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Can Ozone Alter the Chemical Composition and Membrane Integrity of *in vitro Melissa officinalis* Shoots?

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Ozone affects volatile organic compounds that protect plants from biotic and abiotic stress. *In vitro Melissa officinalis* shoots have been exposed to ozone (200 ppb, 3 h) in controlled environmental conditions: leaf pigments, membrane integrity and headspace composition have been assayed during fumigation and after the recovery period (3 h from the beginning of the exposure, FBE). At the end of the exposure, no injury has been observed in untreated and treated shoots, although an evident increase of lipid peroxidation was reported (+38.5 and +37.2% of TBARS levels in comparison with controls, respectively after 1 and 3 h FBE). The levels of total carotenoids significantly raised as normal response mechanism to oxidative stress. SPME-GS-MS analysis showed that, as a consequence of the fumigation, the trends of non-terpenoid compounds increased after 1 and 3 h FBE. This suggests that the concentration and the duration of the treatment have been enough to cause a breakdown of cells (as evidenced by increased TBARS levels) and involves an association between volatile products of lipoxygenase pathway (LOX products) and membrane degradation.

Keywords: essential oils, leaf pigments, lemon balm, lipid peroxidation, micropropagation, oxidative stress, SPME-GC-MS, volatile organic compounds.

Volatiles are low-molecular weight lipophilic compounds produced by plants, mainly constituted by isoprenoids as monoterpenes and sesquiterpenes, with high vapor pressure at ambient temperatures [1]. Due to their physical properties, these compounds easily cross cellular membranes and can be released into the surrounding environment [2]. Isoprenoids are constitutively produced by some plant families and stored in specific structures such as glandular trichomes or resin ducts [3]. Volatile organic compounds (VOCs) play a role in many eco-physiological functions and facilitate multiple interactions between plants and environment, from attracting pollinators to protecting themselves from natural enemies such as pathogens and herbivores [1]). In addition, VOCs have important functions in protecting plants from abiotic insults, such as oxidative stress [4]. Stress conditions may induce the synthesis of some volatiles that play important roles in atmospheric chemistry. During recent years, there has been a growing interest in assessing the atmospheric and ecological effects of ozone (O_3) on VOCs emissions. Isoprenoids affect the formation of O_3 in the troposphere and, at the same time, they are readily oxidized by O_3, thus resulting in a series of new compounds, e.g. isoprene [5].

Ozone is an important air pollutant that causes many negative physiological and biochemical effects in plants [6], due to its strong oxidative potential. Denaturation of lipid membranes, alteration of stomatal conductance and photosynthetic process are associated to O_3-stressed leaves [7]. Protection mechanisms include the synthesis of antioxidant substances, like carotenoids, phenolics, as well as other secondary metabolites [8].

Recently, O_3 stressed *in vitro* shoot cultures of *Melissa officinalis* L. (lemon balm) which exhibited an activation of enzymes of phenolic pathway and increased levels of lignin and rosmarinic acid [9], leading to a new role of O_3 as plant elicitor for the stimulation of secondary responses. Rosmarinic acid increased during the O_3 treatment and decreased after the recovery in filtered air [9]. Rosmarinic acid is widely distributed in leaf extracts of *Lamiaceae* [10] and is the dominant active phenolic compound in *M. officinalis*. Other important compounds present in lemon balm are essential oils, which are principally constituted by the monoterpenes citronellal and citral, (neral and geranial), β-ocimene and citronellol, accompanied by sesquiterpenes β-caryophyllene, germacrene D [11-12]. Lemon balm constituents have beneficial effects for their antioxidant, antibacterial, antiviral, sedative and spasmolytic properties [13-14].

It is known that O_3 modifies the VOCs composition in field grown plants [4], but poor evidences have been gained on *in vitro* plant cultures. The increased concentration of phenolic compounds observed in *M. officinalis in vitro* shoots [9] lead to a new investigation of the involvement of O_3 in essential oil composition in *in vitro* cultures. Recently, it has been demonstrated that the headspace composition of the *in vitro* medicinal plants *Crithmum maritimum*, *Rosmarinus officinalis*, *Salvia officinalis* and *S. hortensis* resulted very similar to that of the corresponding field-grown plants [15]. So, the aim of this study was to verify the effect of O_3 in the headspace composition of *in vitro M. officinalis* shoots.

At the end of the fumigation, no injury was observed in untreated and treated shoots, although membrane integrity was significantly affected by O_3 (Figure 1). During the exposure, an evident increase of lipid peroxidation was observed [+38.5 and +37.2% of thiobarbituric acid reactive substances (TBARS) levels in comparison with controls, respectively after 1 and 3 h from the beginning of the exposure (FBE)].

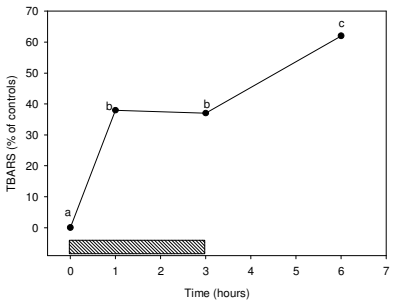

Figure 1: Percentage increases in TBARS (thiobarbituric acid reactive substances) levels in *in vitro Melissa officinalis* shoots exposed to ozone (200 ppb, 3 h) in comparison to controls maintained in filtered air. Variation coefficients were lower than 10%. Different letters indicate significant differences ($P \leq 0.05$, one-way ANOVA). The dashed box shows the fumigation period.

Similar findings have been obtained by Tonelli et al. [9] in *M. officinalis* shoots cultures exposed to O_3 (200 ppb, 3 h). At the end of the recovery time, the percentage rose to 62.8%, confirming the occurrence of lipid peroxidation following the treatment, as shown in other investigations conducted on whole leaves of naturally grown lemon balm [7, 16].

O_3 induced a marked increase in lutein, neoxanthin and violaxanthin content starting from 1 h FBE (+46.2, +79.5 and +58.5% in comparison to controls, respectively) and this rise was maintained at the end of the treatment and during the recovery time (Table 1). Among the oxygenated carotenoids, anteraxanthin showed significant difference in treated shoots in comparison to controls after 3 h FBE (+66.6%). During the treatment, a significant decrease of the β-carotene levels was observed (-27.2 and -30.9% after 1 and 3 h FBE, respectively) followed by a sharp increase to constitutive values during the recovery period. Oxidative stress induced an evident increase in total content of carotenoids starting from 1 h FBE (+30.5% in comparison to controls), reaching the maximum values of 45.8% and 41.8% at the end of the treatment and during the recovery time, respectively.

Table 1 Single and total carotenoids content (μg g^{-1} FW) in *in vitro Melissa officinalis* shoots maintained in filtered air (control) or exposed to ozone (200 ppb, 3 h). Data are shown as mean ± standard deviation. The measurements were carried out 1, 3 and 6 hours form the beginning of exposure. In each row, results of one-way ANOVA are reported and different letters indicate significant differences ($P \leq 0.05$).

		Ozone		
compounds	**control**	**1 h**	**3 h**	**6 h**
Lutein	0.13 ± 0.017 a	0.19 ± 0.002 b	0.23 ± 0.002 c	0.26 ± 0.003 d
Neoxanthin	0.44 ± 0.007 a	0.79 ± 0.008 b	0.90 ± 0.014 c	0.98 ± 0.008 d
Violaxanthin	0.53 ± 0.039 a	0.84 ± 0.065 c	0.87 ± 0.053 c	0.60 ± 0.029 b
Anteraxanthin	0.12 ± 0.024 a	0.09 ± 0.001 a	0.20 ± 0.013 c	0.15 ± 0.032 b
β-carotene	0.55 ± 0.024 b	0.40 ± 0.012 a	0.38 ± 0.002 a	0.52 ± 0.019 b
Total carotenoids	1.77 ± 0.110 a	2.31 ± 0.084 b	2.58 ± 0.080 c	2.51 ± 0.007 c

Since O_3 stress produced changes in the composition of primary isoprenoid metabolites, like carotenoids, which share the same biochemical precursors as the secondary isoprenoid metabolites (mono- and sesquiterpenes), we suppose that the increased carbon demand brought on by this pollutant might influence not only the amount of carbon allocated to secondary compounds, but also the carbon partitioning between several classes of isoprenoids, making one pathway more competitive than another.

In our experiment, patterns of volatiles have been characterized during the O_3 treatment and recovery under O_3-free air (Table 2). In untreated shoots (control), 14 compounds were identified accounting for 96.0% of the total volatiles. All the compounds were mono- and sesquiterpenes (both hydrocarbons and oxygenated ones) with some non-terpenes, such as C_9-C_{10} straight-chain aldehydes. In particular, oxygenated monoterpenes (10 compounds) were the most represented class of constituents, accounting for 85.1% of the total emission, the main one being geranial (67.1%), followed by geraniol (7.6%) and neral (5.7%). Sesquiterpene hydrocarbons were the second most represented class (3 compounds, 8.3%), β-caryophyllene being the main one (7.6%). Globally, in untreated shoots oxygenated compounds were more abundant than hydrocarbon ones (86.0 vs. 9.3%).

Table 2 - Main volatile compounds and classes of compounds (%) emitted by *in vitro Melissa officinalis* shoots maintained in filtered air (control) or exposed to ozone (200 ppb, 3 h). The measurements were carried out 1, 3 and 6 hours from the beginning of exposure.

			Control	Ozone		
		l.r.i.		**1 h**	**3 h**	**6 h**
nt	(*E*)-2-Hexanal	854	tr	0.4 ± *0.1*	2.1	1.5
nt	(*E*)-3-Hexenol	860	tr	0.5 ± *0.2*	2.3 ± *0.1*	4.5
nt	1-Octen-3-ol	982	tr	0.3	0.4	0.6 ± *0.2*
mh	Myrcene	993	1.0 ± *0.5*	1.0 ± *0.4*	0.5 ± *0.1*	0.6 ± *0.1*
nt	(*Z*)-3-Hexenol-acetate	1007	tr	tr	0.4 ± *0.1*	1.1 ± *0.1*
nt	Hexyl-acetate	1008	tr	0.2	0.1	0.2
nt	(*E*)-2-Hexenol-acetate	1018	tr	0.5	tr	2.1 ± *0.5*
om	Linalool	1101	tr	0.1	tr	0.1
nt	Nonanal	1102	0.3 ± *0.1*	tr	tr	0.1
om	Camphor	1143	tr	0.8 ± *0.3*	1.9	1.7 ± *0.3*
om	*cis*-Chrysanthenol	1162	0.6 ± *0.1*	0.8 ± *0.2*	1.2 ± *0.1*	1.2 ± *0.3*
om	*trans*-Mentha-1,7,8-dien-2-ol	1190	0.7 ± *0.2*	0.8 ± *0.2*	1.4 ± *0.2*	1.4 ± *0.4*
nt	Dodecene	1200	0.2	0.2	0.1	0.2 ± *0.1*
nt	*n*-Decanal	1204	0.2 ± *0.1*	0.2	0.1	0.4
om	Nerol	1229	1.0 ± *0.1*	2.6	0.4 ± *0.1*	2.1 ± *0.6*
om	Neral	1240	5.7 ± *1.4*	7.6 ± *1.0*	7.6 ± *0.3*	9.4 ± *2.0*
om	Geraniol	1252	7.6 ± *0.5*	5.1 ± *0.6*	4.0 ± *0.6*	5.4 ± *0.1*
om	Geranial	1269	67.1 ± *0.3*	70.6 ± *16.0*	64.7 ± *2.3*	55.4 ± 7.9
om	Methyl geranate	1322	1.5 ± *0.3*	0.7 ± *0.1*	0.8	1.4 ± *0.1*
om	Geranyl acetate	1382	0.9 ± *0.5*	0.1	tr	0.6
sh	β-Caryophyllene	1418	7.6 ± *0.3*	5.5 ± *2.9*	5.6 ± *1.0*	4.7 ± *0.1*
sh	α-Humulene	1450	0.2	0.2	0.3	0.2
sh	Allo-Aromadendrene	1461	0.5 ± *0.2*	2.3 ± *0.6*	4.2 ± *1.3*	4.5 ± *1.0*
os	Allo-Aromadendrene epoxy	1600	0.9 ± *0.3*	0.7 ± *0.2*	0.1	0.3 ± *0.1*
	Total (%)		**96.0**	**101.2**	**98.2**	**99.7**
om	Oxygenated monoterpenes		85.1	89.2	82.0	78.7
sh	Sesquiterpene hydrocarbons		8.3	8.0	10.1	9.4
os	Oxygenated sesquiterpenes		0.9	0.7	0.1	0.3
nt	Non-terpenoid substances		0.7	2.3	5.5	10.7
mh	Monoterpene hydrocarbons		1.0	1.0	0.5	0.6

Legend: l.r.i. = linear retention indices (DB-5 column); mh = monoterpene hydrocarbons; nt = non-terpenoid substances; om = oxygenated monoterpenes; os = oxygenated sesquiterpenes; sh = sesquiterpene hydrocarbons, tr = percentage <0.1%. Data are shown as mean (n=3) ± standard deviation.

In treated shoots, the compounds identified accounted for XX.X - 98.2% (1 and 3 h FBE, respectively). During the recovery period, 24 compounds were identified, accounting for 99.7% of the total volatiles.

O_3 altered the percentage of identified VOCs emission during and at the end of the exposure in comparison to controls.
According to previous results, the total VOCs emission significantly increased by a factor of about 1 in O_3 fumigated individuals in comparison to controls.
Heiden et al. [17] reported an increase of the total emission of a factor of about 2 in an O_3 resistant cultivar of *Nicotiana tabacum* (Bel-B) and up to a factor of 56 for a sensitive one (Bel-W3) within one day after O_3 treatment (120-170 ppb, 5 h).

The emission of total mono- and sesquiterpenes did not change significantly during the treatment, although the single compounds showed evident alterations. In particular, the oxygenated monoterpene neral increased to 7.6% after 1 and 3 h FBE. Similarly, the hydrogenate sesquiterpene allo-aromandendrene was present 2.3 and 4.2% instead of 0.5% in the control shoots.

The release of highly reactive VOCs may be involved in the mechanism by which O_3 damages the plants, according to the results obtained by Pellegrini et al. [18] in poplar leaves exposed to chronic O_3 (80 ppb, 10 consecutive days, 5 h d^{-1}).
As a consequence of the exposure carried out in this work (200 ppb, 3h) trends related to global non-terpenoid compounds were increasing (2.2% and 5.5% after 1 and 3 h FBE, respectively) in the lemon balm shoots, suggesting that the concentration and the duration of the treatment were sufficient to cause a breakdown of cell membranes (as evidenced by increased TBARS levels) and implying a relation between volatile products of lipoxygenase pathway (LOX products) and membrane degradation [19].
The emission of four LOX products [(*E*)-2-hexanal, (*E*)-3-hexenol, (*Z*)-3-hexenol-acetate and (*E*)-3-hexenol-acetate] has been observed. In detail, (*E*)-2-hexanal and (*E*)-3-hexenol, that in control shoots were present at trace levels, reached the content of 0.4 and 2.1%, 0.5 and 2.3% (after 1 and 3 h FBE, respectively) in fumigated leaves. This suggests that these compounds are the main LOX products originated from free fatty acids released by phospholipases from membranes in response to O_3 [20].

As a consequence of the exposure, the content of non-terpenoid compounds strongly raised (about 15-fold higher than controls, after 6 h FBE).
This finding suggests that (*i*) the composition of LOX products was affected by the severity of O_3 stress, and (*ii*) the most important biochemical pathway was altered during the recovery time.
Heiden et al. [21] demonstrated that corn plants exposed to O_3 (200 ppb, 2 days) have different emission patterns of C6 compounds in comparison to controls. The emission of total mono- and sesquiterpenes did not change significantly following the fumigation, although the single compounds showed evident alterations: in the oxygenated monoterpenes class, the most relevant increases were detected for nerol (about 2-fold higher than controls). Similarly, in sesquiterpene hydrocarbons, the most important increases were detected for allo-aromandendrene [21]..

Our results obtained in shoots cultures are consistent with the "opportunistic hypothesis" stating that the conditions (in this case O_3 exposure) affecting the synthesis of carotenoids alter the production and the emission of specific volatile non-essential isoprenoids together with the hypothesis that the pool size of carotenoids is connected to the emission capacity of VOCs.

Experimental

Plant material, culture conditions and ozone treatment. Explants were obtained from *in vitro* cultures of *M. officinalis* previously established in our laboratory. The shoots were placed on Murashige and Skoog (MS) medium [22] supplemented with 0.5 mg l^{-1} 6-benzylaminopurine (BAP), 3% (w/v) sucrose and 0.8% (w/v) agar [9]. Before the O_3 treatment, 3 week-old shoots were placed on MS medium deprived of BAP for one week to avoid any interaction of phytoregulators. Cultures were maintained in a growth chamber at 22±1 °C under 16 h photoperiod. Uniformly sized shoots (one month old) were placed in a controlled environment fumigation facility [23] under the same climatic conditions as in the growth chamber. O_3 fumigation was performed according to Tonelli et al. [9]. Plants were exposed for 3 h to a target O_3 concentration of 200 ppb (for O_3 1 ppb = 1.96 µg m^{-3}, at 20 °C and 101.325 kPa) in form of a square wave from to the 2nd to the 5th hour of the light period. After the end of the fumigation, plants were left to recover in the growth chamber under O_3-free air. Shoot samples were taken at 0, 1, 3 h FBE and after 3 h of recovery. Control shoots were exposed only to charcoal-filtered air in Perspex chambers. Plant material was stored at -80 °C until the time of analysis.

SPME analyses. Emitted volatiles were analyzed using a Supelco SPME device coated with polydimethylsiloxane (PDMS, 100 µm) in order to sample the headspace of 1 g of fresh plant constituted by *in vitro* plantlets maintained in filtered air or exposed to O_3. Each sample was introduced into a 50 ml glass conical flask and allowed to equilibrate for 30 min. After the equilibration time, the fiber was exposed to the headspace for 10 min at room temperature. Once sampling was finished, the fiber was withdrawn into the needle and transferred to the injector of the GC and GC-MS system, where the fiber was desorbed.

Gas Chromatography-FID. GC analyses were accomplished using an HP-5890 Series II instrument equipped with HP-WAX and HP-5 capillary columns (30 m × 0.25 mm, 0.25 µm film thickness), working with the following temperature program: 60 °C for 10 min, rising at 5 °C min^{-1} to 220 °C; splitless injection mode, injector temperature, 250 °C; carrier gas, nitrogen (2 ml min^{-1}); detector, dual FID. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their linear retention indices (l.r.i.) relative to the series of n-hydrocarbons, and on computer matching against commercial (NIST 98 and ADAMS) besides home-made library mass spectra built up from pure substances and components of known oils and MS literature data. Results were expressed as mean percentage obtained by FID peak area normalization.

Gas Chromatography-Mass Spectrometry. GC-MS analyses were performed with a Varian CP-3800 gas chromatograph equipped with a DB-5 capillary column (30 m × 0.25 mm; coating thickness = 0.25 µm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions: splitless injection mode, injector temperature at 250 °C; oven temperature was programmed from 60 °C to 240 °C at 3 °C min^{-1}; carrier gas, helium at 1 ml min^{-1}; injection of 0.2 µl (10% hexane solution). Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their l.r.i. relative to the series of *n*-hydrocarbons, and on computer matching against commercial (NIST 98 and ADAMS) and home-made library mass spectra built up from pure substances, components of known oils and MS literature data [24-29].

TBARS. TBARS assay, that quantifies oxidative stress by measuring the peroxidative damage to membrane lipids occurring with free radical generation and resulting in the production of MDA (malondialdehyde), was carried out according to Tonelli et al. [9].

Pigment analysis. Pigment analysis was performed by HPLC according to Döring et al. [16]. Thirty mg of leaves were homogenized in 3 ml 100% HPLC-grade methanol and incubated overnight at 4 °C in the dark. The HPLC analysis was performed at room temperature with a reverse-phase Dionex column (Acclaim 120, C18, 5 µm particle size, 4.6 mm internal diameter × 150 mm length). The pigments were eluted at a flow rate of 1 ml min^{-1} using 100 % solvent A (acetonitrile/methanol, 75/25, v/v) for the first 14 min to determine all xanthophylls, also the separation of lutein from zeaxanthin, followed by a 3 min linear gradient to 100% solvent B (methanol/ethylacetate, 68/32, v/v), 15 min with 100% solvent B for the elution of ß-carotene. The pigments were detected at 445 nm. Pure authentic standards were used to quantify the pigment content of each sample.

Statistical analysis. A minimum of four plants per treatment were used in each of the three repeated experiments. Following performance of the Shapiro-Wilk W test, biochemical data was analyzed using one-way analysis of variance (ANOVA) and comparison among means was determined by LSD post-test ($P \leq 0.05$). Since data obtained by control plants maintained in filtered air did not show significant differences (data not shown) during time course, comparison among means was carried out using controls measured at once before the beginning of fumigation. Analyses were performed by NCSS 2000 Statistical Analysis System Software.

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References

[1] Loreto F, Dicke M, Schnitzler JP, Turlings TCJ. (**2014**) Plant volatiles and the environment. *Plant, Cell and Environment*, ***37***, 1905–1908.
[2] Pichersky E, Sharkey TD, Gershenzon J. (**2006**) Plant volatiles: a lack of function or a lack of knowledge? *Trends in Plant Science, **11***, 421.
[3] McGarvey DJ, Croteau R. (**1995**) Terpenoid metabolism. *Plant Cell*, ***7***, 1015–1026.
[4] Loreto F, Schnitzler JP. (**2010**) Abiotic stresses and induced BVOCs. *Trends in Plant Science, **15***, 154–166.
[5] Calfapietra C, Mugnozza GS, Karnosky DF, Loreto F, Sharkey TD. (**2008**) Isoprene emission rates under elevated CO_2 and O_3 in two field-grown aspen clones differing in their sensitivity to O_3. *New Phytologyst, **179***, 55–61.
[6] Van Goethem TMWJ, Azevedo LB, van Zelm R, Hayes F, Ashmore MR. (**2013**) Plant species sensitivity distributions for ozone exposure. *Environmental Pollution, **178***, 1-6.
[7] Pellegrini E, Carucci MG, Campanella A, Lorenzini G, Nali C. (**2011**) Ozone stress in *Melissa officinalis* plants assessed by photosynthetic function. *Environmental Experimental Botany, **73***, 94-101.
[8] Castagna A, Ranieri, A. (2009) Detoxification and repair process of ozone injury: from O_3 uptake to gene expression adjustment. *Environmental Pollution*, ***157***, 1461–1469.
[9] Tonelli M, Pellegrini E, D'Angiolillo F, Petersen M, Nali C, Pistelli L, Lorenzini G. (**2015**) Ozone-elicited secondary metabolites in shoot cultures of *Melissa officinalis* L. *Plant Cell Tissue and Organ Culture, **120***, 617-629,
[10] Petersen M. (**2013**) Rosmarinic acid: new aspects. *Phytochemistry Reviews*, ***12***, 207-227.
[11] Tittel G, Wagner H, Bos R. (**1982**) Chemical composition of the essential oil from Melissa. *Planta Medica*, **46**, 91-98.
[12] Saeb K, Gholamrezaee S. (**2012**) Variation of essential oil composition of *Melissa officinalis* L. leaves during different stages of plant growth. *Asian Pacific Journal of Tropical Biomedicine*, 547-S549.
[13] Ribeiro MA, Bernardo-Gil MG, Esquivel MM. (**2001**) *Melissa officinalis* L., study of antioxidant activity in supercritical residues. *Journal of Supercritical Fluids, **21***, 51–60.
[14] Huang L, Abuhamdah S, Howes MJR, Elliot MSJ, Ballard C, Holmes C, Burns A, Perry EK, Francis PT, Lees G, Chazot PL. (**2008**) Pharmacological profile of essential oils derived from *Lavandula angustifolia* and *Melissa officinalis* with anti-agitation properties: focus on ligand-gated channels. *Journal of Pharmacy and Pharmacology*, ***60***, 1515–1522.
[15] Pistelli La, Noccioli C, D'Angiolillo F, Pistelli Lu. (**2013**) Volatile composition of micropropagated and field grown aromatic plants from Tuscany Islands. *Acta Biochimica Polonica, **60***, 43-50.
[16] Döring AS, Pellegrini E, Campanella A, Trivellini A, Gennai C, Petersen M, Nali C, Lorenzini G. (**2014**) How sensitive is *Melissa officinalis* to realistic ozone concentrations? *Plant Physiology and Biochemistry, **74**,* 156-164.
[17] Heiden AC, Hoffmann T, Kahl J, Kley D, Klockow D, Langebartels C, Mehlhorn H, Sandermann H Jr, Schraudner M, Schuh G, Wildt J. (**1999**) Emission of volatile organic compounds from ozone-exposed plants. *Ecological Applications, **9**,* 1160-1167.
[18] Pellegrini E, Cioni PL, Francini A, Lorenzini C, Nali C, Flamini G. (**2012**). Volatiles emission patterns in poplar clones varying in response to ozone. *Journal of Chemical Ecology, **38**,* 924-932.
[19] Hartikaine K, Nerg AM, Kivimäenpää M, Kontunensoppela S, Mäenpää M, Oksanen E, Rousi M, Holopainen T. (**2009**) Emissions of volatile organic compounds and leaf structural characteristic of European aspen (*Populus tremula*) grown under elevated ozone and temperature. *Tree Physiology, **29**,* 1163-1173
[20] Liavonchanka, A., Feussner, N. (**2006**) Lipoxygenases: occurrence, functions and catalysis. *Journal of Plant Physiology, **163**,* 348-357.
[21] Heiden AC, Kobel K, Langebartels C, Schuh-Thomas G, Wildt J. (**2003**) Emissions of oxygenated volatile organic compounds from plants. Part I: emissions from lipoxygenase activity. *Journal of Atmospheric Chemistry, **45**,* 143-172.
[22] Murashige T, Skoog F. (**1962**) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum,* **15**, 473-493.
[23] Nali C, Pucciariello C, Mills G, Lorenzini G. (**2005**) On the different sensitivity of white clover clones to ozone: physiological and biochemical parameters in a multivariate approach. *Water Air Soil Pollution, **164**,* 137-153.
[24] Adams RP. (1995) In *Identification of essential oil components by gas chromatography-mass spectroscopy*. Allured, Carol Stream, Illinois.
[25] Connolly JD, Hill RA. (**1991**) In *Dictionary of terpenoids.* Chapman & Hall, London.
[26] Jennings W, Shibamoto T. (**1980**) In *Qualitative analysis of flavor and fragrance volatiles by glass capillary gas chromatography*. Academic Press, New York.
[27] Massada Y. (**1976**) In *Analysis of essential oils by gas chromatography and mass spectrometry*. Wiley & Sons, New York.
[28] Stenhagen E, Abrahamsson S, McLafferty FW. (**1974**) In *Registry of mass spectral data*. Wiley & Sons, New York.

[29] Swigar AA, Silverstein RM. (**1981**) In *Monoterpenes*. Aldrich Chem. Comp., Milwaukee.