JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Leaf Volatile Compounds of Seven Citrus Somatic Tetraploid Hybrids Sharing Willow Leaf Mandarin (*Citrus deliciosa* Ten.) as Their Common Parent

ANNE-LAURE GANCEL,[†] PATRICK OLLITRAULT,[†] YANN FROELICHER,[‡] FELIX TOMI,[§] CAMILLE JACQUEMOND,[‡] FRANCOIS LURO,[‡] AND JEAN-MARC BRILLOUET*,[†]

Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Département FLHOR, TA50/16, F-34398 Montpellier Cedex 5, France; Station de Recherches Agronomiques (INRA-CIRAD), F-20230 San Ghjulianu, France; and Université de Corse, Equipe Chimie et Biomasse, UMR CNRS 6134, Route des Sanguinaires, F-20000 Ajaccio, France

Volatile compounds were extracted by a pentane/ether (1:1) mixture from the leaves of seven citrus somatic tetraploid hybrids sharing mandarin as their common parent and having lime, Eurêka lemon, lac lemon, sweet orange, grapefruit, kumquat, or poncirus as the other parent. Extracts were examined by GC-MS and compared with those of their respective parents. All hybrids were like their mandarin parent, and unlike their nonmandarin parents, in being unable to synthesize monoterpene aldehydes and alcohols. The hybrids did retain the ability, although strongly reduced, of their nonmandarin parents to synthesize sesquiterpene hydrocarbons, alcohols, and aldehydes. These results suggest that complex forms of dominance in the mandarin genome determine the biosynthesis pathways of volatile compounds in tetraploid hybrids. A down-regulation of the biosynthesis of methyl *N*-methylanthranilate, a mandarin-specific compound, originates from the genomes of the nonmandarin parents. Statistical analyses showed that all of the hybrids were similar to their common mandarin parent in the relative composition of their volatile compounds.

KEYWORDS: ***Citrus deliciosa*; *Citrus aurantifolia*; *Citrus limon*; *Citrus sinensis*; *Citrus paradisi*; *Fortunella margarita*; *Poncirus trifoliata*; Rutaceae; tetraploid somatic hybrids; leaf volatile compounds; statistical analyses**

INTRODUCTION

Somatic hybridization by fusion of diploid parental protoplasts has been successfully applied to the *Citrus* genus to generate new allotetraploid hybrids (*1*). These hybrids could serve as breeding parents for the production, via crossing with diploid individuals, of seedless triploid cultivars (*2*−*4*). Aside from morphology, color, acidity, and sugar content, aroma compounds are major determinants of the sensory characteristics of not only fresh fruit but also derived products such as juices and essential oils. Despite fruit being already available from certain tetraploid hybrids (*5*), to our knowledge only three studies concerning the composition of leaf essential oils from the citrus somatic hybrids (sweet orange + “Femminello” lemon) (*5*), (“Milam” lemon + “Femminello” lemon) (*6*), and (lime + grapefruit) (*7*) have recently been published. These studies showed that somatic hybridization does not result in a simple addition of parental traits with regard to the biosynthesis of aroma compounds. In some cases pathways are repressed [e.g., aliphatic aldehydes in the (lime + grapefruit) hybrid vs the lime parent], whereas in other cases there is massive overproduction of a compound compared with both parents [e.g., citronellal in the (lime + grapefruit) hybrid] (*7*). To improve our knowledge of the aroma biosynthesis inheritance rules and thereby define strategies for obtaining hybrids possessing good sensory characteristics, more systematic and extensive work is needed on the leaf and peel oil compositions of somatic hybrids compared with their parents.

Tetraploid hybrids having the Willow Leaf mandarin, *Citrus deliciosa* Ten., as their common parent are bred at the Station de Recherches Agronomiques INRA-CIRAD (San Ghjulianu, Corsica, France). With the aim of establishing common inheritance rules, we analyzed the composition of leaf volatile compounds from somatic hybrids of mandarin and, respectively, lime [*Citrus aurantifolia* (Christm.) Swing.], lemon [*Citrus limon* (L.) Burm., two cultivars], sweet orange [*Citrus sinensis* (L.) Osb.], grapefruit (*Citrus paradisi* Macfayden), kumquat [*Fortunella margarita* (Lour.) Swing.], and poncirus [*Poncirus trifoliata* (L.) Raf.]. Leaves from the eight parents were also analyzed, and the results are presented hereafter.

* Author to whom correspondence should be addressed [telephone 33-(0)4-67-61-75-81; fax 33-(0)4-67-61-44-33; e-mail brillouet@cirad.fr].
[†] Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD).
[‡] Station de Recherches Agronomiques (INRA-CIRAD).
[§] Université de Corse, Equipe Chimie et Biomasse.

10.1021/jf0345090 CCC: $25.00
Published on Web 08/29/2003

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: mandarin (cv. Willow Leaf; hereafter designated WLM in tables and figures), lime (cv. Mexican lime, ML), lemon (cv. Eurêka, EUR), lemon (cv. lac, lemon apireno Cantinella, LAC), sweet orange (cv. Shamouti, SO), grapefruit (cv. Star Ruby, SRG), kumquat (cv. Nagami, NK), and poncirus (cv. Pomeroy, PT). We also analyzed 1-year old somatic tetraploid hybrids, obtained by the fusion of protoplasts from the nucellar callus line of mandarin (the common parent) and callus-derived protoplasts of lime (WLM + ML), lac lemon (WLM + LAC), sweet orange (WLM + SO), and grapefruit (WLM + SRG) and leaf-derived protoplasts of lemon (WLM + EUR), kumquat (WLM + NK), and poncirus (WLM + PT). 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, when available, 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.

GC and GC-MS Analysis. Solvent extracts were analyzed by GC-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) (30 m × 0.32 mm i.d. × 0.25 μm film). 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 (DB-1) 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}. Detector temperature was 245 °C. Hydrogen was the carrier gas at 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, Folsom, CA) fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film). Oven temperature was increased from 40 °C at a rate of 3 °C min^{-1} 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}. Detector temperature was 245 °C. Helium was the carrier gas at 1.1 mL min^{-1}. Electron impact mass spectra were recorded in the 40−600 amu range at 1 s $interval^{-1}$. Injected volumes were 1 μL of concentrated extract. Compounds were identified on the basis of linear retention indices on both columns (DB-Wax and DB-1) (*14*) and EI mass spectra (Wiley 275.L library) 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, esters) were determined and found to range from 0.85 to 1.2 versus *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 micrograms of *n*-hexanol equivalent per gram of dry weight. Linear retention indices were calculated with reference to *n*-alkanes (C_5–C_{22}). Concentrations (see **Tables 1** and **2**) are given as the average of data from three individual shrubs. The total contents in volatile compounds of the leaves from hybrids and their parents were 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. For each combination, Euclidean distances were calculated (@DARwin 4.0 software, CIRAD, Montpellier, France) between the mandarin and nonmandarin parents, between the mandarin parent and the hybrid, and between the hybrid and the nonmandarin parent (**Figure 1**). Calculations were based on the average concentrations of each volatile compound (see **Table 1**) from leaves of three individual shrubs. Principal component analysis (PCA) was conducted using XLSTAT 4.2 software (Addinsoft, Paris, France) where variables were the different classes of volatile compounds (see **Table 2**) expressed as micrograms per gram of dry weight. **Figure 2A** 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 regard to the parents (**Figure 2B**).

RESULTS AND DISCUSSION

Our major objective was to qualitatively and quantitatively analyze the volatile compounds extracted from leaves of young citrus somatic hybrids produced by the fusion of protoplasts from the nucellar callus line of mandarin (the common parent) with the callus-derived protoplasts of lime, lac lemon, sweet orange, and grapefruit or with leaf-derived protoplasts of Eurêka lemon, kumquat, and poncirus. The seven hybrids were shown to be allotetraploid ($4n = 36$) hybrids by flow cytometry and isozyme analysis (*8*). The volatile compounds of leaves from the eight parents [lime (ML), Eurêka lemon (EUR), lac lemon (LAC), sweet orange (SO), grapefruit (SRG), kumquat (NK), poncirus (PT), and mandarin (WLM)] were also analyzed. Due to limited amounts of leaves from the 1-year-old somatic hybrids planted at the Station de Recherches Agronomiques INRA-CIRAD (San Ghjulianu), we aimed at developing an extraction procedure adapted to limited amounts of plant material. We preliminarily tested different extraction procedures on parent leaves that were finely ball-milled in liquid nitrogen. These procedures included hydrodistillation, simultaneous distillation−extraction (SDE) at atmospheric pressure, solid-phase microextraction (SPME), and direct solvent extraction with pentane/ether (1:1). Solvent extraction was the most appropriate to our study because, of all tested methods, it provided the largest quantities of extracted components and was feasible with a small number of leaves. Hydrodistillation, which requires large quantities of leaves, provided lower amounts of sesquiterpenes such as (*E*)-β-caryophyllene, whereas SDE drastically affected the monoterpene aldehydes neral and geranial. The conditions of sample preparation (e.g., the duration of ball-milling in liquid nitrogen and extraction by pentane/ether) were also optimized before being applied to the present plant materials.

The total contents in volatile compounds of leaves (percent dry weight) from the parents were lime, 1.33; Eurêka lemon, 0.95; lac lemon, 0.70; sweet orange, 0.54; grapefruit, 0.31; kumquat, 1.29; poncirus, 1.01; and mandarin, 1.38. The leaf volatile contents of hybrids were (mandarin + lime), 0.68;

Table 1. Volatile Compounds of Leaves (Micrograms per Gram of Dry Weight) from Parents and Their Tetraploid Hybrids

no.	compound	RI DB-Wax	RI DB-1	ML[a]	EUR[b]	LAC[c]	SO[d]	SRG[e]	NK[f]	PT[g]	WLM[h]	WLM + ML	WLM + EUR	WLM + LAC	WLM + SO	WLM + SRG	WLM + NK	WLM + PT	reliability of identification[i]
1	α-pinene	1017	927	20	63	20	60	50	1	60	92	79	94	58	36	63	356	141	1
2	α-thujene	1019	921	–[j]	5	2	10	7	–	–	45	33	30	21	13	19	166	68	2
3	hexanal	1072	771	6	20	–	53	22	45	9	13	4	6	1	9	3	3	15	1
4	β-pinene	1097	964	22	628	40	58	61	–	–	89	66	87	77	23	38	308	120	1
5	sabinene	1112	963	20	194	44	1257	1091	–	57	12	11	25	20	8	11	41	16	1
6	δ-3-carene	1140	998	4	138	298	394	–	6	–	–	–	–	–	–	–	43	–	1
7	1-penten-3-ol	1151	–	–	–	–	–	–	5	–	–	–	–	–	–	–	–	11	2
8	β-myrcene	1157	984	123	156	134	188	94	10	975	39	49	132	102	47	88	138	306	1
9	α-phellandrene	1158	991	–	–	–	15	–	–	285	–	–	–	–	–	–	–	–	1
10	α-terpinene	1167	1002	1	–	–	–	–	–	–	7	13	10	7	–	3	67	20	1
11	limonene	1191	1020	3579	3056	2068	655	220	34	93	1118	1646	5080	4590	2155	3820	1039	655	1
12	β-phellandrene	1195	1014	12	36	12	34	13	–	678	–	4	11	28	16	8	18	4	1
13	1,8-cineole	1198	1021	15	35	7	27	–	–	–	–	–	7	16	–	–	–	–	1
14	(*E*)-2-hexenal	1200	827	16	34	–	35	21	10	10	25	8	13	8	14	4	5	24	1
15	(*Z*)-β-ocimene	1227	1031	53	32	17	10	10	2	8	19	3	19	20	14	13	7	5	1
16	γ-terpinene	1235	1047	4	1	1	3	–	–	–	1049	722	606	424	238	397	2861	875	1
17	(*E*)-β-ocimene	1244	1041	267	168	82	326	192	43	220	39	23	94	76	30	44	221	115	1
18	*p*-cymene	1254	1006	–	–	5	11	–	–	20	117	77	42	25	25	26	460	293	1
19	α-terpinolene	1271	1075	5	22	18	41	–	–	–	28	33	29	18	5	11	157	61	1
20	octanal	1277	984	17	–	–	1	–	–	–	1	–	1	–	1	2	–	–	1
21	*cis*-2-penten-1-ol	1310	–	3	5	–	15	9	5	7	4	–	–	1	1	–	3	9	2
22	6-methyl-5-hepten-2-one	1323	969	2	1	–	–	1	–	–	–	–	–	–	–	–	–	–	1
23	*cis*-3-hexen-1-ol	1373	–	5	–	–	5	2	3	–	–	2	5	2	2	2	2	–	2
24	nonanal	1380	1083	6	2	–	1	–	–	–	1	1	1	1	–	1	–	–	1
25	2-hexen-1-ol	1394	–	4	–	–	–	1	–	–	–	1	–	–	–	3	–	–	1
26	*cis*-limonene oxide	1426	1116	2	9	5	–	–	–	–	–	–	–	2	–	–	–	–	1
27	acetic acid	1433	–	5	–	2	–	–	–	41	–	–	–	1	–	–	–	–	1
28	*trans*-limonene oxide	1439	1121	2	6	5	–	–	–	–	–	–	–	1	–	–	–	–	1
29	epoxyterpinolene	1447	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	2
30	α-cubebene	1448	1332	–	–	–	–	–	15	–	–	–	–	–	–	–	4	–	1
31	*trans*-sabinene hydrate	1456	1050	2	8	7	25	13	–	–	4	1	3	1	–	–	–	–	2
32	δ-elemene	1460	1320	4	–	–	–	–	74	–	–	–	–	–	–	–	11	–	2
33	citronellal	1464	1131	61	239	442	257	384	–	–	–	–	3	–	–	3	–	–	1
34	α-ylangene	1470	1351	–	–	–	–	–	41	–	–	–	–	–	–	–	3	–	2
35	α-copaene	1478	1355	–	–	–	4	6	38	–	–	–	–	–	–	–	11	–	1
36	decanal	1485	1184	54	2	4	3	1	–	–	–	2	1	–	2	10	–	–	1
37	β-bourbonene	1502	1362	19	–	–	–	–	80	–	–	–	–	–	–	–	5	–	2
38	β-cubebene	1524	1367	–	–	–	5	6	33	–	–	–	–	–	–	–	10	–	2
39	linalool	1539	1087	61	84	49	341	162	3	12	11	15	10	13	4	5	33	37	1
40	*trans*-α-bergamotene	1575	1414	141	38	65	–	–	–	–	–	–	4	–	–	–	–	–	2
41	β-elemene	1575	1370	–	–	–	37	28	97	–	–	–	–	–	–	4	50	2	2
42	thymyl methyl ether	1575	1216	–	–	–	–	–	–	–	3	–	–	–	–	–	–	–	2
43	(*E*)-β-caryophyllene	1580	1391	924	506	433	229	137	65	5000	211	276	255	114	92	46	590	353	1
44	3,7-guaiadiene	1590	1414	–	–	–	–	–	179	–	–	–	–	–	–	–	7	–	2
45	sesquiterpene[k]	1603	1414	–	–	–	–	–	117	–	–	–	–	–	–	–	5	–	
46	β-guaiene	1621	1482	–	–	–	–	–	22	–	–	–	–	–	–	–	–	–	2
47	α-humulene	1650	1423	101	38	32	76	37	168	331	17	24	20	9	6	7	70	35	1
48	citronellyl acetate	1658	1333	2	18	235	33	81	–	–	–	–	–	–	–	–	–	–	1
49	(*E*)-β-farnesene	1660	1438	29	–	–	79	47	125	78	–	–	–	–	7	7	37	53	1
50	neral	1663	1214	2072	1163	549	147	12	–	–	–	–	–	–	–	–	–	–	1
51	γ-selinene	1672	–	11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2
52	methylgeranate	1678	1298	–	–	37	13	–	–	–	–	–	–	–	–	–	–	–	2
53	α-terpineol	1682	1168	13	19	8	16	5	–	–	18	7	10	9	4	5	–	2	1

Table 1. (Continued)

54	germacrene D	1690	1457	170	–	–	4	4	5266	470	–	7	–	–	–	–	1708	623	2
55	aromadendrene	1696	1419	–	–	–	–	–	112	–	–	–	–	–	–	–	47	–	2
56	β-selinene	1698	1458	40	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2
57	α-selinene	1703	1467	43	–	–	–	–	34	–	7	15	4	–	–	–	50	21	2
58	neryl acetate	1717	1340	37	265	–	28	30	–	–	–	–	–	8	3	–	–	–	1
59	geranial	1719	1246	3420	1828	1184	208	21	–	–	–	–	–	–	–	–	–	–	1
60	bicyclogermacrene	1719	1468	33	44	17	19	22	32	381	13	12	29	14	–	21	429	53	2
61	sesquiterpene	1719	1492	223	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
62	α-bisabolene	1720	1493	54	50	109	–	–	–	–	–	–	–	–	–	–	–	–	2
63	α-muurolene	1729	1493	–	–	–	–	–	20	–	–	–	–	–	–	–	–	–	2
64	(*E*,*E*)-α-farnesene	1740	1490	334	–	–	–	–	–	–	–	29	–	–	–	–	–	–	2
65	germacrene A	1741	1476	313	–	–	313	118	582	165	–	8	–	–	11	9	106	62	2
66	geranyl acetate	1744	1358	176	237	555	54	67	–	–	–	–	–	–	–	–	–	–	1
67	germacrene C	1754	1493	127	–	–	–	–	294	94	–	5	–	–	–	–	151	42	2
68	δ-selinene	1756	1509	–	–	–	–	–	868	–	–	–	–	–	–	–	–	–	2
69	citronellol	1757	1214	–	17	102	24	43	–	–	–	–	–	–	–	–	–	–	1
70	nerol	1786	1214	51	154	151	15	8	–	–	–	–	–	–	–	–	–	–	1
71	germacrene B	1805	1528	415	–	–	–	–	1810	810	–	11	–	–	–	–	142	261	2
72	geraniol	1895	1246	119	153	239	30	17	–	–	–	–	–	–	–	–	–	–	1
73	*cis*-caryophyllene oxide	1955	–	10	6	–	8	13	10	12	–	–	–	–	–	–	5	–	1
74	δ-cadinol	1957	1555	–	–	–	–	–	291	–	–	–	–	–	–	–	10	–	2
75	sesquiterpenol[l]	1960	1539	–	–	–	–	–	36	–	–	–	–	–	–	–	4	–	
76	*trans*-caryophyllene oxide	1962	1580	–	18	–	27	7	18	13	–	–	–	–	–	–	4	–	1
77	(*E*)-nerolidol	2026	1544	–	5	13	5	–	112	–	–	–	–	–	–	–	112	–	1
78	MW = 220	2028	–	–	–	–	–	–	–	114	–	–	–	–	–	–	–	–	
79	methyl *N*-methylanthranilate	2035	1375	–	–	–	–	–	–	–	10768	3570	5194	7612	2869	3474	1678	447	2
80	sesquiterpenol	2052	1560	–	–	–	–	–	107	–	–	–	–	–	–	–	10	–	
81	elemol	2058	1519	–	–	–	–	–	54	–	–	–	–	–	–	–	11	–	2
82	sesquiterpenol	2068	–	–	–	–	–	–	32	–	–	–	–	–	–	–	2	–	
83	10-epi-γ-eudesmol	2074	–	–	–	–	–	–	50	–	–	–	–	–	–	–	10	–	2
84	sesquiterpenol	2125	–	–	–	–	–	–	64	–	–	–	–	–	–	–	29	–	
85	7-epi-γ-eudesmol	2137	–	–	–	–	–	–	38	–	–	–	–	–	–	–	–	–	2
86	γ-eudesmol	2143	1631	–	–	–	–	–	12	–	–	–	–	–	–	–	12	–	2
87	sesquiterpenol	2150	–	–	–	–	–	–	136	–	–	–	–	–	–	–	46	–	
88	sesquiterpenol	2154	1588	–	–	–	–	–	505	–	–	–	–	–	–	–	103	–	
89	thymol	2154	1280	–	–	–	–	–	–	–	13	–	–	–	–	–	–	–	1
90	sesquiterpenol	2159	1594	–	–	–	–	–	143	–	–	–	–	–	–	–	70	–	
91	sesquiterpenol	2161	1596	–	–	–	–	–	71	–	–	–	–	–	–	–	20	–	
92	sesquiterpenol	2168	–	–	–	–	–	–	18	–	–	–	–	–	–	–	–	–	
93	sesquiterpenol	2176	–	–	–	–	–	–	67	–	–	–	–	–	–	–	4	–	
94	sesquiterpenol	2185	–	–	–	–	–	–	86	–	–	–	–	–	–	–	30	–	
95	methyl anthranilate	2189	1332	–	–	–	–	–	–	–	15	–	–	–	–	–	–	–	1
96	α-eudesmol	2191	1619	–	–	–	–	–	284	–	–	–	–	–	–	–	33	–	2
97	β-eudesmol	2198	1613	–	–	–	–	–	390	–	–	–	–	–	–	–	129	–	2
98	β-sinensal	2200	1664	–	–	–	168	81	–	–	–	–	–	–	18	26	–	–	2
99	spathulenol	2218	1560	–	–	–	–	–	104	–	–	–	–	–	–	–	8	–	2
100	α-sinensal	2268	1716	–	–	–	31	–	–	–	–	–	–	–	–	–	–	–	2
101	(*E*,*E*)-farnesol	2291	1696	–	–	–	–	–	–	134	–	–	–	–	–	–	–	–	1

[a] Lime. [b] Eurêka lemon. [c] Lac lemon. [d] Sweet orange. [e] Grapefruit. [f] Kumquat. [g] Poncirus. [h] Mandarin. [i] Key for reliability of identification: 1, identified by linear retention index and mass spectra of reference compounds; 2, tentatively identified by linear retention index and mass spectrum similar to mass libraries. [j] Not detected. [k] MW = 204. [l] MW = 222.

Table 2. Classes of Leaf Volatile Compounds (Micrograms per Gram of Dry Weight) from Parents and Their Tetraploid Hybrids

	ML[a]	EUR[b]	LAC[c]	SO[d]	SRG[e]	NK[f]	PT[g]	WLM[h]	WLM + ML	WLM + EUR	WLM + LAC	WLM + SO	WLM + SRG	WLM + NK	WLM + PT
monoterpenes	4110	4499	2741	3062	1738	96	2396	2654	2759	6259	5466	2610	4541	5882	2679
sesquiterpenes	2981	676	656	766	405	10072	7329	248	387	312	137	116	94	3436	1505
total hydrocarbons	**7091**	**5175**	**3397**	**3828**	**2143**	**10168**	**9725**	**2902**	**3146**	**6571**	**5603**	**2726**	**4635**	**9318**	**4184**
monoterpene aldehydes	5553	3230	2175	612	417	–	–	–	–	3	–	–	3	–	–
monoterpene alcohols	259	462	556	453	235	3	12	29	22	27	38	8	10	33	39
monoterpene esters	215	520	827	128	178	–	–	–	–	–	8	3	–	–	–
sesquiterpene aldehydes	–	–	–	199	81	–	–	–	–	–	–	18	26	–	–
sesquiterpene alcohols	–	5	13	5	–	2496	134	–	–	–	–	–	–	635	–
aliphatic aldehydes	99	58	4	93	44	55	19	40	15	22	10	26	20	8	39
total oxygenated compounds	**6126**	**4275**	**3575**	**1490**	**955**	**2554**	**165**	**69**	**37**	**52**	**56**	**55**	**59**	**676**	**78**
methyl *N*-methylanthranilate	–	–	–	–	–	–	–	10768	3570	5194	7612	2868	3474	1678	447
others	35	53	22	80	46	145	187	39	4	8	8	3	5	22	20

[a] Lime. [b] Eurêka lemon. [c] Lac lemon. [d] Sweet orange. [e] Grapefruit. [f] Kumquat. [g] Poncirus. [h] Mandarin.

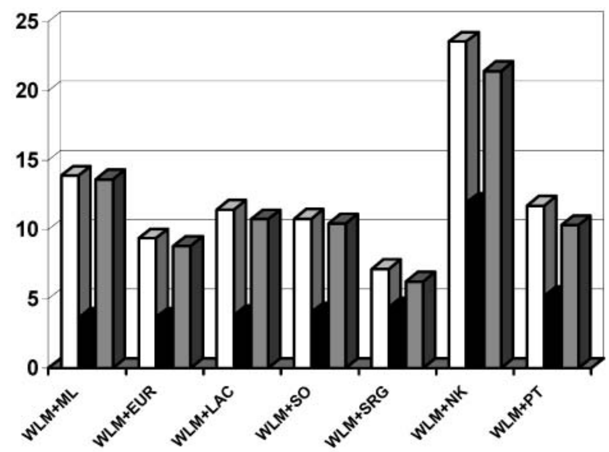

Figure 1. Euclidean distances between mandarin and the nonmandarin parents (white bars), between the mandarin parent and the hybrid (black bars), and between the hybrid and the nonmandarin parent (gray bars). WLM = mandarin; ML = lime; EUR = Eurêka lemon; LAC = lac lemon; SO = sweet orange; SRG = grapefruit; NK = kumquat; PT = poncirus.

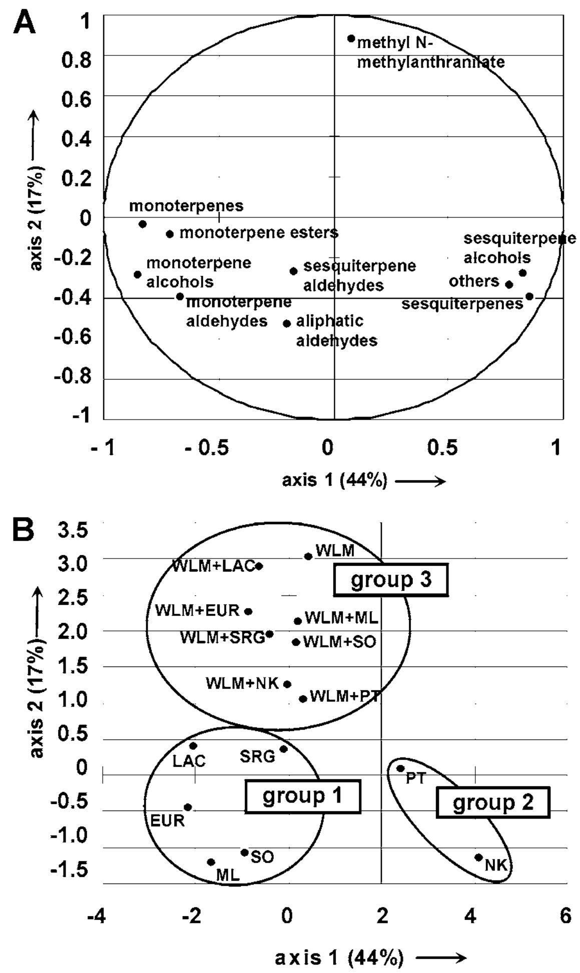

Figure 2. Results from PCA analysis: (A) distribution of variables; (B) three suggested groupings of individuals (groups 1–3). WLM = mandarin; ML = lime; EUR = Eurêka lemon; LAC = lac lemon; SO = sweet orange; SRG = grapefruit; NK = kumquat; PT = poncirus.

(mandarin + Eurêka lemon), 1.18; (mandarin + lac lemon), 1.33; (mandarin + sweet orange), 0.57; (mandarin + grapefruit), 0.82; (mandarin + kumquat), 1.17; and (mandarin + poncirus), 0.47. Contents measured in the hybrid leaves are systematically lower than the sum of the contents of their respective parents (by 35–80%). When compared with the average leaf volatile content of parents, the leaf volatile contents of the hybrids form two different groups:

- Some hybrids have a leaf volatile content quasi-equal to the average of their parents: (mandarin + Eurêka lemon), 1.18 versus 1.17; (mandarin + lac lemon), 1.33 versus 1.04; (mandarin + grapefruit), 0.82 versus 0.85, and (mandarin + kumquat), 1.17 versus 1.34.
- Other hybrids have a leaf volatile content that is about half the average of their parents: (mandarin + lime), 0.68 versus 1.35; (mandarin + sweet orange), 0.57 versus 0.96; and (mandarin + poncirus), 0.47 versus 1.15.

These data show that no general rule can be drawn with regard to the leaf volatile content of hybrids from that of their parents. The leaf volatile content of hybrid leaves was never equal to the sum of their parents.

The composition of leaf extracts from the hybrids and their parents is given in **Table 1**. Each component is given as micrograms of *n*-hexanol equivalent per gram of leaf (dry weight), response factors being taken as 1.0 for all compounds with reference to the internal standard.

Monoterpene Aldehydes, Monoterpene Alcohols, and Their Esters. Aldehydes (citronellal, neral, and geranial), alcohols (citronellol, nerol, geraniol, linalool, and α-terpineol), and acetyl esters of citronellol, nerol, and geraniol were present in five of the seven nonmandarin parents (lime, Eurêka lemon, lac lemon, sweet orange, and grapefruit) but, except for linalool and α-terpineol, were absent in the mandarin parent. Concentra-

Table 3. Classes of Leaf Volatile Compounds (Percent) from Parents and Their Tetraploid Hybrids

	ML[a]	EUR[b]	LAC[c]	SO[d]	SRG[e]	NK[f]	PT[g]	WLM[h]	WLM + ML	WLM + EUR	WLM + LAC	WLM + SO	WLM + SRG	WLM + NK	WLM + PT
monoterpenes	30.7	47.0	39.0	56.0	55.0	0.7	23.7	19.2	40.8	52.9	41.2	46.1	55.5	50.0	55.3
sesquiterpenes	22.3	7.1	9.3	14.0	12.8	78.3	72.5	1.8	5.7	2.6	1.0	2.1	1.2	29.2	31.1
total hydrocarbons	**53.0**	**54.1**	**48.3**	**70.0**	**67.8**	**79.0**	**96.2**	**21.0**	**46.5**	**55.5**	**42.2**	**48.2**	**56.7**	**79.2**	**86.4**
monoterpene aldehydes	41.5	33.8	31.0	11.2	13.2	–	–	–	–	–	–	–	–	–	–
monoterpene alcohols	1.9	4.8	7.9	8.3	7.4	–	0.1	0.2	0.3	0.2	0.3	0.1	0.1	0.3	0.8
monoterpene esters	1.6	5.4	11.8	2.3	5.6	–	–	–	–	–	0.1	0.1	–	–	–
sesquiterpene aldehydes	–	–	–	3.6	2.6	–	–	–	–	–	–	0.3	0.3	–	–
sesquiterpene alcohols	–	0.1	0.2	0.1	–	19.4	1.3	–	–	–	–	–	–	5.4	–
aliphatic aldehydes	0.7	0.6	0.1	1.7	1.4	0.4	0.2	0.3	0.2	0.2	0.1	0.5	0.2	0.1	0.8
total oxygenated compounds	**45.7**	**44.7**	**51.0**	**27.2**	**30.2**	**19.8**	**1.6**	**0.5**	**0.5**	**0.4**	**0.5**	**1.0**	**0.6**	**5.8**	**1.6**
methyl *N*-methylanthranilate	–	–	–	–	–	–	–	78.1	52.7	43.9	57.1	50.6	42.5	14.3	9.2
others	0.3	0.5	0.3	1.5	1.5	1.0	1.9	0.3	0.1	0.1	0.1	0.1	0.1	0.2	0.4
total identified	**99.0**	**99.3**	**99.6**	**98.7**	**99.5**	**99.8**	**99.7**	**99.9**	**99.8**	**99.9**	**99.9**	**99.9**	**99.9**	**99.5**	**97.6**

[a] Lime. [b] Eurêka lemon. [c] Lac lemon. [d] Sweet orange. [e] Grapefruit. [f] Kumquat. [g] Poncirus. [h] Mandarin.

tion ranges were as follows (**Table 2**): monoterpene aldehydes from ~420 μg g^{-1} for the grapefruit to ~5500 μg g^{-1} for the lime; monoterpene alcohols from ~230 μg g^{-1} for the grapefruit to ~550 μg g^{-1} for the lac lemon; monoterpene esters from ~130 μg g^{-1} for the sweet orange to ~830 μg g^{-1} for the lac lemon. The corresponding hybrids were deprived of the same compounds that were absent in their mandarin parent. Because the only monoterpene alcohol extracted from kumquat and poncirus parents was linalool, the (mandarin + kumquat) and (mandarin + poncirus) hybrids were likewise devoid of other monoterpenoid alcohols and aldehydes.

This almost complete inhibition of the biosynthesis of monoterpene oxygenated compounds in hybrid leaves is probably due to the presence of the mandarin genome in the somatic hybrid.

Linalool was present in mandarin and all nonmandarin parents as well as in the seven hybrids. However, different parent−hybrid behaviors were nonetheless observed. When the amount of linalool in the nonmandarin parent was higher than in the mandarin (i.e., lime, Eurêka lemon, lac lemon, sweet orange, and grapefruit), the amount of linalool in hybrids was reduced to a level similar to that in the mandarin parent. Conversely, in kumquat and poncirus, where the level of linalool was lower than or equal to its concentration in mandarin, linalool was overproduced in the corresponding hybrids. Similar behavior was observed for some monoterpenes (i.e., β-pinene, α-thujene, α-pinene, α-terpinene, and α-terpinolene; see further).

Sesquiterpene Hydrocarbons, Sesquiterpene Alcohols, and Sesquiterpene Aldehydes. The amount of sesquiterpene hydrocarbons in the leaves of the eight parents varied from ~250 μg g^{-1} for mandarin to ~10000 μg g^{-1} for kumquat (**Table 2**). In the seven hybrids, their concentration ranged from ~90 μg g^{-1} for (mandarin + grapefruit) to ~3400 μg g^{-1} for (mandarin + kumquat). It can be calculated from **Table 2** that this class of compounds was between ~55%, in the (mandarin + lemon) hybrid, and ~ 87%, in the (mandarin + lime), lower than in the nonmandarin parent. Overall, hybrids were on average ~75% lower than their nonmandarin parent. However, this decrease was not the same for all sesquiterpene hydrocarbons. In most cases, when a sesquiterpene was not detected or only a small quantity was found in the mandarin parent, it was likewise not detected or weakly represented in the corresponding hybrid (**Table 1**) despite being present in the other parent. This was the case in the (mandarin + lime) hybrid for β-bourbonene (−100%/lime parent), *trans*-α-bergamotene (−100%), germacrene A (−97%), and (*E*,*E*)-α-farnesene (−91%). However, in the case of the (mandarin + kumquat) hybrid, the biosynthesis of sesquiterpenes that were present at high concentrations in the kumquat parent (e.g., the germacrene family) was not fully inhibited in the hybrid leaves, with concentrations that were between ~18% (for germacrene A) and ~51% (for germacrene C) of those of the kumquat parent. Sesquiterpene alcohols were found at high concentrations in kumquat leaves (2500 μg g^{-1}) (**Table 2**) but were reduced by ~75% in the (mandarin + kumquat) hybrid, which is to be related to its similarly reduced concentration in sesquiterpene hydrocarbons. It must be mentioned that sesquiterpene alcohols can also be directly synthesized from farnesyl pyrophosphate by sesquiterpenol synthases (*9*).

β-Sinensal, a sesquiterpene aldehyde detected in the leaves of sweet orange and grapefruit, was also found in their corresponding hybrids but at lower levels (~−90%/orange parent and ~−70%/grapefruit parent) (**Table 1**).

Thus, it seems that a down-regulation of the biosynthesis of this family of compounds originates from the mandarin genome. However, unlike most oxygenated monoterpene compounds (other than linalool), which are not produced in hybrids, the production of sesquiterpene hydrocarbons, alcohols, and aldehydes is less affected by somatic hybridization.

Methyl *N*-Methylanthranilate. This compound was observed in the leaves of the mandarin parent and in leaves from the seven hybrids but was absent in the leaves of nonmandarin parents (**Table 1**). However, although it represents 78% of the volatile compounds (~11000 μg g^{-1}) in mandarin leaves (**Table 3**), as previously reported for other cultivars of *Citrus deliciosa* (*10*), its concentration is reduced by between ~30% in the (mandarin + lac lemon) hybrid and ~96% in the (mandarin + poncirus) hybrid, with an average reduction of ~70% for all hybrids. It should be noted that this compound is reduced to a far greater extent in the two hybrids having parents from *Fortunella* and *Poncirus* genera (kumquat and poncirus) than in those having parents from the *Citrus* genus (**Tables 2** and **3**). Thus, somatic hybridization of a mandarin with other members of the *Citrus*, *Fortunella*, and *Poncirus* genera results in a systematic reduction of the concentration of this component in hybrid leaves. Unlike terpenoids, which are synthesized from isopentenyl pyrophosphate and dimethylallyl pyrophosphate through geranyl and farnesyl pyrophosphates (*11*, *12*), the C7 compound methyl *N*-methylanthranilate derives from the phenol biosynthesis pathway by the addition of erythrose-4-phosphate to phosphoenolpyruvate and then successive conversion to shikimic acid, chorismic acid, and finally anthranilic acid (*13*).

Monoterpene Hydrocarbons. The concentration of these compounds in the leaves of the eight parents varied from ~100 μg g^{-1} for kumquat to ~4500 μg g^{-1} for Eurêka lemon (**Table 2**). In hybrids, they were found in concentrations either equal to the sum of those of both parents [(mandarin + Eurêka lemon), (mandarin + lac lemon), and (mandarin + grapefruit)] or similar to those of the mandarin parent [(mandarin + lime), (mandarin + sweet orange), and (mandarin + poncirus)]. In the case of the (mandarin + kumquat) hybrid, the concentration of monoterpenes was found to be twice that of the mandarin parent, the kumquat being very poor in these components.

The behavior of individual monoterpene hydrocarbons varied (**Table 1**). Concentrations of β-pinene and sabinene, two major monoterpenes of Eurêka lemon leaves, were greatly reduced in the corresponding hybrid to levels close to those of the mandarin parent. These two compounds were similarly found in the (mandarin + lime) hybrid at concentrations resembling those of their mandarin parent. Conversely, β-pinene was absent in kumquat and poncirus leaves but was found in the hybrids at levels higher than in mandarin leaves; this is also the case for α-thujene, α-pinene, α-terpinene, and α-terpinolene. Sabinene, a major monoterpene of sweet orange and grapefruit leaves, is lowered by ~99% in the corresponding hybrids to levels resembling that of the mandarin. The production of γ-terpinene was found to be reduced in six of the seven hybrids compared to the mandarin parent, whereas it was overproduced in the (mandarin + kumquat) hybrid.

Limonene, the major monoterpene hydrocarbon of lime, Eurêka lemon, and lac lemon (*14*), was produced in variable concentrations in their corresponding hybrids. In the (mandarin + lime) hybrid its concentration was between those of both parents and similar to that of mandarin, whereas in the (mandarin + Eurêka lemon) and (mandarin + lac lemon) hybrids it was overproduced compared with the parents. In the cases of sweet orange, grapefruit, kumquat, and poncirus, in which limonene was a minor constituent, it was produced at higher concentrations in the corresponding hybrids than in the nonmandarin parents, with levels ranging from 3 times that of sweet orange to 30 times that of kumquat.

Thus, although our data regarding monoterpene hydrocarbons seem to be more confusing than for other classes of volatile compounds, one can generally say that when a nonmandarin parent is poor or devoid of a monoterpene (e.g., kumquat and some monoterpenes of poncirus), the corresponding hybrids overproduce this monoterpene compared to the mandarin parent. Conversely, when a nonmandarin parent is richer in a monoterpene than the mandarin (e.g., lime, Eurêka lemon, lac lemon, and sweet orange), the corresponding hybrids tend to produce this monoterpene in amounts closer to that of the mandarin parent.

Statistical Analyses. The concentrations of volatile compounds were used to calculate Euclidean distances between the mandarin and nonmandarin parent, between the hybrid and the mandarin parent, and between the hybrid and the nonmandarin parent (**Figure 1**). The shortest distances are clearly those between the hybrids and the mandarin parent, implying that the volatile component profiles of hybrids are closest to those of the mandarin parent. In contrast, the distances between the hybrids and their nonmandarin parent are almost as high as those between the mandarin and nonmandarin parents. Thus, with regard to their volatile compound composition, hybrids are as differentiated from their nonmandarin parent as their mandarin and nonmandarin parents are differentiated from each other.

PCA was used to examine the relative distribution of hybrids and their parents according to their production of different classes of volatile compounds (**Figure 2**).

The distribution of variables is shown in **Figure 2A**; it can be seen that the principal factorial plane (constructed with axes 1 and 2) summarizes 61% of the whole variability. Furthermore, two opposite groups of variables are very well represented on axis 1: the monoterpene hydrocarbons, alcohols, esters, and aldehydes, on the one hand, and sesquiterpene hydrocarbons and alcohols, on the other hand. This would mean that when one group is present in high concentration, the other one is weakly represented and reciprocally. This suggests a reciprocal regulation of their biosynthesis pathways.

Moreover, it appears that methyl *N*-methylanthranilate is very well represented on axis 2. Therefore, we can conclude that this compound seems to be totally independent of both previous groups, which could be explained by their two different biosynthesis pathways.

Figure 2B is the representation on the principal factorial plane of the parents and hybrids, the latter ones being projected afterward. Three different groups can be observed:

Group 1 includes lemons, lime, orange, and grapefruit. These individuals are characterized by the production of monoterpene hydrocarbons, monoterpene esters, alcohols, and aldehydes.

Group 2 is defined by two variables, the sesquiterpenes and the sesquiterpene alcohols. This group includes kumquat and poncirus parents. It should be noted that these two parents do not belong to the *Citrus* genus.

Group 3 is fully characterized by axis 2, which is defined by one compound, methyl *N*-methylanthranilate. Large quantities of this volatile compound are produced by the mandarin parent. All hybrids are included in this group. Actually, they also produce this compound but in smaller amounts. This and the absence of monoterpene alcohols and aldehydes explain their close proximity to the mandarin parent.

These statistical analyses seem to confirm that the hybrids are close to the mandarin parent with regard to their qualitative production of volatile compounds. Therefore, all data reported in this paper suggest, in the tetraploid hybrids, complex forms of dominance of the mandarin genome in biosynthesis pathways of volatile compounds.

ACKNOWLEDGMENT

We thank X. Perrier (CIRAD-FLHOR, Montpellier, France) for helpful discussions.

LITERATURE CITED

(1) Grosser, J. W.; Gmitter, F. G., Jr.; Sesto, F.; Deng, X. X.; Chandler, J. L. Six new somatic citrus hybrids and their potential for cultivar improvement. *J. Am. Soc. Hortic. Sci.* **1992**, *117*, 169−173.

(2) Grosser, J. W.; Gmitter, F. G., Jr.; Louzada, E. S.; Chandler, J. L. Production of somatic hybrid and autotetraploid breeding parents for seedless citrus development. *HortScience* **1992**, *27*, 1125−1127.

(3) Ollitrault, P.; Dambier, D.; Sudahono; Mademba-Sy, F.; Vanel, F.; Luro, F.; Aubert, B. Biotechnology for triploid mandarin breeding. *Fruits* **1998**, *53*, 307−317.

(4) Guo, W. W.; Deng, X. X.; Yi, H. L. Somatic hybrids between navel orange (*Citrus sinensis*) and grapefruit (*C. paradisi*) for seedless triploid breeding. *Euphytica* **2000**, *116*, 281−285.

(5) Fatta Del Bosco, S.; Palazzolo, E.; Scarano, M. T.; Germanà, M. A.; Tusa, N. Comparison between essential oil yield and constituents of an allotetraploid somatic hybrid of *Citrus* and its parents. *Adv. Hortic. Sci.* **1998**, *12*, 72−77.

(6) Alonzo, G.; Fatta Del Bosco, S.; Palazzolo, E.; Saiano, F.; Tusa, N. *Citrus* somatic hybrid leaf essential oil. *Flavour Fragrance J.* **2000**, *15*, 258−262.

(7) Gancel, A.-L.; Ollé, D.; Ollitrault, P.; Luro, F.; Brillouet, J.-M. Leaf and peel volatile compounds of an interspecific citrus somatic hybrid [*Citrus aurantifolia* (Christm.) Swing. + *Citrus paradisi* Macfayden]. *Flavour Fragrance J.* **2002**, *17*, 416−424.

(8) Ollitrault, P.; Dambier, D.; Froelicher, Y.; Carreel, F.; d'Hont, A.; Luro, F.; Bruyère, S.; Cabasson, C.; Lofty, S.; Joumaa, A.; Vanel, F.; Maddi, F.; Treanton, K.; Grisoni, M. Apport de l'hybridation somatique pour l'exploitation des ressources génétiques des agrumes. *Cahiers Agric.* **2000**, *9*, 223−236.

(9) Mercke, P.; Crock, J.; Croteau, R.; Brodelius, P. E. Cloning, expression, and characterization of *epi*-cedrol synthase, a sesquiterpene cyclase from *Artemisia annua* L. *Arch. Biochem. Biophys*. **1999**, *369*, 213−222.

(10) Lota, M.-L. Les huiles essentielles d'agrumes: caractérisation par RMN du carbone-13, CPG-IK et CPG/SM. Thesis, Université de Corse, France, 1999.

(11) McGarvey, D. J.; Croteau, R. Terpenoid metabolism. *Plant Cell* **1995**, *7*, 1015−1026.

(12) McCaskill, D.; Croteau, R. Isopentenyl diphosphate is the terminal product of the deoxyxylulose-5-phosphate pathway for terpenoid biosynthesis in plants. *Tetrahedron Lett*. **1999**, *40*, 653−656.

(13) Romero, R. M.; Roberts, M. F.; Phillipson, J. D. Anthranilate synthase in microorganisms and plants. *Phytochemistry* **1995**, *39*, 263−276.

(14) Lota, M.-L.; de Rocca Serra, D.; Tomi, T.; Jacquemond, C.; Casanova, J. Volatile components of peel and leaf oils of lemon and lime species. *J. Agric. Food Chem.* **2002**, *50*, 796−805.

Received for review May 16, 2003. Revised manuscript received July 22, 2003. Accepted August 3, 2003. We thank the Collectivité Territoriale de Corse for granting this study (Grants MB/PPDB/FO/AP/00.1055, MB/FO/ER/01.842, and MB/SN/FO/AP/02.1037)

JF0345090