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Article

Essential Oil Composition of *Melissa officinalis* L. *in vitro* Produced under the Influence of Growth Regulators

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Foram investigados os efeitos de ácido indol acético (11,42 μmol L^{-1}) e benzilaminopurina (8,87 μmol L^{-1}) sobre o crescimento e composição do óleo essencial de plantas *in vitro* de *Melissa officinalis*. Plantas desenvolvidas em meio de Murashige e Skoog (MS) apresentaram o incremento de 1,4 vezes na proporção de nerol e de 4,1vezes de geraniol, quando comparadas às plantas *ex vitro.* Tratamentos com 11,42 μmol L^{-1} de ácido indol acético mais 8,87 μmol L^{-1} de benzilaminopurina resultaram em aumentos de 1,7 e 2,2 vezes na proporção de nerol e geraniol, respectivamente, em plantas de 60 dias. Estes aumentos podem estar associados à ação dos reguladores de crescimento, por estimularem o desenvolvimento vegetal (na organogênese e alongamento de brotos) e retardarem a oxidação de álcoois em aldeídos.

It was investigated the effects of indole-3-acetic acid (11.42 μmol L^{-1}), benzylaminopurine (8.87 μmol L^{-1}) on essential oil composition and on the growth of *Melissa officinalis in vitro* plants. *In vitro* plantlets developed on MS media, showed 1.4 times in the proportion of nerol and 4.1 of geraniol, when compared with *ex vitro* plants. Treatments with 11.42 μmol L^{-1} indole-3-acetic acid plus 8.87 μmol L^{-1} benzylaminopurine led to 1.7 and 2.2 fold in proportion of nerol and geraniol, respectively in 60-day-old whole plants. These increases might be associated with the action of growth regulators wich stimulate plant growth (shoot organogenesis and elongation) and delaying the alcohol oxidation to aldehydes.

Keywords: *Melissa officinalis*, essential oil composition, VOCs, GC/MS, growth regulators, tissue culture

Introduction

Melissa officinalis Lam. (Lamiaceae) also known as lemon balm, is a perennial herb that presents a lemon flavor. The infusion of its leaves is used in folk medicine due to its sedative and antispasmodic properties. The chemical composition of the essential oil of the *M. officinalis* leaf (0.02-0.3% dry weight) has been previously studied, being the major compounds citronellal (2-40%) and citral (mixture of neral and geranial: 10-30%), followed by β-caryophyllene, germacrene D, ocimene and citronellol.[1]

The growth of sprouts and roots and the composition of the essential leaf oil can be altered by addition of selected phytohormones into the nutrition medium.[2] Culture media supplemented with high concentrations of BA induced *Melissa officinalis* plants to accumulate more than 10% alloaromadendrene.[3]

In order to identify possible factors affecting the oil production and its quality, *M. officinalis* was cultured *in vitro* and the effect of growth regulators on the essential oil yield and composition were evaluated by gas chromatography and gas chromatography/mass spectrometry.

Experimental

Plant material

Seeds acquired from ISLAR, batch Nº 8250 were used to obtain the plant material. Erika Von Sohsten Medeiros

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and Angela Vaz (Jardim Botânico do Rio de Janeiro– RB, Brazil) undertook the taxonomic identification of this plant. A voucher nº RB – 365926 is deposited at the Herbarium of Jardim Botânico do Rio de Janeiro. The cultures were established according Silva.[4] The nodal segments were inoculated in the basal media Murashige and Skoog (MS),[5] MS without growth regulators (MS0) and were maintained under white light illumination (Sylvania fluorescent tubs) under 1.6 W m^{-2}, 30 μmol m^{-2} s^{-1} daily photoperiod of 16 hours at 25 ± 1 °C. Those plantlets were used as explants donors for growth regulators test effects. The methodology used follows Kreis and Mosandl.[1]

Treatment with growth regulators

Nodal segments of *in vitro* plantlets were cultured in four different media composition: MS0; MS plus 11.42 μmol L^{-1} Indole-3-acetic acid (IAA); MS plus 8.87 μmol L^{-1} Benzylaminopurine (BA) and MS + 11.42 μmol L^{-1} IAA plus 8.87 μmol L^{-1} BA (n = 100 per treatment) during 60 days.

Acclimatization

Plantlets grown in MS medium were transferred to small pots filled with soil. The *in vitro* raised plants were hardened in a greenhouse and transplanted to the field after 90 days. The acclimatized plants were maintained in the soil during one year (complete vegetative cycle).

Extraction

The *ex vitro* and *in vitro* plantlets fresh aerial parts, 100 g each, were submitted to hydrodistillation for 1.40 hour, in a Clevenger-type apparatus[6] in replicate (n = 2). The time between the isolation and analysis was the same in all experiments to preclude differences in composition due to external factors.[7]

Gas Chromatographic and Gas Chromatography-Mass Spectrometric analyses

Gas chromatography with flame ionization detection (GC/FID) was carried out on a Varian Star Model 3350 instrument using a capillary column coated with DB-5 (30 m x 0.25 mm i d , 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA). The GC oven was heated using the following program: 40 ºC to 220 ºC at 4 ºC min^{-1} with an initial isothermal period of 1 min, splitless. The detector and injector temperatures were held at 280 ºC. Hydrogen was used as carrier gas. The injection consisted of 1.0 μL of distilled oil diluted with hexane. The GC/MS analyses were carried out on a Hewlett-Packard (Agilent Technologies, Avondale, USA) Model 5972 MSD coupled to a HP 5890 GC. The GC conditions were the same as above, except that helium was used as carrier gas. The mass spectrometer was operated on electron impact mode at 70 eV. Molecular assignments were performed with the help of the Wiley 275 standard library of mass spectra, literature data,[8] authentic geraniol standard (Sigma, Part Number: G5135) and mass spectra interpretation besides the comparison with previously published elution order.[1] Quantification was performed from GC profiles using area percent, since in the literature the FID response factor for most of monoterpenes is determined as 1.[9]

Results and Discussion

Data from development in different culture media were compared with those obtained from plantlets treated with MS0.

The addition of 8.87 μmol L^{-1} BA or 11.42 μmol L^{-1} of IAA plus 8.87 μmol L^{-1} of BA to the MS medium resulted in a significant increase of shoot number per explant. When 11.42 μmol L^{-1} IAA was used as unique growth regulator, there was a significant increase in the number of shoots and in the number of new nodes per plantlet (Table 1); all other parameters were not significantly affected.

Table 1. Effect of type and concentration of plant growth regulators on growth, shoot proliferation after six weeks of culture: MS0 (control), MS + 11.42 μmol L^{-1} of IAA, MS + 8.87 μmol L^{-1} BA, MS + 11.42 μmol L^{-1} IAA + 8.87 μmol L^{-1} BA

Culture media	Number of shoots *per* explant	Number of nodes *per* plantlet	Shoot length (cm)	Root frequency (%)
MS0 (100)	1.17:1^{b}	5.20^{b}	5.73^{b}	98^{a}
IAA (100)	1.45:1^{b}	7.54^{a}	7.60^{a}	100^{a}
BA (100)	2.57:1^{a}	5.69^{b}	8.76^{a}	74^{b}
IAA+BA (100)	1.84:1^{c}	5.48^{b}	7.70^{a}	70^{b}

n = 100 *per* treatment. Different letters showed significant difference. p = 0.05

According to GC essential oil analyses there is a direct dependence on the composition content of growth regulators in culture media (Table 2). The principal components of essential oil of the aerial parts of *M. officinalis* are presented on Table 2: geranial (38.1; 43.6; 43.1; 35.5 and 30.6%), neral (26.7; 31.7; 29.9; 27.4 and 23.3%), geraniol (3.3; 15.3; 17.9; 18.3 and 18.0%) and nerol (1.2; 1.9; 3.1; 3.3 and 3.1%) in plants cultured *ex vitro* and on the media MS0; MS+IAA; MS+BA and MS+IAA+BA, respectively (Figure 3). Plantlets developed *in vitro*, on MS media, showed an increase of 1.4 fold in

Table 2. Major (over 0.1%) volatile organic components of aerial parts (100 g) from 45 days old *in vitro* plantlets cultivated in different media: MS0 (control), MS + 11.42 μmol L^{-1} IAA, MS + 8.87 μmol L^{-1} BA, MS + 11.42 μmol L^{-1} IAA + 8.87 μmol L^{-1} BA and *ex vitro* (6 months in the field)

Compounds	Mean peak area%				
	Ex vitro	*In vitro* Plantlets			
		Control – MS0	IAA	BA	IAA + BA
β-Myrcene	0.9	0.9	0.5	0.8	0.5
Linalool	0.3	0.3	0.2	0.3	0.3
2,6-Octadienoic, 4,5-dimethyl	2.3	1.4	0.9	0.8	0.5
Pulegone	3.3	1.4	1.0	0.9	0.6
Pujone	5.1	3.1	1.9	1.7	1.1
Nerol	1.2	1.9	3.1	3.3	3.1
Neral	26.7	31.7	29.9	27.4	23.3
Geraniol	3.3	15.3	17.9	18.3	18.0
Geranial	38.1	43.6	43.1	35.5	30.6
2,6-Octadienoic acid, 3,7-dimethyl-methyl ester	-	-	0.6	0.2	0.3
Geranyl acetate	-	-	0.3	0.3	0.2
trans-caryophyllene	0.3	0.3	0.3	0.2	0.2
Total	81.5	99.9	99.8	89.7	78.7

the proportion of nerol and 4.1 of geraniol, when compared with *ex vitro* cultured plants. Comparative analysis of the chromatographic profiles showed high percentage of neral and geranial in relation to nerol and geraniol in *ex vitro* plantlets and in MS0 (Figure 2), while in plantlets submitted to the growth regulators it was observed a decrease of these levels. However, when the medium was supplemented with MS+IAA, it was observed an increase in the percentage of neral and geranial, when compared with other treatments.

Due to growth conditions employed in this work, the amount of light, temperature, mineral composition of growth regulators, pH and age of the explants could be completely controlled.

In this way, the results obtained show that the addition of these regulators in the culture medium induced the delay of alcohol oxidation to aldehydes.

Comparison between *in vitro* plantlets cultivated in MS medium without growth regulators (control) and in MS medium plus different growth regulators shows that the presence of 8.87 μmol L^{-1} BA and 11.42 μmol L^{-1} IAA in the medium resulted in a significant increase of geraniol level (Table 2 and Figures 1 and 2).

Growth of sprouts and roots, and the composition of the essential leaf oil of *M. officinalis* can be altered by addition of selected phytohormones to the nutrition medium. The essential oil is normally dominated by sesquiterpene hydrocarbons. By addition of appropriate amounts of napthalene acetic acid (NAA), the composition changes to a specific essential oil, dominated by oxygenated monoterpenes. The accumulation of monoterpenes is reduced; sesquiterpene accumulation can also be enhanced by abscisic acid (ABA).[3]

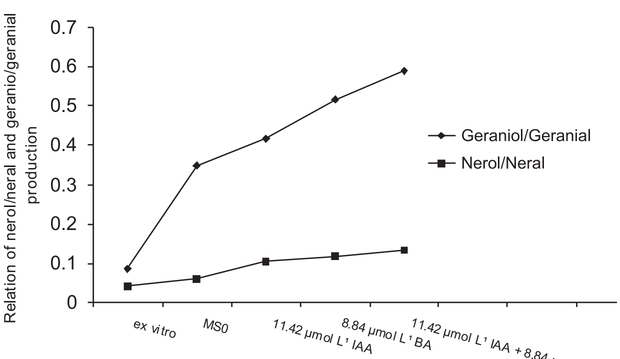

Figure 1. Relation of nerol/neral and geraniol/geranial production, in plants produced with and without growth regulators.

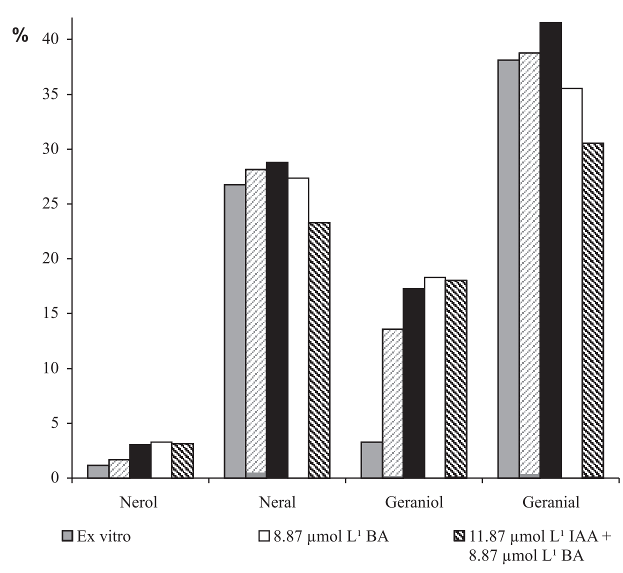

Figure 2. Percentage (%) of the most abundant components of Melissa *officinalis* essential oil.

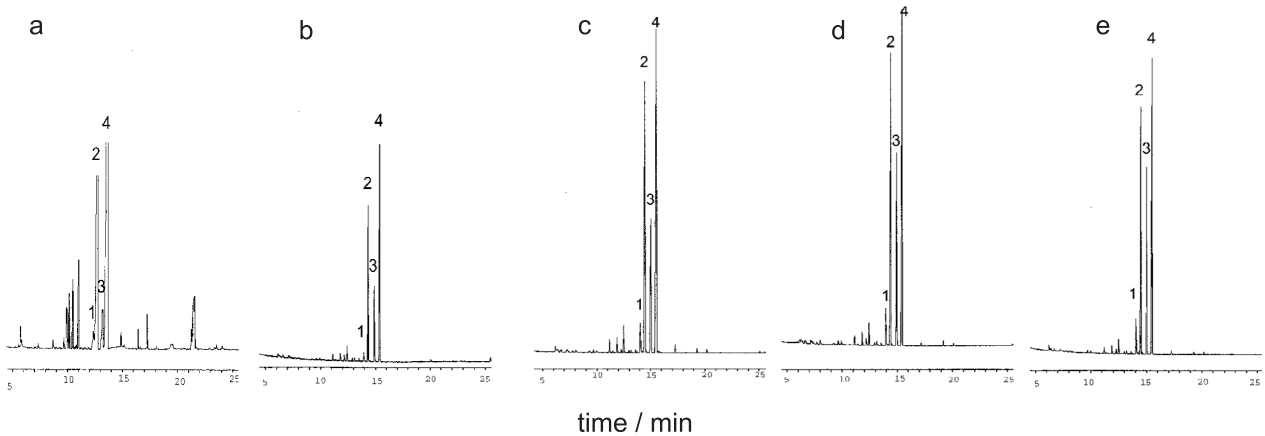

Figure 3. Gas chromatographic profiles of the essential oil of *in vitro Melissa officinalis* cultured in: A) *ex vitro*, B) MS0, C) MS + 11.42 μmol L^{-1} of IAA, D) MS + 8.87 μmol L^{-1} of BA and E) MS + 11.42 μmol L^{-1} IAA + 8.87 μmol L^{-1} BA, during 60 days. (**1**) nerol, (**2**) neral, (**3**) geraniol, (**4**) geranial.

The effects of auxin on gene activity, especially in the epidermis, explains the quantitative variation promoted by addition of auxin.[12] Auxins may affect the kind of proteins formed in a plant cell before or as soon as growth promotion starts, which could explain changes in the level of some substances through the modification of the cell enzymatic pattern.[13]

The effect of cytokinins on the levels of secondary metabolites was already observed. Cytokinins are able to stimulate the synthesis of betacyanins [12] and indole alkaloids.[13]

Field plants are exposed to considerable more stressful conditions, such as lower relative humidity, higher light levels, and herbivory those are the more stressful. The latter conditions tend to favor a greater essential oil accumulation.[11] On the other hand plantlets grown *in vitro* have been continuously exposed to a unique microenvironment that has been selected to provide minimal stress and nearly optimal conditions for plant multiplication.

The results obtained in the present study suggest that growth regulators (IAA and BA) influence on *M. officinalis* oil composition promote the delaying of the alcohol oxidation to aldehydes. It is very interesting, since geraniol has a great commercial value and is mainly utilized as fragrance fixative in the flavor industry.

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