Journal of Essential Oil Bearing Plants
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/teop20

Characterization of Leaf Essential Oil Composition of Homozygous and Heterozygous Citrus clementina Hort. Extan. and its Ancestors

Maria Antonietta Germanà [a] , Eristanna Palazzolo [a] , Benedetta Chiancone [a] & Filippo Saiano [a]

[a] Dip. SAF , Università degli Studi Palermo. , Viale delle Scienze ed 4 - 90128 Palermo, Italy
Published online: 16 May 2013.

To cite this article: Maria Antonietta Germanà , Eristanna Palazzolo , Benedetta Chiancone & Filippo Saiano (2013): Characterization of Leaf Essential Oil Composition of Homozygous and Heterozygous Citrus clementina Hort. Extan. and its Ancestors, Journal of Essential Oil Bearing Plants, 16:1, 92-101

To link to this article: http://dx.doi.org/10.1080/0972060X.2013.764173

Taylor & Francis
Taylor & Francis Group

ISSN Print: 0972-060X
ISSN Online: 0976-5026

Characterization of Leaf Essential Oil Composition of Homozygous and Heterozygous *Citrus clementina* Hort. Extan. and its Ancestors

Maria Antonietta Germanà , Eristanna Palazzolo, Benedetta Chiancone, Filippo Saiano*
Dip. SAF, Università degli Studi Palermo. Viale delle Scienze ed 4 - 90128 Palermo, Italy

Received 26 May 2011; accepted in revised form 30 October 2011

Abstract: Clementine is a natural tangor, resulting from an interspecific cross between mandarin and sweet orange. Gametic embryogenesis, allowing the single-step development of complete homozygous line from the heterozygous parents, increases the efficiency of perennial crop breeding programs. Tri-haploids have been regenerated through pollen embryogenesis (specifically, by anther culture) of *Citrus clementina* Hort. ex Tan., cv. Nules. Two of them (HOMO1 and HOMO2) have been acclimatized and grafted *in vivo* in 2000. Research regarding the chemical characteristics of plant regenerated by pollen embryogenesis or gynogenesis are useful to study the "gametoclonal variation", as well as for further application of haploidy technology in *Citrus* breeding. For these reasons and with the aim to characterize these homozygous clementine genotypes, in this study leaf essential oils were extracted by Clevenger apparatus and their chemical composition was investigated by capillary GC-MS, comparing them with oils obtained from the heterozygous plant and from its ancestors (clementine is a hybrid between sweet orange and mandarin). The obtained oil compositions, submitted to a statistical analysis, are partitioned into groups using principal components analysis (PCA) and, compared to those of both parents, distinguished in three major chemotypes sabinene/linalool, γ-terpinene and methyl N-methylanthranilate.

Key words: *Citrus clementina* Hort. ex Tan., GC-MS analysis, essential oils, pollen embryogenesis.

Introduction

Clementine is of great importance in the Mediterranean citrus industry. Regarding its origin, clementine results to be a natural tangor, and the Webber's hypothesis on the interspecific cross between mandarin and sweet orange, is supported by results obtained using molecular markers [3,18]. Haploid technology, making possible through gametic embryogenesis the single-step development of complete homozygous lines from heterozygous parents, is a particularly interesting breeding method. Haploids, plants with a gametophytic set of chromosomes in the sporophyte, and homozygous doubled haploids (DHs) are important in the fields of genetic and developmental studies, as well as for plant breeding. In fact, they have a potential use in mutation research, selection, genetic analysis, transformation and in the production of homozygous genotypes [11,12]. Particularly in woody plants, generally characterized by a long reproductive cycle, a high degree of heterozygosity, large size and, sometimes, self-incompatibility, it is not possible to obtain homozygous breeding lines through conventional methods involving several generations of selfing and homozygous DHs produced through gametic embryogenesis, provide new opportunities for genetic studies and plant breeding [9-11]. Considerable research has been carried out since the 1970s to obtain haploids for

*Corresponding author (Filippo Saiano)
E-mail: < fsaiano@unipa.it >

fruit tree breeding through gametic embryogenesis, but they were not always successful [9,10,19]. Many haploids and DHs are under observations in many fruit tree crops such as citrus, apple, papaya and peach, but it takes considerable time to characterize them [7,8,13,20,21,22,25,26]. Often *in vitro* regenerated plants show differences in their morphological and biochemical characteristics, as well in chromosome number and structures. Particularly, tri-haploids have been regenerated through pollen embryogenesis (specifically, by anther culture) of *Citrus clementina* Hort. ex Tan., cv. Nules [8]. Two of them, (HOMO1 and HOMO2), have been acclimatized and grafted *in vivo* in 2000.

In this study, the composition of the essential oils, extracted by Clevenger apparatus, from the leaves of pollen-derived homozygous plants compared with those obtained from the heterozygous clementine and from its ancestors, sweet orange and mandarin, has been investigated with the purpose of obtaining information on the "gametoclonal variation", that is the variation produced through the gametic embryogenesis process.

Materials and methods

Plant material

Young and healthy leaves were collected from two tri-haploid plants (HOMO1 and HOMO2) of *Citrus clementina* Hort. ex Tan, cv. Nules, from one Clementine heterozygous plant (HETERO, the genotype that provided the flowers for the pollen embryogenesis experiment), and from the two clementine ancestors: sweet orange (cvs. Bonanza and Salustiana) and mandarin (cvs. Avana and Mandarino Tardivo di Ciaculli, MTC). The homozygous plants object of study have been obtained through pollen embryogenesis (particularly by anther culture) of *Citrus clementina* Hort. ex Tan, cv. Nules [6], and they resulted, as most of regenerants evaluated (82 % of 94 regenerants), triploids at the flow cytometry analysis, other than homozygous through microsatellite marker analyses [8]. The tri-haploid plants (HOMO1 and HOMO2), obtained *in vitro* by anther culture, were acclimatized *in vivo* and grafted *in vivo* in 2000, at the same time of the heterozygous plant (HETERO). The clementine plants (HOMO1, HOMO2 and HETERO) were of the same age of grafting (8 years) and they were grown in plastic pots. The clementine ancestors (sweet oranges and mandarins) were mature and older plants, cultivated in the soil. All the seven genotypes were cultivated in the same place under the identical environmental and cultivation conditions.

Leaf oils extraction

For each genotype, about 100 g of leaves were collected in spring around the canopy, early in the morning and with dry weather. Fresh leaves were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus collecting the oil in hexane. Because the extraction conditions were identical for all samples, the influence of technical parameters on the chemical composition of essential oils were considered negligible [15,16,23,24].

GC-MS analyses

An Agilent 6890 gas chromatograph instrument, equipped with the mass spectrometer detector Agilent 5975 B was used for the chromatographic analyses. A fused silica capillary column SLB-5MS (length 30 m, internal diameter 0.25 mm, 0.25 μm film thickness of silphenylene polymer equivalent in polarity to poly-5 % diphenyl / 95 % dimethyl siloxane phase) from Supelco, Italy, was the stationary phase. The injector in splitless mode had a temperature of 250°C. Experimental chromatographic conditions were as follows: Helium carrier gas at 1 ml/min; oven temperature program: 5 min isotherm at 40°C followed by a linear temperature increase of 4°C min^{-1} up to 200°C held for 2 min. MS scan conditions were: ionization technique, electronic impact (EI) at 70 eV, source temperature 230°C, interface temperature 280°C, mass scan range 33-350 *m/z*. The sample injected 1/50 diluted in pentane was 1 ml. For quantitative results each sample was analyzed in GC-FID [2,23]. The instrumental conditions for the gas chromatograph were the same as above reported. The FID detector was set at 250°C and 1ml of neat oil was injected. The quantitative composition was obtained by peak area normalization, the response factor for each

component was considered equal to 1 and three replicates of each sample were made. Internal standard was undecane.

Identification of the individual components was based (i) on comparison of their GC retention indices (RI), determined relatively to the retention time of a series of *n*-alkanes with linear interpolation, with those of authentic compounds, (ii) on computer matching with mass spectral libraries (NIST 05) and (iii) comparison with spectra of authentic samples or literature data [1].

Data analyses

Data were processed by cluster analysis using hierarchical clustering (Ward's technique and Euclidean distance measure) and were submitted to factor analysis for principal components. These processing were performed with the *SPSS* software (SPSS inc.).

Results and Discussion

Leaf oil composition of all seven genotypes with component amount $\geq$ 0.1 % is reported in Table 1, according their increasing retention index (Linear Retention Index, L.R.I.).

The total of seventy-eight identified components accounted for 97.0-99.6 % of the oils. A great variability in the amount of sabinene (tr-36.8 %), γ-terpinene (tr-10.4 %), methyl *N*-methylanthranilate (tr-92.2 %) and linalool (0.1-21.8 %), terpinen-4-olo (0.1-10.4 %) and one acyclic oxygenated sesquiterpene α-sinensal (tr-25.9 %) has been observed. α-pinene (tr-1.2 %), limonene (tr-4.2 %) and caryophyllene oxide (0.1-9.3 %) were found at a moderate concentration in most of the oils. The compound methyl *N*-methylanthranilate represents more than 80 % of the volatile compounds in the two mandarin leaves, while it was absent in the leaves of sweet orange and in the HETERO leaves. Thus, the natural hybridization of a mandarin with orange resulted in a drastic reduction of the content of this component in HETERO leaves, as well as in leaves of HOMO1 and HOMO2, regenerated by pollen embryogenesis of HETERO. Aldehydes (citronellal, neral, and geranial), alcohols (citronellol, nerol, geraniol, and terpinene-4-ol), acetyl esters of citronellol, nerol, and geraniol were absent in the ancestor mandarins, except for slight amount of linalool and α-terpineol, while in sweet orange ancestors these compounds were all present in higher amount. Differently, the amount of linalool in HETERO, HOMO1 and HOMO2 resulted higher than in oranges and much higher than in the two mandarins, where the linalool was present only in traces. Overall considering oxygenated monoterpene, the clementine HETERO as well as HOMO1 and HOMO2 resulted more similar to the two sweet oranges (Tab.1). α-Sinensal, a sesquiterpene aldehyde detected in the leaves of sweet oranges, but absent in mandarin, was found in the hybrid HETERO, at lower levels in HOMO1, but overproduced in HOMO2. Sabinene, the major monoterpene of the ancestor sweet orange that is present only in traces in mandarins, decreased in the HETERO. On the contrary, it showed a very high content in HOMO1 regenerant, while in HOMO2 was present in traces as well as in mandarins. Compared to the mandarins, the production of γ-terpinene was found to be greatly lower in HETERO, as well as in HOMO1 so resembling sweet oranges. However, for the entire class of monoterpene hydrocarbons, as such as for oxygenated sesquiterpene, HOMO1 and HETERO showed their similitude with sweet orange while HOMO2 did not resemble none of its parents. In conclusion, the essential oils of the seven genotypes appear mainly distinguished with respect to the relative percentage contents of methyl *N*-methylanthranilate, α-sinensal and sabinene. By the cluster analysis, the existence of two principal clusters has been evidenced and the dendrogram, reported in Fig. 1, and factor analysis, reported in Fig. 2, confirmed this distinction.

Cluster I: Methyl N-methylanthranilate chemotype

The two mandarins Tardivo di Ciaculli and Avana, which belong to this chemotype, define clearly cluster I. As reported by Dugo and Di Giacomo [4], their oil composition was dominated by methyl *N*-methylanthranilate (>80 %) with appreciable amounts of γ-terpinene (10.4 and 4.3 %), limonene (3.6 and 1 %) and p-cymene (1.6

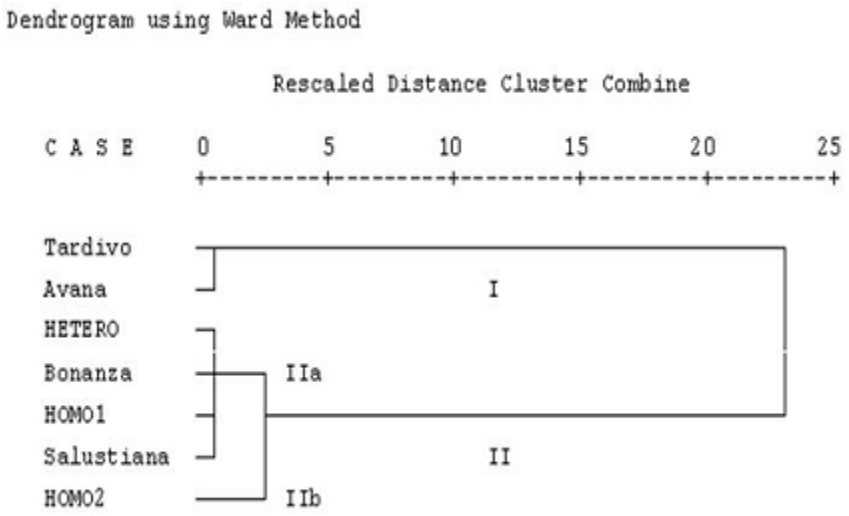

Figure 1. Dendrogram obtained from the cluster analysis of the seven *Citrus* genotypes leaf oils. Samples are clustered using Ward's technique with a squared Euclidean distance measure

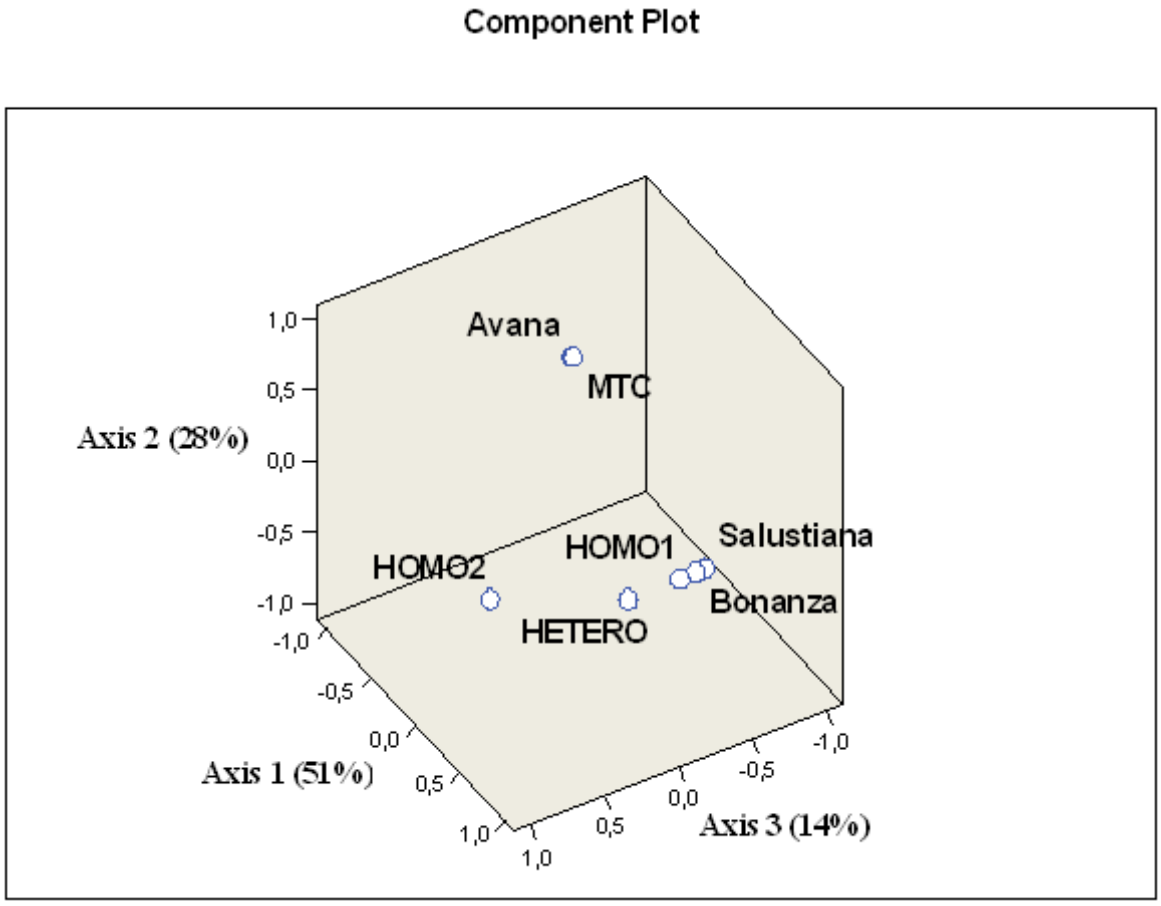

Figure 2. 3D load plot. Distribution of the seven *Citrus* genotypes leaf oils, according to the three principal components

Table 1. Chemical composition[a] of leaf oils extracted from the homozygous plants compared to those ones obtained from the heterozygous clementine and its ancestors: sweet oranges (Bonanza and Salustiana) and mandarins (MTC and Avana).

No.	LRI[b]	Name	I		Ia			IIb	
			MTC	Avana	Salustiana	Bonanza	HETERO	HOMO1	HOMO2
1	931	α-Thujene	0.2	tr	0.2	0.1	0.1	0.2	tr
2	939	α-Pinene	0.4	tr	1	0.4	0.5	1.2	tr
3	976	Sabinene	0.6	0.1	30.6	16.3	12.8	36.8	tr
4	981	β-Pinene	tr	tr	tr	tr	0.3	tr	tr
5	991	Myrcene	0.2	tr	2.6	2.2	2.3	2	tr
6	1005	α-Phellandrene	tr	tr	0.3	0.3	0.3	tr	tr
7	1011	δ-3-Carene	tr	tr	2.9	4.2	5.7	tr	tr
8	1018	α-Terpinene	0.1	tr	0.5	0.5	0.2	tr	tr
9	1022	o-Cymene	tr	tr	tr	0.1	0.1	1.4	tr
10	1026	p-Cymene	1.6	0.6	0.2	0.3	1	1.4	tr
11	1031	Limonene	3.6	1	2.7	3.4	4.1	4.2	tr
12	1040	(Z)-Ocimene	0.1	tr	0.2	0.3	0.4	0.1	tr
13	1050	(E)-Ocimene	0.2	0.1	4.1	7.5	4.9	1.5	tr
14	1062	γ-Terpinene	10.4	4.3	1.1	1.2	0.7	0.2	tr
15	1068	Sabinene hydrate cis	tr	tr	0.7	0.3	0.3	0.8	0.4
16	1074	*cis*-Linalool oxide	tr	tr	tr	tr	0.1	0.2	0.1
17	1088	*trans*-Linalool oxide	0.4	0.2	0.9	1.6	tr	0.2	0.1
18	1088	Terpinolene	tr	tr	0.4	tr	2.1	tr	tr
19	1095	Sabinene hydrate trans	tr	tr	tr	tr	0.1	1	0.5
20	1098	Linalool	0.2	0.1	10.6	8.7	21.8	21.2	19.9
21	1121	*cis*-p-Menth-2-en-1-ol	tr	tr	0.2	0.3	0.2	0.6	0.3
22	1140	*trans*-p-Menth-2-en-1-ol	tr	tr	0.1	0.2	0.2	0.7	0.2
23	1153	Citronellal	tr	tr	1.7	4	2.3	tr	tr
24	1156	Sabina ketone	tr	tr	tr	tr	tr	0.5	0.1
25	1177	Terpinene-4-ol	0.1	0.1	3.4	4.9	4.3	10.4	5.3
26	1181	Thuj-3-en-10-al	tr	tr	tr	tr	tr	0.3	0.1
27	1189	α-Terpineol	0.2	0.1	0.7	1.4	3.4	1.8	2.2
28	1193	*cis*-Piperitol	tr	tr	0.1	0.1	0.1	0.1	0.2
29	1194	Myrtenol	tr	tr	tr	tr	tr	0.3	tr

table 1. (continued).

No.	LRI[b]	Name	I	Ia			IIb	
			MTC	Avana	Salustiana	Bonanza	HETERO	HOMO1	HOMO2
30	1204	Decanal	tr	tr	0.2	0.1	0.1	tr	0.1
31	1205	*trans*-Piperitol	tr	tr	0.1	0.1	0.1	0.6	0.3
32	1217	*trans*-Carveolo	tr	tr	tr	tr	tr	0.2	0.1
33	1235	Myrtenyl acetate	tr	tr	0.1	0.1	tr	0.4	0.2
34	1233	Citronellol	tr	tr	0.9	3.9	5.6	0.2	0.5
35	1240	Neral	tr	tr	1.8	6.3	1.1	tr	tr
36	1255	Geraniol	tr	tr	0.4	1.5	3.3	0.2	0.6
37	1270	Geranial	tr	tr	1.9	7.6	1.5	tr	tr
38	1282	α-Terpinen-7-al	tr	tr	tr	tr	tr	0.2	tr
39	1285	*trans*-Linalool oxide acetate	tr	tr	tr	tr	tr	tr	0.2
40	1287	p-Cymen-7-ol	tr	tr	tr	tr	tr	0.3	tr
41	1298	Carvacrol	0.2	0.2	0.1	0.1	0.1	tr	0.1
42	1300	Varamolo	0.1	0.1	1.6	0.2	tr	0.9	6.7
43	1301	Methyl geranate	tr	tr	0.1	0.3	0.4	tr	tr
44	1339	*cis*-1,3-trans-1,4-p-Menthane-3,8-diol	tr	tr	tr	0.1	0.2	tr	tr
45	1350	α-Terpinyl acetate	tr	tr	tr	tr	tr	tr	1.3
46	1354	Cytronellyl acetate	tr	tr	0.3	0.7	0.1	tr	0.1
47	1365	Neryl acetate	tr	tr	0.3	1.6	tr	tr	tr
48	1383	Geranyl acetate	tr	tr	0.4	1.3	0.5	tr	0.3
49	1391	β-Elemene	tr	tr	5.2	2.6	tr	tr	0.4
50	1402	N-Methyl methylanthranilate	80.4	92.2	0.1	0.1	0.1	tr	tr
51	1418	β-Caryophyllene	tr	tr	1.5	1.7	1.2	1.8	5.9
52	1432	β-Gurjunene	tr	tr	0.2	0.1	tr	tr	0.9
53	1454	α-Humulene	0.2	0.2	2.2	1.7	0.4	0.2	0.8
54	1458	β-Farnesene	tr	tr	tr	tr	0.8	tr	tr
55	1477	γ-Muurolene	tr	tr	tr	tr	tr	tr	0.4
56	1480	Germacrene D	tr	tr	tr	tr	0.1	tr	0.4
57	1494	Bicyclogermacrene	0.2	0.2	1	0.7	0.2	tr	2.4
58	1499	α-Muurolene	tr	tr	tr	tr	0.1	tr	0.3
59	1503	Germacrene A	tr	tr	2.9	2.5	tr	tr	tr

table 1. (continued).

No.	LRI[b]	Name	I	Ia			IIb	
			MTC	Avana	Salustiana	Bonanza	HETERO	HOMO1	HOMO2
60	1508	(E,E)-α-Farnesene	tr	tr	tr	tr	0.4	0.2	2.3
61	1514	Cubebol	tr	tr	0.1	tr	0.1	tr	0.5
62	1524	δ-Cadinene	tr	tr	tr	tr	tr	tr	0.4
63	1524	Sesquiphellandrene	tr	tr	0.3	0.2	tr	tr	tr
64	1549	Elemol	tr	tr	0.2	0.1	0.1	0.1	0.6
65	1556	Germacrene B	tr	tr	tr	tr	tr	tr	0.2
66	1564	Nerolidol	tr	tr	0.3	0.2	0.5	0.4	1.6
67	1576	Spathulenol	tr	tr	0.1	0.1	0.1	0.3	1
68	1581	Caryophyllene oxide	0.1	0.1	0.1	0.3	0.3	4	9.3
69	1580	*trans*-Sesquisabinene hydrate	tr	tr	0.2	0.1	0.2	tr	tr
70	1606	Humulene epoxide II	tr	tr	tr	0.1	tr	0.3	0.9
71	1641	Epi-α-muruulol	tr	tr	tr	tr	0.4	tr	0.9
72	1645	α-muruulol	tr	tr	0.1	tr	tr	tr	0.7
73	1649	β-Eudesmol	tr	tr	tr	tr	tr	0.5	0.9
74	1653	α-Cadinolo	tr	tr	0.3	0.1	0.3	tr	0.9
75	1695	β-Sinensal	tr	tr	9.8	4.5	5.7	tr	0.5
76	1714	Nerolidol acetate	tr	tr	tr	tr	0.1	tr	0.5
77	1693	α-Bergamotol	tr	tr	tr	tr	0.8	tr	1.1
78	1752	α-Sinensal	tr	0.1	1.7	1.3	5.4	1.3	25.9
		Total	99.3	99.7	99.5	98.5	98.5	99	99.6
		Monoterpene hydrocarbons	17.4	6.1	46.5	36.8	35.3	49.1	tr
		Oxygenated monoterpene	1.1	0.7	24.5	44.7	45.4	40.7	32.9
		Sesquiterpene hydrocarbons	0.5	0.3	13.3	9.6	3.4	2.2	14.7
		Oxygenated sesquiterpenes	0.1	0.1	12.9	6.9	14.4	6.7	45.1
		Others	80.6	92.3	2.1	0.8	0.7	0.9	6.9

[a] Average quantitative composition by peak area normalization, three replicate for each replicate; response factor for each component was considered equal to 1.

[b] LRI = Linear Retention Index on SLB-5MS column.

and 0.6 %). The other compounds altogether contribute for a 5 % (Table 1). This chemotype represent the 51% of total variability detected.

Cluster II: α -sinensal/sabinene chemotype

Oils obtained from leaves of HETERO, Bonanza, Salustiana, HOMO1 and HOMO2 belong to this chemotype (cluster II). Moreover, this cluster can be divided into two subgroups on the basis of α-sinensal and sabinene amounts. The first subgroup (IIA), to which belonged HOMO1, Bonanza, Salustiana and HETERO oils, showed for the first three genotypes, sabinene as the main component (respectively 36.8, 16.3 and 30.6 %) and a lower content of linalool (21.2, 8.7 and 10.6 %). On the contrary in the HETERO oil, linalool was the main component (21.8 %), together with sabinene (12.8 %). HOMO2 oil belongs to the second subgroup (IIB), characterized by a high content of α-sinensal (25.9 %) and linalool (19.9 %), whereas the content of sabinene was below the 0.1 %. The two subgroups I and II represent respectively the 28 and 14 % of total variability detected.

Differences in the amount of minor compounds

Terpinen-4-ol was present at an appreciable content in the II cluster, whereas it was almost absent (0.1 %) in the cluster I. Moreover, myrcene content (2.0-2.6 %) and (E)-β-ocimene (1.5-7.5 %) was appreciable in the subgroup IIA and was very low (0.1-0.6 %) in the two others (IIB and I). Lastly, the content of α-terpineol and (E)-caryophyllene accounted for 0.7-3.4 % and 1.2-5.9 % respectively, in the cluster II, while these compounds were absent in the cluster I.

The differences observed between HOMO1 and HOMO2 can be explained because the two new genotypes have been produced through two different regeneration processes starting from different microspores (contained in diverse *in vitro* cultured anthers), that, resulting from both meiotic division, can show a large variation. In fact, ‘‘gametoclonal variation’’, that is the variation observed among plants regenerated from cultured gametic cells and consisting in genetic (chromosome number and structures), morphological, and biochemical differences [5,17], is different from ‘‘somaclonal variation’’, that is due only to the *in vitro* plant regeneration procedure from cultured cells or tissue [14].

Conclusions

In our knowledge, this is the first time that results regarding the chemical characteristics of leaf essential oils extracted from homozygous clementine plants, from the heterozygous mother plant and from its ancestors (mandarins and sweet oranges), have been reported.

Because the samples of all the genotypes have been collected from plants growing under the same environmental and cultural conditions and because experimental protocols (leaf oils extraction and analyses) were the same, quantitative and qualitative differences in the essential oil compositions can be attribute to the genetic basis. Phytochemical compounds resulted useful to characterize homozygous clementine in comparison with the heterozygous mother plant and with its ancestors (mandarins and oranges). Particularly, quali-quantitative analysis of the essential oils showed as HOMO1 has more similarities with HETERO and sweet orange than HOMO2 which is different regarding the α-sinensal content and the entire classes of monoterpene hydrocarbon and oxygenated sesquiterpene. Beside, HETERO, HOMO1 and HOMO2 plants are completely different from mandarin ancestor for the content of its principal component methyl-anthranylate. The single-step development of complete homozygous lines from heterozygous parents obtained through gametic embryogenesis, makes feasible and shortens the time required to produce completely homozygous lines compared to conventional breeding. Results obtained in this research are interesting, not only to deep the knowledge of the mechanism through which the essential oils metabolic pathways are transmitted during the meiosis process, because of the particular method (pollen embryogenesis) of producing homozygous plants, but also to study the “gametoclonal variation”, for further useful applications of haploidy technology in *Citrus* and fruit crop breeding.

References

1. **Adams, R.P. (2001).** Identification of essential oil components by gas chromatography/quadrupole Mass Spectroscopy. Allured Publishing Corporation USA, Carol Stream, Il.
2. **Bicchi, C., Liberto, E., Matteodo, M., Sgorbini, B., Mondello, L., d'Acampora Zellner, B., Costa, R. and Rubiolo, P. (2008).** Quantitative analysis of essential oils: a complex task. Flavour Fragr. J. 23: 382-391
3. **Deng, Z.N., Gentile, A., Nicolosi, E., Continella, G., Tribulato, E. (1996).** Parentage determination of some citrus hybrids by molecular markers. Proc. of the Int. Soc. Citr. 2: 849-854.
4. **Dugo, G., Di Giacomo, A. (2002).** Citrus, ed. Taylor and Francis, pp. 440-446.
5. **Evans, D,A, (1989)** Somaclonal variation-genetic basis and breeding applications. Trends Genet 5: 46-50.
6. **Germanà, M.A., Wang, Y.Y., Barbagallo, M.G., Iannolino, G., Crescimanno, F.G. (1994).** Recovery of haploid and diploid plantlets from anther culture of *Citrus clementina* Hort. ex Tan. and *Citrus reticulata* Blanco. J. Hort. Sci. 69: 473-480.
7. **Germanà, M.A., Crescimanno, F.G., Reforgiato, G., Russo, M.P. (2000).** Preliminary characterization of several doubled haploids of *Citrus clementina* cv. Nules, in: Goren, R., Goldschmidt E.E. (Eds.), Proc. First Int. Symp. Citrus Biotech., Eilat, Israel, Acta Hortic. 535*:* 183-190
8. **Germanà, M.A., Chiancone, B., Lain, O., Testolin, R. (2005).** Anther culture in *Citrus clementina*: a way to regenerate tri-haploids. Austr. J. Agr. Res. 56: 839-845.
9. **Germanà, M.A. (2006).** Doubled Haploid production in fruit crops. Plant Cell Tiss. Org. Cult. 86: 131-146.
10. **Germanà, M.A. (2009).** Haploid and Doubled Haploids in Fruit Trees: In Touraev A., (Forster B. and Jain M. Eds.) Advances in Haploid Production in Higher Plants. (pp. 241-263). Springer
11. **Germanà, M.A. (2011a)** Gametic embryogenesis and haploid technology as valuable support to plant breeding. Plant Cell Reports 30, 5: 839-857.
12. **Germanà, M.A. (2011b).** Anther culture for haploid and doubled haploid production. Plant Cell, Tissue and Organ Culture 104, 3: 283-300.
13. **Höfer, M., Gomez, A., Aguiriano, E., Manzanera, J.A., Bueno, M.A. (2002).** Analysis of simple sequence repeat markers in homozygous lines in apple. Plant Breeding 121: 159-162.
14. **Larkin, P.J., Scowcroft, W.R . (1981).** Somaclonal variation-a novel source of variability from cell culture for plant improvement. Theor Appl Genet 60: 197-214.
15. **Lota, M.L., de Rocca Serra, D., Tomi, F., Casanova, J. (2000).** Chemical variability of peel and leaf essential oils of mandarins from *Citrus reticulata* Blanco. Biochemical Systematics and Ecology 28: 61-78.
16. **Lota, M.L., de Rocca Serra, D., Tomi, F., Casanova, J. (2001).** Chemical variability of peel and leaf essential oils of 15 species of mandarins. Biochemical Systematics and Ecology 29: 77-104.
17. **Morrison, R., Evans, D. A. (1987)** Gametoclonal variation. Plant Breed. Rev. 5: 359-391.
18. **Nicolosi, E., Deng, Z.N., Gentile, A., La Malfa, S., Continella, G., Tribulato, E. (2000).** Citrus phylogeny and genetic origin of important species as investigated by molecular markers. Theor. Appl. Gen. 100: 1155-1166.
19. **Ochatt, S.J., Zhang, Y.X. (1996).** *In vitro* haploidization of fruit trees, in: Jain, S., Sopory, S.K., Veilleux, R.E., (Eds), *In Vitro* Haploid Production In Higher Plants, Dordrecht, The Netherlands: Kluwer Academic Publisher, Vol. 3: pp 193-210.
20. **Pooler, M.R., Scorza, R. (1995a).** Aberrant transmission of RAPD markers in haploids, doubled haploids, and F1 hybrids of peach: observations and speculation on causes. Sci. Hort. 64: 233-241.
21. **Pooler, M.R., Scorza, R. (1995b).** Occurrence of viable eggs in haploid peach. Fruit Varieties J,

49, 4, 239-241.
22. **Rimberia, F.K., Adaniya, S., Ishimine, Y., Etoh, T. (2007).** Morphology of papaya plants derived via anther culture. Sci. Hort. 213-219.
23. **Rubiolo, P., Sgorbini, B., Liberto, E., Cordero C. and Bicchi, C. (2010).** Essential oils and volatiles: sample preparation and analysis. A review. Flavour Fragr. J. 25: 282-290
24. **Tomi, F., Barzalona, M., Casanova, J. and Luro, F. (2008).** Chemical variability of the leaf oil of 113 hybrids from *Citrus clementina* (Commun) × *Citrus deliciosa* (Willow Leaf). Flavour Fragr. J. 23: 152-163
25. **Vanwynsberhe, L., De Witte, K., Coart, E., Keulemans, J. (2005).** Limited application of homozygous genotypes in apple breeding. Plant Breeding, 124: 399-403.
26. **Yahata, M., Harusaki, S., Komatsu, H., Takami, K., Kunitake, H., Yabuya, T., Yamashita, K., Toolapong, P. (2005)**. Morphological characterization and molecular verification of a fertile haploid pummelo (*Citrus grandis* Osbeck). J. Am. Soc. Hort. Sci. 130: 1: 34-40.