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Influence of the Rootstock and the Ploidy Level of the Scion and the Rootstock on Sweet Orange (*Citrus sinensis*) Peel Essential Oil Yield, Composition and Aromatic Properties

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Abstract: Rootstock is widely used for the cultivation of citrus fruits because it brings resistance or tolerance to diseases or environmental constrains and modulates the fruit quality. Polyploidization is a widespread improvement strategy in citrus. The objective was to evaluate the effect of rootstock and ploidy level on the composition of essential oils. Two trials were conducted, one displaying a 'Navelina' orange grafted on three rootstocks and a second combining two ploidy levels (di and tetraploid) of scion ('Pineapple' orange) and rootstock ('Carrizo' citrange). The composition of peel essential oil (PEO) was analyzed by gas chromatography coupled with mass spectrometry, and a panel of experts analyzed its flavor variation with a triangle test approach. The rootstock influenced the yield and composition of the orange PEO, with a low impact on flavor. Neither the rootstock nor the scion ploidy level affected the PEO yield. Only the tetraploid level of the scion significantly modified the PEO composition, reducing the oxygenated compound fraction. Sensitive significant differences were detected between the reference sample (diploid scion–diploid rootstock) and the three other combinations. These results suggest that for the profiling of an aromatic flavor, the rootstock is a key element as is the ploidy level of the scion.

Keywords: gas chromatography; sensorial analysis; mass spectrometry; diploid; autotetraploid; scion and rootstock interaction; oxygenated compounds

1. Introduction

Citrus, like most fruit trees, are grown as associated trees where a productive fruit variety (scion) and a rootstock (root system) are combined [1]. Rootstock plays an essential role in resistance to several diseases and adaptation to abiotic conditions. Although grafting existed for many centuries prior to the B.C. period, in citrus fruits, its use was generalized only during the 19th century following the dissemination of *Phytophthora gummosis*, a fungal disease [2]. The rootstock used was sour orange (*C. aurantium*), which was not only resistant to this disease but also very tolerant to different soil compositions, such as basic pH, calcareous soil, heavy soils, etc. However, citrus trees using sour orange as a rootstock with orange, mandarin, grapefruit and clementine are sensitive to the tristeza viral disease, which spread in the 20th century in many countries where the citrus industry extended [3,4]. Other citrus genotypes are now used as rootstocks for citrus cultivation in various countries, such as the most commonly used 'Volkamer' lemon (*C. jambhiri*),

'Rangpur' lime, citranges (*C. sinensis* × *Poncirus trifoliata*), citrumelos (*C. paradisi* × *P. trifoliata*) or 'Alemow' (*C. macrophylla*) [5].

The choice of the rootstock depends on the cultivar, its association with the cultivar (compatibility and vigor), the composition of the soil, the climatic conditions and the sanitary pressures. Apart from the contribution to resistance and adaptation, the rootstock also modifies the fruit production of the scion in terms of yield and quality (juiciness, sweetness, acidity, fruit size) [6]. However, these traits are strongly dependent on the environment and its interaction with scion and rootstock genotypes. Some rootstocks also induce fast production of the grafted variety [7].

Rootstock can also affect the peel essential oil (PEO) composition and yield of citrus fruit, as demonstrated by several studies [8–13]. The influence of rootstock on leaf essential oil composition and flower essential oil has also been demonstrated [10,14]. The influence of various rootstocks on fruit volatiles, fruit sensory quality, total soluble solids and acidity levels, indicates that the effect of rootstocks on the flavor of citrus fruits is a rather complex phenomenon that greatly depends on specific interactions between the rootstock, the environment and each particular scion variety [15].

Multiplied by seedlings, the citrus fruit being used as a rootstock must also be able to produce somatic embryos (apomictic reproduction) to ensure its clonal propagation. Rootstocks in production orchards are usually diploids. Breeding programs have experimented with the use of tetraploid rootstocks for approximately 20 years to improve resistance or tolerance, even if they slow the growth of trees [16–25]. These tetraploid rootstocks are either doubled diploids, somatic hybrids or sexual hybrids of somatic hybrids called 'tetrazygs'. Spontaneous double-diploid plantlets are frequent in seedlings of diploid apomictic *Poncirus* and its intergeneric hybrids, and their frequency is influenced by environmental factors such as temperature during blooming [26]. A tetraploid somatic hybrid was produced by fusion between protoplasts of *C. reticulata* and *P. trifoliata*, called 'FLHORAG1', which has been evaluated in several Mediterranean and tropical areas [18]. Associated with different cultivars, this somatic hybrid was tested as a rootstock for 15 years in trials with drastic conditions.

In citrus, polyploidization has characteristic morphological effects on ungrafted plants: slower growth, broader and thicker leaves and more compact trees [24,27]. Consequences on the fruits are as follows: thicker rinds, deeper color, fewer seeds and various influences on sweetness and acidity [24,27–29]. Hussain et al. (2012) [28] showed that tetraploid trifoliate orange rootstocks reduce scion canopy development and fruit yield; however, it does not affect the clementine quality criteria, such as sugar content, acidity, juiciness or carotenoid content, with the exception of the hesperidin content, which was higher for clementine scions grafted onto tetraploid rootstocks.

To our knowledge, very few studies have been performed on the influence of the ploidy level on citrus peel essential oil composition. Comparing diploid and tetraploid lines of seven citrus varieties, Cameron and Scora identified fourteen compounds whose contents were affected by the ploidy level [27].

Rémy Cointreau is a French spirits group that elaborates liqueurs based on sweet and bitter orange peels produced in various countries. This company wishes to control the organoleptic quality of the essential oil of citrus fruits to avoid any aromatic variation in their liqueurs. The type of rootstock varies depending on the country that supplies the company with citrus. Moreover, polyploidy appears to be a promising way to improve the citrus culture against the biotic and abiotic constraints present in these countries. This study therefore aimed to investigate the influence of rootstock and ploidy level on the yield, composition and aromatic quality of orange peel essential oil.

2. Materials and Methods

2.1. Biological Material

All the biological materials used in this study came from orchards of INRAE-Cirad in San Giuliano, France (latitude 42°17′ N, longitude 9°32′ E; Mediterranean climate, average

rainfall 840 mm per year and average temperature 15.2 °C; soil derived from alluvial deposits and classified as fersiallitic, pH range 5.0–5.6) [30].

The first experiment was based on fifteen trees of the sweet orange (*C. sinensis*) 'Navelina' cultivar grafted in three rootstocks ('Carrizo' citrange, *P. trifoliata* ('Pomeroy' cultivar) and 'FLHORAG1' (originating from fusion of protoplasts of *P. trifoliata* and *C. deliciosa*) [18]. All trees were of the same age (9 years) and cultivated under identical conditions.

The second experiment was conducted on twenty trees combining the 'Pineapple' sweet orange (*C. sinensis*) and the 'Carrizo' citrange rootstock with two ploidy levels (diploid and tetraploid) for the scion and the rootstock with five trees for each scion–rootstock ploidy combination (2X/2X, 2X/4X, 4X/2X, 4X/4X). All the tetraploid genotypes that came from chromosome doubling during the mitosis of somatic nucellar embryos were selected from diploid seedlings by flow cytometer as described in Dambier et al. (2011) [18]. All the trees were of the same age (10 years) and cultivated in identical conditions.

2.2. Essential Oil Analysis

2.2.1. Raw Material

To perform peel essential oil extraction in order to study the influence of the rootstock, the fruits were harvested in mid-December. Three replicates of five fruits per tree were picked and hand-peeled, and then the fresh peel was stored at −20 °C before further analysis.

To perform peel essential oil extraction to study the influence of the level of ploidy on the scion and the rootstock, the fruits were harvested in mid-February. Five fruits per tree were picked and hand-peeled, and then the fresh peel was stored at −20 °C before further analysis.

For both experiments, three fruits representative of fruit size, shape and color were picked from each tree, and the peel dry matter percentage was calculated using an oven until the weight of all samples stabilized. The yield calculation based on dry peel weight was used for this study because it is more suitable and reliable than the yield based on the fresh weight, which may be affected by the state of turgescence of the fruit. In addition, tetraploid plants are known to have a higher water content in all parts of the plant compared with diploid plants, and we removed this bias by calculating the yield using the dry weight [24].

2.2.2. Hydrodistillation

Before hydrodistillation, 200 g of fruit peel material was blended with distilled water for one minute using a blender (Blender 1300 W, Magimix®, Paris, France).

The samples were introduced to a 2 L wide-neck flask reaction (QFR2LF, Quickfit®, Fisher Scientific, Pittsburgh, PA, USA) with a final volume of one liter (sample and distilled water) and heated for two and a half hours using a heating mantle (EM2000/CE, Electrothermal®, Vernon Hills, IL, USA). The essential oil was collected using a classical Clevenger apparatus. The Clevenger apparatus was cooled using a refrigerated fluid (mix of glycol–water) cooled at 4°C and moved by a Minichiller® (C20, Huber®, Freiburg, Germany).

Then, the essential oils were put in overfull 300 μL tainted vials and stored at −20 °C before further analysis. For sensorial analysis, the peel oil of all samples for each condition was mixed in equal proportions immediately after distillation, transferred to overfull 5 mL tainted vials, and stored at −20 °C.

2.2.3. Gas Chromatography (GC) Analysis

GC analyses were performed on a Perkin Elmer Clarus 500 gas chromatograph (FID, Perkin Elmer, Courtaboeuf, France) equipped with 2 fused silica gel capillary columns (50 m, 22 mm id, film thickness 0.25 μm), BP-1 (polydimethylsiloxane) and BP-20 (polyethylene glycol). The oven temperature was programmed from 60 to 220 °C at 2 °C/min and then held isothermally at 220 °C for 20 min, with an injector temperature of 250 °C, detector temperature of 250 °C, carrier gas hydrogen (1.0 mL/min), and split of 1/60. RIs were

determined relative to the retention times of a series of n-alkanes (C7-C28) with linear interpolation ('Target Compounds' software of PerkinElmer).

The quantitative proportions of the oil constituents were expressed in g per 100 g obtained by peak area normalization using response factors (RF) for each class of compounds as described in Bicchi et al. (2008) [31]. The internal reference compound used was nonane, and each oil sample was prepared with the following volumetric proportions: 1.00/11.75/487.25 (nonane, PEO, chloroform) (Sigma-Aldrich, Saint-Louis, MO, USA).

2.2.4. Gas Chromatography–Mass Spectrometry Analysis (GC–MS)

Gas chromatography was coupled with mass spectrometry (GC/MS); the EOs were analyzed with a PerkinElmer TurboMass detector (quadrupole, PerkinElmer, Courtaboeuf, France) directly coupled with a PerkinElmer Autosystem XL equipped with a fused silica gel capillary column (50 m, 0.22 mm id, film thickness 0.25 μm) (BP-1 polydimethylsiloxane). The analysis was performed with the following parameters: helium as the carrier gas at 0.8 mL/min; split of 1/75; injection volume, 0.5 μL; injector temperature of 250 °C; oven temperature programmed from 60 to 220 °C at 2 °C/min and then held isothermally (20 min); ion source temperature of 250 °C; energy ionization of 70 eV; electron ionization mass spectra were acquired over the mass range of 40–400 Da.

2.2.5. Identification of Components

The components were identified first by comparison of their GC retention indices (RIs) on polar and apolar columns, determined relative to the retention times of a series of n-alkanes with linear interpolation with those of authentic compounds and literature data. The components were also identified by computer matching against the National Institute of Standards and Technology (NIST) commercial mass spectral library and by comparison of spectra with literature data; for further details, we referred to Luro et al. (2019) [32].

2.2.6. Sensorial Analysis

Oil sensorial differences were tested using the triangle method. This method was adapted to detect small differences among a low number of samples. The panel was composed of thirteen panelists (familiar with sensorial analysis studies) aged between 24 and 56 with a mean age of 41.6 (6 men and 7 women). The three samples (two identical and one different) were presented in a randomized order and coded with three-digit numbers, making them unidentifiable over experiments by panelists. Panelists smelled each sample using a test strip and then pointed out the different samples. This experiment was conducted on the three possible combinations.

Sensorial differences between different combinations of ploidy levels were tested with the 'A not A' (with reminder) method. The sample with diploid scion and diploid rootstock (2X/2X) was considered the reference sample. The panel was composed of ten panelists (familiar with sensorial analysis studies) aged between 24 and 56 with a mean age of 42.7 (5 men and 5 women). The panelists had to compare the reference with unknown samples (2X/2X, 2X/4X, 4X/2X, 4X/4X) and tick if they perceived a difference or not. The samples were randomly presented.

2.3. Statistical Analysis

Statistical differences within rootstocks and within ploidy combinations were tested for each chemical compound and on the yield parameter using one-way analysis of variance (ANOVA) followed by Tukey's test using R software (v 4.0.3) [33] with the 'agricolae' package [34].

The overall chemical structure of the sweet orange grafted on the three rootstocks was represented by the proportion of thirteen main compounds and the nine main compounds of the 4 ploidy combinations using principal component analysis (PCA) with R packages 'FactoMineR' and 'factoextra' [35,36].

Sensorial differences within the peel oil of different rootstocks were statistically identified using the discrimination test using the R software 'sensR' package [37]. Sensorial differences within peel oil of separate ploidy combinations were statistically quantified using the AnotA function (equivalent to Fisher's exact test for the estimation of the *p* value) of the 'sensR' package.

3. Results

3.1. Influence of the Rootstock on Essential Oil Yield, Composition and Aromatic Profile

The mean yield of peel essential oil of 'Navelina' sweet orange varied significantly between trees grafted onto 'Carrizo' citrange (7.72 g/100 g of dry peel) and 'FLHORAG1' (5.50 g/100 g of dry peel). The trees grafted on trifoliate orange produced an intermediate quantity of PEO (6.85 g/100 g of dry peel) (Figure 1).
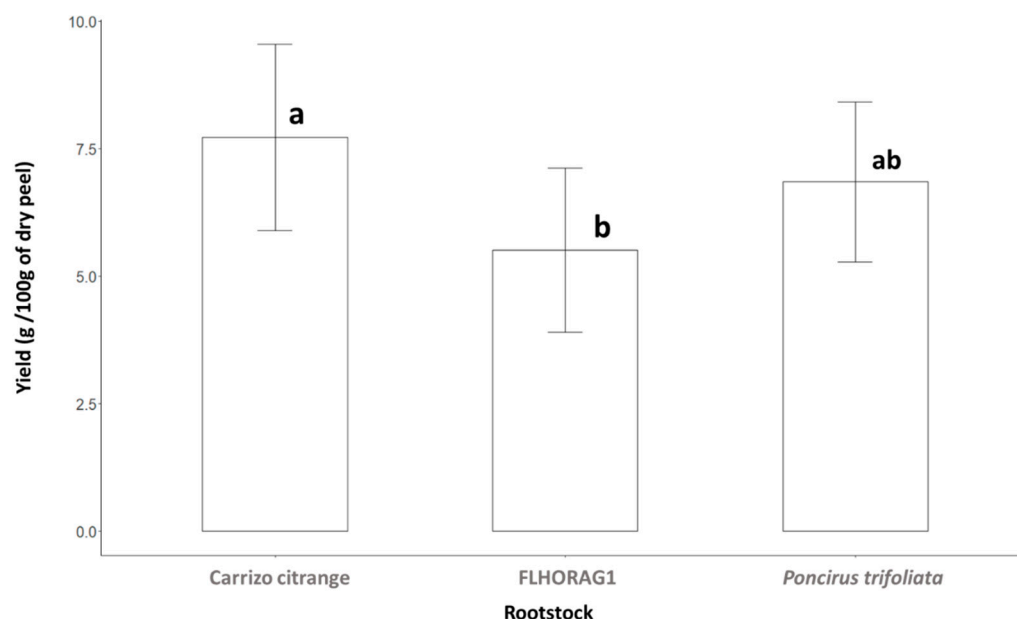
Figure 1. Bar plot representing the mean peel oil yield and the standard deviation for each rootstock. The letter on the top of the bar plot represents the statistical group of yield according to Tukey's test.

Twenty-eight compounds were identified, accounting for 100% of the total composition for each sample. The composition was almost exclusively composed of 21 monoterpenes, representing between 98.55 and 99.76 g/100 g of the total oil weight. The other class of compounds was aliphatic aldehydes (2-hexenal, octanal, nonanal, decanal), accounting together for 1.23 to 1.38 g/100 g of the essential oil. Limonene was the ultradominant compound, accounting for between 94.02 and 94.37 g/100 g′ followed by myrcene at 2.09–2.14 g/100 g. The 26 remaining compounds accounted for 4 g/100 g of essential oil. One aliphatic alcohol (nonan-1-ol), one sesquiterpene (β-elemene) and one diterpene (geranyl α-terpinene) were identified at trace concentrations.

Among the twenty-eight identified compounds, seventeen were statistically significant in different proportions between the three rootstocks (Table 1).

Thirteen compounds were found in different proportions in 'FLHORAG1' and *Poncirus trifoliata*, and nine compounds for each of the other two combinations. Once the main compounds underwent principal component analysis, with an ellipse representing the barycenter of all samples for each group, the influence of the rootstock was clear (Figure 2). The ellipse of the samples corresponding to the Carrizo citrange rootstock on axis 2 is clearly different from those of the other two rootstocks, and this difference is mainly due to α-pinene, α -3-carene, linalool and neral. The difference in effect between 'Carrizo' citrange and *Poncirus trifoliata* is also visible on PCA axis 1, due to the major contribution of limonene, nonanal, decanal, octanal, α -terpineol, sabinene and geranial. However, the

low level of variance seen in the first two axes (lower than 48%) indicates a low diversity among samples. In addition, the distance between the three ellipses representing the three rootstocks is short, and the overlapping of two ellipses (*Poncirus trifoliata* and 'FLHORAG1') indicates that the overall composition between these two modalities is very close.

Using the triangle method with thirteen expert panelists, significant differences were identified between the aromatic profiles of the same sweet orange grafted on three different rootstocks (Figure 3). The discrimination test indicates significant differences between sensory profiles of 'FLHORAG1'/'Carrizo' citrange and 'Carrizo' citrange/*Poncirus trifoliata*. The EOs of the orange grafted on 'FLHORAG1' and on *Poncirus trifoliata* were considered to have the closest sensory profile. This result is in accordance with the overlap of ellipses on the PCA, suggesting that both compositions are similar.

Table 1. The mean concentration (g/100 g) of the seventeen compounds of sweet orange, which varied statistically with the rootstock.

Compound	Ria [1]	Rip [2]	ROOTSTOCK Carrizo Citrange Mean ± sd [3]	FLHORAG1 Mean ± sd	*P. trifoliata* Mean ± sd	Method [4]
2-hexenal	825	1225	0.02 ± 0.02 [ab]	0.02 ± 0.02 [a]	0.00 ± 0.01 [b]	RI, MS
α-thujene	922	1017	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	0.05 ± 0.03 [a]	RI, MS
α-pinene	930	1017	0.48 ± 0.01 [a]	0.47 ± 0.01 [ab]	0.47 ± 0.02 [b]	RI, MS
sabinene	965	1125	0.41 ± 0.10 [a]	0.29 ± 0.07 [b]	0.17 ± 0.07 [c]	RI, MS
β-pinene	970	1114	0.01 ± 0.02 [a]	0.00 ± 0.01 [a]	0.01 ± 0.02 [a]	RI, MS
myrcene *	980	1163	2.13 ± 0.06 [ab]	2.14 ± 0.04 [a]	2.09 ± 0.06 [b]	RI, MS
octanal *	980	1294	0.64 ± 0.15 [a]	0.59 ± 0.10 [a]	0.62 ± 0.14 [a]	RI, MS
δ-3-carene	1005	1150	0.29 ± 0.09 [a]	0.25 ± 0.09 [a]	0.23 ± 0.04 [a]	RI, MS
p-cymene	1011	1275	0.04 ± 0.03 [a]	0.00 ± 0.00 [b]	0.05 ± 0.05 [a]	RI, MS
limonene *	1023	1208	94.02 ± 0.41 [b]	94.29 ± 0.37 [ab]	94.37 ± 0.39 [a]	RI, MS
β-phellandrene *	1023	1215	0.21 ± 0.01 [b]	0.23 ± 0.01 [a]	0.19 ± 0.02 [c]	RI, MS
(E)-β-ocimene	1036	1253	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	RI, MS
γ-terpinene	1048	1248	0.00 ± 0.01 [a]	0.00 ± 0.01 [a]	0.02 ± 0.03 [a]	RI, MS
trans sabinene hydrate	1055	1467	0.01 ± 0.02 [a]	0.01 ± 0.01 [ab]	0.00 ± 0.00 [b]	RI, MS
terpinolene	1078	1286	0.01 ± 0.02 [a]	0.01 ± 0.01 [a]	0.00 ± 0.01 [a]	RI, MS
nonanal	1082	1397	0.09 ± 0.01 [a]	0.09 ± 0.01 [a]	0.08 ± 0.02 [a]	RI, MS
linalool	1083	1549	0.49 ± 0.06 [b]	0.60 ± 0.07 [a]	0.53 ± 0.07 [b]	RI, MS
cis-limonene-1,2-epoxide	1117	1450	0.03 ± 0.04 [b]	0.00 ± 0.00 [b]	0.07 ± 0.07 [a]	RI, MS
trans-limonene-1,2-epoxide	1121	1462	0.13 ± 0.05 [b]	0.00 ± 0.01 [c]	0.24 ± 0.13 [a]	RI, MS
citronellal	1131	1492	0.00 ± 0.02 [a]	0.00 ± 0.01 [a]	0.00 ± 0.00 [a]	RI, MS
nonan-1-ol	1155	1301	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.01 ± 0.02 [a]	RI
terpinen-4-ol	1162	1604	0.02 ± 0.03 [a]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	RI, MS
α-terpineol	1173	1698	0.11 ± 0.02 [a]	0.10 ± 0.04 [a]	0.10 ± 0.02 [a]	RI, MS
decanal	1184	1501	0.58 ± 0.09 [b]	0.68 ± 0.12 [a]	0.53 ± 0.08 [b]	RI, MS
neral	1216	1685	0.12 ± 0.05 [a]	0.04 ± 0.04 [b]	0.08 ± 0.06 [a]	RI, MS
geranial	1243	1735	0.13 ± 0.06 [a]	0.10 ± 0.04 [a]	0.10 ± 0.03 [a]	RI, MS
β-elemene	1386	1590	0.02 ± 0.03 [ab]	0.04 ± 0.04 [a]	0.01 ± 0.02 [b]	RI, MS
geranyl α-terpinene	1941	2219	0.00 ± 0.00 [b]	0.03 ± 0.03 [a]	0.00 ± 0.00 [b]	RI
Total			100.00	100.00	100.00	
Olefins			97.62 [a]	97.77 [a]	97.65 [a]	
Oxygenated			2.38 [a]	2.23 [a]	2.35 [a]	
Aliphatic aldehydes			1.33 [a]	1.38 [a]	1.23 [a]	

[1] Retention index calculated on apolar column, [2] Retention index calculated on polar column, [3] Alphabetic letters correspond to statistical group according to Tukey's test, [4] Identification based on retention index of standards (RI) or mass spectrum (MS), * The quantification was made using the polar column.
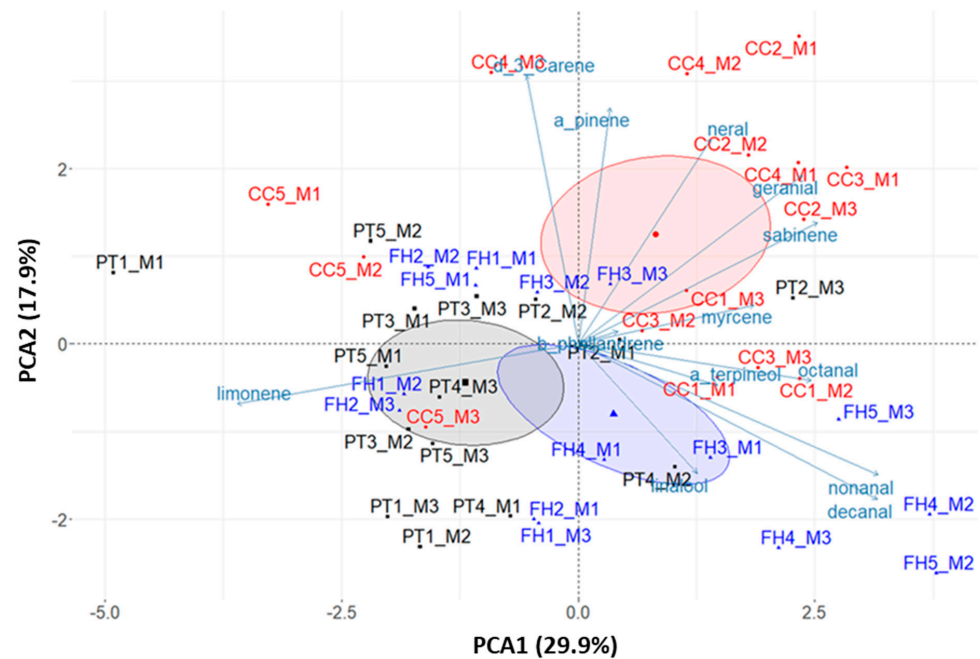
Figure 2. Principal component analysis based on the thirteen main compounds of the peel essential oil. Samples (3 replicates of) in red, blue and black correspond to sweet orange grafted on 'Carrizo' citrange (CC), 'FLHORAG1' (FH) and *Poncirus trifoliata*, (PT), respectively. The acronyms of each rootstock are followed by a number corresponding to the 5 trees and by M1, M2 or M3, corresponding to the 3 replicates. The ellipses represent the position of the gravity center of each group with a 0.95 probability. Gray arrows indicate the contribution of each compound to the two axes of the PCA.
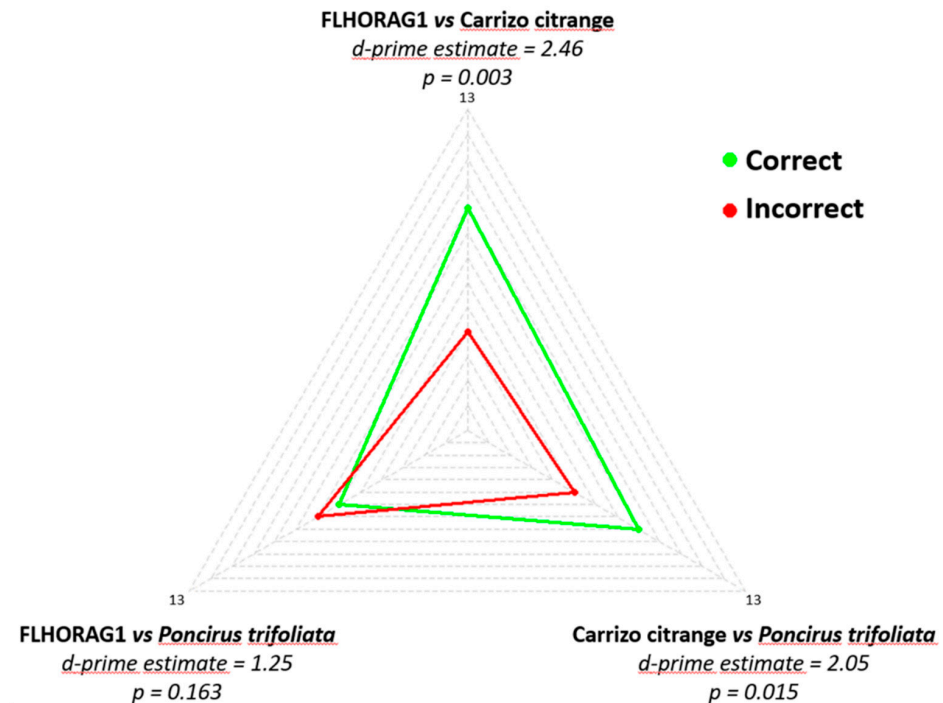
Figure 3. Radar graph summarizing the responses of the thirteen panelists to the triangle test. Correct answers (in green) indicate that the panelist chose the unique sample and not one of the duplications. An incorrect (in red) answer indicates that the panelist chose one of the duplications instead of the unique sample. The *p* value of the discrimination test is indicated under each comparison, and the d-prime estimate indicates the level of difference within samples according to signal detection theory.

3.2. Influence of Ploidy Level on Essential Oil Yield, Composition and Aromatic Profile

The mean peel essential oil yield of the 'Pineapple' sweet orange cultivar was 8.45, 8.63, 9.16 and 8.80 g/100 g of dry peel for 2X/2X, 2X/4X, 4X/2X and 4X/4X, respectively. In these four ploidy conditions, the mean was considered equivalent every time according to Tukey's test.

Twenty-five compounds were identified, accounting for 100% of the total composition for each sample (Table 2). The composition was almost exclusively composed of monoterpenes (19), representing between 99.34 and 99.68 g/100 g. Limonene was the predominant compound, accounting for 93.94 to 95.92 g/100 g, followed by myrcene (1.83–2.04 g/100 g), linalool (0.47–1.07 g/100 g), sabinene (0.31–0.95 g/100 g) and α-pinene (0.40–0.58 g/100 g). The 14 remaining compounds accounted for approximately 1 g/100 g. The other class of compounds was aliphatic aldehydes (hexanal, 2-hexenal, octanal, nonanal, decanal) varying between 0.21 and 0.55 g/100 g. The third and last class of compounds representing the sesquiterpenes was represented by only one compound, and the valencene varied between 0.00 and 0.21 g/100 g.

Table 2. The mean concentration (g/100 g) of 25 compounds of sweet orange for each ploidy rootstock–scion combination.

| | | | PLOIDY OF SCION/ROOTSTOCK | | | | |
| | | | 2X/2X | 2X/4X | 4X/2X | 4X/4X | |
Compound	Ria [1]	Rip [2]	Mean ± sd [3]	Mean ± sd	Mean ± sd	Mean ± sd	Method [4]
hexanal	776	1087	0.00 ± 0.00 [a]	0.01 ± 0.02 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	RI, MS
2-hexenal	828	1225	0.01 ± 0.01 [a]	0.03 ± 0.03 [a]	0.03 ± 0.03 [a]	0.01 ± 0.01 [a]	RI, MS
α-thujene	922	1017	0.01 ± 0.02 [a]	0.02 ± 0.04 [a]	0.00 ± 0.00 [a]	0.02 ± 0.04 [a]	RI, MS
α-pinene	930	1017	0.51 ± 0.07 [a]	0.53 ± 0.01 [a]	0.51 ± 0.02 [a]	0.51 ± 0.01 [a]	RI, MS
sabinene	965	1125	0.73 ± 0.19 [a]	0.65 ± 0.16 [ab]	0.46 ± 0.04 [bc]	0.40 ± 0.13 [c]	RI, MS
β-pinene	970	1114	0.03 ± 0.03 [a]	0.02 ± 0.02 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	RI, MS
myrcene *	980	1163	1.94 ± 0.07 [a]	1.96 ± 0.07 [a]	1.96 ± 0.05 [a]	1.94 ± 0.06 [a]	RI, MS
octanal *	980	1294	0.15 ± 0.03 [a]	0.17 ± 0.05 [a]	0.10 ± 0.05 [a]	0.13 ± 0.06 [a]	RI, MS
δ-3-carene	1005	1150	0.09 ± 0.07 [a]	0.09 ± 0.03 [a]	0.04 ± 0.05 [a]	0.02 ± 0.03 [a]	RI, MS
p-cymene	1011	1275	0.10 ± 0.10 [a]	0.07 ± 0.12 [a]	0.02 ± 0.04 [a]	0.03 ± 0.05 [a]	RI, MS
limonene *	1023	1208	94.49 ± 0.48 [a]	94.6 ± 0.29 [a]	95.77 ± 0.10 [b]	95.63 ± 0.29 [b]	RI, MS
β-phellandrene *	1023	1215	0.21 ± 0.02 [a]	0.22 ± 0.02 [a]	0.22 ± 0.01 [a]	0.21 ± 0.02 [a]	RI, MS
γ-terpinene	1048	1248	0.05 ± 0.06 [a]	0.03 ± 0.05 [a]	0.02 ± 0.04 [a]	0.05 ± 0.07 [a]	RI, MS
nonanal	1082	1397	0.01 ± 0.02 [a]	0.01 ± 0.01 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	RI, MS
linalool	1083	1549	0.87 ± 0.13 [a]	0.89 ± 0.10 [a]	0.63 ± 0.13 [b]	0.70 ± 0.09 [ab]	RI, MS
cis-limonene-1,2-epoxide	1117	1450	0.07 ± 0.07 [a]	0.07 ± 0.11 [a]	0.00 ± 0.00 [a]	0.03 ± 0.07 [a]	RI, MS
trans-limonene-1,2-epoxide	1121	1462	0.01 ± 0.03 [a]	0.02 ± 0.05 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	RI, MS
citronellal	1131	1492	0.01 ± 0.02 [a]	0.01 ± 0.02 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	RI, MS
terpinen-4-ol	1162	1604	0.06 ± 0.06 [a]	0.04 ± 0.04 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	RI, MS
α-terpineol	1173	1698	0.07 ± 0.04 [a]	0.05 ± 0.04 [ab]	0.00 ± 0.01 [b]	0.01 ± 0.02 [b]	RI, MS
decanal	1184	1501	0.27 ± 0.03 [a]	0.26 ± 0.04 [a]	0.17 ± 0.03 [b]	0.19 ± 0.02 [b]	RI, MS
trans-carveol	1198	1836	0.00 ± 0.00 [a]	0.00 ± 0.01 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	RI, MS
neral	1216	1685	0.07 ± 0.06 [a]	0.05 ± 0.07 [a]	0.01 ± 0.01 [a]	0.02 ± 0.04 [a]	RI, MS
geranial	1243	1735	0.09 ± 0.02 [a]	0.09 ± 0.01 [a]	0.00 ± 0.01 [b]	0.02 ± 0.03 [b]	RI, MS
valencene	1486	1716	0.15 ± 0.04 [a]	0.11 ± 0.04 [a]	0.08 ± 0.05 [a]	0.07 ± 0.04 [a]	RI, MS
Total			100.00	100.00	100.00	100.00	
Olefins			98.31 [a]	98.30 [a]	99.06 [b]	98.89 [b]	
Oxygenated			1.69 [a]	1.70 [a]	0.94 [b]	1.11 [b]	
Aliphatic aldehydes			0.44 [a]	0.47 [a]	0.30 [c]	0.33 [bc]	

[1] Retention index calculated on apolar column, [2] Retention index calculated on polar column, [3] alphabetic letters correspond to statistical group according to Tukey's test, [4] Identification based on retention index of standards (RI) or mass spectrum (MS), * the quantification was made using the polar column.

It appears that the ploidy level of the rootstock has no influence on the peel oil composition of scion-produced fruit. Furthermore, the ploidy level of the scion has a strong influence on the essential oil composition. (Table 2 and Figure 4). Significant differences

according to Tukey's test were identified for five compounds (sabinene, limonene, linalool, α-terpineol, decanal and geranial) between the diploid and tetraploid scions (Table 2). It seems that the diploid scion produced slightly more oxygenated compounds than its tetraploid homologous scion, whereas tetraploid scions tended to produce more limonene (Figure 4 and Table 2).
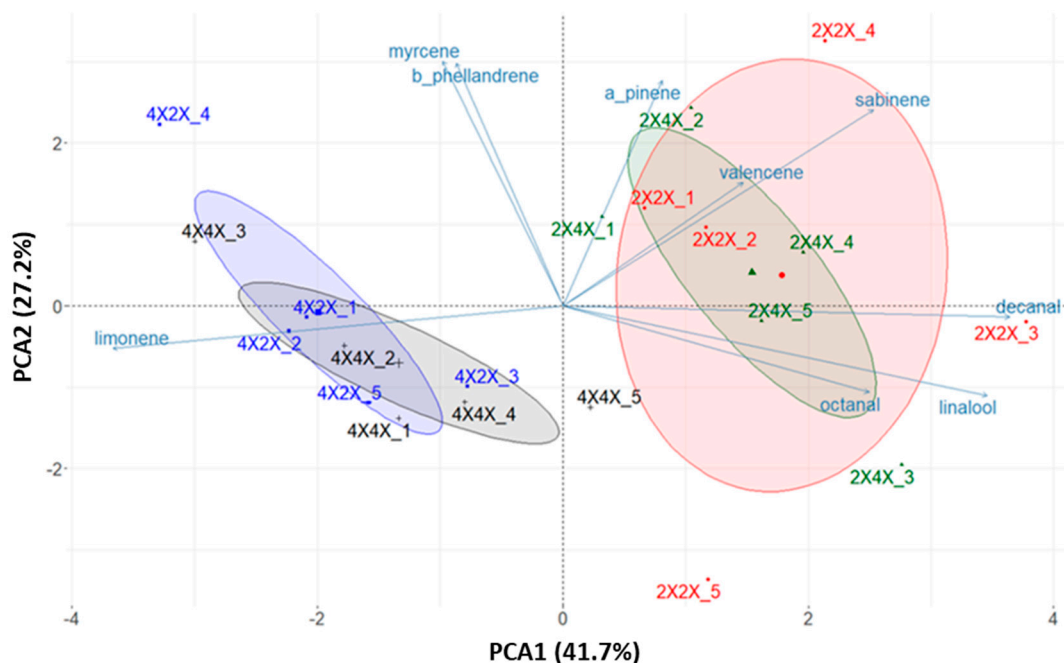
Figure 4. Biplot of a principal component analysis based on the nine main compounds of the fruit peel essential oil of 'Pineapple' sweet orange. Samples (five replicates for each combination) in red, green, blue and black correspond to 2X/2X, 2X/4X, 4X/2X and 4X/4X combinations, respectively. The ellipses represent the position of the gravity center of each group with a 0.95 probability. Gray arrows indicate the contribution of each compound to the two axes of the PCA.

As an initial control, 80% of the answers of the panelists were successful in the comparison of the reference sample (2X/2X) with itself. This rate indicates that our set was reliable (Table 3). The maximum identified difference from the reference was found for the 4X/4X sample, with all the panelists considering those two samples to be different. Nine out of ten considered 2X/4X to be different from the reference. The 4X/2X sample was identified as the least different by panelists with a split decision, and only five out of ten considered it to be different. The *p* value from the 'A not A' test (one-tailed Fisher's exact test) was relevant for the comparison of 2X/4X (0.003) with 4X/2X (0.175). In terms of sensory magnitude (confusability of the product with the reference) according to the Thurstonian model, the d-prime values were 2.12 and 0.84 for 2X/4X and 4X/2X, respectively.

Table 3. Response to 'A not A' test (with reminder). The reference sample (2X/2X) was compared to the three other ploidy combinations and itself by the ten panelists. Fisher's exact test was used for the estimation of the *p* value.

Reference	Test	Ploidy of Scion/Rootstock			
		2X/2X	**2X/4X**	**4X/2X**	**4X/4X**
2X/2X	Identical	8	1	5	0
	Different	2	9	5	10
	p-value		0.003	0.175	0

4. Discussion

The PEO composition of all our samples was consistent with that described in the literature [38]. We identified significant differences in the orange PEO yield and composition when it was grafted onto different rootstocks. This result is in accordance with previous studies conducted by Bitters and Scora (1970) on 'Valencia' sweet orange and Zouaghi et al. (2019) on 'Maltaise demi sanguine' sweet orange [8,12]. However, Verzera et al. (2003) and Pedruzzi et al. (2004) concluded that rootstock had little or no effect on the PEO composition of bergamot and mandarin, respectively [9,10]. Darjazi et al. (2011) proposed that the influence of the rootstock on the yield and composition of PEO was related to differences in water and mineral absorption by the rootstock, permitting higher photosynthesis activity by the scion [14]. Volatile compound precursors come from products of photosynthesis, so an increase in precursors may increase the production of these compounds [39,40]. The quantitative variation observed in PEO composition was small but perceptible by expert panelists, and the difference in sensory profile could be explained by these variations in the composition. We were unable to perform quantitative sensory analysis because variations within samples were too low to permit this kind of analysis. Concerning the variation observed in the PEO yield, it would be interesting to conduct studies on whole-tree fruit yield and peel essential oil yield (in dry weight) to determine which rootstocks are the most suitable for peel oil production. Indeed, it has been demonstrated that rootstock can significantly influence fruit yield per tree [2,41].

The variation in ploidy levels of the scion and rootstock has no influence on the PEO yield. This result was not expected because doubled diploids have been shown to produce fruit with thicker rinds and larger oil glands but are less dense than diploids [27]. An experiment conducted on clementine showed that autotetraploid *Poncirus trifoliata* rootstocks tended to reduce fruit yield, thus certainly reducing PEO yield per tree [28]. Considering their dwarfing effect, which facilitates cultural practices and fruit harvest, their adoption requires higher-density plantations than those currently used with diploid rootstocks to maintain a good PEO yield by hectare [18,19]. Allotetraploid hybrids obtained by somatic hybridization as well as some doubled-diploid rootstocks of interspecific origin do not display such vigor reduction when compared to their diploid parents and are very promising in their potential to tackle the challenge of increasing biotic and abiotic constraints [18,20]. Our results show that the adoption of allotetraploid rootstocks, such as 'FLHORAG1', for PEO production should not greatly affect the PEO yield and composition relative to dry peel weight. Autotetraploid scions are known to be less vigorous and tend to produce fewer fruits per tree [42]. Our results indicate that tetraploid scions have little promise for PEO yield improvement.

The ploidy of the scion influences the composition of PEO but not the ploidy of the rootstock. Differences between diploid and tetraploid sweet oranges mainly manifested in higher amounts of aliphatic aldehydes and oxygenated monoterpenes for diploid scions counterbalanced by a higher amount of limonene in tetraploid scions. These differences could be explained by phenomena such as locus silencing reducing transcriptome activity in polyploids [43]. This phenomenon of transcriptome adulteration induced by autotetraploidization has been observed in *Arabidopsis thaliana* and *Citrus limonia* Osbeck [44,45].

The chemical profiles of sweet oranges differed in the two experiments. Six compounds from the first experiment were not detected in the second: (E)-β-ocimene, trans sabinene hydrate, terpinolene, nonan-1-ol, β-elemene and geranyl α-terpinene. Three compounds were specific to the second experiment: hexanal, trans-carveol and valencene. With the exception of valencene, none of these compounds exceeded 0.05%, and most often they achieved a value less than 0.01%. Valencene is an indicator of advanced maturity in sweet orange, thus the differences could likely be explained by the two-month difference in the sampling date between the two experiments [46]. The cultivar effect could also be a factor of variation as well as the detection threshold, mainly for the compounds with very low proportions (below 0.01%) [47].

Expert panelists perceived significant sensorial differences between the reference sample (2X/2X) and the three other combinations. However, we were unable to link a difference in aromatic profile with a variation in composition even if significant differences in composition (limonene and oxygenated compounds) were quantifiable. It is interesting to note that significant reductions in oxygenated compounds for tetraploid scions do not deeply affect the aromatic sensorial profile, whereas these compounds are known to be fundamental components in the olfactive properties of the oil [48].

Interestingly, the panelists judged that the aromatic profiles of sweet orange grafted on 'FLHORAG1' and *Poncirus trifoliata* were similar (*p* value = 0.163). Even if the 13 compounds had significantly different concentrations, the overall chemical profile was somewhat similar according to the overlapping ellipses of both combinations, which may explain the conclusion of the panelist.

It is also worth noting that no significant difference was identified between the sensory profiles of 2X/2X and 4X/2X. Considering that the ellipses of both combinations are clearly separated on the principal component analysis, these two results seem opposite. However, the panelists were split between these two samples, as demonstrated by a low *p* value (0.175) for the 'A not A' test, which could be explained by the fact that even if these two samples are distinct, they are still close enough to be mistakable. Although a significant difference was detected in the proportion of aliphatic aldehydes between 2X/2X and 4X/2X, it is possible that some of these differences are neutral for the overall profile of sweet orange PEO [48,49]. In addition, the major citrus volatiles were not major influences of the citrus flavor. These compounds, possibly under our gas chromatography detection threshold, could be responsible for the differences perceived by panelists and GC analysis [50]. This result suggests that aromatic profiling must be conducted by sensorial analysis rather than composition analysis.

5. Conclusions

In this study, the influence of three different rootstocks and the level of ploidy of the scion and the rootstock on the yield, composition and aromatic profile of sweet orange peel essential oil were studied. The rootstock influenced the yield of PEO, whereas the level of ploidy of the rootstock and the scionseemed to have no influence. The peel essential oil composition was significantly influenced by the rootstock genotype but not by its ploidy level. Contrary to the rootstock, the ploidy level of the scion influenced the composition of the peel essential oil, reducing the proportions of oxygenated compounds in autotetraploid sweet orange. The genotype of the rootstock and the level of ploidy of both scion and rootstock lightly modified the aromatic profile, but these differences were fairly insignificant. Considering these results, it would be interesting to perform multisite experiments (with soils of various natures) with rootstocks from different genetic origins and different ploidy levels over a couple of years. Thus, it is important to determine which rootstock is the most suitable for peel essential oil production and to profile the PEO according to the cultivation environment, including the rootstock.

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