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Metabolic Profiling of *Varronia curassavica* Jacq. Terpenoids by Flow Modulated Two-Dimensional Gas Chromatography Coupled to Mass Spectrometry

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Abstract: In this study, a metabolomic approach was used to investigate the effect of seasonality on the chemical composition and yield of anti-inflammatory active principle, α-humulene, in the essential oil of three genotypes of *Varronia curassavica* Jacq. (Syn. *Cordia verbenaceae*). The essential oils were extracted by hydrodistillation and analyzed by comprehensive two-dimensional gas chromatography coupled to mass spectrometry (GC×GC-MS). The GC×GC approach a three-fold improvement in qualitative analysis (48 compounds were identified by GC-MS versus 135 by GC×GC-MS). The improved resolving power of GC×GC resolved important coelutions and enabled the detection of unusual substances in *V. curassavica* essential oil. The chromatographic data was analyzed by using peak table-based chemometrics, namely, principal component analysis (PCA) and hierarchical cluster analysis (HCA). The metabolic study showed that seasonality has a significant effect on the chemical composition. The α-humulene content was affected by genotype and season. Spring and summer were the best harvest seasons for the yield of the active ingredient, found in higher concentrations in the VC2 genotype. The proposed metabolomic workflow was successfully applied to terpene analysis found in *V. curassavica* essential oil, and such results have broadened our understanding of the influence of seasonal factors on the specialized metabolism of the species.

Keywords: *Varronia curassavica*; essential oil; seasonality; gas chromatography (GC); comprehensive two-dimensional gas chromatography (GC×GC); flow-modulated

1. Introduction

Essential oils (EOs) are sources of biologically active compounds that can be applied in food, agronomic, cosmetic, and pharmaceutical industries for the elaboration of perfumes, cosmetics, pharmaceuticals, and used as adjuvant in the formulation of medicines. An important example of this application is the essential oil of *Varronia curassavica* Jacq. (syn. *Cordia verbenacea*)—a Brazilian native species, which is mainly found in the Atlantic Rainforest biome, popularly known as 'erva-baleeira' [1].

V. curassavica is a medicinal and aromatic plant from the Boraginaceae family with significant economic importance. The EO produced from its leaves is used in the formulation of the topic anti-inflammatory medicine *Acheflan*®, a phytotherapeutic product fully developed with Brazilian technology and currently exported to Japan, Chile, Mexico, Costa Rica, Equator, Peru, the United States, and Canada [2,3]. In Brazil, the product is the leading prescription drug for chronic tendonitis and muscle pain, being responsible for more than 25% of the market sales [3]. The active principle responsible for anti-inflammatory activity of the EO is the sesquiterpene α-humulene [4–6]. The substance α-humulene is considered a chemical marker in the pharmaceutical industry for the quality control of *V. curassavica* essential oil. Besides its anti-inflammatory activity, the species has been demonstrated to

have other medicinal properties, such as anti-allergenic, antiulcerogenic, antioxidant, antitumor [5,7–11], antibacterial, and antifungal [12,13].

The world's growing demand for natural products, including essential oils, especially from the pharmaceutical industry, depends on standardizing and elucidating the chemical profile of essential oils safely and effectively. Along with the genetic make-up, environmental factors, including seasonality can coordinate or modify the production rate and composition of essential oil [14–18], influencing EO effectiveness, safety, and stability.

Gas chromatography coupled to mass spectrometry (GC–MS) is the most frequently used hyphenated separation technique for the characterization and identification of volatile organic compounds found in such oils [19–22]. However, the complex nature of the samples and the existence of coelutions are a challenge for qualitative and quantitative analysis [23,24]. Even with high-resolution GC using narrow bore capillary columns, the separation of complex mixtures such as essential oils is still incomplete, with a high number of overlapping chromatographic peaks. [25,26].

In this context, comprehensive two-dimensional gas chromatography (GC×GC) is a well-established technique for the resolution of complex mixtures of volatile organic compounds. The enhanced resolving power of GC×GC is attained by coupling two capillary columns interfaced by the modulator [27–31]. Among the available modulators, the cryogenic modulator is currently the most popular solution [32]. Flow modulators are an interesting alternative for GC×GC experiments, since their separation efficiency may be equivalent to those obtained by thermal modulators [32,33]. For instance, Cordero et al. [34] evaluated the potential of GC×GC with differential flow modulator in medium and high complexity essential oil samples. Mint, lavender and vetiver essential oils were used for this evaluation. The authors reported increased peak capacity attained by differential flow modulation for the analysis of EOs. Accordingly, the benefits of GC×GC have led to an ever-increasing application of the composite technique for the separation and identification of volatile organic compounds in many other fields [35–40], including metabolomics [41–43].

Metabolic profiling of plants is informative to gain an insight into the potential of natural products for commercial use. For instance, the composition of EO is impacted by important factors like geography (soil, altitude, and humidity), seasonality (temperature and light conditions), and genotype. Hence, the chemical composition must be somewhat constant and predictable for quality assurance of the final product for pharmaceutical purposes. All reports characterized individual samples of *V. curassavica* using 1D-GC-based methods [44,45]. The seasonal effect of essential oil composition was explored using GC-MS [46–48], but without taking into consideration the contribution of plant genotype [48,49]. Marques et al. [48] previously evaluated the seasonal variation of EO from *V. curassavica* Jacq. accessions for an entire year. However, the authors could not factor into the investigation the accurate contribution of the multiple genotypes, because the biological material was harvested from native plants found in different geographical locations. Furthermore, careful inspection of the 1D-GC chromatograms seemed to indicate significant coelutions, which may have omitted important information regarding EO composition. In this work, we investigated *V. curassavica* plants with three different genotypes commercially available. All plants were cultivated in the same green house to eliminate the interference of geographical conditions. In addition, a GC × GC-MS-based metabolomic approach was used for *V. curassavica* EO characterization to improve the chemical analysis, especially for terpenes with overlapping peaks and low-intensity peaks. More specifically, flow modulated comprehensive two-dimensional gas chromatography coupled with mass spectrometry (FM-GC × GC-MS) and chemometric tools were combined in order to interpret the metabolic profile. These results improved our understanding of the influence of seasonal factors on the specialized metabolism of the species and are important to establish reliable data regarding *V. curassavica* crop behavior to fully explore the potential of this plant for commercial use. Lastly, we have confirmed the benefits of using GC × GC for EO analysis by highlighting previously overlapped peaks in 1D-GC, which were detected for the first time by GC × GC.

2. Material and Methods

2.1. Plant Material

Three genotypes of *V. curassavica* (VC1, VC2, and VC3) were evaluated, comprising a total of nine plants. The plants, obtained from commercial matrices and propagated by stables (VC1 and VC2) and tissue culture (VC3), were kept in 20-L plastic roofed pots at the Center of Plant Genetic Resources of the Agronomic Institute (IAC), located in the city of Campinas, State of São Paulo (22°52′ south latitude, 47°04′ west longitude, and 677 m altitude). The regional climate, according to the Köppen classification, is classified as Cwa—subtropical climate with hot summers and dry winters. The climatological data for the sampling period were obtained from the meteorological station of the Agrometric Information Center (Ciiagro) [50]. The aerial parts were harvested in winter (16 August 2018), spring (21 November 2018), summer (21 February 2019) and autumn (30 May 2019). The harvested leaves of *V. curassavica* were manually separated from the stems and dried at 40 °C in an air-circulation oven for 72 h.

2.2. Extraction of Essential Oil

Essential oils were extracted by hydrodistillation in a Clevenger apparatus for a period of two hours. The essential oil was stored in a freezer (−4 °C) for further characterization of the chemical composition.

2.3. Comprehensive Two-Dimensional Gas Chromatographic Analysis (GC×GC-FID/MS)

Analyses of the essential oils from *V. curassavica* were performed on a GC×GC system, which comprised of a TRACE 1300 GC equipped with a flame ionization detector (FID) and an ISQ single transmission quadrupole mass spectrometer (QMS) (Thermo Scientific—Waltham, MA, USA). Differential flow modulation using the reverse fill/flush configuration was attained using INSIGHT modulator (SepSolve Analytical—Frankfurt, Germany). Data digitalization using FID was attained at 120 Hz. A 40–450 m/z scanning range were used producing 31 scans s^{-1}. The transfer line and the ion source were operated at 300 °C and 220 °C, respectively. Helium was used as carrier gas and auxiliary gas at constant flow rates of 0.5 mL min^{-1} and 20.0 mL min^{-1}. The column set consisted of a primary 30 m × 0.25 mm-id × 0.25 µm (β of 250) MEGA-5HT and a secondary 5 m × 0.25 mm-id × 0.25 µm (β of 250) HP-50 + wall coated open tubular (WCOT) capillary columns (Agilent Technologies—Santa Clara, CA, USA). Deactivated fused silica capillaries were used as transfer lines for simultaneous MS (5 m × 0.18 mm-id) and FID (5 m × 0.32 mm-id) detection. This setup ensured a reproducible splitting of the ^{2}D effluent to FID (70%) and MS (30%). Sample introduction was performed using a split ratio of 1:20. The GC oven was programmed from 60 to 240 °C at 3 °C min^{-1}. Modulation period was set to 5.0 s with a re-injection (flush) pulse of 100 ms. The chromatographic analyses were performed in triplicate.

Xcalibur software (Thermo Scientific-Waltham, MA, USA) was used for data acquisition and processing was performed using GC Image software (Zoex-Houston, TX, USA). Identification of the terpenes was performed comparing the substance mass spectra with NIST database and filtered by the retention index (LTPRI) using the NIST WebBook and published literature [51]. Retention indices were determined by the injection of a homologous series of C_8-C_{20} n-alkanes (04070-1ML) (Merck-St. Louis, MO, USA) using the Van den Dool and Kratz equation [52]. The analytes were tentative identified through comparative analysis of mass spectra considering a minimum similarity correspondence of 80% and a deviation of ±25 LTPRI from the NIST and Adams [51].

2.4. Data Analysis

Analysis of variance (ANOVA) followed by Tukey test ($p < 0.05$) was applied to verify the differences between essential oil yields and α-humulene contents. The results of the chemical analyses were submitted to multivariate statistical analyses, namely, principal components analysis (PCA), and hierarchical clustering analysis (HCA). Such models were built using the software XLSTAT—2020

version (Addinsoft–Bordeaux, France). Heatmap were constructed using the on-line tool MetaboAnalist 4.0 [53].

3. Results and Discussions

3.1. Essential Oil Content

The average yield of the essential oils of the three genotypes harvested during the winter, spring, summer, and autumn seasons is shown in Table 1. The yield of essential oils varied from 0.22 to 0.60% (*v*/*w*). The yields are in agreement with those reported in other studies, which showed values ranging from 0.16 to 2.74%. [46–48].

Table 1. Average yield (%) of the essential oil of the three genotypes of *Varronia curassavica* extracted by hydrodistillation. Distinct uppercase letters (A, B and AB) for the rows and lowercase (a) for columns represent statistically significant differences ($p < 0.05$).

Genotype	Season			
	Winter ^{B}	Spring ^{A}	Summer ^{AB}	Autumn ^{AB}
VC1 ^{a}	0.22 ± 0.04	0.42 ± 0.04	0.32 ± 0.08	0.41 ± 0.03
VC2 ^{a}	0.36 ± 0.06	0.60 ± 0.03	0.46 ± 0.02	0.52 ± 0.02
VC3 ^{a}	0.29 ± 0.04	0.58 ± 0.02	0.45 ± 0.03	0.44 ± 0.04

The analysis of variance (ANOVA) showed that there was a significant difference in the yield of essential oils ($p < 0.05$) depending on the season. Spring was the season with the highest production (0.533%) and winter the season with the lowest production (0.291%). Between summer and autumn there was no significant difference (0.407 and 0.397%, respectively). The VC2 genotype showed the highest production of essential oil (0.60 ± 0.03%) in spring, a period that coincides with the flowering of the plant. Although this species blooms at any time of the year, it flowers with higher intensity during spring.

The correlation of the results presented in Table 1 with the meteorological data (Figure 1) allows us to ascertain that the climatic conditions also constitute a strong factor. The rainfall exhibited a significant variation, since the accumulated rainfall indexes that varied from 75 to 484 mm. Matias and collaborators [46] also observed the influence of rainfall on the production of *V. curassavica* essential oil, which corroborates our findings.

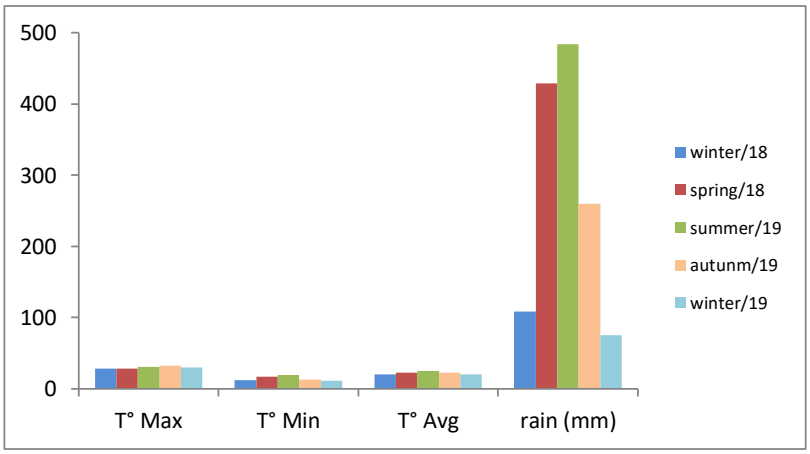

Figure 1. Meteorological data for the period of four seasons sampling. T° Max—Maximum temperatures from July 2018 to July 2019; T° Min—low temperatures from July 2018 to July 2019; T° Avg—average temperature from July 2018 to July 2019; rain (mm)—pluviometric precipitation in the period July 2018 to July 2018. Fonte: CIIAGRO [50].

3.2. Characterization of the Essential Oil

The chemical compositions of the EOs of the three genotypes of *V. curassavica*, harvested in four different seasons, were analyzed by GC-MS and GC×GC-MS, as shown in Figure 2. Many substances that were not apparent in 1D-GC, due to overlapping peaks or because they are found in low intensities, are clearly revealed in the GC×GC chromatogram. An example of peaks that were co-eluted in the first dimension (^{1}D) and were resolved in the second dimension (^{2}D), as shown in Figure 3. The composite system resolved the coelution between germacrene D, (*E*)-β-ionone and *tau*-elemene (Figure 3A) and between δ-cadinene, cadin-1(2),4-diene, and two unidentified compounds (Figure 3B).

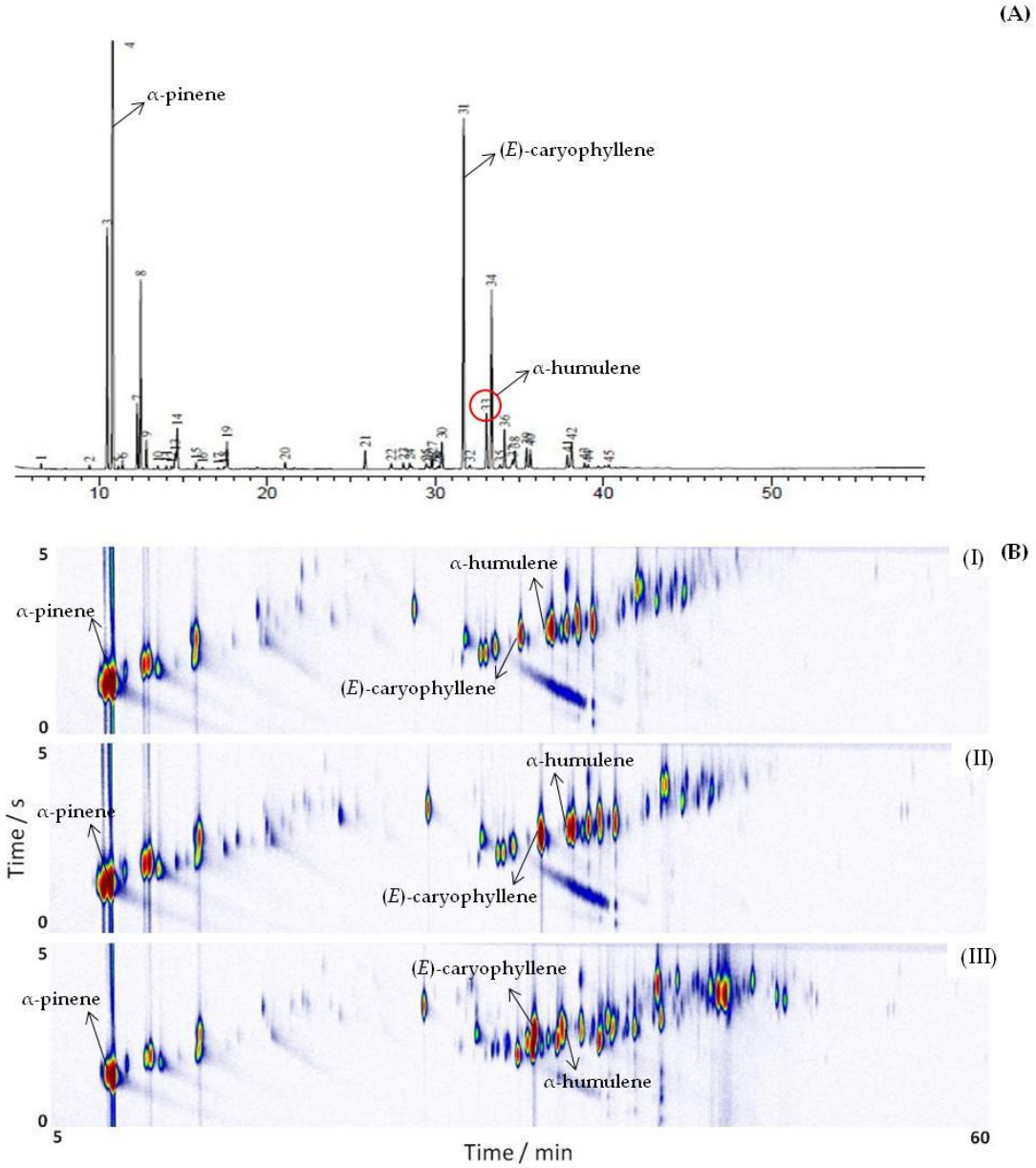

Figure 2. GC-MS chromatograms (**A**) and GC×GC-MS chromatograms (**B**) of the essential oil from three sample of *V. curassavica* (**I**) genotype VC1; (**II**) genotype VC2; (**III**) genotype VC3.

The GC×GC analysis enabled the tentative identification of 135 peaks compared to 48 peaks with GC-MS resulting in a three-fold improvement in qualitative analysis. Previous reports corroborated this improved feature for qualitative analysis [37,38,40,54]. For instance, Santos et al. [24] evaluated the essential oil of two species of Piperaceas (*Manekia obtusa* and *Piper cubataonum*). In *M. obtuse* oil, 80 compounds were identified by GC×GC-MS, while 22 only were assigned by GC-MS. For the leaf

and branch oils of *P. cubataonum*, 57 and 66 compounds were identified by GC×GC compared with only 14 and 20 compounds by GC/MS, respectively.

It is important to note that although 168 compounds were detected in the GC×GC analysis, only 135 (~80%) were tentatively identified. The presence of very similar mass spectra imposed restraints to fully elucidate the compounds present in the EO.

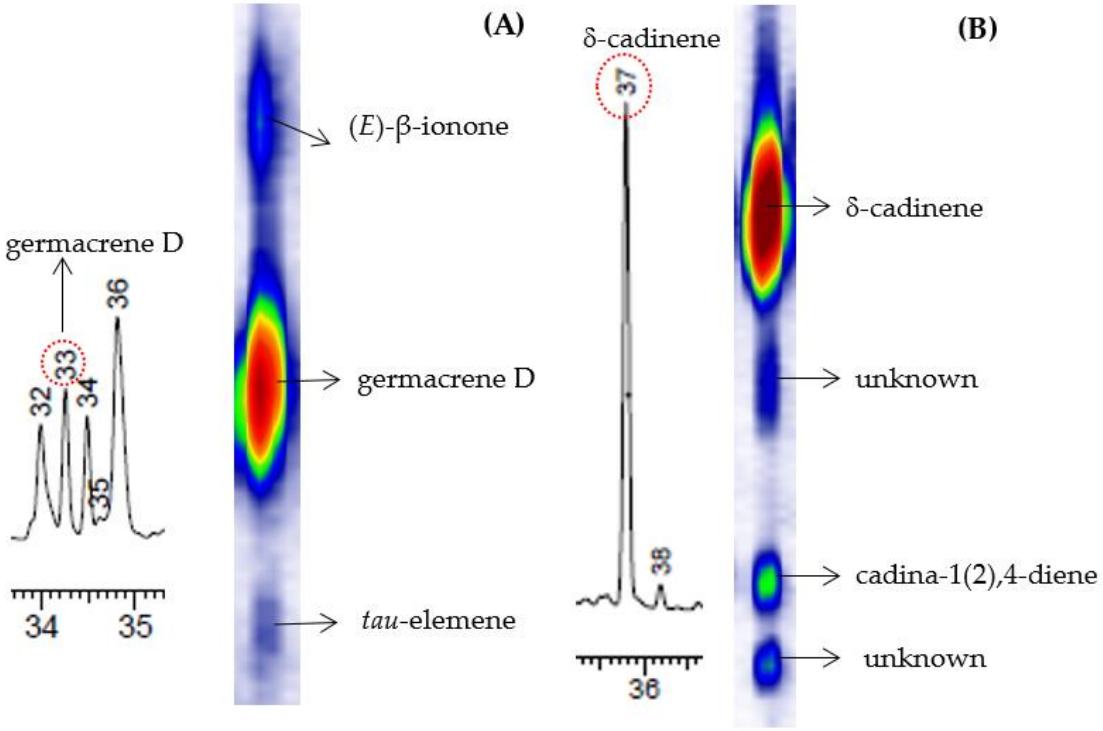

Figure 3. Example of improved peak capacity illustrating the resolution of two overlapping clusters (#33 and #37) in the first dimension of the chromatograms for essential oil *V. curassavica*. GC×GC resolved germacrene D (**A**) and δ-cadinene (**B**) as shown in the contour plots. The identified compounds and their average relative proportions are shown in Table 2. The chemical classes of the analytes found in the EO of *V. curassavica* were monoterpenes and sesquiterpenes (~96% of the oil).

The analysis of essential oils by GC×GC-FID/MS showed that α-pinene (27.1–58.9%) and (*E*)-caryophyllene (7.1–23.9%) were the terpenes with higher relative proportions in all EO of the three genotypes, regardless of the harvesting season. However, α-pinene and (*E*)-caryophyllene exhibited different relative proportions across all genotypes. Other terpenes were also detected in high intensities, such as α-tujene, sabinene, β-pinene, 1,8-cineol, and germacrene D. Noteworthy, allo-aromadendrene, and spathulenol were found only in the VC1 and VC2 genotypes. Terpenes like α-santalene, (*E*)-α-santalal, and (*E*)-α-bergamotenal were detected only in the VC3 genotype (Table 2).

Among the identified compounds, neryl acetate was detected only in genotypes VC2 and VC3 in the winter samples. β-copaene was detected in genotype VC1 (winter and autumn) and genotype VC2 (summer only). Terpene cis-muurol-5-en-4-ol was detected only in genotype VC2 (winter and spring), while cis-sesquisabinene hydrate was found only in VC3 (winter and spring). This variation in EO composition with seasonality is supported by previous reports [55,56]. Furthermore, different genotypes cultivated under the same conditions may exhibit differential expressions resulting in distinct chemical profiles, including the occurrence or absence of metabolites [57].

Table 2. Chemical composition (relative %) of *Varronia curassavica* essential oils harvested in different seasons and analyzed by GC×GC-FID/MS. LTPRI - Linear temperature programmed retention indices; Exp.–LTPRI experimental obtained by the injection of a homologous series of C8-C20 n-alkanes using the Van den Dool and Kratz equation [52]; Lit. - LTPRI obtained from literature [51] and NIST WebBook. (*)—LTPRI not found; (**)—Substance identified by comparison with Sciarrone and collaborators [44]; (ta)—Trace amounts (≤0.003); (-)—Absence of the metabolite.

Substance	LTPRI		Genotype/Season											
			VC1				VC2				VC3			
	Exp.	Lit.	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
tricyclene	930	926	0.464	ta	ta	ta	1.074	ta	ta	ta	0.091	ta	ta	ta
α-thujene	939	930	8.046	5.929	6.047	9.273	9.369	7.188	7.154	9.392	7.479	ta	0.032	0.078
α-pinene	947	939	28.920	35.236	33.769	48.178	27.102	29.058	33.985	33.062	33.029	41.899	45.805	58.864
thuja-2,4(10)-diene	956	951	ta	ta	0.036	ta	0.026	ta	0.067	ta	-	-	-	-
camphene	963	954	0.233	0.212	0.161	0.142	0.288	0.252	0.181	0.182	0.245	0.172	0.158	0.133
sabinene	983	975	2.048	1.353	1.401	1.621	2.557	1.387	1.321	2.333	0.614	0.372	0.414	0.305
β-pinene	990	979	3.179	2.734	2.535	3.178	5.997	5.341	4.898	5.702	2.106	1.530	1.517	1.585
myrcene	998	990	0.547	0.320	0.403	0.367	0.829	0.427	0.359	0.647	0.442	0.113	0.296	0.212
α-phellandrene	1019	1002	0.038	0.079	0.079	0.124	0.088	0.079	0.074	0.128	0.027	0.019	0.043	ta
α-terpinene	1027	1017	ta	ta	0.036	ta	0.031	ta	0.053	0.051	-	-	-	-
o-cymene	1037	1022	0.122	ta	0.044	ta	0.123	ta	0.049	ta	0.026	ta	0.006	ta
limonene	1024	1024	ta	ta	ta	0.147	ta	ta	ta	0.147	-	-	-	-
β-phellandrene	1029	1025	ta	0.043	ta	ta	ta	0.030	ta	ta	ta	0.532	ta	ta
sylvestrene	1038	1025	0.492	0.556	0.590	0.310	0.356	0.367	0.392	0.349	0.635	ta	0.721	0.399
1,8-cineole	1041	1026	2.016	1.412	1.019	1.455	2.269	1.112	0.702	1.855	1.039	0.853	0.854	0.600
(E)-β-ocimene	1044	1049	ta	ta	0.015	ta	ta	ta	0.025	ta	ta	ta	0.025	ta
γ-terpinene	1070	1059	0.040	0.078	0.107	0.085	0.100	0.076	0.154	0.077	0.021	ta	0.023	ta
cis-sabinene hydrate	1081	1070	0.069	0.043	0.026	0.030	0.119	0.034	0.042	ta	0.018	ta	0.012	ta
terpinolene	1102	1088	ta	ta	0.014	ta	ta	0.019	0.026	ta	-	-	-	-
linalool	1108	1096	0.057	ta	ta	0.052	0.031	ta	ta	ta	-	-	-	-
α-pinene oxide	1108	1097	0.078	ta	ta	ta	0.021	ta	ta	ta	0.105	tr	tr	tr
trans-sabinene hydrate	1111	1098	0.029	0.034	ta	ta	0.108	0.034	0.027	ta	0.009	tr	tr	tr
n-nonanal	1115	1110	0.444	ta	0.243	ta	0.344	ta	0.181	ta	0.119	tr	0.095	tr
(E)-3(10)-caren-4-ol	1130	*	0.018	ta	ta	ta	0.043	ta	ta	ta	-	-	-	-
cis-p-menth-2-en-1-ol	1134	1124	-	-	-	-	0.023	ta	ta	ta	-	-	-	-
α-campholenal	1126	1139	ta	ta	0.033	ta	0.025	ta	0.028	ta	0.015	0.012	0.018	tr
trans-pinocarveol	1152	1139	0.036	ta	0.051	ta	ta	ta	0.041	ta	0.028	tr	0.019	tr
isopinocarveol	1151	1140	ta	0.034	0.016	ta	ta	0.026	0.019	ta	tr	0.032	0.019	tr
trans-sabinol	1157	1142	0.061	0.017	ta	ta	0.049	ta	0.036	ta	0.034	tr	tr	tr
camphor	1157	1146	0.029	0.034	0.028	ta	-	-	-	-	-	-	-	-

Table 2. *Cont.*

Substance	LTPRI		Genotype/Season											
			VC1				VC2				VC3			
	Exp.	Lit.	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
pinocarvone	1174	1164	0.032	ta	0.039	ta	0.030	ta	ta	ta	0.015	0.009	0.011	tr
borneol	1182	1169	ta	ta	0.009	ta	ta	0.012	ta	ta	0.010	0.015	0.008	tr
terpinen-4-ol	1190	1177	0.105	0.108	0.103	0.074	0.166	0.077	0.180	0.112	0.033	0.017	0.022	tr
α-terpineol	1209	1188	0.033	ta	0.016	ta	0.034	ta	0.022	ta	0.006	tr	tr	tr
β-cyclocitral	1229	1219	ta	ta	0.018	ta	ta	ta	ta	ta	0.007	tr	tr	tr
citronellol	1223	1229	ta	ta	0.012	ta	ta	ta	0.017	ta	-	-	-	-
bornyl acetate	1290	1285	0.857	0.466	0.451	0.373	1.096	0.720	0.830	0.633	0.706	0.557	0.671	0.537
trans-pinocarvyl acetate	1302	1298	-	-	-	-	-	-	-	-	0.005	0.014	tr	tr
myrtenyl acetate	1329	1326	-	-	-	-	-	-	-	-	0.013	tr	0.009	tr
δ-elemene	1338	1338	0.010	0.022	0.019	0.057	0.006	0.024	0.012	ta	-	-	-	-
α-cubebene	1351	1351	0.061	0.069	0.064	0.052	0.037	0.042	0.034	ta	0.005	0.017	tr	tr
citronellyl acetate	1353	1352	0.592	0.178	0.143	0.159	0.434	0.098	0.113	0.222	0.315	0.087	0.217	0.069
neryl acetate	1361	1365	-	-	-	-	0.008	-	-	-	0.011	-	-	-
cyclosativene	1373	1371	0.909	0.669	ta	0.420	ta	0.472	ta	0.356	0.072	0.061	ta	ta
α-Ylangene	1375	1373	ta	ta	0.511	ta	0.566	ta	0.272	ta	ta	ta	0.035	ta
α-copaene	1380	1376	1.568	1.340	1.278	0.879	0.716	0.671	0.537	0.417	0.079	0.078	0.037	0.042
β-bourbonene	1387	1388	0.069	0.193	0.212	0.125	0.127	0.117	0.148	0.081	0.006	0.014	ta	ta
β-cubebene	1393	1388	1.616	1.499	1.206	1.057	0.755	0.705	0.507	0.571	-	-	-	-
7-epi-sesquithujene	1389	1391	-	-	-	-	-	-	-	-	0.036	0.019	0.051	0.022
β-longipinene	1410	1403	0.033	0.032	ta	0.060	0.041	0.034	ta	ta	-	-	-	-
sesquithujene	1404	1405	-	-	-	-	-	-	-	-	1.438	1.473	1.192	1.027
(Z)-caryophyllene	1410	1408	-	-	-	-	-	-	-	-	0.017	0.008	0.010	ta
cis-α-bergamotene	1418	1412	-	-	-	-	-	-	-	-	1.125	1.179	0.998	0.860
α-santalene	1425	1417	-	-	-	-	-	-	-	-	10.213	11.887	9.140	9.379
(E)-caryophyllene	1427	1419	7.481	10.997	11.679	7.119	18.026	23.999	22.384	21.548	11.917	14.800	12.330	11.453
β-copaene	1435	1432	0.111	-	-	0.051	-	-	0.076	-	-	-	-	-
trans-α-bergamotene	1435	1434	-	-	-	-	-	-	-	-	0.197	0.271	0.145	0.082
α-guaiene	1451	1439	0.024	ta	0.021	ta	-	-	-	-	-	-	-	-
aromadendrene	1457	1441	0.020	0.172	0.070	ta	0.110	0.197	0.021	ta	-	-	-	-
(Z)-β-Farnesene	1445	1442	-	-	-	-	-	-	-	-	0.212	0.207	0.171	0.105
epi-β-santalene	1451	1447	-	-	-	-	-	-	-	-	ta	0.026	0.020	0.026
cis-muurola-3,5-diene	1450	1449	ta	ta	ta	ta	0.010	0.033	ta	ta	0.035	ta	ta	ta
trans-muurola-3,5-diene	1457	1453	ta	0.030	ta	ta	ta	0.034	ta	ta	ta	ta	ta	ta
sesquisabinene	1454	1454	-	-	-	-	-	-	-	-	ta	ta	1.652	ta
α-humulene	1463	1454	1.470	2.383	2.442	1.378	2.649	3.821	3.934	3.284	2.892	3.456	2.880	1.574
(E)-β-farnesene	1457	1456	-	-	-	-	-	-	-	-	1.862	1.835	0.057	1.004
allo-aromadendrene	1469	1460	11.904	12.781	13.821	11.513	10.687	8.632	9.879	12.300	-	-	-	-
dehydro-aromadendrene	1471	1462	0.541	0.312	0.573	ta	0.218	ta	0.276	ta	0.154	0.232	ta	ta

Table 2. *Cont.*

Substance	LTPRI		Genotype/Season											
			VC1				VC2				VC3			
	Exp.	Lit.	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
γ-muurolene	1480	1479	0.840	0.589	0.532	0.290	0.256	0.251	0.168	ta	0.023	0.017	ta	ta
γ-elemene	1480	1488	ta	ta	0.036	0.204	-	-	-	-	-	-	-	-
germacrene D	1488	1481	1.716	2.965	3.879	1.585	0.015	3.548	4.855	2.066	0.592	0.498	1.170	0.383
tau-elemene	1484	1484	0.383	0.539	0.216	0.139	0.175	0.163	ta	ta	0.264	ta	ta	ta
(E)-β-ionone	1488	1488	0.168	ta	ta	ta	0.050	ta	ta	ta	0.059	0.244	ta	ta
cis-β-guaiene	1492	1490	ta	ta	0.085	0.087	ta	ta	0.042	ta	ta	ta	ta	ta
α-zingiberene	1500	1493	-	-	-	-	-	-	-	-	0.020	ta	0.054	0.037
trans-muurola-4(14),5-diene	1488	1493	ta	ta	ta	ta	2.062	ta	ta	ta	ta	ta	0.021	ta
γ-amorphene	1496	1493	ta	ta	ta	ta	0.069	ta	ta	0.084	ta	ta	ta	ta
epi-cubebol	1493	1494	ta	ta	0.698	0.121	ta	ta	0.160	0.124	-	-	-	-
biciclogermacrene	1502	1500	1.283	ta	6.183	3.822	1.051	ta	2.166	1.690	-	-	-	-
α-muurolene	1504	1500	1.016	ta	ta	ta	ta	ta	ta	0.087	0.142	ta	0.057	0.037
trans-β-guaiene	1504	1502	0.845	ta	0.022	ta	0.563	ta	0.017	ta	-	-	-	-
β-bisabolene	1512	1505	-	-	-	-	-	-	-	-	2.498	ta	2.438	0.070
guaia-1(10),11-diene	1516	1509	0.178	0.169	ta	ta	0.072	0.079	ta	ta	-	-	-	-
(Z)-γ-bisabolene	1514	1514	-	-	-	-	-	-	-	-	ta	ta	0.274	0.034
cadina-1(2)4-diene	1524	1519	0.023	ta	ta	ta	0.021	ta	ta	ta	-	-	-	-
cubebol	1514	1521	ta	ta	1.186	1.107	ta	ta	0.552	0.317	-	-	-	-
β-sesquiphellandrene	1521	1525	-	-	-	-	-	-	-	-	ta	ta	0.287	0.208
δ-cadinene	1524	1530	7.217	5.800	3.640	2.924	2.998	1.969	0.830	0.838	0.636	ta	ta	ta
(E)-γ-bisabolene	1531	1531	-	-	-	-	-	-	-	-	1.002	1.390	0.886	0.487
trans-cadina-1,4-diene	1541	1534	0.045	0.067	0.029	0.037	0.026	0.032	0.016	ta	-	-	-	-
(E)-α-bisabolene	1545	1545	-	-	-	-	-	-	-	-	0.023	ta	0.020	0.038
α-calacorene	1551	1545	0.045	0.020	0.018	ta	-	-	-	-	-	-	-	-
elemol	1557	1549	0.035	0.047	0.017	ta	-	-	-	-	-	-	-	-
cis-muurol-5-en-4-ol	1561	1551	-	-	-	-	0.048	0.047	-	-	-	-	-	-
cis-sesquisabinene hidrate	1551	1559	-	-	-	-	-	-	-	-	0.110	0.053	-	-
germacrene B	1567	1561	0.277	0.185	0.063	0.083	0.133	0.086	0.036	ta	-	-	-	-
trans-sesquisabinene hydrate	1561	1565	-	-	-	-	-	-	-	-	0.486	0.345	ta	ta
(E)-nerolidol	1567	1565	ta	ta	0.037	ta	ta	ta	ta	ta	0.039	ta	0.039	ta
β-calacorene	1573	1565	0.011	ta	0.004	ta	ta	ta	ta	ta	ta	ta	ta	ta
germacrene D-4-ol	1586	1575	ta	0.059	0.057	0.041	ta	0.018	0.025	ta	ta	ta	ta	ta
spathulenol	1588	1578	3.738	1.003	1.422	0.527	0.985	0.171	0.555	0.185	-	-	-	-
caryophyllene oxide	1594	1583	0.726	0.424	0.438	0.198	2.524	0.502	0.636	0.274	2.509	1.031	0.901	0.644
7-epi-trans-sesquisabinene hydrate	1598	*	-	-	-	-	-	-	-	-	ta	ta	0.629	0.327
salvial-4(14)-en-1-one	1604	1594	0.122	ta	ta	ta	ta	0.015	0.021	ta	ta	ta	ta	ta
ledol	1615	1602	0.716	0.437	0.410	ta	0.370	0.254	0.282	ta	-	-	-	-

Table 2. *Cont.*

Substance	LTPRI		Genotype/Season											
			VC1				VC2				VC3			
	Exp.	Lit.	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
humulene epoxide II	1621	1608	0.349	0.067	0.083	ta	0.302	0.043	0.083	ta	0.284	0.075	0.049	ta
isoaromadendrene epoxide	1612	1620	ta	ta	ta	0.237	ta	ta	ta	0.192	-	-	-	-
muurola-4,10(14)-dien-1-b-ol	1636	1631	0.426	0.123	0.173	0.097	-	-	-	-	-	-	-	-
cis-Cadin-4-en-7-ol	1636	1636	ta	ta	ta	ta	0.143	0.087	0.069	0.061	ta	0.017	ta	ta
isospathulenol	1643	1639	0.263	0.162	0.100	ta	0.165	0.027	0.073	ta	-	-	-	-
caryophylla-4(12),8(13)-dien-5α-ol	1649	1640	ta	ta	ta	ta	0.121	ta	ta	ta	ta	0.017	ta	ta
tau-cadinol	1640	1640	ta	ta	0.045	ta	ta	ta	0.056	0.035	ta	ta	ta	ta
allo-aromadendrene epoxide	1651	1641	0.054	0.108	ta	ta	-	-	-	-	-	-	-	-
cubenol	1655	1646	ta	ta	0.015	ta	ta	0.064	ta	ta	-	-	-	-
α-muurolol	1657	1646	1.067	0.500	0.333	0.200	0.366	0.134	0.160	0.093	0.070	0.034	ta	ta
α-cadinol	1666	1654	0.113	0.081	0.151	ta	0.051	0.040	0.102	ta	-	-	-	-
cis-calamenen-10-ol	1668	1661	0.300	ta	ta	ta	ta	ta	ta	ta	ta	ta	ta	ta
intermedeol	1670	1666	0.123	0.070	ta	ta	ta	0.018	ta	ta	ta	ta	ta	ta
trans-10-hydroxycalamenene	1679	1675	0.035	ta	0.025	ta	-	-	-	-	-	-	-	-
ledene oxide (II)	1687	1682	0.093	0.045	0.045	ta	-	-	-	-	-	-	-	-
(2Z,6Z)-farnesal	1684	1684	ta	ta	0.022	ta	ta	ta	0.008	ta	ta	ta	ta	ta
Z-α-trans-bergamotol	1668	1690	-	-	-	-	-	-	-	-	0.491	0.218	ta	ta
(E)-α-bergamotenal	1679	**	-	-	-	-	-	-	-	-	4.098	3.311	4.171	2.212
(E)-α-Santalal	1689	**	-	-	-	-	-	-	-	-	6.003	5.618	7.227	3.679
eudesm-7(11)-en-4-ol	1707	1700	0.113	0.081	ta	ta	0.064	0.026	ta	ta	-	-	-	-
(Z)-β-santalol	1716	1716	-	-	-	-	-	-	-	-	0.048	0.021	0.034	ta
β-santalol	1723	1727	-	-	-	-	-	-	-	-	ta	ta	0.014	0.036
(E)-β-santalol	1732	1739	-	-	-	-	-	-	-	-	0.030	0.172	0.168	0.126
(Z)-α-santalol acetate	1780	1778	-	-	-	-	-	-	-	-	0.296	0.139	0.132	0.092
(Z)-α-trans-bergamotol acetate	1795	1794	-	-	-	-	-	-	-	-	0.207	ta	ta	ta
(Z)-β-santalol acetate	1821	1819	-	-	-	-	-	-	-	-	0.012	0.017	ta	ta
hexahydrofarnesyl acetone	1845	1844	0.075	ta	ta	ta	0.009	ta	ta	ta	0.014	ta	ta	ta
isopimara-9(11),15-diene	1946	1905	0.028	0.014	ta	ta	0.019	0.014	ta	ta	ta	0.008	ta	ta
isopimara-8,15-diene	1956	1947	0.032	0.017	ta	ta	0.022	0.014	ta	ta	0.020	0.007	ta	ta
sandaracopimara-8(14),15-diene	1963	1969	0.075	ta	ta	ta	0.014	ta	ta	ta	0.021	ta	ta	ta
Monoterpene hydrocarbons			**44.129**	**46.541**	**45.223**	**63.426**	**47.939**	**44.206**	**48.712**	**52.070**	**44.717**	**44.637**	**49.040**	**61.576**
Oxygenated monoterpenes			**2.561**	**1.681**	**1.382**	**1.611**	**2.918**	**1.315**	**1.141**	**1.967**	**1.319**	**0.937**	**0.964**	**0.600**
Sesquiterpene hydrocarbons			**39.683**	**40.834**	**46.600**	**31.881**	**41.388**	**44.908**	**46.209**	**43.322**	**35.460**	**37.470**	**33.926**	**26.870**
Oxygenated sesquiterpenes			**8.284**	**3.208**	**5.262**	**2.527**	**5.137**	**1.445**	**2.781**	**1.282**	**14.680**	**11.068**	**13.364**	**7.117**
Others			**2.271**	**0.676**	**0.837**	**0.532**	**1.996**	**0.847**	**1.124**	**0.855**	**1.281**	**0.916**	**0.993**	**0.607**
Total identified			**96.93**	**92.94**	**99.30**	**99.98**	**99.38**	**92.72**	**99.97**	**99.50**	**97.46**	**95.03**	**98.29**	**96.77**

The substance α-humulene is the chemical marker in the pharmaceutical industry for the quality control of *V. curassavica* essential oil, which varied from 1.47% to 3.93%. Variance analysis (ANOVA) demonstrated significant differences ($p < 0.05$) between the seasons and between the genotypes evaluated (Table 3). Spring and summer were the seasons with the highest average production of the active principle α-humulene (3.82% and 3.93%, respectively), while autumn was the season with the lowest average production (1.38%). The highest average relative proportion was observed in the VC2 genotype during summer (3.93 ± 0.10) (Table 3). Besides environmental factors, genetic characteristics can also determine and modify the production rate of essential oils [58–60].

Table 3. Contents of α-humulene (% Relative and SE Average) in the EO obtained from *V. curassavica* genotypes harvested at different seasons and analyzed by GC×GC-MS. Distinct uppercase letters (A, B and AB) for the rows and lowercase (a, b and ab) for columns represent statistically significant differences ($p < 0.05$).

Genotype	α-Humulene			
	Winter ^{AB}	Spring ^{A}	Summer ^{A}	Autumn ^{B}
VC1 ^{b}	1.47 ± 0.45	2.38 ± 0.39	2.44 ± 0.19	1.38 ± 0.49
VC2 ^{a}	2.65 ± 0.06	3.82 ± 0.31	3.93 ± 0.10	3.28 ± 0.21
VC3 ^{ab}	2.89 ± 0.14	3.46 ± 0.09	2.88 ± 0.16	1.57 ± 0.42

The heatmap plot (Figure 4), generated from hierarchical clustering, provides an overview of *V. curassavica* metabolic profile in response to the seasonal cycle and plant genetics. It is possible to observe that the genotype was responsible for most of the variance observed in the samples, highlighting two main blocks. The first block (upper eighteen rows) comprises a group of metabolites that are most abundant and present/absent in the VC3 genotype. For instance, α-pinene (2) is most abundant in the samples of VC3 genotype. The (*E*)-α-bergamotenal (53), (*E*)-α-santalal (54), e α-santalene (27) are present only in the VC3 genotype. The second block showed similar metabolic profiles, which is the case for the VC1 and VC2 genotypes, which is ascertained by the results attained with PCA analysis, as presented in the following paragraphs.

The most different chemical composition was found for the VC3 genotype (Figure 4), suggesting the contribution of other regulatory levels, besides environmental and genetic, in the chemical composition of the essential oils, e.g., physiological cycle of the plant and interaction between genotype and environment. The observations on the intra-class variance may contribute to *V. curassavica* breeding programs aiming at increasing the production of substances with biological activity.

To investigate the effects of seasonality on the volatile compounds of *V. curassavica*, a peak table-based multivariate approach, namely, principal component (PCA) was adopted for pattern recognition of the three evaluated genotypes. A two-component PCA model expressed 71.8% of the total variance with PC1 being responsible for describing 61.8% and PC2 10% of the variance. The scores graph illustrated in Figure 5A indicated that the information contained in PC1 may be used to distinguish the samples into two groups (VC3 and VC1 + VC2). The loadings plot (Figure 5B) describes the most important variables that contributed to such pattern.

The substances responsible for the observed clustering between the samples were α-pinene (2), found in a higher relative proportion in the VC3 genotype samples, and (*E*)-α-bisabolene (42), (*E*)-β-santalol (56), (*E*)-α-bergamotenal (53), (*E*)-α-santalal (54), α-santaleno (27), cis-α-bergamotene (26), (*Z*)-β-Santalol acetate (59), (*E*)-α-bergamotene (29), (*Z*)-β-farnesene (31), (*E*)-β-farnesene (33), (*E*)-γ-bisabolene (40), (*Z*)-β-Santalol (55), (*Z*)-caryophyllene (25), (*Z*)-α-Santalol acetate (57), (*Z*)-α-trans-bergamotol (52), and (*Z*)-α-trans-bergamotol acetate (58).

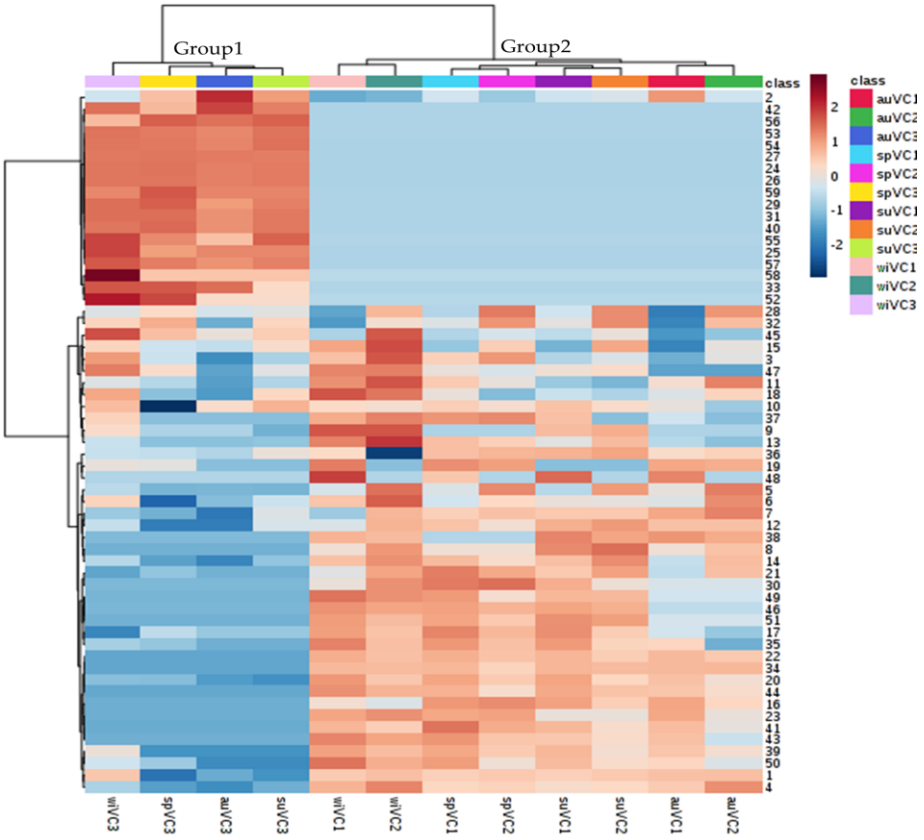

Figure 4. Heatmap generated from hierarchical clustering of the chemical composition of the essential oils of the three genotypes of *V. curassavica* as a function of the seasons. (Distance measure using Euclidean, and clustering algorithm using Ward's method).

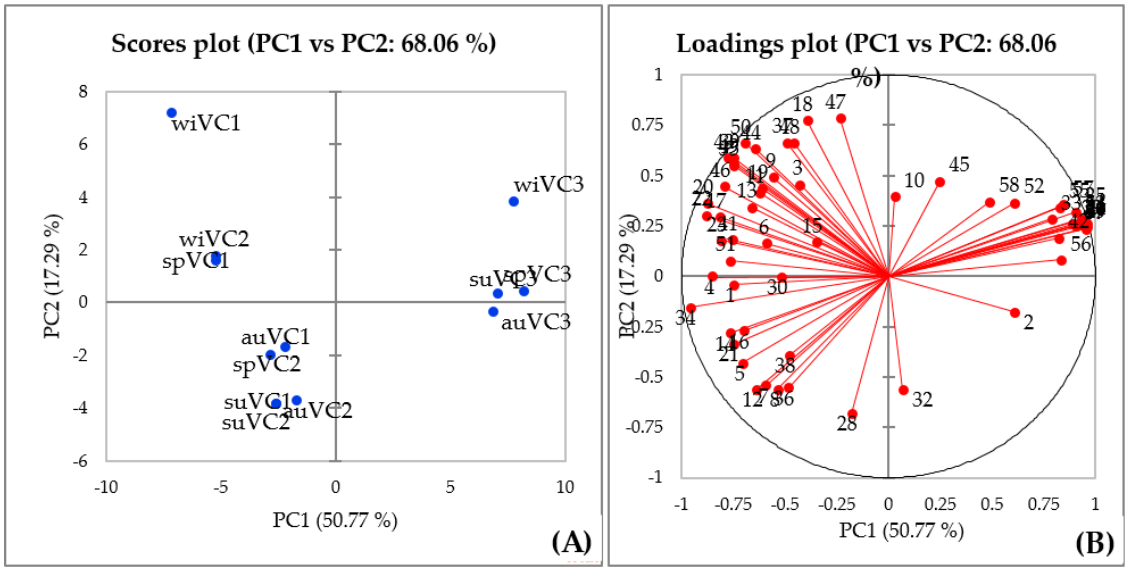

Figure 5. Scores plot (**A**) and loadings plot (**B**) obtained from PCA of data on the chemical composition of the *V. curassavica* essential oil.

In the current study, our results suggested that the genetic make-up is likely the most important factor contributing to the chemical composition of *V. curassavica* essential oils, whereas seasonal contributes to modulate the metabolite profile. Several factors have been demonstrated to contribute

to the metabolic profile of plant essential oils [61,62], such as abiotic stress [63–65], genetics [58,59], and plant-pathogen interactions [60,66–68]. Furthermore, environmental biotic and abiotic conditions modulate several regulatory levels determined by the plant genotype affecting the profile of specialized metabolites of essential oils [57,69]. The determination of the contribution of distinct factors to the chemical profile of plant essential oils may help breeding and agricultural strategies to increase the yield of metabolites of interest, as well as in the selection of genotypes for the development of breeding programs.

4. Conclusions

Two-dimensional gas chromatography has proved to be a versatile tool for analyzing complex mixtures such as *V. curassavica* essential oil. GC×GC-QMS allowed the identification of 135 constituents in the essential oil, three times more when compared to 1D-GC. The improved resolving power of GC×GC has resolved co-eluting peaks facilitating the detection of trace substances in the essential oil of *V. curassavica*. The metabolic study of volatile constituents showed that the metabolic profile and the content of the anti-inflammatory active principle, α-humulene, were significantly influenced by the genotype and seasonality. The content of the active principle was higher during spring and summer, being recommended for leaf harvest. Among the investigated genotypes, VC2 displayed higher amounts of the active principle.

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Conflicts of Interest: The authors declare no conflicts of interest.

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