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Changes in the essential oil composition of *Melissa officinalis* in response to elicitors methyl jasmonate and methyl salicylate

Simone da Silva[1], Cláudio Barbosa Moreira[2], Maria Apparecida Esquibel[2], Rosane Aguiar da Silva San Gil[3], Carlos Alberto da Silva Riehl[3], Alice Sato[4]

[1] Centro de Biotecnologia da Amazônia – CBA, Laboratório de Cultura de Tecidos Vegetais. E-mail: simonydasilva@gmail.com;
[2] Universidade Federal do Rio de Janeiro, Instituto de Biofísica Carlos Chagas Filho. E-mail: claudiobmoreira@gmail.com; esquibelma@gmail.com;
[3] Universidade Federal do Rio de Janeiro – UFRJ, Instituto de Química. E-mail: rsangil@iq.ufrj.br; riehl@iq.ufrj;
[4] Universidade Federal do Estado do Rio de Janeiro – UNIRIO, Departamento de Ciências Naturais. E-mail: alicesato@unirio.br.

Abstract

It was investigated the effects of methyl jasmonate (MeJa) and methyl salicylate (MeSA) on essential oil composition of *Melissa officinalis in vitro* plants. MeJA treatments showed an increase of 13% of geranial proportion during 24 h of exposition, when compared with the control plants, in MS0 medium. After 48h of treatment, in addition to the presence of 2-methyl-2-cyclopenten-1-one (8.5% of the oil), it was observed an increase of 1,531.2% of verbenol and 1,114.8% of citronellal. After 96 hours of MeJA exposition, any effect was observed on plant secondary metabolites production since the essential oil composition was similar to the control (MS0). Regarding MeSa treatments, with 72 hours of exposure, presented an accumulation of geranial 21.4% greater than the control.
Keywords: Secondary metabolites, tissue culture, aldehydes, alcohols

Resumo

Mudanças na composição do óleo essencial de *Melissa officinalis* em resposta aos eliciadores jasmonato de metila e salicilato de metila. Foram investigados os efeitos do jasmonato de metila (MeJa) e salicilato de metila (MeSa) na composição do óleo essencial de plantas *in vitro* de *Melissa officinalis*. Os tratamentos com MeJa mostraram um aumento de 13% da proporção de geranial, durante 24 h de exposição, quando comparado com o controle, em meio MS0. Após 48 horas de tratamento, além da presença de 2-metil-2-ciclopenten-1-ona (8,5% do óleo), foi observado um aumento de 1.531,2% de verbenol e 1.114,8% de citronelal. Após 96 horas de exposição ao MeJA, nenhum efeito foi observado na produção de metabólitos secundários, uma vez que a composição do óleo essencial foi semelhante a do controle (MS0). Com relação aos tratamentos com MeSa, em 72 horas de exposição, apresentou-se um acúmulo de geranial 21,4% maior que o controle.
Palavras chave: Metabólitos secundários, cultura de tecidos, aldeídos, alcoóis

Introduction

The essential oil of a plant is internationally defined as the volatile fraction of the plant obtained by hydrodistillation, steam distillation, dry distillation or a suitable mechanical process without heating plant materials (Simmonds, 2014). The parameters of the quantity and composition of the oils vary among species, beyond the influence of the environment where they develop. Act as allelopathic or irritants in plant protection against predation by insects and parasites infestation. Essential oils and their constituents have high potential pesticide (Marongiu et al., 2004; Dudareva & Negre, 2005; Phillips et al., 2006), in addition to having biological application as antimicrobial agents (Siani et al., 2000; Phillips et al., 2006).

When infected by pathogenic microorganism, plants respond with rapid activation of various spatially and

temporally regulated defense reactions. These responses include oxidative cross linking of cell wall proteins, production of phytoalexins, hydrolytic enzymes, incrustation of cell wall proteins with phenolics and finally hypersensitive death of plant cell. The molecules that stimulate the production of secondary metabolites are termed as elicitors (Namdeo, 2007).

Melissa officinalis L. (Lamiaceae) is a well-known herb used to give fragrance to different food and beverage products. It has also been used as a medicinal plant for treatment of headaches, gastrointestinal disorders, nervousness, and rheumatism (Carnat et al., 1998). The essential oil is a known antibacterial and antifungal agent and, it is also responsible for the mild depressive and spasmolytic properties of the plant (Mimica-Dukic et al., 2004).

Melissa essential oil is obtained by hydrodistillation and its low yield (0.02 to 0.40%) makes it from one of the most valuable classes of essential oils. Coupled with low incomes, important pharmacological properties, make lemon balm occupies a prominent place on the list of medicinal plants, making the price of its oil extremely high when compared to the essential oils of rose and orange blossom (Sorensen, 2000). The chemical composition of the essential oil of *Melissa officinalis* leaf (0.2-1% on dry weight) has been studied and the major compounds (10-40%) are citral (neral and geranial), followed by limonene, cineole, geraniol, ß-caryophyllene and spathulenol (Carnat et al., 1999).

Tissue culture provides means of rapid propagation of a large number of uniform plants while maintaining their genotype. Beneficial uses of tissue culture for the purpose of extraction of secondary metabolites include avoidance of collection of endangered wild species and production of secondary metabolites due to rapid growth of *in vitro* cultures (Arikat et al., 2004). *In vitro* development of plantlets of *Melissa officinalis* on MS medium related to *ex vitro* cultured plants, presented an increase of 1.4 fold of nerol proportion and 4.1 of geraniol (Silva et al., 2005).

Jasmonates are a class of plant hormones that mediate various aspects of gene and metabolic regulation, defense, stress responses, reproduction, and possibly communication. The role of JA in plants has received considerable attention, and various modes of action for JA and its methyl ester (MeJa) have been proposed (Farmer, 2007; Farmer and Ryan, 1990).

Methyl Jasmonate (MeJa) was first identified as a component of the essential oil of several plant species. Studies showed that exogenous jasmonates can promote senescence and act as growth regulators. Endogenous jasmonates are derived principally from linolenic acid and had been considered as controllers of secondary metabolites as well as defence systems (Turner et al., 2002). Subsequent research revealed that wounding and elicitors could cause jasmonates accumulation in plants. These results implied a role for jasmonates in plant defense that has been confirmed (Creelman and Mullet, 1997).

Jasmonates have also been associated with the increased production of volatile organic compounds (Van Poecke and Dicke, 2004). The exogenous application of the methyl ester, MeJa, has also been described as triggering a twofold increase in monoterpene and sesquiterpene accumulation in needles and a fivefold increase in total terpene emissions in the foliage of Norway spruce (Martin et al., 2003), as well as dramatic increases in terpenoid emissions in Nicotiana attenuate (Halitschke and Baldwin, 2004).

Methyl salicylate (MeSA) is known to be released by stressed plants (Peñuelas et al., 2007). It has been demonstrated that methyl salicylate, a derivative of salicylic acid that activates defense signaling pathways against pathogens in plants (Ryals et al., 1996, Tjeerd et al., 2010), also induce lima bean leaves to produce volatile compounds involved in HIPVs (Ozawa et al., 2000). As well it was shown that airborne MeSa may drive plants to produce and emit monoterpenes in a similar way of defense-related genes in other plants (Peñuelas et al., 2007).

Although MeJa and MeSa have been shown to be an inducer of secondary metabolites in many plants, the effect of them on secondary metabolites in *Melissa officinalis* plants have not been reported. Therefore, the main objective of this study was to estimate the effect of MeJa and

MeSa on the essential oil composition of *Melissa officinalis in vitro* cultured.

Materials and methods

Plant material

Seeds purchased from ISLA®, batch Nº 8250 were used to obtain the plant material. The taxonomic identification was deposited at the herbarium of the Instituto de Pesquisas do Jardim Botânico of Rio de Janeiro, Brazil (RB 365.926). The cultures were established according to Silva et al. (2005). The nodal segments were grown in the Murashige and Skoog basal medium – MS0 - (Murashige and Skoog, 1962) and were maintained under white light illumination (Sylvania fluorescent tubes) 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 (1994).

Methyl jasmonate and methyl salicilate treatments

MeJa and MeSa solution was purchased from Sigma-Aldrich added to ethanol (EtOH) in 1:9 proportions (RODRIGUEZ-SAONA et al., 2001). It was used 20 µL of the solution in cotton peaces that were put with the plants with 60 days of culture in MS0. The essential oil composition was evaluated 24, 48, 72 and 96 h after adding MeJa or MeSa and compared with control cultures. Control cultures were untreated during that time.

Extraction

The fresh aerial parts of *in vitro* plantlets (15 g each) were submitted to hydro distillation for 2 hours, in a Clevenger type apparatus (Rezende et al., 2004) 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 (Silva et al., 2004).

Gas chromatographic and gas chromatography-mass spectrometric analyses

Gas chromatography with flame ionization detection (GC/FID) was carried out using a Varian Star Model 3350 instrument with 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 using a VARIAN Model CP3800 coupled to a VARIAN 1200L MS/MS quadruple mass spectrometer, with VF-5MS (5% phenyl, 95% methyl polysiloxane) (30m x 0.25 mm x 0.25 µm) capillary column. 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 (ADAMS, 1995), authentic geraniol standard (Sigma, Part Number: G5135) and mass spectra interpretation besides the comparison with previously published elution order (Kreis and Mosandl, 1994).

It was injected the hydrocarbonets (C7-C26) moisture, in the same conditions. The constituents were identified by comparing linear Kováts Index (KI), their retention times (RT) and mass spectra with those obtained from the MS library.

Results and discussion

Essential oil compounds evaluation of plants submitted to methyl jasmonate (MeJa) treatment during 24, 48, 72 and 96 hours

Essential oil composition from plants submitted to different time expositions to MeJa treatment were compared with the one obtained from control plants. It has an essential oil

composition modification after 48 and 72 h. On the other hand, it was observed the reduction of alcohols (nerol and geraniol) accumulation in all treatments. In relation to the aldehydes, it was observed neral reduction in all treatments and an increase of geranial (13.1%) in 24h of treatment, in relation to the control (Figure 1 and Table 1).

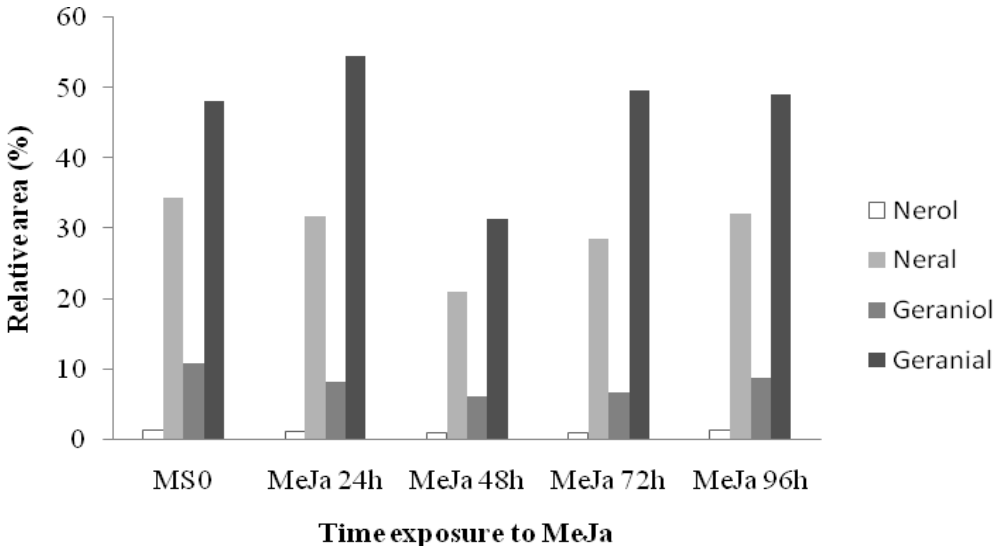

Figure 1. Effect of Methyl Jasmonate on essential oil compounds of *Melissa officinalis in vitro* plants.

Geraniol is a mixture of the two cis-trans isomers properly named geraniol (trans) and nerol (cis) (Chen and Viljoen, 2010). It has characteristic rose-like odour and the taste (at 10 ppm) is described as sweet floral rose-like, citrus with fruity, waxy nuances (Burdock, 2009). This monoterpene alcohol is a widely used fragrance material. A survey of consumer products revealed that it is present in 76% of investigated deodorants on the European market, included in 41% of domestic and household products and in 33% of cosmetic formulations based on natural ingredients and its production exceeds 1000 metric tons per annum (Rastogi et al., 1996, 1998, 2001). In addition, geraniol exhibits various biochemical and pharmacological properties. Researchers have shown geraniol to be an effective plant-based insect repellent (Barnard and Xue, 2004) and its potential as an antimicrobial agent has been highlighted in several studies (e.g. Bard et al., 1988). Geraniol exerts *in vitro* and *in vivo* antitumour activity against murine leukemia, hepatoma and melanoma cells (Burke et al., 1997; Yu et al., 1995a,b).

Citral is the name given to a natural mixture of two isomeric acyclic monoterpene aldehydes: geranial (*trans*citral, citral A) and neral (*cis*-citral, citral B) (Lewinsohn et al., 1998). Because of its characteristic lemon aroma, citral is of considerable importance in the food and flavor industry. Citral is also an important raw material used in the pharmaceutical, perfumery and cosmetics industries, especially for the synthesis of Vitamin A and ionones (Dawson, 1994). Citral possesses antifungal activity against plant and human pathogens (Yousef at al., 1978; Rodov et al., 1995), inhibits seed germination (Dudai et al., 1994), and has bactericidal (Asthana et al., 1992; Kim et al., 1995) and insecticidal properties (Rice and Coats, 1994).

Table 1. Major volatile organic constituents (over 0.1%) of aerial parts (15 g) from 60 days old *in vitro* plantlets, cultivated in MS0 (control) and submitted to MeJa exposure, during 24, 48, 72 and 96 hours.

Constituents	MS0 60 (Control)		MeJa 24h		MeJa 48h		MeJa 72h		MeJa 96h		KI
	RI	RA	RI	RA	RI	RA	RI	RA	RI	RA	
2-methyl-2-cyclopenten-1-one	-	-	-	-	-	8.5	-	9.15	-	-	-
dihydro-linalool	1134	0.26	1134	0.14	-	-	-	-	-	-	1134
trans-verbenol	1144	0.93	1144	0.88	1144	14.24	1144	0.91	1144	0.99	1144
Citronellal	1153	1.35	1153	1.29	1153	15.05	1153	0.96	-	-	1153
methyl salicylate	-	-	-	-	-	-	-	-	1190	4.34	1190
Nerol	1228	1.40	1228	1.10	1228	0.87	1228	0.98	1228	1.28	1228
Neral	1240	34.41	1240	31.69	1240	21.04	1240	28.46	1240	32.13	1240
Geraniol	1255	11.02	1255	8.20	1255	6.20	1255	6.73	1255	8.85	1255
Geranial	1270	48.21	1270	54.53	1270	31.49	1270	49.68	1270	49.05	1270
propanoic acid	1350	0.62	1350	0.56	1350	0.32	1350	0.65	1350	0.86	1350
geranyl acetate	1383	0.22	-	-	-	-	-	-	-	-	1383
cariophillene-<z->	-	-	1404	0.20	1404	0.54	1404	0.48	-	-	1404
α-humulene	-	-	-	-	1454	0.66	1454	0.58	-	-	1454
D-germacrene	1480	0.29	1480	0.77	1480	0.20	1480	0.27	-	-	1480
		98.71		99.36		99.11		98.85		97.5	

RI: Retention index; KI: Kovats index (Adams, 1995); RA: Relative area (%)

Between 24-72 h of MeJa exposure, it was detected the presence of z-caryophyllene. After 48 h of MeJa exposition, it was observed larger components diversity than the control (Figure 1 and Table 1).

After 48 h of treatment, it was observed 2-methyl-2-ciclopenten-1-one presence, corresponding to 8.5% of the total oil composition. This is a compound emitted by plants when they are ataqued for herbivores. Resistance against pathogens can also be increased by modified emission of green-leaf volatiles initially released upon herbivory (Shiojiri et al, 2006). The JA-mediated wound response following herbivore attack leads to indirect (volatile emissions) and direct defence (formation of defence proteins and small compounds) [Pieterse et al., 2006], and even mechanical wounding is sufficient to give a volatile pattern similar to that caused by herbivore attack (Mithöfer et al., 2005).

With 96 h, it was observed the presence of methyl salicylate in 4.3% of the total essential oil composition. Plants containing methyl salicylate produce this organic ester (a combination of an organic acid with an alcohol) most likely as an anti-herbivore defense. If the plant is infested with herbivorous insects, the release of methyl salicylate may function as an aid in the recruitment of beneficial insects to kill the herbivorous insects (James and Price, 2004). Aside from its toxicity, methyl salicylate may also be used by plants as a pheromone to warn other plants of pathogens such as tobacco mosaic virus (Shulaev et al., 1997). These results indicate that this compounds production was induced by the presence of MeJa in *M. officinalis* cultures.

The observed pattern of increased MeSa emissions was also found by Martin et al. (2003) in MeJA-treated Norway spruce leaves. The results of Ament et al. (2004) with *JA-synthesis* mutant tomato plants suggest that induced emissions of MeSA depend on JA at the transcriptional level. Methyl salicylate has also been found to be released after herbivore damage in other species, and to attract the enemies of herbivores (Dicke et al., 1999; Kessler and Baldwin, 2001). However, Heil (2004) reported a decrease in MeSa emission in Lima bean leaves sprayed with a dose of JA

(double the dose used in this study), and attributed this decrease to downregulation between these two antagonist phytohormones.

Both the JA-induced increase and decrease in MeSA emissions are possible outcomes of the complex interactions between these two hormones and their metabolic pathways. The response of MeSa emissions to JA appears to depend on the JA dose and on plant conditions. Jasmonic acid and salicylic acid (SA) appear to regulate separate biochemical pathways and have different functions (Karban and Baldwin, 1997), although their signalling pathways can function independently or can interact through crosstalk in additive or negative ways (Stotz et al., 2002). Significant induction of MeSa has been found to require an optimal concentration of JA in a *JA-synthesis* mutant tomato plant (Ament et al., 2004). In the same study, the authors also stated that the optimal dose of exogenous JA required to induce MeSa emission may depend on the amounts of other plant defence intermediates (such as SA) present at the moment of application, as SA may antagonize downstream JA responses. In any case, in our study, after the application of exogenous JA in plants that apparently were not stressed, we have found a JA-mediated volatilization of MeSa that provides evidence of crosstalk between JA and SA.

In previous studies with tobacco suspensions cells, the activity of the sesquiterpene cyclase were mensured by MeJa treatment, and it was demonstrated that the highest activity were observed 48 h after the treatment, with an improvement of capsidyol (a sesquiterpenoid known by a tobacco plants fytoalexin) quantity (Dicke, 1999).

Quantitatively, it was observed that it occur a TRANS-verbenol and citronellal increases in 1.531 and 1.115%, respectively, after 48 h. After, 72 h of treatment, the amount returned to the control level (Table 1).

In 72 hours of treatment, also qualitative alteration in the oil composition was observed (Figure 1 and Table 1): the relative 2-methyl-2-ciclopenten-1-one quantity increased to 9.2% and α-humulene to 0.6% of the total *M. officinalis* essential oil composition. In 96 h of treatment, the oil composition was similar to the control (Figure 1 and Table 1). These results assume that after 96 h of MeJa exposition, this already does not make any effect on elicitation of plant secondary metabolites production.

Mithöfer et al. (2005) used MeJa aspersion (0.01; 0.1 and 0.5 mM), during 1, 2, 3, and 4 days, on field basil. Compared with the control, the eugenol and L-linalool amounts in basil plants treated with 0.5 mM MeJa had an increase of 56 and 43%, respectively, on the fourth day (Mandujano-Chavez et al., 2000).

Essential oil compounds evaluation of plants submitted to methyl salicylate (MeSa) treatment during 24, 48, 72 and 96 hours

In the case of plants subjected to treatment with methyl salicylate, there was a reduction in alcohol levels (nerol and geraniol) and neral. But it was observed the content of geranial increasing. The best period for obtaining plants with highest geranial production, is in 72 hours, with an increase of 21.4% when compared with control plants, MS0 medium (Figure 2 and Table 2).

Qualitatively, it was observed the presence of z-cariophillene and α-humulene in 0.2 and 0.5% of the total oil composition, respectively, in 72 hours of treatment (Table 1). Treatment with MeJa has also been reported to induce an increase in enzyme activities and elevated levels of transcripts of monoterpene synthases and diterpene synthases in Norway spruce (Fäldt et al., 2003). In *Quercus ilex* different monoterpene synthases appear to produce different monoterpenes (Staudt et al., 2004), and JA treatment altered neither the composition nor the relative abundance of the emitted monoterpenes. Thus it seems that JA would affect the activity of all these enzymes or their gene transcription similarly. However, there has been some evidence suggesting that the effect of JA on terpenoid emission is related to its impact on the substrate supply feeding its biosynthesis pathway, instead of a JA elicitation of the kinetics of these pathway enzymes (Ferrieri et al., 2005).

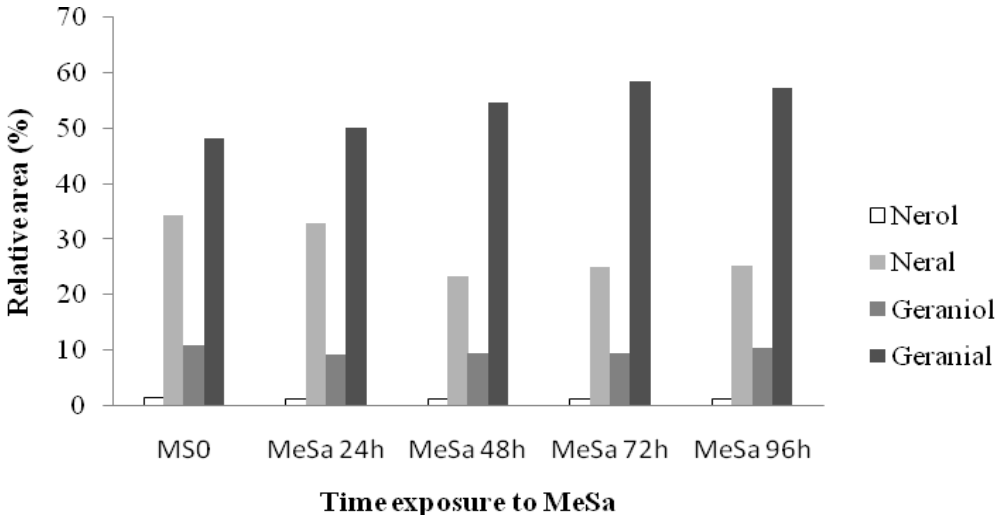

Figure 2. Effect of Methyl Salicylate on essential oil compounds of *Melissa officinalis in vitro* plants.

Table 2. Major volatile organic constituents (over 0.1%) of aerial parts (15 g) from 60 days old *in vitro* plantlets cultivated in MS0 (control) and submitted to MeSa exposure, during 24, 48, 72 and 96 hours.

Constituents	MS0 60 (Control)		MeSa 24h		MeSa 48h		MeSa 72h		MeSa 96h		KI
	RI	RA	RI	RA	RI	RA	RI	RA	RI	RA	
2-methyl-2-cyclopenten-1-one	-	-	-	-	-	-	-	-	-	-	-
dihydro-linalool	1134	0.26	-	-	-	-	-	-	-	-	1134
trans-verbenol	1144	0.93	1144	0.72	1144	0.65	1144	0.59	1144	0.72	1144
Citronellal	1153	1.35	1153	1.01	1153	0.91	1153	0.80	1153	0.96	1153
methyl salicylate	-	-	-	-	-	-	-	-	-	-	-
Nerol	1228	1.40	1228	1.31	1228	1.28	1228	1.25	1228	1.25	1228
Neral	1240	34.41	1140	32.85	1140	23.5	1240	25.02	1240	25.28	1240
Geraniol	1255	11.02	1255	9.05	1255	9.61	1255	9.45	1255	10.52	1255
Geranial	1270	48.21	1270	50.15	1270	54.58	1270	58,51	1270	57.29	1270
propanoic acid	1350	0.62	1350	0.88	1350	0.33	1350	0.45	1350	0.55	1350
geranyl acetate	1383	0.22	1383	0.53	1383	0.82	-	-	1383	0.27	1383
cariophillene-<z->	-	-	-	-	-	-	1404	0.18	-	-	1404
α-humulene	-	-	-	-	-	-	1454	0.52	-	-	1454
D-germacrene	1480	0.29	1480	0.43	1480	0.14	-	-	1480	0.14	1480
		98.71		96.93		91.82		96.77		96.98	

RI: Retention index; KI: Kovats index (ADAMS, 1995); RA: Relative area (%)

The role of these JA-mediated increases in monoterpene emissions may be related to their properties in helping to protect against both abiotic and biotic stresses, given that these isoprenoids may be of particular relevance in the adaptation of plant species to adverse environmental conditions (Peñuelas and Munné-Bosch, 2005). Monoterpenes can act as antioxidants protecting plant membranes against peroxidation and reactive oxygen species such as singlet oxygen, as has been described for *Quercus ilex* (Loreto et al., 2004), because of their double bonds.

Furthermore, given that they are small lipophilic molecules, and they may also assist hydrophobic interactions in membranes that result in their stabilization (Velikova et al., 2004).

Conclusions

The present study used the *in vitro* culture of *Melissa officinalis* plants in order to analyze the effect of Methyl jasmonate and Methyl salycilate in quantitative and qualitative production of essential oil.

With the use of MeJA, after 48 hours of treatment, it was observed the presence of 2-methyl-2-cyclopenten-1-one (making up 8.5% of oil), there was an increase of 1,531.2% of verbenol and 1,114.8% of citronellal, and after 72 hours of exposure, showed an increase in the relative amount of 2-methyl-2-cyclopenten-1-one (7.6%) of the total composition of *M. officinalis* essential oil. At 96 hours of exposure to methyl jasmonate, the oil composition was similar to the control plants (MS0).

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