Abiotic elicitation of *Trifolium pratense* L. Suspension culture

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Received: 4. June 2007 / Accepted: 23. August 2007

SUMMARY

Abiotic elicitation of *Trifolium pratense* L. Suspension culture
Important substances of secondary metabolism include flavonoids and isoflavonoids. The *Trifolium pratense* L. (*Fabacae*) suspension culture is characterized by low production of these metabolites and therefore we tried to influence the production output with elicitation. From their origin point of view, the elicitors are divided into two groups – biotic and abiotic. The latter group includes, for instance, the salts of heavy metals. Our work was aimed at observing the effect of the copper sulphate abiotic elicitor on the production of the *Trifolium pratense* L. suspension culture (variety DO-8 and variety DO-9) that was cultivated in Gamborg media supplemented with 2 $mg.1^{-1}$ of 2,4-dichlorophenoxyacetic acid and 2 $mg.\ 1^{-1}$ of 6-benzylaminopurine. The maximum increase in the flavonoid production took place, when compared with the test check, during the 168-hour application of the 100 μmol concentration.
The DO-8 variety isoflavonoids production was stimulated namely during the 48-hour application of the 1 μmol concentration; the best elicitation effect of the DO-9 variety was achieved with the 168-hour application of the 10 μmol concentration.
Key words: *Trifolium pratense* L. suspension culture – flavonoids and isoflavonoids – abiotic elicitation – heavy metals

Čes. slov. Farm., 2007; 56, 225–229 *Má*

SOUHRN

Abiotická elicitace suspenzní kultury *Trifolium pratense* L.
Mezi významné látky sekundárního metabolismu patří flavonoidy a isoflavonoidy. Suspenzní kultura *Trifolium pratense* L. (*Fabaceae*) má však nízkou produkci těchto metabolitů, a proto byla snaha ovlivnit její elicitací. Podle původu se elicitory rozdělují na biotické a abiotické elicitory, mezi které lze zařadit např. soli těžkých kovů. Cílem práce bylo sledovat vliv abiotického elicitoru síranu měďnatého na produkci suspenzní kultury *Trifolium pratense* L. (varieta DO-8 a varieta DO-9), která byla kultivovaná na mediu podle Gamborga s přídavkem 2 $mg.l^{-1}$ 2,4-dichlorfenoxyoctové kyseliny a 2 $mg.l^{-1}$ 6-benzylaminopurinu. Maximální zvýšení produkce flavonoidů oproti kontrole vyvolala u obou variet 168hodinová aplikace koncentrace 100 μmol. Produkci isoflavonoidů u variety DO-8 stimulovala zejména 48hodinová aplikace koncentrace 1 μmol, u variety DO-9 měla největší elicitační účinek 168hodinová aplikace koncentrace 10 μmol.
Klíčová slova: suspenzní kultura *Trifolium pratense* L. – flavonoidy a isoflavonoidy – abiotická elicitace – těžké kovy

Čes. slov. Farm., 2007; 56, 225–229

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Explant cultures of plants can synthesize secondary metabolites that are typical of the mother plant. Their production, compared to intact plants, is usually lower and this fact makes us look for other methods and procedures to increase the production output. One of the ways to go is the elicitation method since plants react defensively against the influence of pathogens, or other environmental impacts, and accumulate many secondary substances [1]. Factors that evoke the plant's defensive reaction are called "elicitors" or "stressors" and can be divided into the biotic group (for instance, fungi, bacteria, viruses) and the abiotic group (for instance, heavy metals, radiation, algidity) [2].

Flavonoids and isoflavonoids, characterized by a wide spectrum of biological effects, are two of the most important secondary metabolism defensive substances (phytoalexins) [3–8]. Isoflavonoids are, due to their capability to interact with the estrogen receptors and their structural similarity with the 17 ß-estradiol, one of the most important phytoestrogenes [9].

At present, we are looking for other possible sources of these substances to supplement the soya plant source. Red clover (*Trifolium pratense* L., *Fabaceae*), containing isoflavonoids, formononetin, biochanin A, daidzein, and genistin, appears to be a very promising plant [10]. Red clover extracts show a potential medical effect, for instance in cases of different tumour type ailments; they also ease the menopausal discomfort symptoms and help in cases of cardio-vascular ailments and osteoporosis [11].

The objective of our work was to observe the effect of the abiotic elicitor of copper sulphate on the production of flavonoids and isoflavonoids by the *Trifolium pratense* L. suspension cultures (variety DO-8 and variety DO-9).

EXPERIMENTAL PHASE

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 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 cultures were derived from callus cultures. First by mechanical loosening on the shaker followed by the cultivation in the Gamborg nutrient media with the 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 subcultivation interval lasted 14 days [13].

Elicitation

In the 10th passage, on the 21st day of the cultivation [13], the media volume (volume of 25 ml) was supplemented with 1.0 ml of the studied copper sulphate concentrations (0.1 μmol; 1 μmol; 10 μmol and 100 μmol). The application period of the elicitor lasted 6, 24, 48, and 168 hours.

Determining the flavonoids and isoflavonoids

The collected cells were separated from the media by vacuuming, rinsed in distilled water and dried at the laboratory temperature. The elicited and the inspection samples underwent photometric determination of flavonoids in accordance with the Czech Pharmacopoeia 2005 [14] and the determination of isoflavonoids via the HPLC method [15]. The HPLC determination process conditions were as follows: a RP-18 Lichrospher column (250 × 4 mm, particles size 5 μm) with a precolumn made of the same material; elution: linear gradient of a mobile phase A (methanol) in a phase B (water containing 0.15 % of phosphoric acid) 30–80 % from 0 to 9 minutes was followed by the isocratic elution mobile phase in composition of 80 % of phase A in phase B from 9 to 15 minutes; the elution speed was 1.1 ml/min; the detection was carried out at the 260 nm wave length. The obtained results were statistically evaluated in the t-test for at least 3 members of the team and at the p=0.05 level of statistical significance.

RESULTS AND DISCUSSION

Successful elicitation depends on many factors, each of them specific to a different elicitor and a different explant culture; for instance, different amounts of concentration and different time lengths of the elicitor's effect. Four water solution concentrations of copper sulphate were tested (0.1 μmol, 1 μmol, 10 μmol, and 100 μmol); these concentrations had been chosen from within the range that is usually applied with this type of elicitor [16-20]. The time periods of the elicitor's effect that were tested (6, 24, 48, and 168 hours) had been based on the results of the already performed tests [21–23] and on different pieces of published information that deal with the increase in the production of the secondary metabolites after the addition of an elicitor in the range of several hours to several days [16, 19, 20]. Inspection cultures were collected after 6 and 168 hours since their production does not change notably in such short time intervals.

Another factor that may influence the success of elicitation is the physiological state of the culture, or its growth phase. The findings of previous experiments imply [13] that elicitation of the *Trifolium pratense* L. suspension culture is best on day 21 of subcultivation; our culture's elicitation was therefore carried out on that day of cultivation.

The results of the elicitation of the *Trifolium pratense* L. (variety DO-8) suspension culture with copper sulphate (Fig. 1) show that namely the strongest concentration of 100 μmol is most suitable and, in the 168 hour application, it induced a statistically significant increase in the production (230 % greater when compared with the check test) – the maximum content of flavonoids was determined at 0.23 %. Statistically significant increase in production (by 43 %) was also recorded at the 24 hour application of 10 μmol concentration.

In the case of the elicitation of the *Trifolium pratense* L. suspension culture (variety DO-9) with cop-

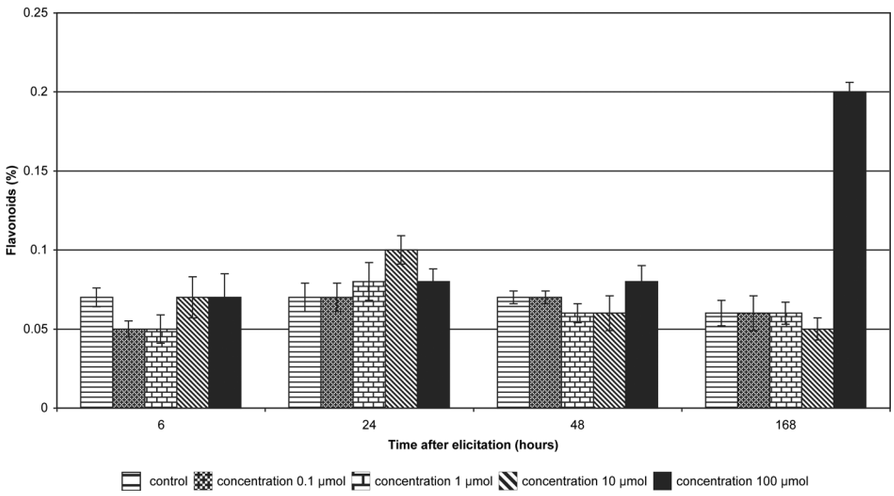

Fig. 1. Production of flavonoids in suspension culture of Trifolium pratense L. (variety DO-8) elicited with copper sulphate

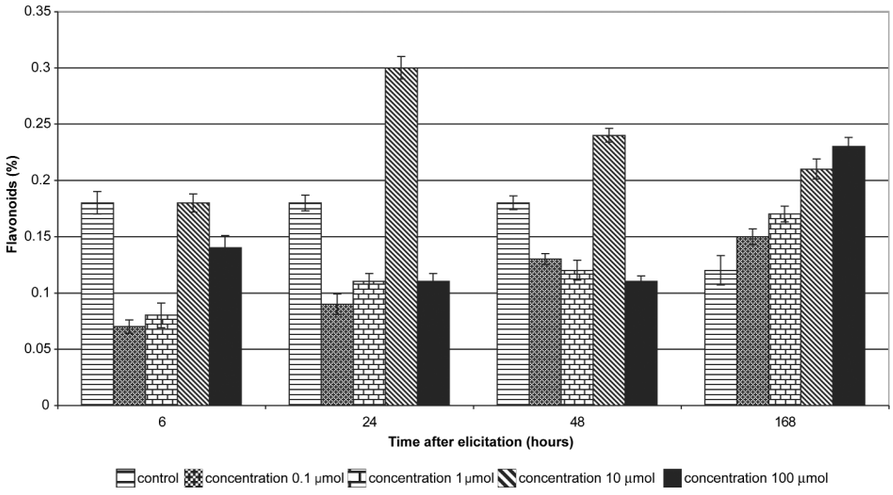

Fig. 2. Production of flavonoids in suspension culture of Trifolium pratense L. (variety DO-9) elicited with copper sulphate

per sulphate (Fig. 2), the best elicitation effect was recorded at the concentration of 10 µmol that brought about, in all tested time intervals (with an exception of the 6-hour application), a statistically significant increase in production when compared with the check test. The highest content of flavonoids (0.30 %) was recorded after the 24-hour application when the production was increased by 67 %. In the cases of other concentrations of the elicitor, a positive impact of elicitation was recorded only after the longest, 168-hour, application of the elicitor and the effect increased in accordance with increased concentration of the elicitor. The flavonoids production was increased by 92 % with the strongest concentration of copper sulphate.

The comparison of the flavonoids production in the elicited *Trifolium pratense* L. suspension cultures (varieties DO-8 and DO-9) confirms that the maximum increase in the production of flavonoids in both cases was induced during the 168-hour application of the strongest concentration of copper sulphate.

The *Trifolium pratense* L. suspension cultures (varieties DO-8 and DO-9) elicited with copper sulphate were also observed for the production of isoflavonoids using the HPLC method. The test check culture of the variety DO-8 contained the following isoflavonoids: genistin, daidzein, genistein, and formononetin (Table 1). The success in the production was namely achieved with the 48-hour application of the 1 µmol concentration which stimulated the production of all these isoflavonoids. The maximum content (0.48 %) and a statistically significant increase in production, in comparison with the check test by 860 %, were recorded in genistin. The daidzein and formononetin production increased by 300 % and the genistein production by 50 %. The content of genistin increased also in the other concentrations of the elicitor; once again namely after the 48-hour application.

The elicitation of the DO-9 variety suspension culture (Table 2) also increased the content of isoflavonoids. The best elicitation effect was achieved after the 168-hour application of the 10 µmol concentration when the highest content of genistin (0.38 %) was recorded. This fact represents a statistically significant increase by 124 %.

Tab. 1. Production of isoflavonoids in suspension culture of Trifolium pratense L. (variety DO-8) elicited with copper sulphate

Elicitor concentration (mmol)	Time after elicitation (hours)	Isoflavonoid contents (%)			
		genistin	daidzein	genistein	formononetin
	6	0.05±0.006	0.02±0.006	0.02±0.003	0.01±0.006
	24	0.05±0.006	0.02±0.010	0.02±0.006	0.01±0.002
control	48	0.05±0.010	0.02±0.006	0.02±0.000	0.01±0.006
	168	0.02±0.006	0.01±0.002	0.01±0.008	0.01±0.006
	6	0.06±0.010	0.01±0.004	0.01±0.006	0.01±0.006
	24	0.05±0.006	0.02±0.005	0.01±0.006	0.01±0.010
0.1	48	0.10±0.015	0.02±0.006	0.02±0.005	0.01±0.006
	168	0.05±0.006	0.02±0.015	0.01±0.006	0.01±0.010
	6	0.09±0.006	0.01±0.004	0.01±0.006	0.01±0.006
	24	0.10±0.025	0.01±0.006	0.02±0.006	0.03±0.004
1	48	0.48±0.015	0.08±0.004	0.03±0.002	0.04±0.007
	168	0.08±0.010	0.01±0.006	0.01±0.000	0.02±0.006
	6	0.06±0.006	0.01±0.003	0.01±0.006	0.02±0.006
	24	0.08±0.006	0.01±0.000	0.02±0.006	0.03±0.008
10	48	0.12±0.010	0.02±0.004	0.02±0.002	0.03±0.004
	168	0.07±0.006	0.01±0.002	0.01±0.006	0.02±0.003
	6	0.06±0.006	0.01±0.002	0.01±0.006	0.01±0.006
	24	0.08±0.006	0.01±0.006	0.01±0.006	0.01±0.000
100	48	0.10±0.008	0.02±0.005	0.02±0.004	0.03±0.012
	168	0.07±0.006	0.01±0.006	0.01±0.010	0.03±0.006

Tab. 2. Production of isoflavonoids in suspension culture of Trifolium pratense L. (variety DO-9) elicited with copper sulphate

Elicitor concentration (mmol)	Time after elicitation (hours)	Isoflavonoid contents (%)			
		genistin	daidzein	genistein	formononetin
	6	0.22±0.008	0.01±0.006	0.01±0.006	–
	24	0.22±0.010	0.01±0.006	0.01±0.006	–
control	48	0.22±0.005	0.01±0.000	0.01±0.000	–
	168	0.17±0.007	0.01±0.010	0.01±0.006	–
	6	0.11±0.012	0.02±0.003	0.01±0.006	0.01±0.006
	24	0.08±0.006	0.01±0.000	0.01±0.006	0.01±0.000
0.1	48	0.08±0.010	0.01±0.003	0.01±0.000	0.01±0.010
	168	0.25±0.015	0.02±0.004	0.02±0.003	0.01±0.006
	6	0.08±0.010	0.02±0.003	0.01±0.006	0.01±0.006
	24	0.08±0.012	0.01±0.006	0.01±0.000	0.01±0.006
1	48	0.11±0.006	0.01±0.003	0.01±0.006	0.01±0.000
	168	0.07±0.012	0.02±0.004	0.02±0.006	0.01±0.006
	6	0.16±0.003	0.03±0.005	0.02±0.006	0.01±0.006
	24	0.36±0.010	0.03±0.003	0.01±0.005	0.01±0.000
10	48	0.36±0.006	0.04±0.006	0.01±0.000	0.02±0.010
	168	0.38±0.010	0.04±0.008	0.02±0.010	0.02±0.000
	6	0.19±0.022	0.02±0.015	–	–
	24	0.22±0.010	0.02±0.006	–	–
100	48	0.21±0.009	0.03±0.012	–	–
	168	0.17±0.013	0.03±0.006	–	–

The very low content of daidzein and genistein in the culture increased after this type of elicitation by 300 % and 100 %; even formononetin induction was achieved (the test check culture actually did not contain formononetin).

The results prove that the production of the monitored secondary metabolites in the *Trifolium pratense* L. suspension culture is low. One of the reasons for this can be the fact that it is a young, fast growing suspension culture which accumulates, in most cases, only small amounts of metabolites. The production may be increased by introducing a certain stress – for instance lowering the concentration of nutrients in the solution, leaving out the growth regulator, or applying an elicitor to the media. The elicitation of the *Trifolium pratense* explantate culture with copper ions confirmed this fact. The utilization of the copper ions as an abiotic elicitor can be documented in other examples [16, 24, 26].

This work was sponsored under the Research Project No. MSM 0021620822.

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EXCERPTA

● Nové druhy chirurgických operačních rukavic

Jistota práce s tímto druhem zdravotnického prostředku je především daná použitým základním materiálem a jeho zpracováním. Vysoké nároky jsou zde dány mechanickými vlastnostmi, hustotou, hmatatelností, komfortností při nošení i biologickou snášenlivostí. Nyní se nově zavádějí dva druhy: PEHA – profile plus a Peha-neon plus puder frei (vyrábí firma Hartmann), které nejsou uvnitř posypány. Oba druhy rukavic mají na povrchu speciální texturu (zvrstvení), která nesnižuje přirozenou citlivost prstů a zajišťuje stabilitu držení chirurgického nástroje, další předností je snadné nasazení i na vlhkou ruku a stejně lehké sundání, při čemž vnitřní stěny rukavic dobře snáší lihový roztok. První je z klasického latexu, neobsahuje ani stopy bílkoviny, druhý typ je vyroben ze syntetického kaučuku – polychloroprenu (ten se pro svoje tepelné izolační vlastnosti využívá na obleky potápěčů, ve zdravotnictví na některá sportovní obinadla a ochranné roušky). Chirurgické rukavice tohoto druhu jsou vhodné pro použití u alergických nemocných, nepoškozují se cementy, jejich povrchová textura zlepšuje uchopení, zaručuje dobrý cit jak při práci tak při šití. Oba druhy rukavic jsou jednotlivě baleny do PE obalů se suchým zipem, vysterilizovány, snadno se z nich vyndavají a není zde nebezpečí jejich mechanického poškození.

Hartmann WundForum, 2007 (2), 13-14.

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