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Elicitation – a tool to improve secondary metabolites production in *Melissa officinalis* L. suspension cultures
Elicitácia ako nástroj na zlepšenie produkcie sekundárnych metabolitov v suspenzných kultúrach *Melissa officinalis* L.

Original research article

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Abstract Our recent study is focussed on the investigation of the influence of biotic (*Botrytis cinerea*, methyl jasmonate) and abiotic (cadmium and cobalt chloride) elicitors on *Melissa officinalis* L. (lemon balm) suspension cultures production properties. The plant material was treated with different concentrations of methyl jasmonate (10 and 100 μmol.l^{-1}), with a phytopathogenic fungus hydrolyzate (*Botrytis cinerea*, 1 and 2 ml with glucose equivalent 24 μg.ml^{-1}), cadmium and cobalt (II) chloride (both at concentrations 100 μmol.l^{-1} and 1 μmol.l^{-1}). Elicited suspension cultures were harvested after 24, 48 and 72 h of elicitor treatment. The elicitation effect was evaluated based on hydroxycinnamic acid derivatives content expressed as rosmarinic acid in dose- and time-dependent manners. The *Botrytis cinerea* hydrolyzate exhibited the best effect on *Melissa officinalis* L. suspension cultures production properties among biotic elicitors (glucose equivalent 24 mg.ml^{-1}, 1 ml, after 72 h of treatment). The hydroxycinnamic acid derivatives content increased in triplicate compared to non-elicited samples. Methyl jasmonate affected the suspension cultures production properties moderately at concentration 10 μmol.l^{-1}. Chlorides salts of cobalt and cadmium stimulated phenolic compounds production effectively at concentrations 100 μmol.l^{-1}. The presence of cobalt and cadmium ions in suspension cultures growth media increased the hydroxycinnamic acid derivatives content twofold and quadruple, respectively.

Slovak abstract Súčasná práca je zameraná na štúdium vplyvu biotických (*Botrytis cinerea*, metyljazmonát) a abiotických elicitorov (chlorid kademnatý a chlorid kobaltnatý) na produkčné vlastnosti suspenzných kultúr medovky lekárskej (*Melissa officinalis* L.). Rastlinný materiál bol elicitovaný metyljazmonátom (10 and 100 μmol.l^{-1}), hydrolyzátom bunkovej steny fytopatogénnej huby *Botrytis cinerea* (1 a 2 ml s glukózovým ekvivalentom 24μg.ml^{-1}), chloridom kademnatým a kobaltnatým (oba v koncentráciách 100 and 1 μmol.l^{-1}). Elicitované kultúry boli vystavené expozícii elicitorov po dobu 24, 48 a 72 h. Efekt elicitácie sa hodnotil na základe obsahu derivátov kyseliny hydroxyškoricovej, vyjadrených ako kyseliny rozmarínová, v závislosti od koncentrácie použitého elicitora a dĺžky jeho pôsobenia. Spomedzi testovaných biotických elicitorov najúčinnejšie podporil produkčné vlastnosti suspenzných kultúr *Melissa officinalis* L. hydrolyzát z *Botrytis cinerea* (glukózový ekvivalent 24 mg.ml^{-1}, 1 ml, doba pôsobenia 72 h). Obsah derivátov kyseliny hydroxyškoricovej sa po elicitácii zvýšil trojnásobne v porovnaní s neelicitovanými kultúrami. Produkčná schopnosť suspenzných kultúr elicitovaných metyljazmonátom sa mierne zvýšila len pri použití koncentrácie 10 μmol.l^{-1}. Chlorid kobaltnatý a kademnatý efektívne stimulovali produkciu fenolových látok pri koncentrácii 100 μmol.l^{-1}. Prítomnosť iónov kobaltu a kadmia v rastových médiách suspenzných kultúr viedla k dvoj-, resp. štvornásobnému zvýšeniu obsahu derivátov kyseliny hydroxyškoricovej.

Keywords *elicitation – Melissa officinalis L. – hydroxycinnamic acid derivatives – methyl jasmonate – Botrytis cinerea – metal ions*

Kľúčové slová: *elicitácia – Melissa officinalis L. – deriváty kyseliny hydroxyškoricovej – metyljazmonát – Botrytis cinerea – ióny ťažkých kovov*

INTRODUCTION

The demand for important active natural compound is still high in the field of pharmacy. Isolation from wild-growing or cultivated plants currently often is the only way to obtain appreciable amounts, as chemical synthesis tends to be difficult and non-profitable (Namdeo, 2007). The production of valuable active secondary metabolites used in therapy in medical plants is usually low (usually less than 1% dry weight), as these substances play no fundamental role in the maintenance of plants life processes (Ramakrishna & Ravishankar, 2011). Low yields of natural compounds from

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intact herbs have led to efforts to find new strategies to enhance their biosynthetic capacity. It seems that production of plant secondary metabolites by cell cultures is an attractive alternative to extraction of whole plants (Oksman-Caldentey & Inzé, 2004). Elicitation techniques provide a way to achieve higher yield of plant secondary metabolites due to controlled stress conditions.

Lemon balm, *Melissa officinalis* L. (Lamiaceae), is a medicinal plant of long tradition with a large variety of uses in European traditional medicine. Therapeutic properties of lemon balm are attributed to essential oil that exhibits antioxidative, antibacterial, antiviral, sedative, carminative and antispasmodic effects (Tóth *et al*., 2003). Phenolic compounds, represented by rosmarinic, caffeic, protocatechuic acids and their methyl esters contribute to many of the activities mentioned as well. *Melissa officinalis* L. subsp. *officinalis* is the only of three known subspecies that is used in pharmaceutical preparations nowadays (Marongui *et al*., 2004). The domestic cultivar 'Citra' belongs to frequently studied taxa due to essential oil production (Fialová *et al*., 2008; Mrlianová *et al*., 2001, 2002, 2011; Tóth *et al*., 2003).

MATERIAL AND METHODS

Plant material

Melissa officinalis L. cv. 'Citra' suspension cultures prepared from long-term calli were used in our experiment, grown in 25 ml of *Murashige and Skoog* liquid medium supplemented with kinetine (0.1 mg.l^{-1}), 2,4-dichlorphenoxyacetic acid (1 mg.l^{-1}) and sucrose (30 g.l^{-1}). Cultures were cultivated at 23°C, in 60 lux light (12 h per day) on a rotary shaker (150 rpm).

Elicitor treatment

Four elicitors were tested, each at two different concentrations. Suspension cultures of *Melissa officinalis* L. were elicited with 1 and 2 ml of *Botrytis cinerea* hydrolyzate (glucose equivalent 24 µg.ml^{-1}). Commercial methyl jasmonate (Sigma, St. Louis, USA) was present in growth media at concentrations 10 and 100 µmol.l$^{-1,}$ respectively. Cadmium and cobalt (II) chlorides were tested at final concentrations 1 and 100 µmol.l^{-1}. For control samples, elicitors were omitted. Elicitation was performed on the 14th day of sub-cultivation under aseptic conditions in a sterile air cabinet. Subsequently, elicited suspension cultures were maintained under the same conditions mentioned above and harvested 24, 48 and 72 h after elicitation.

Determination of total hydroxycinnamic acid derivates according to Arnow's method

Hydroxycinnamic acid derivatives content was determined using Arnow's method (Arnow, 1937) as adopted in the Ph. Eur. 8 (European Pharmacopoeia, 2014) and expressed as rosmarinic acid. Acquired results are the mean of three parallel measurements.

RESULTS

The effect of four elicitors on *Melissa officinalis* L. cell suspension cultures production properties was tested in this study. Final concentrations of methyl jasmonate in treated suspension cultures were 10 and 100 µmol.l^{-1}, respectively. A *Botrytis cinerea* hydrolyzate (glucose equivalent 24 µg.ml^{-1}) was added to suspension cultures in 1 and 2 ml amounts, respectively. Chlorides salts of cadmium and cobalt (II) were present in media at concentrations 100 and 1 µmol.l^{-1} each, respectively. The best effect on hydroxycinnamic acid derivatives production was exhibited by the addition of 1 ml of *Botrytis cinerea* hydrolyzate, among different concentrations of tested biotic elicitors. An increase in the production occurred after 24 h of elicitation and continued up to 72 h (Figure 1). The maximal amount of hydroxycinnamic acid derivatives was approximately triple compared to non-treated suspension cultures. The addition of 2 ml of the *Botrytis cinerea* hydrolyzate did not show the same effect as with 1 ml of it. Even though the content of hydroxycinnamic acid derivatives increased, total amounts quantified in suspension cultures elicited with 1 ml of hydrolyzate were not achieved. Methyl jasmonate showed a moderate increase in phenolic compounds at concentration 10 µmol.l^{-1}. The higher concentration of this elicitor used led rather to a decrease of hydroxycinnamic acid derivatives compared to non-elicited cultures, on the contrary (Figure 2).

Chlorides salts of cadmium and cobalt effectively increased phenolic compounds production at concentrations 100 µmol.l^{-1} each. The highest amount of hydroxycinnamic acid derivatives was quantified after 24 h of treatment with cadmium chloride and the content was almost quadruple compared to non-elicited samples. Prolonging the elicitation time (48, 72 h) led to a decrease in quantity of the studied compounds (Figure 3). Where cadmium chloride stimulated the production of hydroxycinnamic acid derivatives 24 h after elicitation, cobalt (II) chloride exhibited different time course accumulation of phenolic compounds. Maximum amount of hydroxycinnamic acid derivatives was observed in suspension cultures exposed to cobalt (II) chloride during 72 h (Figure 4). Lower concentrations of both metal ions showed a similar time course accumulation of hydroxycinnamic acid derivatives in *Melissa officinalis* L. suspension cultures as higher concentrations.

DISCUSSION

Elicitors have been chosen based on previous work performed at the Department of Cellular and Molecular Biology of Drugs, Faculty of Pharmacy in Bratislava. The *Botrytis cinerea* hydrolyzate is a successful elicitor, the stimulating effect on secondary metabolites production of which has been

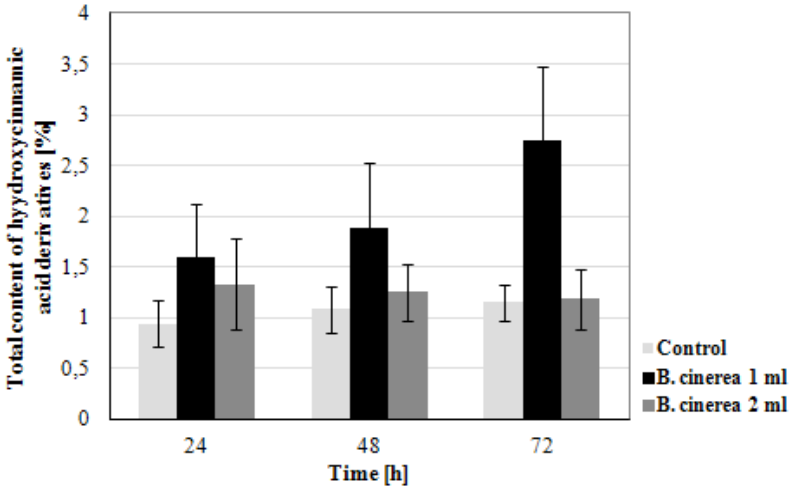

Figure 1. Changes in total hydroxycinnamic acid derivatives content [%] in Melissa officinalis L. suspension cultures after elicitation with Botrytis cinerea hydrolyzate

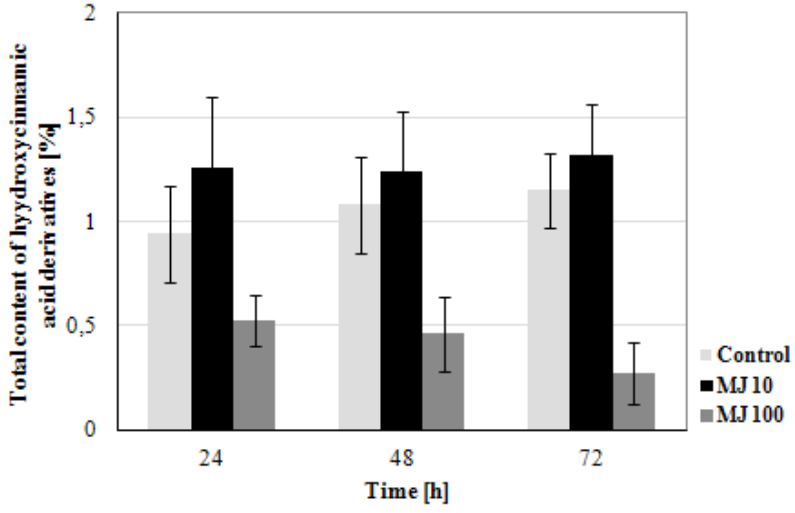

Figure 2. Changes in total hydroxycinnamic acid derivatives content [%] in Melissa officinalis L. suspension cultures after elicitation with methyl jasmonate (MJ)

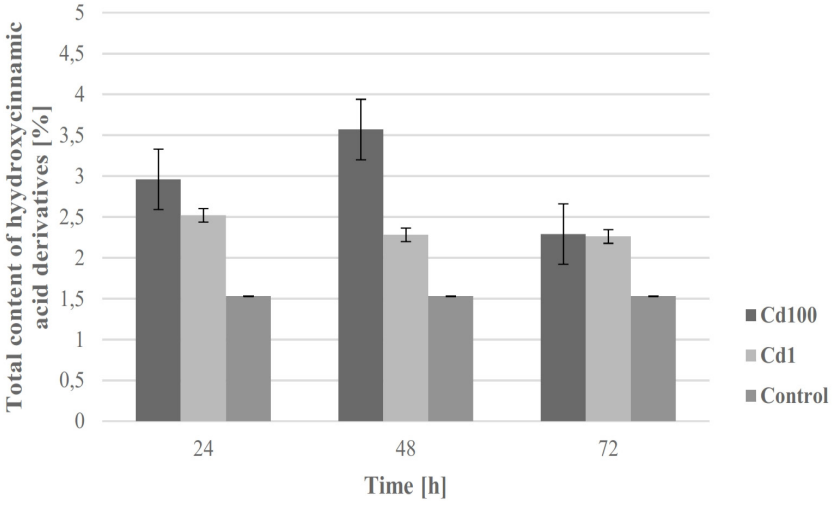

Figure 3. Changes in total hydroxycinnamic acid derivatives content [%] in Melissa officinalis L. suspension cultures after elicitation with cadmium chloride ($CdCl_2$)

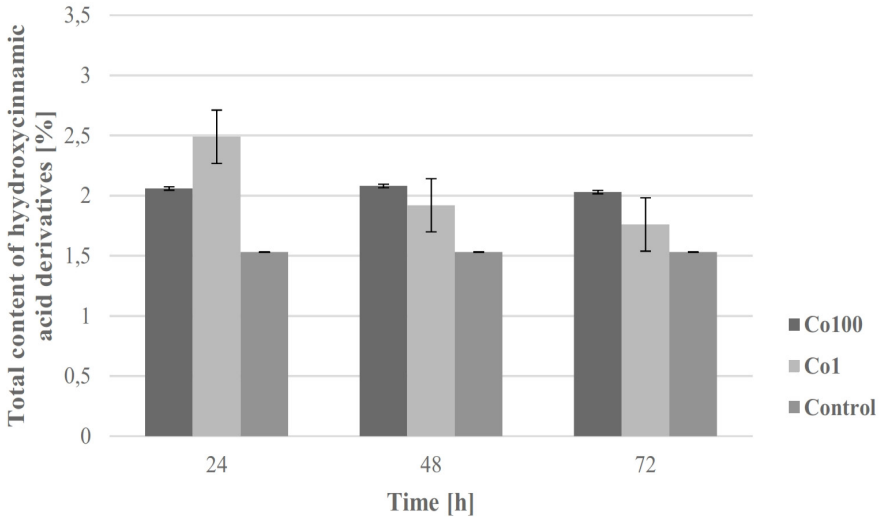

Figure 4. Changes in total hydroxycinnamic acid derivatives content [%] in Melissa officinalis L. suspension cultures after elicitation with cobalt chloride ($CoCl_2$)

confirmed in plant *in vitro* cultures of Papaveraceae family plants before (Balažová *et al*., 2011; Bilka *et al*., 2012). The outcomes of this study are in agreement with the results of these mentioned works. The optimum dosage of *Botrytis cinerea* for the *Melissa officinalis* L. suspension cultures elicitation was found to be 1 ml of hydrolyzate, glucose equivalent 24 µg.ml^{-1}.

The concentration of 100 µmol.l^{-1} methyl jasmonate was frequently reported in literature as a successful tretment to elicit secondary metabolites in various plant species (Ketchum *et al*., 1999; Mizukami *et al*., 1993). We expected similar effects at this concentration on the phenolic compounds formation in *Melissa officinalis* L. suspension cultures. The results of experiments have not confirmed our expectations, however. The total hydroxycinnamic acid derivatives content in elicited suspension cultures was lower than in non-elicited samples. We assume that such a high dosage of methyl jasmonate could induce a hypersensitive response leading to cell death and leakage of cell content into the medium. The presence of methyl jasmonate at 10 µmol.l^{-1} concentration increased the content of hydroxycinnamic acid derivatives during 72-h long elicitation only slightly. The relation between methyl jasmonate dosage and secondary metabolites production has been investigated in many plants (Lee-Parson *et al*., 2004; Ram *et al*. 2013). These works reported that methyl jasmonate effectively increased the production of secondary metabolites at concentrations below 100 µmol.l^{-1}.

Recently, elicitation with heavy metals was studied to reveal their effect on secondary metabolites production in plant suspension cultures (Bhuvaneswari *et al*. 2012; Cai *et al*. 2013). The stimulation of secondary metabolites accumulation by metals salts has been proven in cell cultures of several plants, such as *Eschscholtzia californica* Cham. (Balažová *et al*. 2008), *Papaver somniferum* L. (Bilková *et al*. 2006), *Catharanthus roseus* L. (Srivastava & Srivastava, 2010), *Vitis vinifera* L. (Cai *et al*., 2013). On the other hand, heavy metals can negatively affect biological processes in plant organisms (López-Millán *et al*., 2009). Cadmium chloride at concentration 100 µmol.l^{-1} increased the content of hydroxycinnamic acid derivatives in *Melissa officinalis* L. suspension cultures almost quadruple over non-elicited samples 24 h after elicitor treatment. Compared to cadmium, cobalt (II) chloride showed different time course accumulation of hydroxycinnamic acid derivatives. The maximal amount of phenolic compounds in suspension cultures was reached 72 h after elicitation with cobalt (II) chloride at concentration 100 µmol.l^{-1}. The hydroxycinnamic acid derivatives content has exhibited similar time course accumulation in our study as the triterpenoid saponins content in *Gymnema sylvestre* suspension cultures elicited with cadmium and cobalt salts (Bhuvaneswari *et al*., 2012).

CONCLUSION

Among the tested elicitors, cadmium chloride and *Botrytis cinerea* hydrolyzate have been proven to be the best in enhancing hydroxycinnamic acid derivatives accumulation in *Melissa officinalis* L. suspension cultures. Successful stimulation of secondary metabolites production in cell suspension cultures of medicinal plants by elicitation particularly depends on the type of elicitor, its dosage, and exposure time.

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