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Effect of salicylic acid on essential oil compounds of *Melissa officinalis in vitro* plants

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Abstract

Melissa officinalis (lemon balm) presents essential oils and polyphenols with reported biological activity. Micropropagation represents an important tool for the standardization and selection of elite plants. Production of secondary metabolites in plants depends on the biotic and abiotic conditions of the environment in which they are grown. Plants induced to produce specific biologically active compounds by exogenous molecules may have their nutraceutical value increased. Salicylic acid acts in plants as an inducer of the expression of protective protein and it can be thus included into biotic elicitors. The present paper examined the effects of salicylic acid on the essential oil composition of *M. officinalis* plants grown *in vitro*. The maximal proportion of geranial/geraniol and neral/nerol showed an increase of 2.2 and 1.6 times, respectively, after 24 hours of exposure to SA, when compared to control plants (MS0).
Keywords: Secondary metabolites, tissue culture, aldehydes, alcohols

Resumo

Efeito do ácido salicílico na composição do óleo essencial de *Melissa officinalis* cultivada *in vitro*. *Melissa officinalis* possui óleos essenciais e polifenóis que apresentam atividades biológicas. A micropropagação representa uma importante ferramenta para a padronização e seleção de plantas elite. A produção de metabólitos secundários em plantas depende das condições bióticas e abióticas do ambiente em que são cultivadas. As plantas são induzidas a produzir componentes com atividade biológica específica através de moléculas exógenas, podendo ter seu valor nutracêutico aumentado. O presente trabalho teve como objetivo avaliar o efeito do ácido salicílico na composição do óleo essencial de *Melissa officinalis* cultivada *in vitro*. O ácido salicílico atua em plantas como um indutor da expressão de proteínas protetoras, podendo ser incluído entre os eliciadores bióticos. A proporção máxima de geranial/geraniol e neral/nerol apresentaram um aumento de 2,2 e 1,6 vezes, respectivamente, após 24 horas de exposição ao AS, quando comparadas às plantas controle (MS0).
Palavras chave: Metabólitos secundários, cultura de tecidos, aldeídos, álcoois

Introduction

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. The essential oil is a well-known antibacterial and antifungal agent, and it is responsible for the mild depressive and spasmolytic properties of the plant as well (Carnat et al., 1998). It was reported that methanolic extracts of *Melissa officinalis* have antioxidant properties, which are mostly due to high amount of phenolic acids, revealing significant antimicrobial, particularly antibacterial activity of this essential oil (Mimica-Dukic et al., 2004).

Since the amounts of compounds with biological activity differ significantly in each plant, the main goal of breeding is to select highly productive individuals and to propagate them vegetatively in order to maintain their valuable characteristics (Mészáros et al., 1999). *In vitro* regeneration of adventitious shoots can be an effective tool either for mass cloning of selected genotypes or to establish a model of metabolic pathway in order to enhance production of natural products and synthesis of novel materials (Capell & Christou, 2004).

Aiming the synthesis of essential oils (Arikat et al., 2004) and antioxidant compounds, micropropagation procedures have been applied to many species of the Lamiaceae family, like *Salvia officinalis* (Santos-Gomes et al., 2002); *Hedeoma multiflorum*, utilized in the folk medicine (Koroch et al., 1997) and *Mentha arvensis*, as a menthol source (Bhat et al., 2001). Optimization of *in vitro* culture conditions for *M. officinalis* has been reported by many authors (Silva et al., 2006; Mészáros et al., 1999; Tavares et al., 1996; Gbolade & Lockwood, 1989), establishing efficient procedures for cells and multiple shoot cultures of this species.

Therefore, expositing *in vitro* cultures to elicitors may provide a tool to promote changes on secondary metabolism aiming to determine the necessary conditions to stimulate the production of specific groups of biologically active compounds (Pasternak et al., 2005).

Polyphenols levels in plants change according to the biotic and abiotic conditions of the environment and depend on light type, phenological stage and presence of pathogens (Randhir et al., 2002). Pathogen attack may lead to production of specific secondary metabolites for defense, which might present biological properties (Pasternak et al., 2005), e.g. scopolamine in *Brugmansia candida* (Pitta-Alvarez et al., 2000), hypericin in *Hypericum perforatum* (Sirvent and Gibson, 2002) and scopoletin in *Ammi majus* (Staniszewska et al., 2003). Elicitors of the secondary metabolism have been used to produce valuable compounds (Pasternak et al., 2005).

Salicylic acid (SA) has been reported to be involved in regulating a number of processes in plants (Hayat et al., 2010). One of its well-known functions is that SA plays an important role in plants disease resistance against pathogens. SA is shown to regulate the expression of pathogen protein (PR) genes to mediate a hypersensitive response (HR) and a systemic acquired resistance response (SAR) (Yin et al., 1997). Exogenous use of elicitors like salicylic acid enhanced synthesis of coumarins in *Matricaria chamomilla* (Pastírová et al., 2004). It is also know that elicitors may promote *Glycine max* growth (Gutiérrez-Coronado et al., 1998), reduce the activity of enzymes related to the synthesis of defense secondary metabolites in *Eleocharis tuberosa* (Peng & Jiang, 2006) and promote phenylalanine ammonia-lyase activity in *Prunus avivum* (Yao and Tian, 2005). These findings indicate that elicitors act as signal molecules for gene expression in plants. However, compounds or chemicals (such as elicitors) capable of enhancing the yields of secondary metabolites, such as essential oil, in aromatic plants, are not known in the art presently.

The present work examines how compounds such as the elicitor, salicylic acid, may affect the synthesis of essential oil composition in *in vitro* plants of *Melissa officinalis*.

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).

Treatment with salicylic acid

It was used 18 µL of salicylic acid solution (1.5 mM) in cotton pieces (based on the protocol described by Rodriguez-Saona et al, 2001), that were put with the plants with 60 days of culture in MS0. The compositions of the essential oil produced were evaluated 24, 48, 72 and 96 h after adding SA and compared with control cultures, which were untreated during that time.

Extraction

The fresh aerial parts of *in vitro* plantlets (15 g each) were submitted to hydrodistillation 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 prevent 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 composition of plants after treatment during 24, 48, 72 and 96 hours with salicylic acid (SA)

In general, plants submitted to salicylic acid treatments showed a decrease of alcohols levels (geraniol and nerol). After 24 hours, there was a neral accumulation decrease, in 15.8%; at 48 hours, the increase were 3.0% and in 96 hours, it was obtained the greatest accumulation (increase of 8.9%), compared with control plants, in MS0 (Table 1). In relation to neral production, there was an increase of 63.9% on this component proportion, with 24 hours of SA exposure (Figure 1).

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 exposure to SA during 24, 48, 72 and 96 hours.

Constituents	MS0 60 (Control)		SA 24h		SA 48h		SA 72h		SA 96h		KI
	RI	RA	RI	RA	RI	RA	RI	RA	RI	RA	
dihydro-linalool	1134	0.26	-	-	-	-	-	-	-	-	1134
trans-verbenol	1144	0.93	1144	0.84	1144	1.0	1144	0.88	1144	1.09	1144
Citronellal	1153	1.35	1153	1.18	1153	1.48	1153	1.27	-	1.62	1153

nerol	1228	1.40	1228	0.72	1228	1.02	1228	1.47	1228	1.08	1228
neral	1240	34.41	1240	28.99	1240	35.45	1240	33.88	1140	37.46	1240
geraniol	1255	11.02	1255	6.35	1255	7.93	1255	10.21	1255	9.00	1255
geranial	1270	48.21	1270	60.32	1270	52.32	1270	52.28	1270	48.62	1270
propanoic acid	1350	0.62	1350	0.47	1350	0.43	1350	0.45	1350	0.48	1350
geranyl acetate	1383	0.22	1383	0.16	-	-	-	-	-	-	1383
D-germacrene	1480	0.29	1480	0.38	-	-	-	-	-	-	1480
		98.71		99.41		99.63		100		99.35	

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

These results showed that the best treatment to geranial accumulation, in salicylic acid presence (25%), is with 24 hours of exposure (Table 1). In relation to geranial production, there was an increase of 117.4% on this component proportion, with 24 hours of SA exposure (Figure 1).

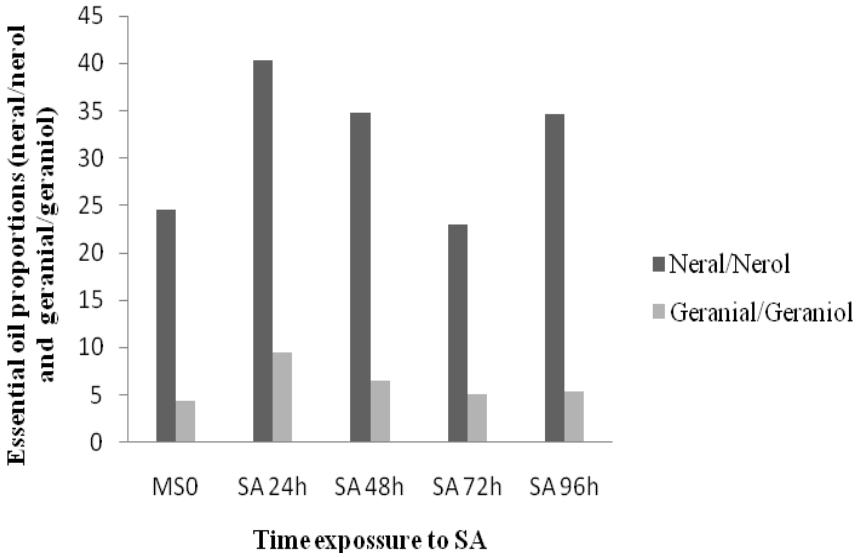

Figure 1. Effect of Salicylic Acid (SA) on *Melissa officinalis* essential oil contents (neral/nerol, geranial/geraniol) production *in vitro* culture.

When plant cells are exposed to elicitor treatment, it initiates a signal transduction from the surface of the plasma membrane that trigger ROS production, which induces plant defense response and enhances the activity of key enzymes which catalyze the biosynthesis of target secondary metabolites (Zhao et al., 2005).

The results obtained in the present study suggest that salicylic acid (SA) promoted the increasing of the alcohols oxidation to aldehydes. These results were similar to those reported by Silva et al. (2006), when the use of indolacetic acid and 6-benzylaminopurine on *M. officinalis* culture promoted the increase of the alcohol oxidation to aldehydes.

According to Mann (1987), the routes of plant secondary metabolites are only activated during some particular growth stages and development or in periods of stress caused by nutritional limitations. The adaptability of plants in stress conditions are influenced by the stress duration and magnitude, beyond the genetic variability. The active ingredient concentration in plants depends on the genetic control, the genotypic interactions and the environment, which can be triggered in conditions of stress, i.e. excess or deficiency of some factor of the environment, such as water, light, temperature, nutrients, among others (Andrade and Casali, 1999). Plants develop protection mechanisms against pathogens (viruses, bacteria, fungi, insects, etc.) producing toxins against the invading agent

and acquired infection resistance (Pinto et al., 2002).

It was discovered the salicylic acid (SA) involvement and its acetylated derivative (acetylsalicylic acid - ASA) in defense reactions against pathogens. The salicylic acid is accumulated in plant tissue after infection, causing an immune response, called systemic acquired resistance (SAR) (Pinto et al., 2002).

Use of salicylic acid in tobacco (*Nicotiana tabacum*) leaves led to a reaction-type SAR, as if the pathogen (in this example, the tobacco mosaic virus) were present. Moreover, it was observed that when salicylic acid formation was blocked, SAR activity disappeared or decreased, weakening the plant resistance (Pinto et al., 2002). In this paper, the exposure to SA during 24h led to an increase of 25% of geranial so, after that period, the levels of this compound gradually decreased until the levels of control.

Conclusion

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

The highest accumulation for geranial (25%) and neral (8.9%) was obtained using SA for 24 and 96 hours, respectively.

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