ORIGINAL ARTICLE

Production of flavonoids and isoflavonoids in jasmonic acid-induced red clover suspension cultures

Produkce flavonoidů a isoflavonoidů v suspenzní kultuře jetele lučního elicitované kyselinou jasmonovou

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Summary

Effect of exogenously applied jasmonic acid (JA) in combination with calcium and verapamil (a calcium channels blocker) on the production of flavonoids and isoflavonoids in suspension cultures of *Trifolium pratense* L. was investigated. The culture was cultivated in Gamborg medium with an addition of 2 mg.l^{-1} of 2,4-dichlorophenoxyacetic acid and 2 mg.l^{-1} of 6-benzylaminopurine, at the temperature of 25 °C, 16-hr light/8-hr dark period. The best effect of jasmonic acid on the production of flavonoids and isoflavonoids was manifested after a 24-hour application of the 50 μmol.l^{-1} concentration. The maximum production of JA-induced suspension culture was observed when cells were treated with a high level of calcium (10 mmol.l^{-1}). The addition of all concentrations of verapamil to JA-induced suspension culture decreased production of flavonoids and isoflavonoids.

Keywords: flavonoids • isoflavonoids • jasmonic acid • elicitation • *Trifolium pratense* suspension culture

Souhrn

Byl sledován vliv exogenní aplikace kyseliny jasmonové (JA) v kombinaci s vápníkem a verapamilem (blokátor vápníkového kanálu) na produkci flavonoidů a isoflavonoidů suspenzní kulturou *Trifolium pratense* L. Kultura byla kultivována při teplotě 25 °C a světelné periodě 16 hodin světlo/8 hodin tma na živném médiu podle Gamborga s přídavkem 2 mg.l^{-1} 2,4-dichlorfenoxyoctové kyseliny a 2 mg.l^{-1} 6-benzylaminopurinu. Nejlepší vliv kyseliny jasmonové na produkci flavonoidů a isoflavonoidů se projevil po 24hodinové aplikaci koncentrace 50 μmol.l^{-1}. Maximální produkce elicitované suspenzní kultury byla zjištěna, když buňky byly ošetřeny vysokou dávkou vápníku (10 mmol.l^{-1}). Přidání všech koncentrací verapamilu k suspenzní kultuře elicitované JA snížilo produkci flavonoidů a isoflavonoidů.

Klíčová slova: flavonoidy • isoflavonoidy • kyselina jasmonová • elicitace • suspenzní kultura *Trifolium pratense*

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Introduction

Red clover (*Trifolium pratense* L., *Fabaceae*) is a rich source of isoflavonoids with multiple potential protective functions, plant secondary metabolites belonging to the group of phenylpropanoids. Red clover contains isoflavones, which have a high affinity to estrogen receptor α (ERα), estrogen receptor β (ERβ), progesterone receptor and androgen receptor. The higher affinity to ERβ compared to ERα has been used as an explanation why red clover extracts function as food additives to treat menopausal disorders and may reduce risk of breast cancer[1]. The main isoflavones present in red clover are formononetin and biochanin A, of which formononetin is more abundantly found in roots[2]. Other isoflavones found in roots include daidzein, genistein, pratensein, pseudobaptigenin, calycosin, methylorobol, irilin B, afrormosin and irilone[3]. Isoflavonoids are synthesised mainly constitutively in intact plant and in suspension culture of *Trifolium pratense* L., but their concentrations may be influenced by biotic or abiotic stresses (elicitors) such as ozone, UV light, pathogens, heavy metals, phytohormones[4–8]. The elicitation method can be an important strategy towards improved production of plant secondary metabolites. Jasmonic acid, plant hormonal regulator, is an integral part of a general signal transduction system regulating inducible defense

genes in plants. Moreover, exogenously applied jasmonic acid and methyl jasmonate induce *de novo* transcription of the gene of the key enzyme of the phenylpropanoid pathway, phenylalanine ammonia lyase. Jasmonic acid and the jasmonates are, therefore, key signal compounds in the elicitation process leading to *de novo* transcription and translation and, ultimately, to the biosynthesis of secondary metabolites in plant cell cultures. Calcium functions as a versatile messenger in mediating responses to hormones, biotic/abiotic stress signals and a variety of developmental cues in plants[9, 10].

Table 1. The effects of jasmonic acid, calcium and verapamil on flavonoids production in Trifolium pratense L. suspension cultures

Time (hour)	Elicitor	Concentration of elicitor (c)	Flavonoid contents (mg.g^{-1} DW)
6	Control	0	1.72 ± 0.08
	Jasmonic acid (JA)	5 µmol.l^{-1}	1.69 ± 0.04
		50 µmol.l^{-1}	1.85 ± 0.07
		500 µmol.l^{-1}	1.74 ± 0.03
	JA (50 µmol) + $CaCl_2$	0.1 mmol.l^{-1}	2.41 ± 0.16
		1 mmol.l^{-1}	3.14 ± 0.11
		10 mmol.l^{-1}	4.35 ± 0.09
	JA (50 µmol) + Verapamil	1 µmol.l^{-1}	1.58 ± 0.13
		10 µmol.l^{-1}	1.47 ± 0.20
		100 µmol.l^{-1}	0.43 ± 0.09
24	Control	0	1.72 ± 0.08
	Jasmonic acid (JA)	5 µmol.l^{-1}	1.89 ± 0.11
		50 µmol.l^{-1}	2.95 ± 0.06
		500 µmol.l^{-1}	2.43 ± 0.05
	JA (50 µmol) + $CaCl_2$	0.1 mmol.l^{-1}	3.12 ± 0.12
		1 mmol.l^{-1}	4.23 ± 0.07
		10 mmol.l^{-1}	5.62 ± 0.08
	JA (50 µmol) + Verapamil	1 µmol.l^{-1}	0.96 ± 0.20
		10 µmol.l^{-1}	0.80 ± 0.06
		100 µmol.l^{-1}	0.52 ± 0.13
48	Control	0	1.72 ± 0.08
	Jasmonic acid (JA)	5 µmol.l^{-1}	0.84 ± 0.10
		50 µmol.l^{-1}	0.95 ± 0.06
		500 µmol.l^{-1}	1.36 ± 0.11
	JA (50 µmol) + $CaCl_2$	0.1 mmol.l^{-1}	1.51 ± 0.10
		1 mmol.l^{-1}	1.45 ± 0.08
		10 mmol.l^{-1}	1.42 ± 0.10
	JA (50 µmol) + Verapamil	1 µmol.l^{-1}	0.69 ± 0.10
		10 µmol.l^{-1}	0.56 ± 0.16
		100 µmol.l^{-1}	0.55 ± 0.04
168	Control	0	1.93 ± 0.06
	Jasmonic acid (JA)	5 µmol.l^{-1}	0.83 ± 0.12
		50 µmol.l^{-1}	0.91 ± 0.04
		500 µmol.l^{-1}	1.08 ± 0.08
	JA (50 µmol) + $CaCl_2$	0.1 mmol.l^{-1}	0.46 ± 0.17
		1 mmol.l^{-1}	0.44 ± 0.08
		10 mmol.l^{-1}	0.22 ± 0.12
	JA (50 µmol) + Verapamil	1 µmol.l^{-1}	0.57 ± 0.05
		10 µmol.l^{-1}	0.54 ± 0.11
		100 µmol.l^{-1}	0.54 ± 0.09

Data represent the mean value ± SD (n = 3).

This experiment investigated the influence of jasmonic acid and the effect of jasmonic acid in combination with calcium and verapamil (a calcium channels blocker) on the production of flavonoids and isoflavonoids in *Trifolium pratense* suspension cultures.

Experimental part

Instruments

A 200S analytical scales made by Sartorius, Göttingen; a PS 20A autoclave, by Chirana, Brno; a HS 31A hot-air sterilizer by Chirana, Brno; a laminar flow workbench by

Table 2. The effects of jasmonic acid, calcium and verapamil on isoflavonoids production in Trifolium pratense L. suspension cultures

Time (hour)	Elicitor	Concentration of elicitor (c)	Isoflavonoid contents (mg.g^{-1} DW)		
			Genistin	Genistein	Daidzein
6	Control	0	0.14 ± 0.02	0.22 ± 0.01	0.20 ± 0.02
	Jasmonic acid (JA)	5 µmol.l^{-1}	0.15 ± 0.04	0.20 ± 0.05	0.19 ± 0.08
		50 µmol.l^{-1}	0.42 ± 0.03	0.20 ± 0.03	0.20 ± 0.05
		500 µmol.l^{-1}	0.20 ± 0.04	0.10 ± 0.07	0.20 ± 0.02
	JA (50 µmol) + $CaCl_2$	0,1 mmol.l^{-1}	0.47 ± 0.07	0.25 ± 0.05	0.22 ± 0.10
		1 mmol.l^{-1}	0.53 ± 0.02	0.32 ± 0.08	0.28 ± 0.05
		10 mmol.l^{-1}	0.68 ± 0.06	0.41 ± 0.06	0.27 ± 0.07
	JA (50 µmol) + Verapamil	1 µmol.l^{-1}	0.40 ± 0.03	0.20 ± 0.04	0.19 ± 0.03
		10 µmol.l^{-1}	0.40 ± 0.03	0.18 ± 0.02	0.20 ± 0.04
		100 µmol.l^{-1}	0.35 ± 0.06	0.12 ± 0.05	0.16 ± 0.03
24	Control	0	0.14 ± 0.02	0.22 ± 0.01	0.20 ± 0.02
	Jasmonic acid (JA)	5 µmol.l^{-1}	0.81 ± 0.07	0.40 ± 0.08	0.22 ± 0.06
		50 µmol.l^{-1}	0.95 ± 0.04	0.50 ± 0.04	0.50 ± 0.04
		500 µmol.l^{-1}	0.73 ± 0.02	0.25 ± 0.05	0.23 ± 0.02
	JA (50 µmol) + $CaCl_2$	0,1 mmol.l^{-1}	1.05 ± 0.03	0.62 ± 0.03	0.58 ± 0.08
		1 mmol.l^{-1}	1.28 ± 0.08	0.78 ± 0.09	0.64 ± 0.04
		10 mmol.l^{-1}	1.50 ± 0.05	0.80 ± 0.04	0.82 ± 0.02
	JA (50 µmol) + Verapamil	1 µmol.l^{-1}	0.32 ± 0.04	0.28 ± 0.02	0.21 ± 0.07
		10 µmol.l^{-1}	0.25 ± 0.05	0.18 ± 0.03	0.28 ± 0.04
		100 µmol.l^{-1}	0.12 ± 0.02	0.10 ± 0.02	0.12 ± 0.03
48	Control	0	0.14 ± 0.02	0.22 ± 0.01	0.20 ± 0.02
	Jasmonic acid (JA)	5 µmol.l^{-1}	0.55 ± 0.03	0.50 ± 0.05	0.47 ± 0.06
		50 µmol.l^{-1}	0.62 ± 0.07	0.50 ± 0.02	0.35 ± 0.07
		500 µmol.l^{-1}	0.39 ± 0.04	0.22 ± 0.03	0.22 ± 0.03
	JA (50 µmol) + $CaCl_2$	0.1 mmol.l^{-1}	0.64 ± 0.07	0.66 ± 0.04	0.41 ± 0.06
		1 mmol.l^{-1}	0.63 ± 0.02	0.85 ± 0.07	0.53 ± 0.03
		10 mmol.l^{-1}	0.75 ± 0.06	0.91 ± 0.02	0.49 ± 0.07
	JA (50 µmol) + Verapamil	1 µmol.l^{-1}	0.50 ± 0.03	0.35 ± 0.04	0.35 ± 0.02
		10 µmol.l^{-1}	0.52 ± 0.02	0.22 ± 0.07	0.26 ± 0.04
		100 µmol.l^{-1}	0.48 ± 0.11	0.20 ± 0.03	0.24 ± 0.04
168	Control	0	0.12 ± 0.02	0.18 ± 0.03	0.10 ± 0.01
	Jasmonic acid (JA)	5 µmol.l^{-1}	0.23 ± 0.02	0.22 ± 0.04	0.40 ± 0.08
		50 µmol.l^{-1}	0.37 ± 0.05	0.20 ± 0.08	0.10 ± 0.06
		500 µmol.l^{-1}	0.18 ± 0.07	0.15 ± 0.02	0.10 ± 0.04
	JA (50 µmol) + $CaCl_2$	0,1 mmol.l^{-1}	0.38 ± 0.01	0.32 ± 0.06	0.12 ± 0.10
		1 mmol.l^{-1}	0.41 ± 0.03	0.45 ± 0.03	0.23 ± 0.04
		10 mmol.l^{-1}	0.49 ± 0.06	0.47 ± 0.04	0.31 ± 0.02
	JA (50 µmol) + Verapamil	1 µmol.l^{-1}	0.35 ± 0.10	0.20 ± 0.03	0.11 ± 0.09
		10 µmol.l^{-1}	0.32 ± 0.04	0.22 ± 0.02	0.10 ± 0.07
		100 µmol.l^{-1}	0.30 ± 0.03	0.19 ± 0.06	0.10 ± 0.03

Data represent the mean value ± SD (n = 3).

Fatran LF, Žilina; a roller by Vývojové Dílny AV ČR, Praha; a 2010 shaker by Unimax, Heidolph; a CE 1010 spectrophotometer by Cecil Instruments, Cambridge; a liquid chromatograph (PU-2089 pump, MD-2015 detector, AS-2055 automatic sample injector) by Jasco, Tokyo.

Trifolium pratense L. suspension culture

The suspension culture (variety Sprint) was derived from callus culture mechanically by shaking in the Gamborg liquid nutrient medium[11] supplemented with 2 mg.l^{-1} of 2,4-dichlorophenoxyacetic acid and 2 mg.l^{-1} of 6-benzylaminopurine. The culture was cultivated on a roller at the temperature of 25 °C, and a 16-hr light/8-hr dark photoperiod. The experiments used 4-year-old suspension cultures. The subcultivation interval lasted 14 days[12].

Elicitation

During the elicitation process, on the 21st day of cultivation[12], the suspension culture (volume 25 ml) received 1.0 ml of a jasmonic acid solution (in the concentrations of 5 µmol.l^{-1}, 50 µmol.l^{-1} and 500 µmol.l^{-1} dissolved in 96% ethanol), calcium chloride (in the concentrations of 0.1 mmol.l^{-1}, 1 mmol.l^{-1} and 10 mmol.l^{-1}) and verapamil (in the concentrations of 1 µmol.l^{-1}, 10 µmol.l^{-1} a 100 µmol.l^{-1})[13–16]. The control cultures received 1.0 ml of distilled water. The elicitor application durations were 6, 24, 48, and 168 hours[8, 16]. Inspection cultures were collected after 6 and 168 hours since their production does not change notably in such short time intervals.

Determining the flavonoids and isoflavonoids

The elicited and the inspection samples underwent a photometric determination of flavonoids in accordance with the Czech Pharmacopoeia 2009[17] and a determination of isoflavonoids via the HPLC method[18]. The HPLC conditions were as follows: a RP-18 Lichrospher column (250 × 4 mm, particle size 5 µm) with a precolumn made of the same material; elution: linear gradient of a mobile phase A (methanol) in phase B (water containing 0.15% (v/v) of phosphoric acid) 30–80% (v/v) from 0 to 9 minutes was followed by an isocratic elution with a mixture of 80% (v/v) of phase A in phase B from 9 to 15 minutes; the flow rate was 1.1 ml.min^{-1}; the detection was carried out at the 260 nm wavelength. The contents of the monitored substances were quantified by using the mathematical method of normalization and by comparing with the calibration curve drawn by the external standard of the same substance. The obtained results were statistically evaluated by the t-test at $p = 0.05$.

Results and discussion

The study investigated the effect of exogenously applied jasmonic acid (JA) in combination with calcium and verapamil on the production of flavonoids and isoflavonoids in *Trifolium pratense* suspension cultures. The cultures were treated with jasmonic acid (5, 50 and 500 µmol.l^{-1}). The results show that the best effect of jasmonic acid on the production of flavonoids and isoflavonoids was manifested after a 24-hour application of the 50 µmol.l^{-1} concentration. The maximum content of flavonoids was determined at 2.95 mg.g^{-1} DW and the production was stimulated by 72% in comparison with the control (Table 1). The maximum increase in the content of isoflavonoids was as follows: genistin by 579% (0.95 mg.g^{-1} DW), daidzein by 150% (0.50 mg.g^{-1} DW) and genistein by 127% (0.50 mg.g^{-1} DW) (Table 2).

To study the role of calcium on flavonoids and isoflavonoids production, JA-induced (50 µmol.l^{-1}) suspension cultures of *Trifolium pratense* were treated with $CaCl_2$ (0.1, 1 and 10 mmol.l^{-1}) and verapamil (1, 10, and 100 µmol.l^{-1}). The highest flavonoids and isoflavonoids production was observed when cells were treated with a high level of calcium (10 mmol.l^{-1}). The maximum content of flavonoids (5.62 mg.g^{-1} DW) was induced by a 24-hour application of calcium and the production was increased by 227% in comparison with the control (Table 1). The production of isoflavonoids genistin (1.50 mg.g^{-1} DW) and daidzein (0.82 mg.g^{-1} DW) was best stimulated after a 24-hour application, the increase being 970% and 310%, respectively. The maximum content of genistein (0.91%) was found after a 48-hour application and the production was stimulated by 314% in comparison with the control (Table 2). An addition of all concentrations of verapamil to JA-induced (50 µmol.l^{-1}) suspension cultures decreased the production of flavonoids and isoflavonoids in comparison with the control culture and the JA-induced culture (Tables 1 and 2).

The process of elicitation activates various Ca^{2+} – and calmodulin-dependent protein kinases by increasing the level of free Ca^{2+} in the cytoplasm and triggers the cellular responses, which may include alterations in gene expression. Calcium ions seem to play a role in the control of the production of secondary metabolites but in a different manner depending on the species. It has a stimulatory as well as an inhibitory role in the production of the secondary metabolites in plants. The importance of jasmonic acid, calcium ions, and verapamil in the process of biotic elicitation can be documented in other examples[13–15, 19–23].

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Conflicts of interest: none.

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