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Chemical Components and Biological Activities of Essential Oils of *Mentha* × *piperita* L. from Field-Grown and Field-Acclimated after In Vitro Propagation Plants

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Abstract: In this work, we studied in vitro propagation of three cultivars of *Mentha* × *piperita* L. Murashige and Skoog medium (MS) supplemented with 0.5 mg·L^{-1} BAP was the most optimal medium for micropropagation of the cultivars studied. The ability of peppermint plants field-acclimated after in vitro micropropagation to produce essential oils (EOs) was investigated. EO was obtained by hydrodistillation from dried leaves and flowering shoots from control (field grown) plants and plants acclimated in field conditions after in vitro propagation. The samples were collected at the first and second year of vegetation, and their chemical composition was investigated using gas chromatography-mass spectrometry (GC-MS). Differences were observed in the yield, as well as in the quantitative and qualitative composition of the EOs extracted from the control plants and field-acclimated plants after in vitro propagation. Menthol was the main component of the EO in control plants, while pulegone and menthone were dominant in the EO pattern in field-acclimated in vitro regenerants in the first year of the growing season. However, in the second year of vegetation, the content of the main EO components in field-acclimated peppermint plants was approximately the same as in control plants. The antioxidant activity of EOs extracted from field-acclimated after in vitro micropropagation plants was found to be the same as in control field-grown *M.* × *piperita* plants.

Keywords: peppermint; *Mentha* × *piperita*; essential oil; antioxidant activity; in vitro micropropagation; nodal explants; plant regeneration; acclimation

1. Introduction

Peppermint *Mentha*× *piperita* L., a representative of the genus *Mentha* (*Lamiaceae*), is one of the most widely used aromatic plants in the world, and it has long been used safely in medicines [1–3]. Almost the entire peppermint plant has pharmacologically beneficial

properties such as antioxidant, antiallergic, antiviral and antibacterial actions, thus preventing the development of microorganisms and even suppressing the development of cancer cells [4–6]. In the food industry, dried peppermint leaves are used as mint teas or infusions [7,8]. A number of flavonoids among the secondary compounds in mint have been identified as highly effective antioxidants. These compounds are known to slow down the oxidative degradation of lipids, improving the quality and nutritional value of food. However, the most important biologically active constituents of peppermint are essential oils (EOs). The plant contains up to 250 different volatile components, with menthol being the most important [5,9]. EOs are used to add flavor and aroma to chewing gum, candy, chocolate, liqueurs, or alcohol. The tobacco industry also uses it to make menthol cigarettes [10]. Due to the high demand for peppermint essential oil, this crop has been bred throughout the 19th and 20th century. At present, many mint cultivars with a unique essential oil composition have been registered. The so-called "apple", "cognac", and "lemon" mint are grown commercially, and each of these mint cultivars is characterized by a high content of individual essential oil components, such as limonene, 1,8-cineole (eucalyptol), carvone, linalool, and others. They are valued in the cosmetic and pharmaceutical industries for their refreshing aroma and bioactive properties [11,12]. Due to its complex interspecific hybrid origin, peppermint is almost completely autosterile. On average, only one seed is formed per plant. The use of seedlings from rarely produced seeds leads to complex segregation in the offspring, where plants with an unstable phenotype emerge [13]. All cultivars of this species mainly reproduce vegetatively by dividing rhizomes and rooting shoots, which often slows down its multiplication. For large-scale reproduction of *M.* × *piperita*, the use of cuttings is not ideal due to the long time (two years) required to obtain a developed plant. The production and commercialization of young *M.* × *piperita* plants is limited by a number of factors such as plant contamination with phytopathogens after cutting, seasonal weather conditions, and lack of high-quality raw materials [14,15]. Given the moderate percentage of vegetative propagation of *M.* × *piperita*, micropropagation (in vitro) is proposed as an alternative option for mass production to meet commercial demand, and to preserve commercially valuable genotypes [16–19]. The in vitro method provides the required amount of mint material for planting plantlets. At the same time, the application and validity of this method largely depends on the ability of regenerated plants to produce high yields of essential oil of suitable quality.

In recent years, many experiments with peppermint have shown that the choice of growth regulator plays a crucial role after establishment in the in vitro culture of mint explants. The results obtained indicate that the studied genotypes have a specific response to specific conditions in the plant tissue culture. [16,20–24]. The use of growth regulators increases the production of EOs by peppermint plants and affects the chemical composition of EOs [18,22,25–27]. The amount of the individual components contained in the EOs changes and undesirable substances are not synthesized, which was confirmed by the experiment of Bertoli et al. [20]. However, there are no studies devoted to investigating the changes in the composition of EOs of in vitro multiplied regenerants that were acclimated during a long period in field conditions.

In the present study, we attempted to develop a protocol for the rapid micropropagation of three menthol chemotypes of *M.* × *piperita* (cv Zabava, Tik-Tak and Serebristaya). We also estimated the chemical composition of the EOs in field-acclimated plants, which were micropropagated by in vitro tissue culture, in the first and second year of vegetation and compared the values with those for plants grown in natural conditions.

2. Materials and Methods

2.1. Plant Material

Mentha × *piperita* L. cultivars, Zabava, Tik-Tak and Serebristaya were obtained from the collection of the S.I. Rostovtsev Botanical Garden in Moscow K.A. Timiryazev Agricultural Academy, Russian State Agrarian University (RSAU-MTAA, Moscow, Russian).

Peppermint stock plants of some cultivars were planted in the experimental plot at RSAU-MTAA, Moscow (55°83′ N, 37°58′ E) in late May in furrows at a distance of 12-15 cm between each plant and with row spacing of 50 cm. The soil in the plot had the following agrochemical parameters: pH 6.2; humus content, 8.1%; sand content, 22.5%; nitrate forms of nitrogen −3.5; exchangeable forms of phosphorus, 45.2 mg 100 g^{-1} and potassium, 24.5 mg 100 g^{-1} of soil. The sum of active temperatures in the Moscow region in 2019 amounted to 2258 °C. The annual precipitation was 556 mm in 2019 (the amount of precipitation was 196 mm during the growing season). Control plants of the peppermint varieties were collected during the beginning of flowering (budding phase) in August 2020 for the extraction of essential oil (EO) and for in vitro micropropagation. In 2020 (first vegetation year) and 2021 (second vegetation year), the yield and the EO composition were determined at a similar phase of development in peppermint plants acclimated in soil after in vitro micropropagation.

Cultivars Zabava, Tik-Tak are highly productive, winter-hardy peppermint cultivars [28]. The plant height ranges between 92 and 94 cm. The stem is slightly pubescent, the leaves are light green, elliptical, and not pubescent. The flowers are light purple, and collect in spike-shaped inflorescences. The essential oil content of absolutely dry raw materials is 4.2%; the oil contains 55–70% menthol and 10–20% menthone. The varieties meet the requirements of the production technology of cultivation. They are highly resistant to septoria and rust [29]. Cultivar Serebristaya is a highly productive, highly oily variety [28] with a lant height of 62–68 cm. The shrub is compact and hemispherical. The plants are pubescent; the stems have anthocyanin coloration. The leaves are ovoid on a long petiole and the flowers have a purple coloration. The essential oil content is 3.5%. The essential oil of this variety contains up to 40% menthol and 27–30% menthone. This species is resistant to diseases and pests [29]. Pubescence was assessed visually under a Bresser-Advance ICD 10×–160× binocular microscope (Shenzen Boshida Instrument Co, Ltd., Shenzhen, China).

2.2. Micropropagation

For the establishment of in vitro plants, stem segments 1.0–1.5 cm long with axillary buds from *M.* × *piperita* plants were disinfected with 4% sodium hypochlorite solution containing two drops of Tween 20 for 4 min. This step was followed by treatment with ethanol at 70% for 30 s, and three passages in sterile distilled water. Then, nodal fragments were aseptically planted onto Murashige and Skoog (MS) agar (0.8%) medium supplemented with sucrose (3%) and growth regulator (6-benzylaminopurine (BAP))—0.5 mg·L^{-1} BAP (V-1) and 1.0 mg·L^{-1} BAP (V-2)—and in MS control medium without the addition of plant hormones (V-K) [30]. All media pH values were fixed at 5.6–5.8. The media were steam-sterilized in an autoclave at 121 °C for 20 min culture flasks were kept in a culture chamber set at 23 °C with a photoperiod of 16 h/8 h (light/dark) and 70% humidity level. The experiment was performed in triplicate. After four weeks of cultivation, individual morphobiological parameters were determined: the number and length of shoots, the number of nodes on the shoot, the length of the root, and the frequency of rhizogenesis. The multiplication factor was calculated as the number of microcuttings that can be obtained for one passage; for this, the average number of emerged shoots was multiplied by the average number of nodes on the shoot [31]. In total, 30 plants in each medium variant were taken into account.

Four-weeks-old plantlets from shoot-tip explants were used for the acclimatization of in vitro plants. The plants were removed from the culture flasks and the roots were washed in tap water to remove agar traces. They were then transplanted into pots (7 cm diameter) containing sphagnum moss [32]. Sphagnum moss has aseptic properties and, unlike soil substrates, does not require autoclaving. Moss can be stored indefinitely, absorbs and retains a large amount of liquid, allows air to pass through, and also prevents the development of bacterial and fungal pathogens. The pots were acclimated at 23 ± 2 °C with a 16 h light/8 h dark photoperiod with a light intensity of 160 µM photons m^{-2} s^{-1} and 70% humidity level. The plantlets were moistened by spraying with distilled water 3–4 times

per day during the first week and then irrigated twice a week. Irrigation was carried out by alternating between distilled water and an irrigation mineral solution (1/10 m/v).

2.3. Isolation of the Essential Oil and Determination of Essential Oil Composition

A sample (up to 20 g) was submitted to hydro-distillation for 3 h using a Clevenger apparatus, according to the standard procedure described in the European Pharmacopoeia V Ed. (2010) [33]. Oils were recovered directly from above the distillate without adding any solvent, and stored in the dark at 4 °C. Yield was then estimated on the basis of the dryweight of plant material.

The qualitative composition of the oil was determined by gas chromatography at the Center for Collective Use of the Federal Research Center "Fundamentals of Biotechnology", (RFMEFI62114) (Moscow, Russian) [34]. The identification of essential oil components was obtained by gas chromatography-mass spectrometry (GC-MS). Analyses were carried out on a Shimadzu GS 2010 (Shimadzu Europa GmbH, Duisburg, Germany) gas chromatograph with a GCMS-QP 2010 mass detector. The gas chromatography column was using a SPB-1 nonpolar column (solid-phase-bound methyl silicone) (Supelco, Merck SA, Darmstadt, Germany) (30 m × 0.25 mm ID, 0.25 µm film thickness). The column oven temperature was set at 60 °C for 3 min, increased to 100 °C at a rate of 1.5 °C·min^{-1}, increased to 180 °C at a rate of 4 °C·min^{-1}, and held at 180 °C for 1 min. Next, it was increased to 200 °C at a rate of 10 °C·min^{-1}, increased to 250 °C at a rate of 2.5 °C·min^{-1}, and held at 250 °C for 5 min. Injector, interface and detector temperatures were 180 °C, 205 °C and 250 °C, respectively. The gas chromatography mass analysis was carried out with the same characteristics as used in gas chromatography. A 1.0 µL sample was injected in the split mode, with a split ratio of 1:150. For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. The column oven temperature program was the same as in the GC analysis. Helium was used as a carrier gas at a flow rate of 1.5 mL min^{-1}. Mass range was 30–400 m z^{-1}, while the injector and MS transfer line temperatures were set at 220 and 290 °C, respectively. The percentage composition of the oils was computed by the normalization method from the GC peak areas, which were calculated as the mean values of two injections of each oil sample, without using response factors. The identity of the components was confirmed based on comparisons of the retention index, retention times and mass spectra with those obtained from the libraries Nist 05 and Wiley 07 [35].

2.4. Determination of the Antioxidant Activities of the Essential Oils

The antioxidant activity was assessed by free radical-scavenging activity (DPPH). The free radical-scavenging activity of oils was evaluated with 2,2-diphenyl-β-picrylhydrazyl [36]. Three milliliters of 4.10−5M DPPH were added to 1 mL of the essential oil diluted with ethanol at different concentrations (100, 50, 25, 10, 5 mg·mL^{-1}). The mixture was shaken and allowed to stand at room temperature for 30 min. The decrease in absorbance at 517 nm was measured against a blank using a spectrophotometer (Genesys 20 UV-VIS, Thermo Fisher Scientific, Walham, MA, USA). Butylated hydroxyanisole (BHA) was used as positive control. The radical-scavenging activity of samples, expressed as percentage inhibition of DPPH, was calculated according to the formula: % inhibition = ((AB−AA)/AB) × 100, where AB and AA are the absorbance values of the control and of the test sample, respectively. All analyses were performed in triplicate.·

2.5. Statistical Analysis

All measurements were repeated three times for each assay. Results are presented as mean ± SD. Data were analysed (ANOVA, Tukey test) using Windows SPSS, version 20.0 (IBM Corp., Armonk, NY, USA). For compared treatments, p-values ($p < 0.05$) were regarded as statistically significant.

3. Results and Discussion

3.1. In Vitro Cultures

The qualitative characteristics of explants are often decisive for the successful passage of the sterilization stage and placement in aseptic conditions. It is important that the plants are free from the presence of phytopathogenic microflora and fungal spores, which are inherent in septic conditions in vivo. Factors that can complicate this stage include: pubescence of the surface of plant stems and leaves, the increased surface area of such explants and the complexity of their surface structure.

When introduced into in vitro culture, the peppermint cultivars without noticeable pubescence (Tik-Tak, Zabava) demonstrated an explant viability of 98.8% to 94.4%. Under the same sterilization conditions, the cultivar Serebristaya with strong pubescence showed a viability of 82.2%. When working with explants with strong stem pubescence, it is necessary to either increase the number of explants or increase the exposure time by 1–2 min in sodium hypochlorite.

The statistical analysis showed that the conditions of microclonal propagation had a statistically significant effect on characteristics such as plant height, number of shoots, and root length in all the chemotypes participating in the experiment (cultivars Zabava, Tik-Tak, Serebristaya) (Table 1). The active development of the main shoots (1.1–3.9 shoots per explant) from 3.3 to 6.5 cm in length was observed during micropropagation of cultivars Zabava, Tik-Tak, and Serebristaya. Taller plants were obtained in all cultivars on V-K control medium. Moreover, the studied cultivars showed different reactions: cv Tik-Tak had a maximum growth rate that was 1.3-1.5 times higher than cv Zabava and Serebristaya. Plant height was 10% lower on V-1 medium, and 18–24% lower on V-2 medium in all cultivars studied.

The development of taller plants on the control medium after four weeks of cultivation is associated with the absence of cytokinins in this medium. Cytokinins usually suppress cell elongation and promote cell division in plant shoots. They are primarily involved in the growth and differentiation of cells and affect the apical dominance and growth of the axillary primordia. Cytokinins play a crucial role in the favorable growth of plants under conditions of tissue culture [37–39]. Characteristically, the number of newly formed lateral shoots is increased. The number of explants on the control medium for all three cultivars was 2 and 2.5–3 times less than on the V-1 and V-2 media, respectively. The number of new nodal segments in cultivars Zabava and Tik-Tak was slightly higher on medium V-1, and on medium V-2, in all three cultivars this indicator was statistically significantly lower by 17–21% compared to the control medium. In all studied cultivars, the multiplication factor was the lowest on the control V-K medium (4.3-8.3) and almost twice as high on the experimental media, and did not differ statistically significantly between the V-1 and V-2 media (Figure 1). The cv Tik-Tak had the maximum multiplication factor and cv Serebristaya had the minimum.

Full MS medium supplemented with 1.0 mg·L^{-1} IBA is optimal for in vitro root induction in mint [17,22,40]. A number of studies have found that mint shoots can form roots spontaneously [41,42]. This process was observed in our work as well. Therefore, further treatment with auxins to accelerate rooting was not carried out. This is an advantage of in vitro propagation as it saves significant time and avoids the use of auxins. The frequency of root formation was 80–90%. The greatest number of roots was formed in cv Zabava, and the root length was longer in cv Tik-Tak (Table 1).

Table 1. Average values of indicators after four weeks of in vitro culture of initial explants of peppermint.

Chemotype	Nº of New Lateral Shoots			Plant Height (cm)			Nº of New Nodal Segments			Root Length (cm)		
	V-K	V-1	V-2	V-K	V-1	V-2	V-K	V-1	V-2	V-K	V-1	V-2
Zabava	$1.3 \pm 0.1_{c}$	$2.7 \pm 0.1_{b}$	$3.3 \pm 0.2_{a}$	$5.0 \pm 0.2_{a}$	$4.5 \pm 0.1_{b}$	$4.1 \pm 0.3_{bc}$	$4.1 \pm 0.4_{a}$	$4.2 \pm 0.5_{a}$	$3.5 \pm 0.3_{b}$	$2.1 \pm 0.1_{b}$	$3.5 \pm 0.2_{a}$	$1.5 \pm 0.2_{c}$
Tik-Tak	$1.5 \pm 0.2_{c}$	$3.2 \pm 0.4_{ab}$	$3.9 \pm 0.2_{a}$	$6.5 \pm 0.2_{a}$	$5.8 \pm 0.2_{b}$	$5.1 \pm 0.4_{c}$	$5.5 \pm 0.3_{ab}$	$5.9 \pm 0.4_{a}$	$4.5 \pm 0.6_{b}$	$4.8 \pm 0.3_{b}$	$6.5 \pm 0.3_{a}$	$4.2 \pm 0.2_{bc}$
Serebristaya	$1.1 \pm 0.1_{b}$	$2.5 \pm 0.3_{a}$	$2.8 \pm 0.2_{a}$	$4.3 \pm 0.1_{a}$	$3.9 \pm 0.2_{bc}$	$3.3 \pm 0.3_{c}$	$3.9 \pm 0.2_{a}$	$3.7 \pm 0.5_{a}$	$3.1 \pm 0.3_{b}$	$2.6 \pm 0.2_{ab}$	$3.0 \pm 01_{a}$	$1.8 \pm 0.1_{c}$

Values followed by the same letter within a line are not significantly different for each medium according to Tukey's test at $p \leq 0.05$, that is, a > b > c.

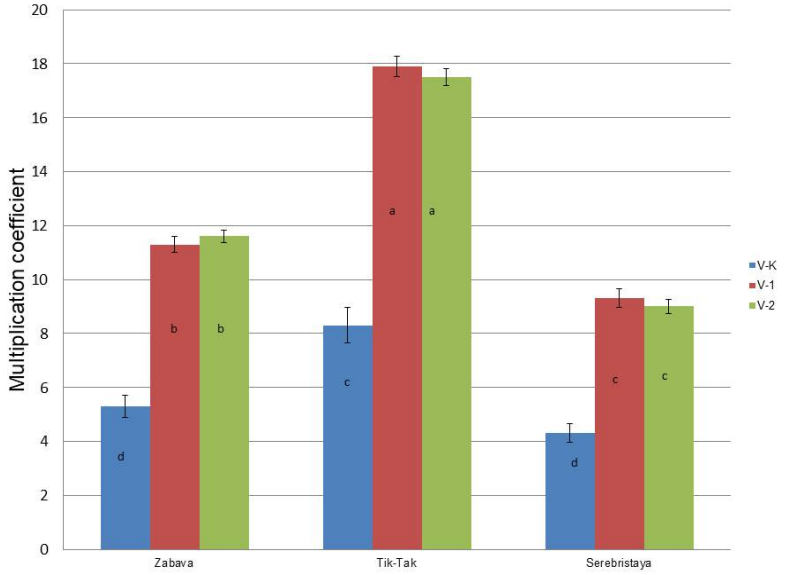

Figure 1. Significant effects of media on the multiplication coefficient in developing peppermint explants. Different letters indicate significant differences between media according to Tukey's test ($p \leq 0.05$).; bars represent standard deviations.

It is rather difficult to compare the effect of two BAP concentrations on the shooting and rooting processes observed in our studies and in the experiments of other researchers, since the original plants differ in genotype and hemotype. However, according to the morphological parameters, our results agree with the data described elsewhere. Therefore, when choosing the most optimal nutrient medium for clonal micropropagation of varieties Zabava, Tik-Tak, and Serebristaya, it was found that V-1 medium containing 0.5 mg·L^{-1} BAP was the most suitable. V-1 medium provides both a high multiplication factor and a maximum frequency of root formation, which makes it possible to exclude the in vitro rooting stage and immediately transfer the rooted plants for in vivo adaptation. The in vitro rooted plants were successfully acclimatized. To optimize the conditions for adaptation of rooted mint shoots to in vivo conditions, a unique natural substrate with high water-holding capacity and aseptic properties, that is, sphagnum moss was used [43,44]. Overall survival rate exceeded 95%.

3.2. Peppermint's Essential Oil Composition

The analysis of EOs by GC-MS in control plants and acclimated regenerants in the first and second year of vegetation revealed a total of 32 compounds, which ranged from 90.5 to 97.8% of the total amount of EOs (Table 2), with a predominance of oxygenated monoterpenes (up to 80%), such as menthol (up to 69.9%) and menthone (up to 35.0%). The predominance of menthol over other components indicates that these varieties can be attributed to the menthol chemotype. The Russian Pharmacopoeia XIII (2013) only determines the menthol content in raw peppermint, that is, from 30 to 60% [44,45]. At the same time, the European Pharmacopoeia (2010) postulates that terpenes in *M.*×*piperita* essential oil used in the pharmaceutical industry in Europe should be within the following ranges: limonene 1.0–5.0%, cineole 3.5–14.0%, menthone 14.0–32.0%, menthofuran 1.0–9.0%, isomenthone 1.5–10.0%, menthyl acetate 2.8–10.0%, isopulegol—maximum 0.2%, menthol 30.0–55.0%, pulegone—maximum 4.0%, and carvone—maximum 1.0% [33]. According to these legal requirements, all three cultivars (Zabava, Tik-Tak and Serebristaya) can be used in the pharmaceutical industry.

Table 2. The content of EO in plant biomass of peppermint cultivars, depending on the growing conditions, %.

Compound	Zabava			Tik-Tak			Serebristaya		
	Control	In vitro		Control	In vitro		Control	In vitro	
		1st Vegetation Year	2nd VegetationYear		1st Vegetation Year	2nd Vegetation Year		1st Vegetation Year	2nd Vegetation Year
α-pinene	0.7 $_{ab}$	0.8 $_{a}$	0.6 $_{b}$	0.6 $_{a}$	0.2 $_{c}$	0.4 $_{b}$	0.9 $_{ab}$	1.0 $_{a}$	0.8 $_{b}$
sabinene	0.3 $_{ab}$	0.3 $_{ab}$	0.4 $_{a}$	0.3 $_{a}$	0.2 $_{ab}$	0.2 $_{ab}$	0.6 $_{a}$	0.6 $_{a}$	0.5 $_{ab}$
β-pinene	0.7 $_{a}$	0.7 $_{a}$	0.8 $_{a}$	0.6 $_{a}$	0.3 $_{b}$	0.3 $_{b}$	1.2 $_{a}$	1.2 $_{a}$	1.0 $_{b}$
myrcene	0.5 $_{ab}$	0.6 $_{a}$	0.4 $_{b}$	0.4 $_{a}$	0.3 $_{a}$	0.3 $_{a}$	0.3 $_{a}$	0.2 $_{a}$	0.2 $_{a}$
3-octanol	0.5 $_{a}$	0.4 $_{a}$	0.4 $_{a}$	0.6 $_{a}$	0.3 $_{b}$	0.5 $_{a}$	0.3 $_{b}$	0.2 $_{bc}$	0.6 $_{a}$
α-terpinene	0.1 $_{a}$	0.2 $_{a}$	0.2 $_{a}$	0.1 $_{a}$	0.1 $_{a}$	0.1 $_{a}$	0.3 $_{a}$	0.2 $_{a}$	0.2 $_{a}$
ρ-cymene	0.1 $_{a}$	0.1 $_{a}$	0.1 $_{a}$	0.1 $_{a}$	t	0.1 $_{a}$	0.4 $_{ab}$	0.1	0.5 $_{a}$
d-limonene	1.5 $_{c}$	2.6 $_{b}$	2.8 $_{a}$	4.0 $_{a}$	1.7 $_{b}$	1.1 $_{bc}$	2.1 $_{bc}$	2.3 $_{b}$	3.0 $_{a}$
1,8-cineole	1.1 $_{b}$	1.2 $_{b}$	2.2 $_{a}$	1.2 $_{c}$	1.8 $_{b}$	2.3 $_{a}$	6.5 $_{a}$	5.5 $_{b}$	5.1 $_{bc}$
trans-ocimene	0.1 $_{a}$	0.1 $_{a}$	t	0.1 $_{b}$	0.3 $_{a}$	0.1 $_{b}$	0.2 $_{a}$	0.2 $_{a}$	0.1 $_{a}$
γ-terpinene	0.1 $_{a}$	0.2 $_{a}$	0.1 $_{a}$	0.1 $_{a}$	0.1 $_{a}$	0.1 $_{a}$	0.6 $_{a}$	0.7 $_{a}$	0.5 $_{b}$
trans-sabinene hydrate	0.2 $_{a}$	0.3 $_{a}$	0.3 $_{a}$	t	t	t	0.4 $_{a}$	0.1	0.3 $_{ab}$
linalool	0.1 $_{a}$	0.2 $_{a}$	0.1 $_{a}$	0.1 $_{a}$	0.1 $_{a}$	0.1 $_{a}$	t	t	t
menthone	8.3 $_{c}$	14.4 $_{a}$	9.9 $_{b}$	10.8 $_{c}$	18.0 $_{a}$	11.2 $_{b}$	27.1 $_{c}$	35.0 $_{a}$	26.8 $_{b}$
menthofuran	0.1 $_{a}$	0.1 $_{a}$	t	t	1.0	t	3.6 $_{c}$	7.3 $_{a}$	4.9 $_{b}$
iso-menthone	3.2 $_{a}$	2.4 $_{b}$	2.0 $_{bc}$	4.0 $_{c}$	11.6 $_{a}$	5.3 $_{b}$	3.2 $_{b}$	4.4 $_{a}$	2.7 $_{c}$
neo-menthol	0.1 $_{a}$	0.1 $_{a}$	0.2 $_{a}$	0.2 $_{bc}$	0.5 $_{a}$	0.3 $_{b}$	0.7 $_{a}$	0.4 $_{bc}$	0.5 $_{b}$
menthol	56.5 $_{a}$	23.3 $_{d}$	51.3 $_{b}$	66.6 $_{a}$	29.8 $_{d}$	59.9 $_{b}$	36.6 $_{a}$	10.6 $_{d}$	32.1 $_{b}$
iso-menthol	3.7 $_{b}$	2.4 $_{c}$	5.5 $_{a}$	1.5 $_{b}$	1.0 $_{c}$	3.1 $_{a}$	2.5 $_{b}$	2.3 $_{bc}$	4.4 $_{a}$
α-terpineol	0.1 $_{b}$	0.3 $_{a}$	0.1 $_{b}$	0.1 $_{a}$	0.2 $_{a}$	0.2 $_{a}$	0.3 $_{a}$	0.3 $_{a}$	0.4 $_{a}$
pulegone	1.0 $_{d}$	25.3 $_{a}$	2.4 $_{c}$	0.7 $_{d}$	22.5 $_{a}$	1.8 $_{c}$	3.1 $_{d}$	19.9 $_{a}$	5.7 $_{c}$
carvone	0.2 $_{a}$	0.1 $_{a}$	0.1 $_{a}$	0.1 $_{a}$	0.1 $_{a}$	t	0.1 $_{c}$	0.9 $_{a}$	0.3 $_{b}$

Table 2. *Cont.*

Compound	Zabava			Tik-Tak			Serebristaya		
	Control	In vitro		Control	In vitro		Control	In vitro	
		1st Vegetation Year	2nd VegetationYear		1st Vegetation Year	2nd Vegetation Year		1st Vegetation Year	2nd Vegetation Year
p-menthane-8-ol	t	t	t	t	t	t	0.1 $_{ab}$	0.2 $_{a}$	0.2 $_{a}$
piperitone	2.9 $_{d}$	4.4 $_{a}$	3.5 $_{c}$	0.1 $_{c}$	1.0 $_{a}$	1.1 $_{a}$	0.5 $_{bc}$	1.6 $_{a}$	0.7 $_{b}$
Menthyl acetate	5.8 $_{a}$	3.3 $_{bc}$	3.9 $_{b}$	0.1 $_{d}$	6.7 $_{a}$	1.2 $_{c}$	0.1 $_{a}$	0.1 $_{a}$	0.1 $_{a}$
β-caryophyllene	0.1 $_{b}$	t	0.3 $_{a}$	0.2 $_{b}$	0.4 $_{a}$	0.4 $_{a}$	t	t	t
germacrene D	0.2 $_{a}$	0.2 $_{a}$	0.2 $_{a}$	0.8 $_{a}$	t	0.7 $_{a}$	t	t	t

t-trace < 0.05%; values followed by the same letter within a row are not significantly different according to Tukey's test ($p \leq 0.05$); 1st vegetation year—first vegetation year (2020); 2nd vegetation year—second vegetation year (2021).

The EO yield depends on many factors, such as in vitro and in vivo growing conditions, harvest date, post-harvest processing and others [46,47]. Thus, the maximum content of EO in control parent plants over the years of the study (2019-2021) was recorded in cv Tik-Tak, which was 3.29 ± 0.14% on average, the minimum was 2.55 ± 0.12% in cv Serebristaya (Figure 2). The content of EO was higher in both the first and in the second year of vegetation in acclimated plants of all cultivars; however, this excess in EOs content was not statistically significant in comparison with the control in cultivars Zabava and Serebristaya. It should be noted that earlier studies have already indicated that the use of growth regulators (in particular, BAP) increases the production of EOs and biomass in peppermint [3]. However, as the total EOs yield increases, the application of BAP often affects the production of secondary metabolites such as menthone, menthol, menthofuran, and pulegone [18,22,25,26]. There were no significant differences in the composition of EOs in the control and acclimatized plants. However, acclimatized plants showed a change in the percentage of specific compounds in the composition of the EOs (Table 2; Figure 3). Acclimation of plants of three peppermint cultivars in field conditions showed that in the first year of vegetation, the content of menthol in the EOs composition decreased 2.2–3.5 times. At the same time, pulegone and menthone predominated in the chromatographic profile of EOs in peppermint plants of all three cultivars. An increase in the content of isomenthone in the cv Tik-Tak and menthofuran in the cv Serebristaya was also noted. Pulegone is known to be the precursor of menthone and isomenthone in the biosynthesis of monoterpenes of the genus *Mentha*, and menthone and isomenthone are precursors of menthol and isomenthol, respectively [20,21]. Therefore, the accumulation of pulegone as the main component of EOs indicates the suboptimal functioning of reducing enzymes that catalyze the transformation of pulegone into menthone, and subsequently into menthol. This observation is consistent with other studies in which microcloned shoots accumulate pulegone as a major component instead of menthone and menthol [42]. A comparative evaluation of EOs in control plants and plants of the second year of vegetation of the three peppermint cultivars showed significant fluctuations in the content of the minor components of the EOs (Table 2). For the major components of the EOs, the variability was lower and was mainly associated with isomenthone, isomenthol, and *d*-limonene. In the EO composition of the three varieties of peppermint plants from the second year of vegetation, menthol (32.1–69.9%) and menthone (9.9–26.8%) prevailed, and the content of pulegone decreased to levels characteristic of the EO profile of control plants. An increase in the percentage of menthofuran was observed in the EOs of acclimated plants of cv Serebristaya.

In the first year of vegetation, the menthofuran content increased 2.5 times; in the second year, it decreased, but still exceeded the level in control plants by 1.5 times. In addition, the content of a minor component such as p-menthane-8-ol (p-menthane lactone) in the EO composition of acclimated plants of this cultivar was increased from 0.06% in control plants, to 0.18% and 0.16% in plants of the first and second year of growing, respectively. It is known that the biosynthetic pathway of p-menthane lactone is closely related to menthofuran [48]. One of the derivatives of p-menthane lactone is menthofurolactone, which has a significant effect on the quality of peppermint EOs. In particular, menthofurolactone has an interesting odor, which has been described as pleasant, fresh, mint, and caramel-spicy [22]. It was demonstrated that despite moderate changes in this EO component, the resulting plants had a more pleasant, fresh mint aroma. This result suggests that the biosynthetic pathway leading to p-menthanlactone derivatives via menthofuran plays a significant role in the formation of the characteristic peppermint aroma. This change in the composition of EOs could provide an attractive opportunity to grow this peppermint variety, which can be used in the cosmetics industry.

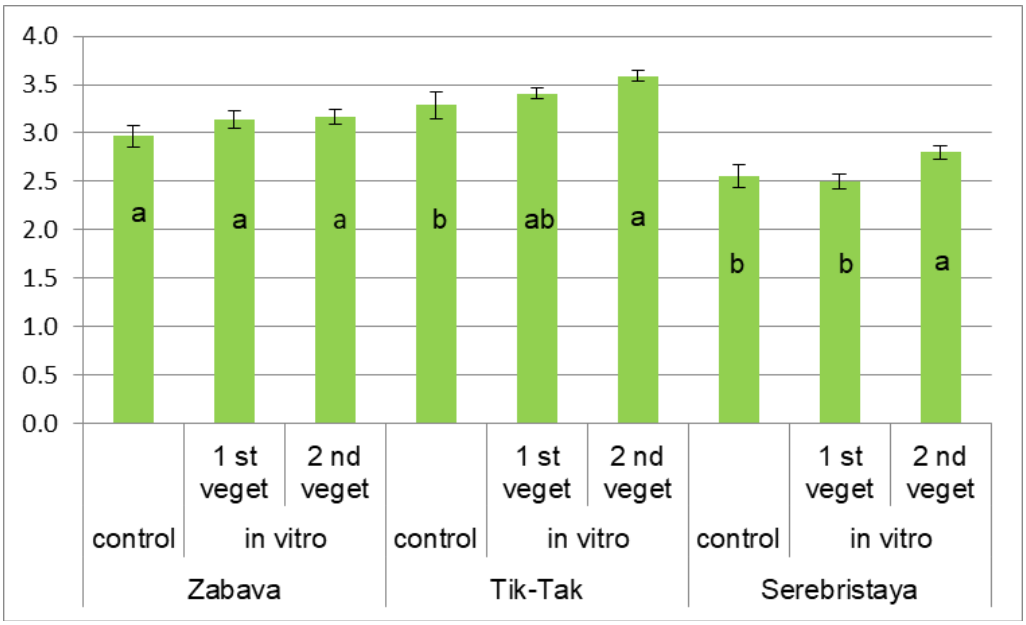

Figure 2. The content of EO in the aerial part of peppermint plant, depending on the growing conditions, %. For each chemotype, columns followed by the same letter are not significantly different according Tukey's test ($p \leq 0.05$); bars represent standard deviations. 1st vegetation year—2020; 2nd vegetation year—2021.

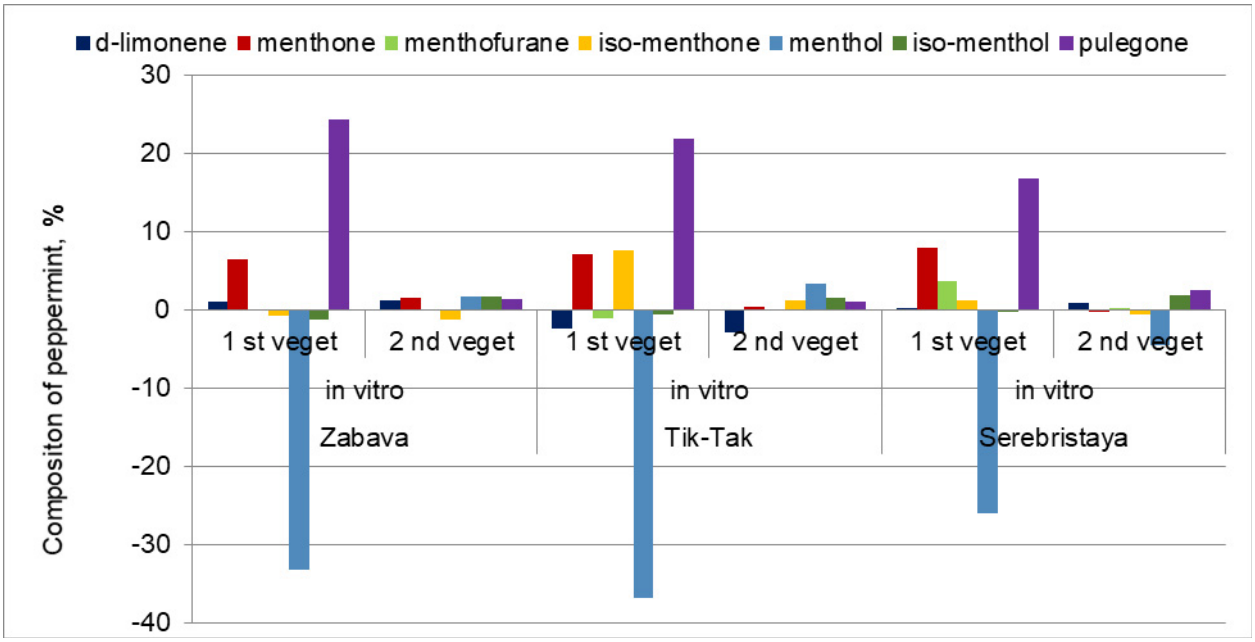

Figure 3. Differences in the content of individual components of EOs extracted from in vitro propagated peppermint cultivars Zabava, Tik-Tak and Serebristaya, which were cultivated in field conditions after acclimation in the 1st (2020) and 2nd (2021) years of vegetation, in comparison with the parental forms grown in the field.

It is generally accepted that the composition of EOs of in vitro regenerants should be restored to the composition in the parental forms 120 days after the transfer of plants to field conditions [16]. Our analyses only confirmed the complete recovery of the EO profiles by the second growing year in all three cultivars. However, it should be noted that one of the reasons for the decrease in menthol and an increase in menthone in the composition of EOs in plants of the three cultivars may be the climatic conditions in the first year of the vegetation season. The air temperature is known to be one of the main regulating factors

during the period of intensive EO biosynthesis. The sum of the effective temperatures for the growing season needs to be 3200–3400 °C to obtain the highest quality EO composition. This indicator is slightly lower (about 2400–2600 °C) in the Moscow region [15,47]. Despite the fact that the weather anomalies observed in recent years have led to a significant climate warming and an increase in the sum of effective temperatures in Central Russia, it is probably the low sum of the effective temperature of the growing season in 2020 (2123 °C) that resulted in a decrease in the yield of menthol in the composition of EOs in acclimatized peppermint plants of three cultivars in the first year of vegetation. On the other hand, the hot summer of 2021 (the sum of the effective temperatures of the growing season in 2021 was 2854 °C) contributed to the high content of menthol in the EOs in peppermint plants of three cultivars in the second vegetation season.

3.3. Antioxidant Activity of Peppermint Essential Oil

EO samples taken from control and acclimated peppermint plants of the second year of vegetation were analyzed to determine their antioxidant activity. The analysis showed that all EO samples of the studied plants function as scavengers of free radicals. The IC_{50} values in control plants varied from 17.99 ± 2.56 to 29.88 ± 1.05 mg·mL^{-1} (Figure 4). The antioxidant activity of EOs in acclimated peppermint plants of three cultivars did not differ statistically significantly from the control samples. The lowest antioxidant activity was recorded in the EOs from plants of cv Serebristaya (18.07 ± 1.95), and the highest was in cv Zabava (31.88 ± 2.06).

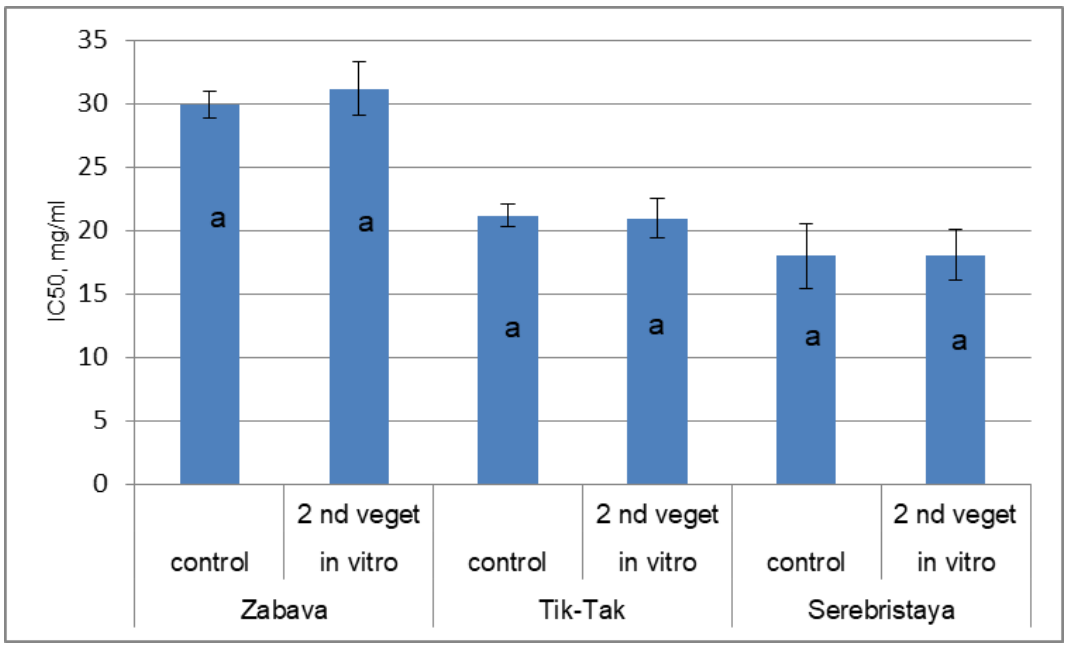

Figure 4. Antioxidant activity of the EOs in peppermint cultivars depending on the growing conditions, IC_{50} (mg·mL^{-1}). 2nd veget—second vegetation year. Values followed by the same letter (a) were not significantly differed for each variant ($p \leq 0.05$).

Our results are generally similar to those of Ramos et al. (2017) [49]. The authors state that peppermint has potent antioxidant properties. The antioxidant activity of peppermint EOs is associated with the presence of menthol and menthone, which have an aromatic ring and a hydroxyl group in their molecular structure [49]. In addition, the high neutralizing activity of peppermint EOs can be explained by the presence of compounds such as 1,8-cineole, piperitone and sesquiterpene hydrocarbons in the EO.

4. Conclusions

Our study on the influence of micropropagation conditions and the process of acclimatization in the field on the composition of the EOs of three varieties of peppermint showed that the use of the growth regulator BAP in a concentration of 0.5 mg·L^{-1} is optimal for micropropagation of the studied cultivars. The content of EOs in peppermint plants of the three cultivars acclimated in field conditions was higher in both the first and second years of vegetation as compared to control plants—in the cv Zabava by 0.17-0.20%, in the cv Tik-Tak by 0.12-0.30%, and in the cv Serebristaya by 0.25%.

The chemotype of cultivars Tik-Tak and Zabava was characterized by a high content of menthol (66.6% and 56.5%, respectively) and menthone (10.8% and 8.3%, respectively). In cv Serebristaya, the content of menthol was 36.6%, menthone was 27.1%, menthofuran was 3.6%, isomenthone was 3.2%, and pulegone was 3.1%. Acclimatization after microclonal propagation of peppermint plants of the three cultivars showed that in the first year of vegetation, the content of menthol in the EOs' composition decreased by 2.2-3.5 times. Pulegone and menthone prevailed in the EO profile of peppermint plants for all three cultivars. Menthol and menthone predominated in the EOs of acclimated peppermint plants of the three cultivars in the second year of vegetation. If the menthol content was 32.1-69.9%, and the menthone content was 9.9-26.8%, the pulegone content decreased to a level characteristic of the EO profile in control plants.

The EOs of the studied *Mentha* × *piperita* cultivars have significant antioxidant activity. Herewith, the antioxidant activity of EOs from peppermint plants acclimatized after microclonal propagation did not differ statistically significantly from the control samples.

Thus, micropropagation of three *M.* × *piperita* cultivars by the proliferation of nodal fragments in vitro is a reliable method for rapid propagation that allows the production of the same secondary metabolites as in control plants.

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