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Yield and Quality of Essential Oils in Hemp Varieties in Different Environments

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Abstract: Due to its possible utilization in cosmetics, medicine and crop protection, as a valuable alternative to petrochemical-derived products, hemp essential oil is now considered a product with high value added and a promising marketing potential. This experiment was conducted with the aim of evaluating the effect of four different locations of Northern Italy during two years (four environments) and three hemp monoecious varieties on the production and quality of essential oils (EOs) obtained by inflorescences harvested at full flowering of female flowers. The highest inflorescence yield was obtained at Maiano 2017, where a superficial groundwater layer (1.5 m) was present, with values that ranged from 1.69 of Fedora to 2.06 t ha⁻¹ of Futura. EOs production ranged between 3.4 and 4.9 L ha⁻¹, affected mainly by the variety effect. The terpene in EOs, very similar between varieties and environments, was mainly composed of sesquiterpenes (caryophyllene and humulene, as the most abundant) rather than monoterpenes (α -pinene, β -myrcene and trans- β -ocimene, in particular). Phytocannabinoids, and in particular cannabidiol (CBD), were not removed from tissues by the steam during hydrodistillation, and if this is confirmed by further experiments, the residual biomass, now considered as waste, could assume significant importance as a source for further utilization.

Keywords: inflorescence yield; essential oil yield; hydrodistillation; terpene composition; phytocannabinoids

1. Introduction

Cannabis sativa L. is one of the oldest plant sources for food, textile fibers and medicine [1]. Hemp, namely industrial hemp or fiber hemp, is an annual and dioecious plant. However, breeders have developed several monoecious cultivars, considered particularly suitable for dual-proposal cultivation (seeds and fiber). There has also recently been growing interest in the utilization of hemp secondary metabolites, extracted from inflorescences of low-THC types cultivated by farmers without restrictions, namely non-psychoactive cannabinoids, terpenoids and flavonoids [2–4].

Unlike phytocannabinoids, which are specific to the genus *Cannabis*, terpenes are common in many plant families [5] and in *Cannabis sativa* essential oils (EOs) are the largest group of chemical compounds, with more than 100 molecules identified. Terpenes are classified in different families depending on the degree of polymerization of isoprene units (C₅); monoterpenes and sesquiterpenes, which include terpenes with 10 and 15 carbon atoms, respectively, are the most common in nature and are also the main constituents of the EOs in hemp [6,7]. Terpenes are responsible for the smell and flavor typical of the different varieties of *C. sativa*, whereas phytocannabinoids are odorless [7]; thus, anti-drug dogs are trained to recognize the β -caryophyllene-epoxides, a sesquiterpene [8]. Biosynthesis and bioaccumulation of these substances take place mainly in the pedunculated glandular trichomes, which are abundant on the perigon bracts at the beginning of flowering, while the sessile glandular trichomes are in all the aerial plant parts from the earliest growth stages [9]. Two pathways are

involved in the biosynthesis of terpenes: the plastidial methylerythritol phosphate (MEP) pathway and the cytosolic mevalonate (MEV) pathway. The former, through eight steps, leads to the synthesis of geranyl diphosphate (GPP) the precursor of monoterpenes. Instead, farnesyl diphosphate (FPP), synthesized through the MEV pathway, is the precursor of sesquiterpenes [7,10]. Terpenes are lipophilic compounds that pass easily through membranes, particularly the blood-brain barrier, and therefore have a wide range of pharmacological properties; among these, the most important are: anticancer, anxiolytic, immune stimulating, anti-inflammatory, analgesic, memory skills improving and gastro protective [7]. Hemp EOs can be used in cosmetics for the production of soaps, shampoos, perfumes, creams and even in food as flavoring for alcoholic and non-alcoholic beverages, or as additives in bakery and catering [11]. Thanks to their antimicrobial properties [12–14] they are used in topical treatments of wounds and skin infections, against pathogens involved in nosocomial infections, in food spoilage and poisoning, intestinal syndromes and against bacterial strains resistant to antibiotics [13]. Hemp essential oil has also proven to be effective as an insecticide [15–17], nematocide [18], fungicide [19], allelopathic [20–22] and anti leishmaniasis [23], justifying its use in crop protection [15] as an alternative to petrochemical-derived products. The quantitative and qualitative composition of *C. sativa* essential oil varies substantially in relation to variety, pedo-climate conditions, harvest time and extraction method [24,25] and for this reason, their terpene profile is often unpredictable and not easily standardized [5,26]. An increase in essential oil yields is possible by preventing pollination: indeed *C. sativa* continues to produce new flowers until it is pollinated, but as documented in studies by Meier and Mediavilla [11], this is feasible only in a greenhouse, utilizing dioecious varieties. In the field, low humidity conditions increase the amount of trichomes in inflorescences [22], as well as the terpene concentration of essential oil [11]. The optimal period to maximize oil production coincides with the maximum amount of intact glandular trichomes in the inflorescence, corresponding to when about 50% of seeds have reached maturity [11]. Moreover, the essential oil concentration seems to be inversely related to the single inflorescence biomass [27] and despite dioecious varieties being higher than that of monoecious varieties, the latter could be preferred because they provide a larger inflorescence yield per hectare [11]. For these reasons, the choice of monoecious varieties is undoubtedly preferable, because if the inflorescences are harvested at full flowering of female flowers, slightly earlier than the above-mentioned optimal period, the plants react by issuing new inflorescences that can provide a satisfactory seed production, improving the sustainability and a multipurpose exploitation of the crop [28]. Hemp essential oil, although considered a product with high value added and a promising marketing potential [29,30], has received little attention from the scientific and industrial community [13]. The multipurpose exploitation of the hemp crop, as already highlighted in a previous trial [28], is the main objective of this experiment, in which the evaluation of the effect of different environments (2 years and four locations of Northern Italy) and three hemp monoecious varieties on the production and quality of EOs were evaluated.

2. Materials and Methods

2.1. Plant Materials

Three different monoecious varieties were utilized, namely, Fedora, Ferimon and Futura. These varieties, of the same origin (France) but with a different cycle duration under these environmental conditions (unpublished data), are listed in the EU database of registered [31] hemp varieties and normally used in field trials in different European environments [32].

2.2. Environments, Trial Description and Characters Analyzed

The trial was conducted at S. Osvaldo, UD (N 46°04'00", E 13°23'00", 109 m a.s.l.) in 2016, Aviano, PN (N 46°03'31", E 12°38'31", 157 m a.s.l.), Campofornido, UD (N 46°01'13", E 13°08'41", 79 m a.s.l.) and Maiano, UD (N 46°12'46", E 13°03'40", 160 m a.s.l.) in 2017. The main soil characteristics at the experimental sites are reported in Table 1.

Table 1. Main soil characteristics (0–0.5 m layer).

Parameters	S. Osvaldo 2016	Campoformido 2017	Aviano 2017	Maiano 2017
Sand (> 0.05 < 2 mm) (%)	43	26	29	43
Loam (> 0.002 < 0.05 mm) (%)	40	59	53	50
Clay (< 0.002 mm) (%)	17	15	18	7
pH (KCl 1:2.5)	7.35	6.4	7.1	7.2
Total calcareous (%)	5.5	1	37	56
Active calcium carbonate (%)	0.2	n/a	1.6	1.4
Organic matter (%)	1.8	2.2	5.8	4.5
Total nitrogen (g kg ⁻¹)	1.85	1.9	3.7	2.8
C/N	10	6.8	9.2	9.4
Phosphorus available (mg kg ⁻¹)	34	47	26	30
Potassium exchangeable (ppm)	164	135	195	98

Sowing was done by a plot seeder (Wintersteiger AG, Ried im Innkreis, Austria), in rows with 0.15 m interrow spacing, using 28 kg ha⁻¹ of seed corresponding to about 130 viable seeds per m², at an average sowing depth of 3 cm in all environments. In order to avoid severe water stress effects on crops, the trials at S. Osvaldo and Aviano, which had a very shallow cultivation soil (about 50 cm), were conducted with irrigation (sprinkler irrigation; Table 2). The main agronomic techniques adopted are reported in Table 2, while the main climatic traits during crop season in comparison with a multi-year period for each location are reported in Figure 1. In each location, the trial was sown in a randomized block with three replications, where the factor of variation was the variety, as fixed effect, in experimental units of approximately 35 m². An area of about 22 m² for each unit, discarding the border rows, was utilized to harvest the inflorescences at the phenological stage corresponding to full flowering of female flowers (50% of the bracts formed), encoded as phase 2302 by Mediavilla et al. [33].

Table 2. Main cropping management techniques adopted.

Environment	Previous Crop	Soil Tillage	Fertilization ¹	Sowing–Emergence Time	Irrigation
S. Osvaldo-2016	Wheat	Ploughing at 30 cm and n.2 pre-sowing harrowing	Pre-sowing: 0-60-0 N-P-K + 80 N in post-emergence	20th–29th April	n.3 of 20 mm each
Aviano-2017	Corn	Ploughing at 30 cm and n.2 pre-sowing harrowing	Pre-sowing: 18-18-25.5 of N-P-K + 50 N in post-emergence	13th–24th April	n.4 of 20 mm each
Campoformido-2017	Corn	Ploughing at 30 cm and n.2 pre-sowing harrowing	Pre-sowing: 5 t ha ⁻¹ of manure	10th–22th April	-
Maiano-2017	Chicory	Ploughing at 30 cm and n.2 pre-sowing harrowing	Pre-sowing: 0-60-0 of N-P-K + 60 N in post-emergence	11th–23th April	-

¹ as kg ha⁻¹ of N, P₂O₅ and K₂O.

The fresh inflorescences were obtained after manually cutting the upper part of the plant stem at the insertion level of the first basal flowers of the inflorescence. A representative sample of each inflorescence was weighed before and after being dried in a forced ventilation oven for 3 hours at 105 °C to determine the dry matter percentage. The inflorescences, immediately after harvest, were placed in plastic bags in a cooler, transported to the laboratory, weighed and stored in the dark at a temperature of –20 °C until further utilization and analyses. The main characters analyzed were dry

matter content in inflorescences (%); essential oils (EOs) concentration in inflorescences (ml 100 g⁻¹ of dry matter); inflorescence yield (kg ha⁻¹ of dry matter); EOs yield (L ha⁻¹); identification and quantification of EOs compounds (%); quantification of phytocannabinoids, as tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN) content in inflorescences and in EOs (mg 100 g⁻¹ of dry matter). Essential oil compounds and phytocannabinoids identification and quantification were only performed in the three environments in 2017: Aviano, Campofornido and Maiano.

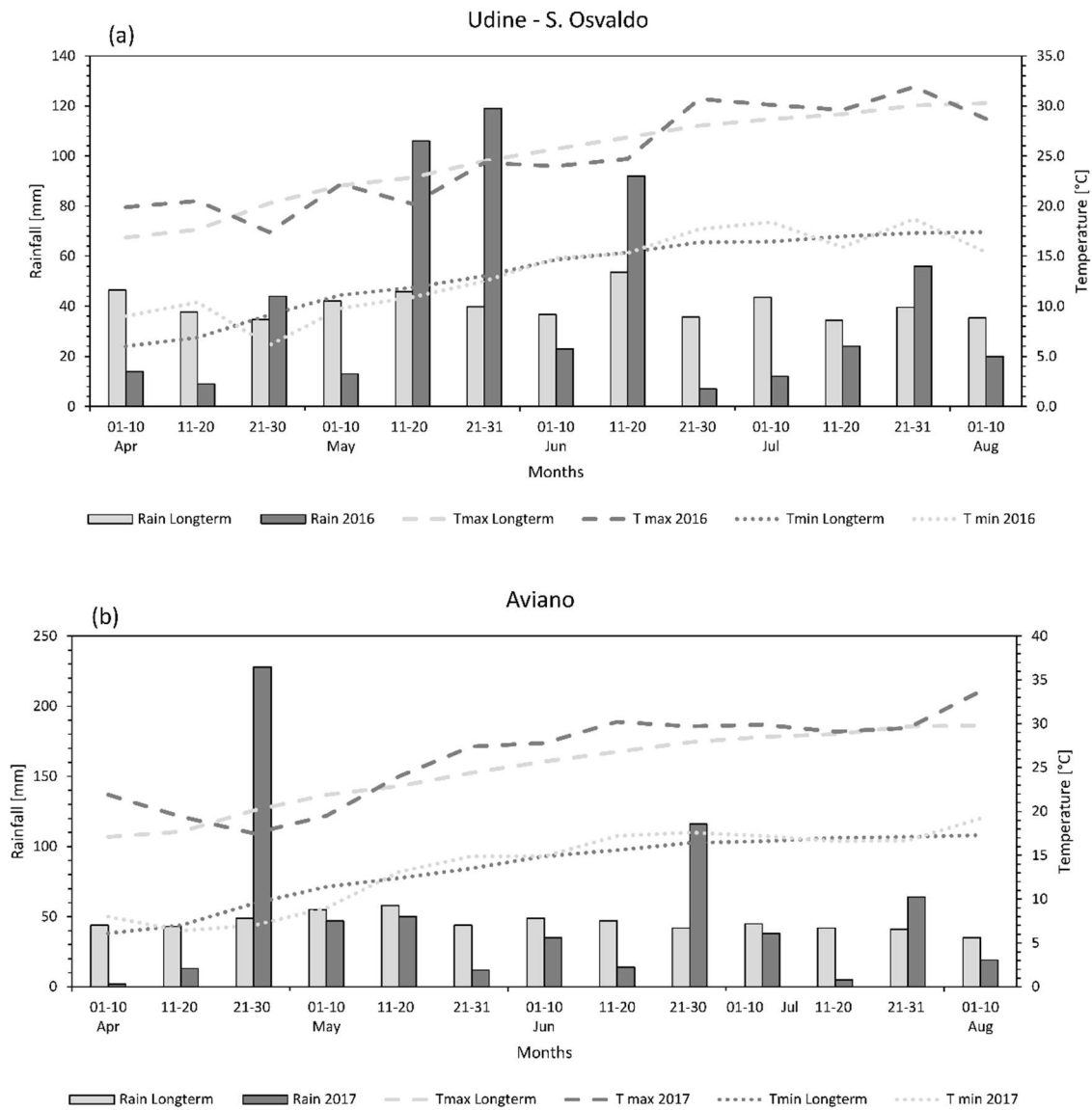


Figure 1. Cont.

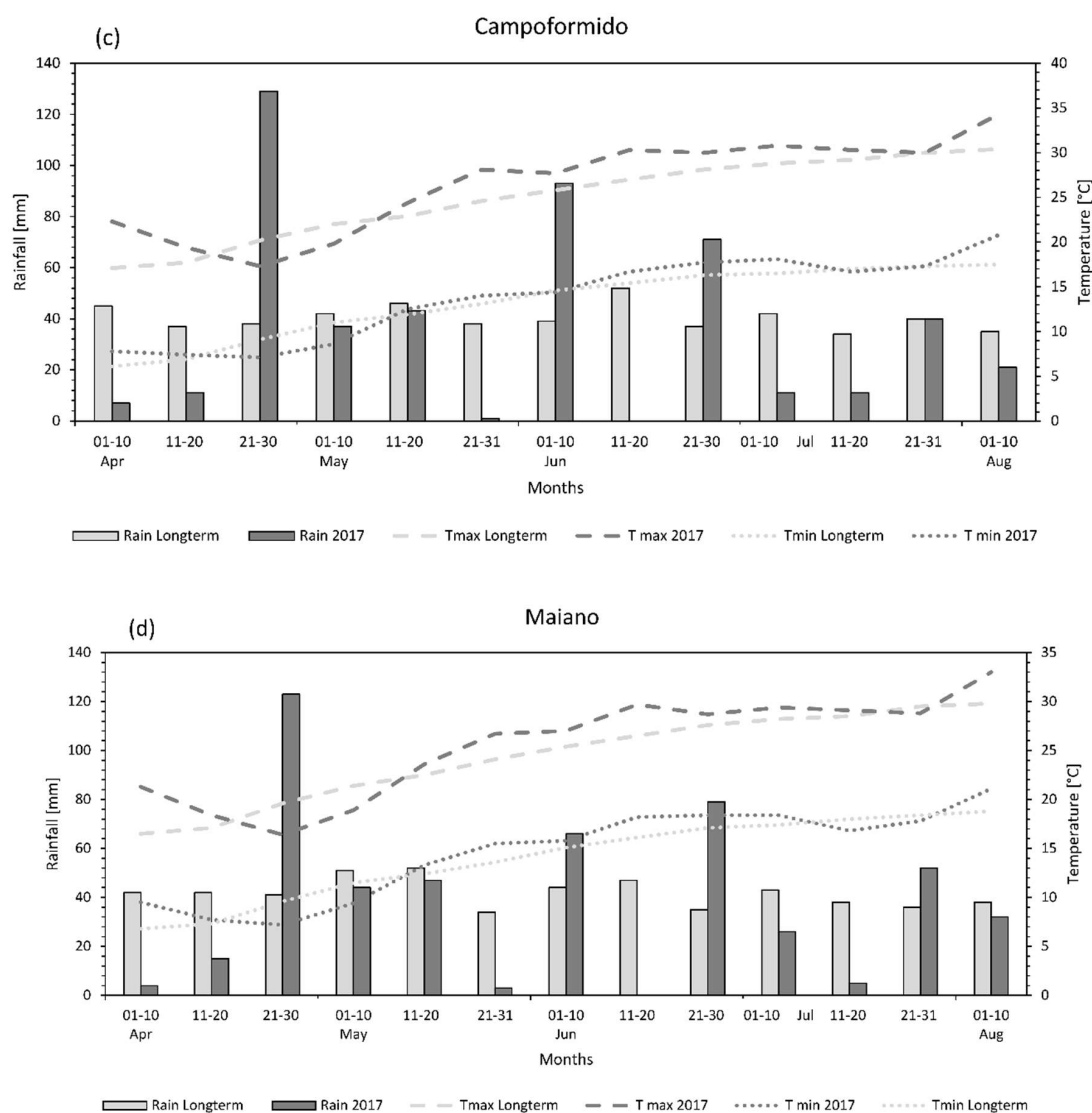


Figure 1. Ten days maximum average temperature (T max), minimum average temperature (T min) and total rainfall during the growing season in Udine (a) during 2016, Aviano (b), Campoformido (c) and Maiano (d) during 2017. Long-term values were the 25-year average.

2.3. Chemicals

Cannabidiol (CBD), cannabinol (CBN), delta-9-tetrahydrocannabinol (THC), delta-8-tetrahydrocannabinol (used as internal standard), N-methyl-(trimethylsilyl) trifluoroacetamide (MSTFA) and trimethylchlorosilane (TMCS) were purchased from Sigma-Aldrich (Milan, Italy). All reagents of analytical grade were purchased from Carlo Erba (Milan, Italy) and the linear hydrocarbons (C₈–C₃₀) used as standards were obtained from Sigma-Aldrich.

2.4. Essential Oil Extraction Method

The frozen fresh inflorescences were defrosted at room temperature and submitted to essential oil extraction by hydrodistillation, through a Clevenger-type apparatus described in the Italian Pharmacopoeia [34]. Briefly, 100 g of fresh inflorescence was chopped, put in a round bottomed flask and 600 mL of distilled water was added. The flask was connected to the condenser and flow was adjusted to 2 mL min⁻¹ of condensate. Distillation continued for 3 hours. Each EO sample obtained, preventively dried over anhydrous MgSO₄ and filtered was expressed as v/w (mL 100 g⁻¹ of dry weight) and analyzed by GC-MS.

2.5. Apparatus, Sample Preparation, Identification and Quantification of Terpenes in EOs

EOs composition was determined by GC-MS (Agilent Technologies GC-MS 5977E, USA) and the amount of each compound was expressed as relative percentage by peak area normalization. One microliter of each EO sample was diluted in 50 μL of *n*-hexane in an amber vial. The gas chromatograph was fitted with a 30 m HP-5MS capillary column (Agilent Technologies, Santa Clara, CA, USA). Helium was used as carrier gas (1.2 mL min^{-1} constant flow). Oven settings were as follows: $40 \text{ }^\circ\text{C}$ for 3 min, steps of $4 \text{ }^\circ\text{C/min}$ up to $280 \text{ }^\circ\text{C}$ and then holding at $280 \text{ }^\circ\text{C}$ for 10 min with a run time of 73 min. One μL of sample was injected in split mode and the split ratio was 1:20. Data acquisition was performed in scan mode by MassHunter software (Agilent Technologies, Santa Clara, CA, USA). The EO compounds were identified by a comparison of their Mass spectra with those reported in the Mass Spectral Library Nist 14 and by means of their Linear Retention Indices (LRI) relative to a series of *n*-alkanes ($\text{C}_8\text{--C}_{30}$) on the same capillary column. LRI obtained were compared with values reported in the literature [26,27,35–40].

2.6. Identification and Quantification of Phytocannabinoides in Inflorescences

Phytocannabinoides identification and quantification were performed on inflorescence and EOs samples from the three environments in 2017: Aviano, Campofornido and Maiano. The analysis of cannabinoids was performed according to Pellegrini et al. [35] with some modifications. The quantification was obtained with the external standard method.

2.7. Statistical analysis

Statistical analysis was conducted using the R v. 3.4.0 (R Foundation, Vienna, Austria) [41]. A two-way ANOVA analysis using environments and varieties as fixed factors was utilized. If the differences were significant, averages were compared through an LSD (least significant difference) test using the package 'Agricolae' of R (Felipe de Mendiburu, Lima, Peru) [42]. Only for EOs compounds, a two-way ANOVA was performed as fixed-effect model with locations and varieties. Significance of each source of variation was evaluated by F-test. When the F-ratio revealed significant differences, means were compared by the LSD at $p \leq 0.05$.

3. Results and discussion

3.1. Weather Conditions

All meteorological data were obtained from the Regional meteorological service (OSMER-ARPA, FVG Region Meteorological Service).

At S. Osvaldo, total rainfall in the period considered (from April to the first 10 days of August) was 539 mm in 2016, similar to the long-term average (526 mm) (Figure 1a). Minimum and maximum average temperatures of the previous long-term period were very close to those of the experimental year ($13.5 \text{ }^\circ\text{C}$ and $24.9 \text{ }^\circ\text{C}$). Maximum average temperature in 2016 was significantly warmer ($30.2 \text{ }^\circ\text{C}$) compared to the average of the previous 24 years ($29.2 \text{ }^\circ\text{C}$). Furthermore, in the same period, rainfall was significantly lower than in the previous 24 years (63%).

At Aviano (Figure 1b) the amount of rainfall between April and early August in 2017 (643 mm) was higher than that of the multi-year average (594 mm). However, it was concentrated particularly in the last ten days of April (228 mm), June (116 mm) and July (64 mm). In 2017, during the period April–early August, maximum average temperature was $1.4 \text{ }^\circ\text{C}$ higher than the corresponding multi-year temperature ($24.7 \text{ }^\circ\text{C}$). In particular, during the flowering period, maximum average temperature was always higher than the corresponding long-term average, with a peak of $33.7 \text{ }^\circ\text{C}$ in the first 10 days of August.

At Campofornido, maximum average temperature was $1.6 \text{ }^\circ\text{C}$ higher than the long-term average (Figure 1c). During the crop cycle, total rainfall was 53.1% more than the long-term average, though not uniformly. Instead, the last 10 days of May and mid-June were almost without any rainfall. During

the crucial period of flowering (mid-June–early August), maximum average temperature was 1.8 °C, higher than the long-term average.

At Maiano (Figure 1d), the cumulated rainfall was 496 mm, lower than the long-term average (543 mm). It had a non-homogeneous trend, unlike those of the long-term average, with peaks in the last 10 days of April (123 mm) and in the first and last 10 days of June (66 and 79 mm respectively). Maximum and minimum average temperatures were of 1.8 and 1.3 °C, respectively, from mid-May until the first 10 days of July, higher than in the multi-year period. Between the last 20 days of June and the first 10 days of August, during the flowering in this environment, rainfall was higher than that of the long-term average (+ 19%), but with a non-homogeneous trend.

3.2. Inflorescences and EOs Yield

Cultivar and environment, as main treatments, showed significant effects for all variables according to the ANOVA analysis, with the exception of environment for Essential oil (EOs) yield, while the two-way year × genotype interaction was significant for all the characters analyzed (Table 3).

Table 3. Analysis of Variance (F-Value) for Essential Oil (EOs) concentration in inflorescence, inflorescence yield and EOs yield.

Source of Variation	Degrees of Freedom	EOs ^a Concentration in Inflorescence	Inflorescence Yield	EOs Yield
Cultivar (C)	2	34.38 ***	21.28 ***	27.13 ***
Environment (E)	3	7.68 **	12.68 **	0.82 ns
C × E	6	5.03 *	5.05 *	4.84 *

*, ** and *** significance at the $p < 0.05$, 0.01 and 0.001 levels, respectively. ns = not significant. ^a EO = Essential Oil.

Ferimon and Futura, as mean across environments, provided a similar yield of inflorescences, significantly higher than that of Fedora, with the exception of Campoformido. Among environments across varieties, the highest inflorescence yield was obtained at Maiano for all varieties, with values that ranged from 1.69 of Fedora to 2.06 t ha⁻¹ of Futura. At S. Osvaldo and Aviano, Ferimon and Futura produced significantly more than Fedora (1.70 and 1.52 against 1.09 t ha⁻¹, respectively); conversely, at Campoformido and Maiano, the three varieties provided an equivalent inflorescence yield. The lowest inflorescence yield was obtained by Fedora at Aviano (1.15 t ha⁻¹) (Figure 2).

The concentration of EOs in inflorescences, as mean of the environments across varieties, was 0.25% (v/w). Futura obtained the maximum value in Campoformido (0.34 %) (Figure 3), showing a significant higher EOs content, with respect to the other cultivars, in environments with the exception of Maiano (Figure 3). Fedora and Ferimon showed very similar concentrations of EOs in inflorescences in all four environments (Figure 3). Maiano, where no difference between varieties was observed, showed the lowest mean value across varieties of the trial (Figure 2). As reported by several authors [5,11–14], the EOs concentration in inflorescences is dependent on the variety and environmental conditions. At Maiano, the effect of the alternation of wet and dry periods, conditions favorable to an increase in the concentration of EOs in hemp [11,22] was lacking due to a superficial groundwater layer (1.5 m) during the summer 2017. The above mentioned alternation of wet and dry periods was instead present in the other environments (Figure 1), even if only partially attenuated by irrigation.

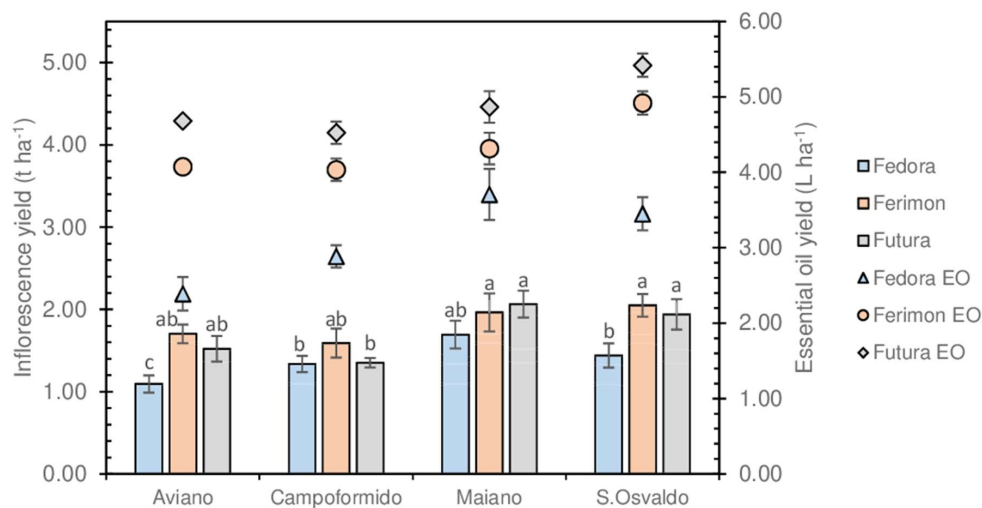


Figure 2. Dry weight inflorescence yield (primary y-axis on the left, columns) and EOs (secondary y-axis on the right, symbols) yield. Means with the same letter are not significantly different. Least significant difference (LSD) at 5% level. Bars represent standard error.

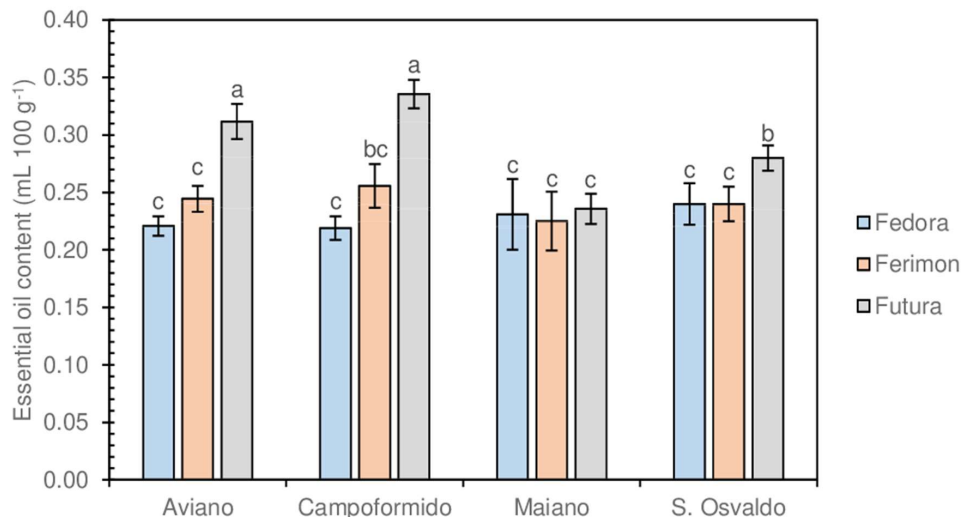


Figure 3. EOs content on hemp inflorescence dry weight. Means with the same letter are not significantly different. LSD at 5% level. Bars represent standard error.

Our results confirmed what was previously reported by Meier and Mediavilla [14]; the concentration of EOs had an opposite trend with respect to inflorescence yield. Indeed, Futura always gave higher EOs content than the other varieties in all locations (Figure 3), with the exception of Maiano, where the highest inflorescence yield was obtained by all varieties (Figure 2).

The average production of EOs was 4.3 L ha⁻¹ as mean of the environments and difference in EOs production is due mainly to the inflorescence yield (Figures 2 and 3). Among varieties, Futura was the most productive, with the highest value of the trial obtained at S. Osvaldo (5.4 L ha⁻¹) and followed by Ferimon (4.9 L ha⁻¹) in the same environment (Figure 2). In 2017, the ranking of the varieties in the three environments was the same as in 2016; Futura showed the highest and very similar yield in all environments (average 4.6 L ha⁻¹), followed by Ferimon (average 4.1 L ha⁻¹) and Fedora, with the lowest yield of the trial obtained at Aviano (2.4 L ha⁻¹) (Figure 3). The EOs yield obtained is lower than that observed by Mediavilla and Steinemann [29] in Switzerland (10 L ha⁻¹), but in our experiment, the inflorescence harvest time was significantly earlier than the optimal period.

Bertoli et al. [27], with other monoecious varieties and in a different environment, showed higher inflorescences (4.5–6 t ha⁻¹) and essential oils (5.3–7.8 kg ha⁻¹) productions than in our experiment.

On the contrary, Nissen et al. [13], using the same Futura variety, obtained inflorescences (6.6 t ha^{-1} as fresh weight) and essential oil (2 kg ha^{-1}) yields very close to these of this trial.

3.3. Phytocannabinoids Determination in Inflorescences and EOs

Phytocannabinoids analysis was performed in the three environments in 2017: Aviano, Camapoformido and Maiano. The concentrations of the main phytocannabinoids THC, CBD and CBN, in the inflorescences and in the EOs obtained by hydrodistillation were determined through the GC-MS analysis (Figure 4). The THC concentration detected in each inflorescence was significantly lower than the limit of 0.2% (data not shown) that the EU regulation states for cannabis plants for industrial uses. ANOVA analysis indicates the absence of the environments and interaction effect on CBD content in the inflorescences. Conversely, the variety effect, as mean across environments, was significant. In particular, Futura registered similar and higher levels in all environments than the other varieties, with an average value of $61.1 \text{ mg } 100 \text{ g}^{-1}$ of dry matter, followed by Fedora (mean 49.0 mg) and Ferimon; this latter showed the lowest CBD concentration (mean 40.3 mg) (Figure 4). The CBD concentration obtained in Fedora (0.49%) was comparable to that obtained by Brighenti et al. [43] (0.32%), but higher than those reported by Mediavilla and Steinemann [29] (0.19%) and by De Backer et al. [44] (0.1%). Similarly, CBD concentration in Futura (0.61%) was almost double what was observed by Brighenti et al. [43]. The 10-based logarithm analysis of the relationship between CBD and THC [41], confirmed that all three varieties have a value between 0.95 and 1.51, and therefore belong to chemotype III, representing non-psychoactive varieties that are normally used for industrial purposes.

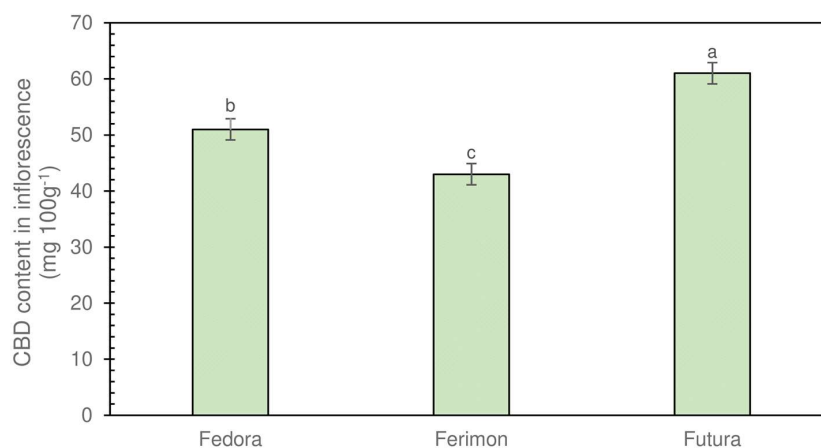


Figure 4. Cannabidiol (CBD) content in the inflorescences. Means with the same letter are not significantly different. LSD at 5% level. Bars represent standard error.

In all the EOs analyzed, THC and CBN content was lower than the instrument's detection limit (1 ppb). Conversely, the CBD content in EOs, expressed as $\text{mg } 100 \text{ g}^{-1}$ of inflorescence as dry matter, showed the highest values in Futura in all environments (values ranging from 0.055–0.068 mg), with a similar trend to that reported for inflorescences, but with values about 1000 times lower. This result means that the steam during the hydrodistillation is not able to remove the phytocannabinoids from the tissues and convey them into the EO. If, as appears from the first evaluations, CBD is not found in the aromatic distillation waters (personal communication), it is very likely that these compounds remain concentrated in the biomass and that the latter could be usefully exploited instead of being considered a waste product.

3.4. Characterization of EOs Composition

As for phytocannabinoids, characterization of EOs was performed in the three environments in 2017: Aviano, Campoformido and Maiano. A profile of compounds characterizing the EOs obtained by hydrodistillation was identified by GC-MS analysis. In total, about 65 different compounds were identified representing from 93 to 97% of all components present in EOs analyzed (Table 4).

Table 4. GC–MS results of the essential oils extracted by hydrodistillation from the hemp inflorescences of the three monoecious varieties cultivated in Northern Italy.

Compound	LRI	Aviano			Campoformido			Maiano		
		Ferimon	Fedora	Futura	Ferimon	Fedora	Futura	Ferimon	Fedora	Futura
α -Pinene	942	10.12 \pm 1.01	11.34 \pm 1.45	11.64 \pm 1.42	10.25 \pm 1.92	10.62 \pm 1.44	11.53 \pm 0.34	10.06 \pm 1.19	11.84 \pm 1.40	11.36 \pm 0.69
Camphene	946	0.19 \pm 0.00	0.25 \pm 0.03	0.28 \pm 0.03	0.22 \pm 0.01	0.24 \pm 0.01	0.30 \pm 0.02	0.21 \pm 0.10	0.23 \pm 0.02	0.27 \pm 0.02
β -Pinene	984	4.49 \pm 0.39	4.96 \pm 0.53	5.24 \pm 0.50	4.67 \pm 0.53	4.68 \pm 0.40	5.14 \pm 0.13	4.43 \pm 0.45	5.17 \pm 0.24	5.15 \pm 0.38
β -Myrcene	992	9.91 \pm 1.33	5.41 \pm 0.79	7.17 \pm 0.79	8.95 \pm 1.42	5.77 \pm 0.56	6.39 \pm 0.34	10.01 \pm 1.08	6.68 \pm 0.58	6.61 \pm 0.36
α -Phellandrene	996	tr	0.22 \pm 0.01	0.28 \pm 0.03	tr	0.24 \pm 0.03	0.25 \pm 0.00	tr	0.23 \pm 0.02	0.25 \pm 0.00
δ 3-Carene	998	tr	1.55 \pm 0.22	0.59 \pm 0.22	tr	1.58 \pm 0.24	0.52 \pm 0.19	0.22 \pm 0.00	1.86 \pm 0.14	0.45 \pm 0.10
α -Terpinene	1010	-	tr	0.25 \pm 0.02	-	tr	0.22 \pm 0.00	-	tr	0.23 \pm 0.01
o-Cymene	1025	tr	0.21 \pm 0.07	-	-	0.31 \pm 0.11	-	-	0.32 \pm 0.07	tr
D-Limonene	1035	-	tr	-	tr	2.55 \pm 1.04	-	-	2.00 \pm 1.15	tr
D-Limonene	1035	2.45 \pm 0.12	2.38 \pm 0.33	2.72 \pm 0.30	3.66 \pm 0.26	2.63 \pm 0.33	2.77 \pm 0.15	3.90 \pm 0.18	2.53 \pm 0.32	2.78 \pm 0.31
+ β -Phellandrene	1037	tr	0.48 \pm 0.03	0.74 \pm 0.26	-	-	-	tr	0.53 \pm 0.31	tr
β -Phellandrene	1037	tr	0.48 \pm 0.03	0.74 \pm 0.26	-	-	-	tr	0.53 \pm 0.31	tr
1,8 Cineol	1040	0.88 \pm 0.09	0.49 \pm 0.04	0.64 \pm 0.10	0.62 \pm 0.04	0.49 \pm 0.07	0.80 \pm 0.06	0.71 \pm 0.08	0.53 \pm 0.04	0.70 \pm 0.12
trans- β -Ocimene	1042	1.44 \pm 0.28	0.76 \pm 0.08	0.45 \pm 0.05	1.40 \pm 0.33	0.90 \pm 0.31	0.43 \pm 0.04	1.40 \pm 0.10	1.08 \pm 0.26	0.52 \pm 0.05
β -Ocimene	1044	8.88 \pm 2.01	4.93 \pm 0.48	3.31 \pm 0.32	6.94 \pm 1.12	4.03 \pm 1.47	2.92 \pm 0.22	8.63 \pm 0.48	4.89 \pm 0.98	3.42 \pm 0.31
γ -Terpinene	1064	tr	tr	0.24 \pm 0.02	tr	tr	0.24 \pm 0.01	tr	tr	0.24 \pm 0.02
cis-Sabinene hydrate	1068	-	-	-	-	tr	0.18 \pm 0.07	-	-	tr
Terpinolene	1079	3.45 \pm 0.42	4.63 \pm 0.49	5.82 \pm 0.56	3.22 \pm 0.83	4.29 \pm 1.85	0.55 \pm 0.11	3.81 \pm 0.40	4.90 \pm 0.67	4.83 \pm 0.64
Linalool	1082	-	tr	0.21 \pm 0.07	tr	tr	0.22 \pm 0.08	-	-	-
trans-(-)-Pinocarveol + 2-Pinen-10-ol	1126	-	tr	0.22 \pm 0.01	tr	0.24 \pm 0.09	0.21 \pm 0.01	tr	tr	tr
p-Cymen-8-ol	1163	-	-	-	-	0.32 \pm 0.12	tr	-	0.31 \pm 0.09	tr
Cosmen-2-ol	1210	tr	-	-	0.32 \pm 0.23	tr	-	0.27 \pm 0.13	-	tr
2-Nonen-4-one, 2-methyl-	1215	-	tr	-	tr	-	-	-	-	tr
β -Curcumene	1389	tr	0.23 \pm 0.08	tr	tr	0.22 \pm 0.01	tr	tr	0.24 \pm 0.14	tr
7-epi-Sesquithujene	1389	tr	0.21 \pm 0.07	tr	tr	tr	tr	tr	tr	tr
β -Elemene, (-)	1387	0.25 \pm 0.02	tr	tr	tr	tr	tr	tr	tr	tr
Isocaryophyllene	1406	0.67 \pm 0.18	0.72 \pm 0.13	0.81 \pm 0.13	0.74 \pm 0.13	0.89 \pm 0.08	0.86 \pm 0.11	0.56 \pm 0.12	0.71 \pm 0.11	0.92 \pm 0.01
cis- α -Bergamotene	1416	0.56 \pm 0.02	0.46 \pm 0.04	0.47 \pm 0.05	0.36 \pm 0.03	0.52 \pm 0.02	0.45 \pm 0.02	0.34 \pm 0.03	0.48 \pm 0.04	0.46 \pm 0.08

Table 4. Cont.

Compound	LRI	Aviano			Campoformido			Maiano		
		Ferimon	Fedora	Futura	Ferimon	Fedora	Futura	Ferimon	Fedora	Futura
Caryophyllene	1420	22.00 ± 1.44	22.40 ± 1.48	20.52 ± 1.13	21.01 ± 0.98	20.72 ± 1.43	20.39 ± 1.31	21.88 ± 0.70	21.12 ± 1.18	21.43 ± 0.76
trans- α -Bergamotene	1434	2.55 ± 0.22	2.98 ± 0.20	3.36 ± 0.25	2.61 ± 0.24	3.28 ± 0.24	3.22 ± 0.10	2.48 ± 0.16	3.19 ± 0.21	3.35 ± 0.25
α -Longipinene	1444	tr	0.31 ± 0.11	0.26 ± 0.05	0.26 ± 0.18	0.27 ± 0.01	0.21 ± 0.00	tr	0.24 ± 0.01	0.32 ± 0.16
cis- β -Farnesene	1454	3.38 ± 0.15	4.46 ± 0.20	4.43 ± 0.27	3.47 ± 0.20	4.51 ± 0.21	4.20 ± 0.14	3.36 ± 0.29	4.51 ± 0.29	4.40 ± 0.27
Humulene	1457	9.20 ± 0.55	9.28 ± 0.29	8.62 ± 0.31	9.07 ± 0.16	8.67 ± 0.23	8.61 ± 0.37	9.18 ± 0.47	8.68 ± 0.16	8.88 ± 0.36
Aromandendrene	1462	1.12 ± 0.11	1.46 ± 0.10	1.29 ± 0.15	1.23 ± 0.27	1.46 ± 0.07	1.55 ± 0.10	1.07 ± 0.10	1.36 ± 0.15	1.20 ± 0.07
β -Patchoulene	1461	tr	-	tr	-	tr	0.34 ± 0.14	-	-	tr
γ -Gurjunene	1461	tr	0.28 ± 0.05	0.39 ± 0.10	0.24 ± 0.17	0.30 ± 0.02	0.34 ± 0.15	tr	0.25 ± 0.04	0.32 ± 0.06
α -Curcumene	1473	-	tr	-	tr	0.33 ± 0.13	tr	-	-	-
β -Selinene	1489	2.12 ± 0.21	3.13 ± 1.12	2.19 ± 0.29	1.78 ± 0.26	3.25 ± 0.26	2.49 ± 0.09	1.76 ± 0.13	2.92 ± 0.27	2.43 ± 0.38
Valencene	1492	tr	1.17 ± 1.85	0.35 ± 0.06	0.23 ± 0.01	0.23 ± 0.00	0.44 ± 0.02	tr	0.23 ± 0.00	0.33 ± 0.05
α -Selinene	1496	1.25 ± 0.15	2.37 ± 0.21	1.55 ± 0.20	1.34 ± 0.21	2.30 ± 0.19	1.76 ± 0.06	1.31 ± 0.08	2.06 ± 0.07	1.70 ± 0.30
α -Farnesene	1503	0.45 ± 0.06	0.83 ± 0.13	0.51 ± 0.10	0.38 ± 0.02	0.73 ± 0.10	0.57 ± 0.03	0.38 ± 0.03	0.68 ± 0.18	0.58 ± 0.05
β -Bisabolene	1506	0.48 ± 0.15	0.71 ± 0.13	0.78 ± 0.13	0.58 ± 0.04	0.68 ± 0.14	0.74 ± 0.16	0.50 ± 0.02	0.64 ± 0.08	0.72 ± 0.10
β -Elemene, (-)	1506	tr	-	-	tr	tr	-	-	-	-
γ -Muurolene	1506	tr	0.25 ± 0.09	0.24 ± 0.02	0.20 ± 0.14	0.22 ± 0.01	0.19 ± 0.01	-	tr	tr
γ -Gurjunene	1506	0.15 ± 0.01	0.24 ± 0.08	0.34 ± 0.07	0.24 ± 0.01	0.21 ± 0.01	0.37 ± 0.08	tr	0.20 ± 0.11	0.32 ± 0.14
γ -Cadinene	1506	-	0.19 ± 0.06	0.27 ± 0.04	-	tr	0.17 ± 0.03	-	tr	tr
Selina -3,7(11)-diene	1507	0.43 ± 0.11	0.36 ± 0.07	0.46 ± 0.13	0.41 ± 0.19	0.42 ± 0.10	0.54 ± 0.13	0.36 ± 0.07	0.37 ± 0.03	0.41 ± 0.06
β -Sesquiphellandrene	1516	0.29 ± 0.03	0.50 ± 0.09	0.43 ± 0.07	0.33 ± 0.01	0.49 ± 0.08	0.41 ± 0.03	0.29 ± 0.01	0.46 ± 0.03	0.41 ± 0.03
(\pm)- β -Acoradiene	1518	tr	0.20 ± 0.07	0.24 ± 0.02	0.20 ± 0.14	0.23 ± 0.00	0.25 ± 0.01	tr	tr	0.23 ± 0.00
δ -Cadinene	1519	0.52 ± 0.25	0.47 ± 0.05	0.76 ± 0.10	0.49 ± 0.03	0.42 ± 0.07	0.88 ± 0.04	0.44 ± 0.07	0.37 ± 0.07	0.78 ± 0.06
Nerolidol	1560	-	tr	tr	-	tr	-	-	-	tr
Germacrene B	1570	0.88 ± 0.18	1.10 ± 0.15	0.92 ± 0.14	1.03 ± 0.33	0.82 ± 0.27	0.91 ± 0.07	0.96 ± 0.17	0.75 ± 0.07	1.03 ± 0.07
Caryophyllene oxide	1584	5.48 ± 0.88	4.29 ± 0.35	4.04 ± 0.65	5.95 ± 1.31	6.07 ± 1.91	4.33 ± 0.14	5.44 ± 0.60	5.03 ± 0.84	4.25 ± 0.34
trans-Sesquisabinene hydrate	1580	tr	0.21 ± 0.07	tr	-	tr	-	-	tr	-
(-)-Globulol	1590	tr	0.27 ± 0.06	0.30 ± 0.03	0.34 ± 0.24	0.27 ± 0.04	0.32 ± 0.02	tr	0.22 ± 0.00	0.26 ± 0.03
Humulene epoxide 2	1592	1.88 ± 0.08	1.20 ± 0.17	1.10 ± 0.22	2.05 ± 0.61	1.45 ± 0.68	1.19 ± 0.06	1.81 ± 0.21	1.36 ± 0.19	1.17 ± 0.12
Aromadendrene oxide-(2)	1594	-	tr	-	tr	0.93 ± 1.01	tr	-	tr	-
β -Guaiene	1599	0.41 ± 0.20	0.29 ± 0.07	0.43 ± 0.10	0.35 ± 0.10	0.35 ± 0.03	0.50 ± 0.05	0.30 ± 0.04	0.24 ± 0.14	0.45 ± 0.03
Isovalencenol	1640	0.33 ± 0.05	0.36 ± 0.13	0.30 ± 0.05	0.35 ± 0.24	0.26 ± 0.03	0.36 ± 0.04	0.24 ± 0.12	tr	0.30 ± 0.03
Isoaromadendrene epoxide	1645	0.44 ± 0.11	0.35 ± 0.11	0.34 ± 0.06	0.48 ± 0.20	0.35 ± 0.06	0.37 ± 0.04	0.34 ± 0.09	0.23 ± 0.00	0.38 ± 0.04

Table 4. Cont.

	LRI		Aviano		Campoformido			Maiano		
Allohimachalol	1652	-	0.19 ± 0.06	tr	-	-	-	-	-	-
α-Bisabolol	1646	-	-	0.22 ± 0.07	-	0.25 ± 0.02	0.12 ± 0.01	-	tr	0.29 ± 0.02
7-epi-cis-sesquibinene hydrate	1660	tr	0.39 ± 0.14	0.30 ± 0.04	0.22 ± 0.15	0.21 ± 0.01	0.12 ± 0.03	tr	tr	0.39 ± 0.19
Cannabidiol	2431	0.25 ± 0.05	0.85 ± 0.15	0.37 ± 0.03	tr	0.22 ± 0.01	0.37 ± 0.04	0.27 ± 0.08	tr	0.97 ± 0.12

LRI = Linear retention indices on DB5-column; tr = traces <0.01%; - = not detected.

There were 23 compounds present in all EOs, representing from 78 to 88% of all components. These data were subjected to two factor statistical analysis (ANOVA), in which variety and location were the factors of variation. The ANOVA analysis showed that the components profiles of the EOs tested were influenced exclusively by the factor variety, of which the mean values are shown in Figure 5.

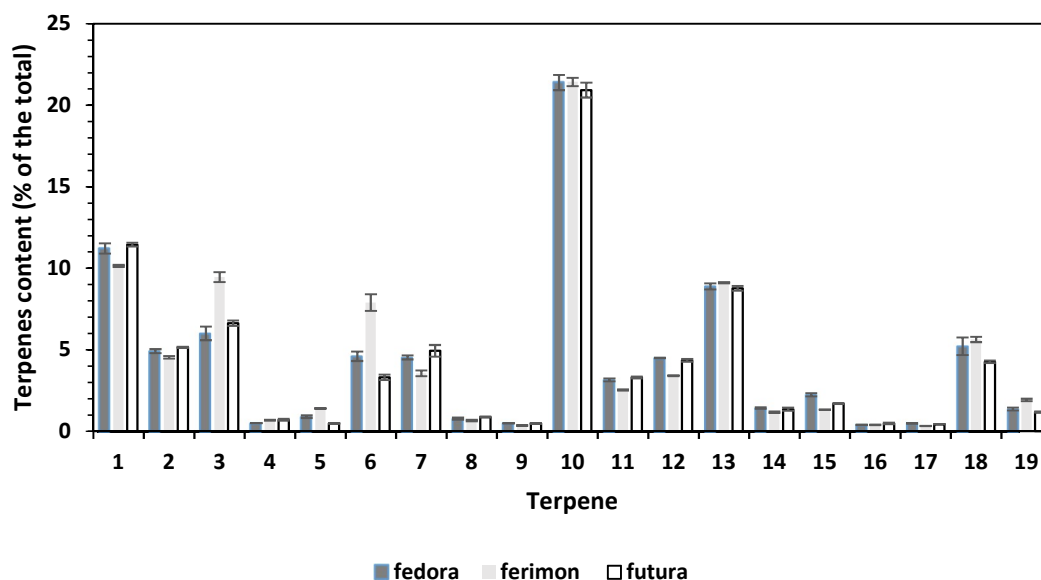


Figure 5. Relative percentage composition of the main and common constituents of EOs of the three varieties studied, as mean within the environments (1: α -Pinene; 2: β -Pinene; 3: β -Myrcene; 4: Eucalyptol 1,8 Cineol; 5: trans- β -Ocimene; 6: β -Ocimene; 7: Terpinolene; 8: Isocaryophyllene; 9: cis- α -Bergamotene; 10: Caryophyllene; 11: trans- α -Bergamotene; 12: cis- β -Farnesene; 13: Humulene; 14: Aromandendrene; 15: α -Selinene; 16: Selina-3,7(11)-diene; 17: β -Sesquiphellandrene; 18: Caryophyllene oxide; 19: Humulene epoxide II). Bars represent standard error.

In all varieties, the sesquiterpenes were higher than monoterpenes (average 33.9%). In particular, sesquiterpenes hydrocarbon concentration fluctuated from 40.8% recorded in Ferimon to 43.9% in Fedora and monoterpenes values ranged between 31.9% in Fedora and 37.4% in Ferimon. On the contrary, in experiments by Bertoli et al. [27] and Nissen et al. [13], the class of compounds present in higher percentages is that of monoterpenes, which reached 55.4% and 64.2%, respectively. The anticipating of the inflorescence harvest time carried out in this experiment, to allow the plants to issue new inflorescences in order to also obtain seed, could explain the differences in terpenoids profile obtained, which is affected by variety, maturity level of female flowers and percentage of fertilized flowers [28].

As reported in Figure 5, between the EOs common to all varieties, the main compound was caryophyllene, a natural bicyclic sesquiterpene, detected with a rather constant concentration between the varieties studied (20.8–21.6%), very close to the mean value (18.8%) obtained by Bertoli et al. [27] in similar experiments with other varieties. This compound is normally used as food flavoring, and has anti-inflammatory, analgesic and antipyretic properties [45].

Other compounds quantitatively relevant and common to all varieties studied were the α -pinene (10.1–11.5%), a monoterpene with a smell similar to that of pine resin [46] used as food flavoring, solvent and as additive of lubricating oils [47], and humulene (8.7–9.1%), a monocyclic sesquiterpene with anti-inflammatory properties [48].

The three varieties showed a very similar terpene composition, with the exception of Ferimon that had a significantly higher concentration of β -myrcene and trans- β -ocimene than the others (Figure 5). The former is an aliphatic monoterpene synthesized from many plants, particularly from hops (*Humulus lupulus* L.), used as a solvent, in the preparation of synthetic fragrances, pharmaceuticals, personal

hygiene products and as food flavoring [49]. While trans- β -ocimene is a monoterpene very widespread in plant species and is one of the components of the pheromones emitted by several insects [48].

EOs terpene profiles of the three different varieties are qualitatively comparable to what was observed by Novak et al. [14] and Mediavilla and Steinemann [11], with the exception of the β -myrcene content that was lower in our study.

Our results do not confirm what was reported by Novak et al. [14] and Verma et al. [16], who stated that the terpinolene concentration in French varieties would be around 16%; in fact, in the present study, although the varieties were of French origin, the concentration of terpinolene reached 5.1% as a maximum value in Ferimon.

Among the 65 compounds identified in the GC-MS analysis, α -terpinene and γ -terpinene were found exclusively in Futura with 0.25% and 0.24%, respectively of total components, while *o*-cymene, *p*-cimen-8-ol and β -curcumene, representing 0.32%, 0.34% and 0.23% of the total, respectively, were found exclusively in Fedora.

The analyzed EOs, along with the main terpenes, showed very interesting minor components that, even if in small quantities, may perhaps contribute to the final aroma and characterize one EO over another. Often, these minor compounds, terpenoids, are a modified class of terpenes with different functional groups, containing oxygen and oxidized methyl moved or removed at various positions. The oxidation, in fact, leads to the formation of highly oxygenated molecules, which are often further rearranged in similar compounds with different chemical reactivity, through a process that is not yet completely clear [50,51]. The antibacterial properties of many EOs are probably due to these minor components [52], which, after the variation of their structures, become biologically active and are used worldwide for the treatment of many diseases. This could be the case of 1,8 cineol (eucalyptol), used in the treatment of bronchial complaints, sinusitis and colds [53] or of linalool, a tertiary alcohol present in very low quantities in EOs, but with antimicrobial activity and a fresh, clean, mild, light floral fragrance with a slight citrus impression [54]. This may explain the particular aroma of some hemp EOs obtained, for example, from Futura at Aviano and Campoformido (Table 4). Instead, caryophyllene oxide is an oxidation derivative of sesquiterpene caryophyllene. It has a strong wooden odor and anti-inflammatory, antioxidant, antiviral, anti-carcinogenic and analgesic properties [55] and in all EOs obtained, it was present in quantities similar to those of some important monoterpenes such as myrcene and β -ocimene (Figure 5). Other oxygenated sesquiterpenes that are found in smaller quantities are humulene epoxide II (derived from humulene), globulol, aromadendrene oxide-(2), allohimachalol and α -bisabolol; all these compounds have anti-inflammatory, analgesic, antibiotic and anticancer activities.

4. Conclusions

The EOs production is interesting, despite the early harvesting of inflorescences, reaching around a maximum of about 5 L ha⁻¹ with Futura. The inflorescence EOs content appeared to be linked to variety and positively influenced by environments with alternating periods of good water availability and moderate water stress. Conversely, environments, as Maiano-2017 in this experiment, with elevated water availability during the crop cycle, affect the inflorescence EOs content and promote high inflorescence biomass yield. The early harvest provided a terpene composition of EOs characterized mainly by sesquiterpenes rather than monoterpenes and among the compounds, caryophyllene, which is used as food flavoring and has anti-inflammatory, analgesic and antipyretic properties [45] is the most abundant. On the contrary, the effects of different varieties and environments on the compounds profile of EOs were very limited. This considerable stability of terpenes obtained in this experiment could be a useful indication for the breeders involved in the selection of new strains especially for marijuana marketplace.

Phytocannabinoids, and in particular CBD, are not removed from tissues by steam during hydrodistillation, unlike terpenes, and probably remain concentrated in the residual biomass now considered as waste. Finally, adopting an early inflorescence harvesting time a good quanti-qualitative

EOs yield utilizing the monoecious varieties is achieved and at the same time, a valuable seed production can be obtained from the plants, as reported by Baldini et al. [28], improving the sustainability of the hemp crop.

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