents while limonene (compound 9) exhibits a reverse behavior. δ-3-Carene (compound 5), a monoterpene absent from grapefruit leaves, is produced in high concentrations in the orange and lemon parents. Sesquiterpene hydrocarbons, such as γ-selinene (compound 38), β-selinene (compound 40), an unknown sesquiterpene (compound 43), and (*E*,*E*)-α-farnesene (compound 45) were exclusively produced in lime leaves while *trans*-α-bergamotene (compound 33) and α-bisabolene (compound 44) were only found in the three acid citrus. On the other hand, α-copaene (compound 30), β-cubebene (compound 32), and β-elemene (compound 34) were present only in orange and grapefruit parents.

Oxygenated compounds were produced at the same relative levels in the three acid citrus (~50%) and in both orange and grapefruit parents (~30%). This difference is due to the high concentrations in monoterpene aldehydes in lime, lemon, and citron, whereas these concentrations were low in orange and grapefruit. Whatever the considered acid citrus, citronellal (compound 16) content was lower than neral (compound 17) and the latter was lower than geranial (compound 18). The same phenomena is observed for the corresponding alcohols, citronellol, nerol, and geraniol (compounds 21−23, respectively). This is not the case in orange and grapefruit leaves where the citronellal is produced in a higher concentration than neral and geranial. We must add that the linalool (compound 19) content is higher in both orange and grapefruit parents than in lime, lemon, and citron. Moreover, we can note that the orange and grapefruit parents are the only parents producing sesquiterpene aldehydes [α- and β-sinensals (compounds 50 and 49) for orange and β-sinensal only for grapefruit]. So among the parents, we can gather the individuals into two groups: the acid citrus group producing as much hydrocarbons as oxygenated compounds and the orange−grapefruit group producing about 2.3× more hydrocarbons than oxygenated compounds.

Leaf Volatile Compounds from Hybrids. The composition of leaf extracts from the hybrids is given in **Table 1.** When an acid citrus (lime, lemon, and citron) is fused with either grapefruit or sweet orange, the relative contents of total hydrocarbons and oxygenated compounds in the hybrids are similar (~50−50%) and close to those of their acid citrus parents. When grapefruit is hybridized with sweet orange, the hybrid, SRG + SO, has an aromatic profile close to both parents (hydrocarbons, ~70%; oxygenated compounds, ~30%), which themselves have similar ratios. In that case, it is worth mentioning again that grapefruit is a hybrid of sweet orange and pummelo [*Citrus grandis* (L.) Osb.] (*15*).

Monoterpene Aldehydes, Monoterpene Alcohols, and Their Esters. Citronellal (compound 16) was produced in the hybrids sharing the grapefruit parent (except SRG + SO) at a lower extent than neral (compound 17), which was in turn lower than geranial (compound 18), like in the acid citrus leaves. The hybrids sharing the orange parent (SO + ML and SO + LAC, except SRG + SO), show a citronellal (compound 16) level similar to neral as in orange leaves. Nevertheless, the total aldehyde concentrations in these latter hybrids are close to those of their acid citrus parent. Concerning SO + ML, we can note that its citronellal (compound 16), citronellol (compound 21), and citronellyl acetate (compound 24) amounts are around 5−7× higher than those of the orange parent. Overproduction of these three compounds has already been observed in leaves of an older SRG + ML hybrid (*18*). In SRG + SO, monoterpene aldehyde concentrations are close to the addition of those of their parents.

Total monoterpene alcohol contents in grapefruit hybrids (including SRG + SO) seem to be similar to the nongrapefruit parent. This is not the case for orange hybrids. The linalool (compound 19) concentrations in grapefruit hybrids are close to those of their acid citrus parent while the linalool concentrations in the orange hybrids seem to be between both parental values. We can note that the geraniol/nerol ratio, which was comprised between ~1.6 and ~2.3 in all of the parents, is between ~1.7 and ~2.0 for the grapefruit hybrids except for SRG + SO and between ~1.0 and ~1.4 for the orange hybrids including SRG + SO.

Table 2. [∑(Geraniol + Geranial + Geranyl Acetate)/∑(Nerol + Neral + Neryl Acetate)] (Ratio 1) and [∑(Geraniol + Geranial + Geranyl Acetate)/∑(Citronellol + Citronellal + Citronellyl Acetate)] (Ratio 2) of Leaf Volatile Compounds from Parents and Their Tetraploid Hybrids

	ML[a]	LAC[b]	CDC[c]	SO[d]	SRG[e]	SRG + ML	SRG + LAC	SRG + CDC	SRG + SO	SO + ML	SO + LAC
ratio 1	1.72	2.83	1.58	1.54	2.10	1.49	1.12	1.45	1.13	1.53	1.25
ratio 2	58.97	2.54	21.60	0.93	0.21	2.11	3.33	2.56	0.45	1.64	1.13

[a] Lime. [b] Lac lemon. [c] Citron. [d] Sweet orange. [e] Grapefruit.

Among the monoterpene esters, we can note the peculiar behavior of neryl acetate (compound 26), which is overproduced with regards to its two parents in the lime- and lac lemon-derived hybrids.

We have also examined the contents in monoterpene oxygenated compounds of hybrids (as compared to their parents) with a new approach. We calculated the following ratios: ∑(geraniol + geranial + geranyl acetate)/∑(nerol + neral + neryl acetate) (ratio 1) and ∑(geraniol + geranial + geranyl acetate)/∑(citronellol + citronellal + citronellyl acetate) (ratio 2) (**Table 2**). It appears that ratio 1 in hybrids was systematically lower than the parent ones with nevertheless similar scale values. Ratio 2 exhibited very different values in the parent leaves. This ratio is leveled in the hybrids (mean value = 1.87). On the basis of ratio 2, we grouped hybrids into three clusters. (i) Hybrids that have a ratio 2 lower than 1: Only one hybrid belongs to this category, SRG + SO. Its parents, orange and grapefruit, are also included in this cluster. (ii) Hybrids that have a ratio 2 comprised between 1 and 2: This is the case of hybrids with the orange parent, except SRG + SO. (iii) Hybrids having a ratio 2 higher than 2: This group includes hybrids sharing grapefruit as their common parent, except SRG + SO.

Sesquiterpene Hydrocarbons and Sesquiterpene Aldehydes. Whatever the hybrid, the sesquiterpene concentrations, except (*E*)-*β*-farnesene (compound 37) and α-bisabolene (compound 44) ones, are systematically lower than that of the parent producing the highest amount of sesquiterpenes (decrease by ∼−37%/SRG for SRG + CDC to ∼−81%/ML for SRG + ML). This first result about sesquiterpene concentrations in hybrids with regards to the parent producing the highest amount of sesquiterpenes has been already observed in seven citrus allotetraploid hybrids, sharing the mandarin as their common parent (*12*): Actually, the amounts of sesquiterpenes were between 55 and 87% lower in the seven hybrids than in their nonmandarin parents. Thus, this latter observation would mean that the production in sesquiterpenes was not specifically affected by the presence of the mandarin genome in above allotetraploid hybrids as it was hypothesized in our previous work but by the chromosomal doubling or by the merger of stranger cells (addition of different genomes). Determination of the concentrations of sesquiterpenes in autotetraploids with regards to their unique parent could allow us to exclude one of the two above hypotheses.

β-Sinensal (compound 49), a sesquiterpene aldehyde detected in the leaves of sweet orange and grapefruit, and α-sinensal (compound 50), detected only in orange leaves, were also found but at lower levels in their corresponding hybrids except in lime-derived ones. In these latter hybrids, sinensals are less affected (*β*-sinensal in SRG + ML) or overproduced (α-sinensal appearing in SRG + ML and α-and *β*-sinensals overproduced in SO + ML).

Monoterpene Hydrocarbons. Several behaviors were observed among monoterpene hydrocarbons: (i) α-Pinene, *β*-pinene, and sabinene (compounds 1, 3, and 4, respectively), produced in higher concentrations in orange and grapefruit leaves than in acid citrus ones, were found at intermediary concentrations in hybrids. (ii) *δ*-3-Carene (compound 5), which is strongly expressed in lemon and in orange leaves and weakly (or even nonexpressed) in other parental leaves, was present at a high concentration in orange hybrids and in SRG + LAC. (iii) Limonene (compound 9), produced by all of the parents, is strongly represented in all hybrids. Whatever the citrus fused with grapefruit, the hybrid limonene contents were between 2000 and 3000 μg g^{-1}. Concerning orange-derived hybrids (except SRG + SO), their limonene contents were close to their nonorange parents. In the case of SRG + SO, limonene was overproduced with regards to each of its parents.

Thus, it seems, from above examples, that none parent appears to dominate the production of volatile compounds in the somatic hybrids. It seems to depend on the parents used for the somatic fusion and also on the considered volatile compound contrarily to the hybrids obtained with the common mandarin (*12*). The production of sesquiterpenes only appears to respond to a regulation linked to the somatic hybridization.

Statistical Analyses. A classification based on the presence/absence (1/0) of each volatile compound (from **Table 1**) was performed using the Sokal and Michener dissimilarity index (**Figure 1**). This index is calculated between individuals by pairs and uses the number of compounds present in both individuals, the number of compounds absent in both individuals, and the number of compounds present in one individual and absent in the other and reciprocally. So, the individuals were clustered in three groups: (i) Group I includes only one individual, the lime parent. The lime isolation can be explained by the fact that the mean index between the lime and the other individuals is higher (∼0.38) than the others (comprised between ∼0.20 and 0.30). This high index is in part due to the presence of many sesquiterpene hydrocarbons such as *β*-bourbonene (compound 31), germacrene B (compound 48), and C (compound 47), which are exclusively synthesized by the lime. (ii) Group II contains the two other acid citrus, lemon and citron, and the SRG + CDC hybrid itself closer to its citron parent than its grapefruit one with regards to the presence and absence of volatile compounds. Indeed, SRG + CDC and its citron parent have only nine unshared compounds, while SRG + CDC and grapefruit have 19. Among them, we can find *trans*-α-bergamotene (compound 33) and *β*-elemene (compound 34). (iii) Group III comprises grapefruit and orange and all hybrids except SRG + CDC. This group is divided into two subclusters: subcluster (i) (as shown in **Figure 1**) includes grapefruit and orange parents and their mutual hybrid (SRG + SO). This result means that orange and grapefruit have very close aromatic profiles; as above-mentioned, grapefruit is a sexual hybrid between sweet orange and pummelo (*15*), implying a genetic proximity between grapefruit and orange, which can partly explain this repartition. Subcluster (ii) (as shown in **Figure 1**) contains all of the hybrids between orange or grapefruit and lime or lemon revealing their aromatic proximity. It seems that these hybrids would be slightly closer to their orange or grapefruit parent than their acid citrus parents with regards to the presence and absence of volatile compounds.

The concentrations of volatile compounds considered individually were used to calculate Euclidean distances between each parent, between the hybrid and the first parent, and between the hybrid and the other parent (**Figure 2**). Qualitative and quantitative data are here integrated. The shorter the distances were, the closer the aromatic profiles between both individuals were. Three different behaviors can be distinguished among hybrids: (i) Hybrids for which distances between the hybrid and the grapefruit or the orange parent are shorter than distances between the hybrid and its other parent. This is the case of lime-derived hybrids, SRG + ML and SO + ML, meaning that hybrid aromatic profiles are closer to the orange and grapefruit parents than to the lime parent with regards to qualitative and quantitative data. (ii) Hybrids for which distances between the hybrid and the grapefruit or the orange parent are slightly higher than distances between the hybrid and its other parent. In this group are included hybrids with lemon and citron, SRG + LAC, SRG + CDC, and SO + LAC. (iii) The SRG + SO hybrid, for which the three calculated distances are quite similar in agreement with the genetic proximity of all these individuals.

This presentation shows that hybrids do not behave haphazardly (similarities can be seen between, respectively, the two lime-derived hybrids and the three hybrids having lemon and citron as one parent). However, and contrary to the mandarin-derived hybrids (*12*), it is difficult to withdraw conclusions with regards to a peculiar form of dominance of one parent or the other in the hybrids.

The last presentation, a principal component analysis, was used to examine the relative distribution of hybrids and their parents according to their production in different classes of volatile compounds (**Figure 3A**,**B**). The distribution of variables is shown in **Figure 3A**; one can see that the factorial space (constructed with axes 1−3) explains 89% of the whole variability. **Figure 3B** shows that principal component analysis did not allow us to discriminate the hybrids since they were distributed in the same area.

Contrary to our previous study on mandarin-derived somatic hybrids (*12*), where the mandarin genome clearly exerted a strong dominance in its somatic hybrids, statistical analyses gave here half-tint results: on the basis of the absence/presence of volatile compounds, most hybrids (except SRG + CDC) appeared closer to the grapefruit−orange couple than to the acid citrus group; on a quantitative basis, this is also true for the lime-derived hybrids. However, a principal component analysis analysis did not allow to discriminate these hybrids.

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