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Influence of $AgNO_3$ Treatment on the Flavonolignan Production in Cell Suspension Culture of *Silybum marianum* (L.) Gaertn

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***Abstract*—**The abiotic elicitation is one of the methods for increasing the secondary metabolites production in plant tissue cultures and it seems to be more effective than traditional strategies. This study verified the use of silver nitrate as elicitor to enhance flavonolignans and flavonoid taxifolin production in suspension culture of *Sylibum marianum* (L.) Gaertn. Silver nitrate in various concentrations ($5.887.10^{-3}$ mol/L, $5.887.10^{-4}$ mol/L, 5.887.10-5 mol/L) was used as elicitor. The content of secondary metabolites in cell suspension cultures was determined by high performance liquid chromatography. The samples were taken after 6, 12, 24, 48, 72 and 168 hours of treatment. The highest content of taxifolin production (2.2 $mg.g^{-1}$) in cell suspension culture of *Silybum marianum* (L.) Gaertn. was detected after silver nitrate ($5.887.10^{-4}$ mol/L) treatment and 72 h application. Flavonolignans such as silybinA, silybin B, silydianin, silychristin, isosilybin A, isosilybin B were not produced by cell suspension culture of S. marianum after elicitor treatment. Our results show that the secondarymetabolites could be released from *S. marianum* cells into the nutrient medium by changed permeability of cell wall.

Keywords*—Silybum marianum* (L.) Gaertn., elicitation, silver nitrate, taxifolin.

I. Introduction

THE milk thistle *Silybum marianum* (L.) Gaertn. (*Asteraceae*) is a well-known medicinal plant used mainly in chronic liver disease as hepatoprotective agent. The fruits of this plant contain silymarin, an isomeric mixture of flavonolignans – silydianin, silychristin A and B, silybin A and B and isosilybin A and B [1]. Taxifolin, quercetin and dehydrokaempferol are also important flavonoids presented in the milk thistle. Silymarin is used to treat liver disorders and shows direct anti-carcinogenic activity against several human carcinoma cells; in addition, silymarin has antidiabetic, hypolipidemic, anti-inflammatory, cardioprotective, neurotrophic and neuroprotective effects [2], [3].

Plant tissue cultures are the most useful and favourable experimental systems for examining various factors on the biosynthesis of desired products and for exploring effective measures to enhance their production. *In vitro* culture of cells and tissues may offer an alternative for the production of silymarin but until now, in all cases, flavonolignan production in *in vitro* cultures is very low and even disappears in prolonged culture [4]. Many traditional strategies can be used to increase the production of secondary metabolites but elicitation is one of the most successful. The principle of elicitation consists in the accumulation of secondary metabolites, as the part of the plant defense against effects caused by pathogens (viruses, bacteria) or by plant environment factors (pH, temperature, draught, heavy metals, etc.). Elicitation strengthens the transcription of genes; which are necessary for synthesis of secondary metabolites [5]. Therefore, elicitation treatment of plant tissue cultures with elicitors is one of the most effective strategies for improving secondary metabolite production in plant tissue cultures [6], [7]. There are several reports where heavy metals elicited different secondary metabolite production in the plant cell culture system. Copper induced the berberine production in *Thallictrum rugosum* suspension culture [8], reserpine production in *Rauwolfia serpentina* culture [9], chromium increased production of dipyranocoumarins in *Calophyllum inophyllum* L. [10], silver nitrate increased tanshinone production in *Salvia miltiorrhiza* [11], heavy metal salts ($CdCl_2$, $CoCl_2$) increased diosgenin production in *Trigonellafoenum-graecum* L.[12].

The present study focuses on the effects of silver nitrate ($AgNO_3$) on the accumulation of flavonolignans, flavonoid taxifolin in cell suspension cultures of *S. marianum* respectively.

II. Materials and Methods

A. Silybum marianum (L.) Gaertn. Cell Suspension Culture

The callus culture was derived from the germinating seeds (germination capacity over 75%) of plant *Silybum marianum* (L.) Gaertn. (*Asteraceae*). Seed was obtained from the Garden of Medicinal Plants, Faculty of Pharmacy in Hradec Kralove. Calluses were cultured on Murashige and Skoognutrient medium [13] supplemented with α-naphtylacetic acid (NAA) as a growth regulator at a concentration of 5.4×10^{-5} mol/L. *S. marianum* callus cultures were cultivated in 30mL of nutrient medium on paper bridges in Erlenmeyer flasks for 25 days and these cultures were incubated in growth chambers at 26 ± 1°C under 16 h photoperiod. White light of intensity of 3.500 lux was used. The cell suspension culture was then derived from the callus culture of *S. marianum* in the 39th–41st

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passages/generation mechanically by shaking in Murashige and Skoog nutrient medium [13] supplemented with α-naphtylacetic acid (NAA) as a growth regulator at a concentration of 5.4×10^{-5} mol/L and 15 mL of cell suspension was cultivated in 250 mL Erlenmeyer flasks with shaking at 120 rpm. Suspensions were kept under the same conditions as callus cultures. The subcultivation interval of both cell suspension cultures lasted 2 weeks.

B. Elicitation

For elicitation experiments silver nitrate in concentrations of (c1) $5.887x10^{-3}$ mol/L; (c2) $5.887x\ 10^{-4}$ mol/L; (c3) $5.887x.10^{-5}$ mol/L was used. 1 mL of elicitor solution in ethanol was added to each flask (containing 30 mL) of the cell suspension culture before the end of the exponential growth phase of the culture on the 16th day of cultivation. 6; 12; 24; 48; 72 and 168 h after elicitor application, the cells from suspension cultures were sampled, dried and the content of flavonolignans was determined. Simultaneously, the controls (without the elicitor treatment) were run for 24 and 168 h. The suspension cells were dissociated from the liquid nutrient medium by filtration through Whatman filter paper (No. 1-6) under vacuum, dried and the content of flavonolignans was determined. The nutrient medium was kept frozen to be used for subsequent analysis. All treatments were triplicated; each data point reported is the mean of three replicate measurements.

C. Determination of Flavonolignans

The content of flavonolignans in *S. marianum* cultures *in vitro* was determined by HPLC on a UNICAM CRYSTAL 200 Liquid Chromatograph, according Czech Pharmacopeia [14].

Dry mass of suspension cells (0.100 g) was extracted twice (in a water bath under reflux cooler) with 10 mL of 80 % (v/v) methanol for 30 min. The extract was filtered via Teflon filter (diameter 22 μm) and 2 mL of this filtrate was analysed by HPLC method. HPLC conditions were developed in our laboratory as follows: a RP-18 Lichrospher column (250 x 4 mm, particles size 5 μm) with a precolumn made from the same material; elution : linear gradient of a mobil phase A (methanol) in a phase B (water containing 0.15% (v/v) of phosphonic acid) 30-80% (v/v) from 0 to 9 minutes was followed by the isocratic elution with a mixture of 80% (v/v) of a phase A in a phase B from 5 to 15 minutes; the flow rate was 1.1 mL/min; the detection was carried out at the 260 nm wavelength. The content of flavonolignans was quantified by using the mathematical method of normalization and by comparing with the calibration curve drawn by the external standard of the same substance. Silymarin, silybin A, B, silychristrin, silydianin, isosilybin A, B were used as standards.

D. Statistical Analysis

All experimental analyses were carried out in a minimum of three independent samples for each elicitation period and each concentration of elicitor. To determine whether there was a difference between values of samples, the T-test was applied. Values of $p\leq0.05$ were considered as significantly different. The differences between means were determined by using Tukey´s multiple comparison test.

III. Results

This experiment is focused on influence of the elicitor concentration and the time duration of the elicitor effect on the flavonolignans and flavonoid content.

Only flavonoid taxifolin was determinted in cell suspension culture of *S. marinanum* after $AgNO_3$ elicitation (Fig. 1). The highest content of taxifolin (2.2 $mg.g^{-1}$ DW) was obtain after silver nitrate treatment in c2 ($5.887x10^{-4}$ mol/L) concentration and 72 h application. The increased content of taxifolin ($1.1mg.g^{-1}$DW) in suspension culture of *S. marianum* was also found after silver nitrate ($AgNO_3$) elicitation in highest concentration c1 $5.887x10^{-3}$ mol/L and 12, 24 h applications and also after silver nitrate elicitation in the lowest concentration c3 $5.887x.10^{-5}$ mol/L and 48, 72, 168 h applications. Flavonolignans such as silybin A, silybin B, silydianin, silychristin, isosilybin A, isosilybin B were not produced by a cell suspension culture of *S. marianum* after elicitor treatment. Control samples (without the elicitor treatment) do not produce monitored secondary metabolites.

Cultures *in vitro* can release secondary metabolites also into nutrient medium. For this reason individual flavonoid and flavonolignans were tested also in nutrient medium of cell suspension cultures. The noticeable excretion of flavonolignans into nutrient medium was determined after elicitation of silver nitrate in concentration c3 (Table I). The maximum contents excreted silydian (3.5 mg/100mL), silybin A (1.8 mg/100mL) and silybin B (1.2mg/100mL) in nutrient medium were found after elicitor treatment in this concentration (c3 $5.887x.10^{-5}$ mol/L) and twelve-hour application. The highest content of taxifolin 1.9 mg/100mL was determined in a nutrient medium after the elicitor treatment in c1 concentration and 48 h application. The highest content of released silychristin 1.7 mg/100mL was determined in a nutrient medium after the elicitor treatment in (c2 $5.887x.10^{-4}$ mol/L) concentration and 168 h application. Other observed secondary metabolites were not found in a nutrient medium.

The control suspension culture (without the elicitor application) released taxifolin (0.1 mg/100mL) in 0 hours also silychristin (0.1 mg/100mL) and silydianin (0.3 mg/100mL) after 168 h into a nutrient medium.

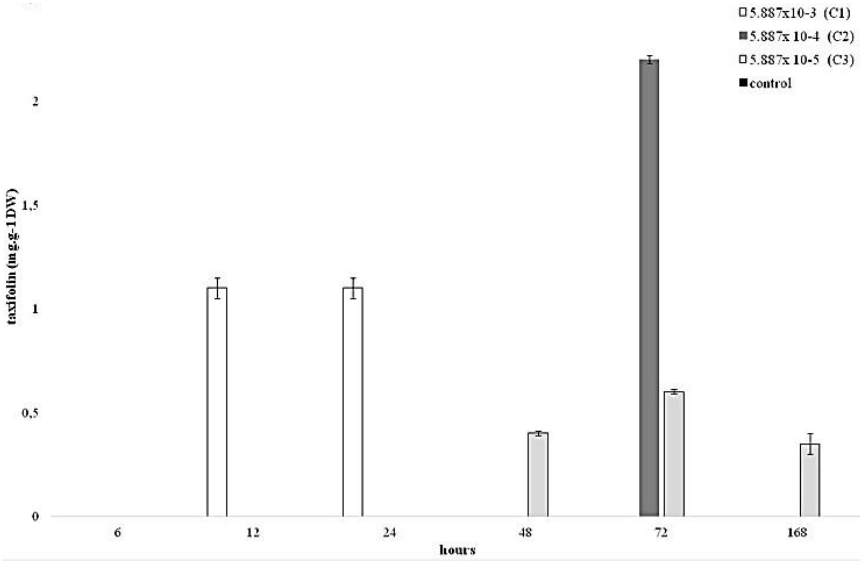

Fig. 1 Flavonoid content (mg.g^{-1} DW) in *Silybum marianum* L. Gaertn. cell suspension culture after silver nitrate ($AgNO_3$) treatment

TABLE I
FLAVONOLIGNAN CONTENT (MG/100 ML) IN *SILYBUM MARIANUM* L. GAERTN. NUTRIENT MEDIUM AFTER $AgNO_3$ TREATMENT

$AgNO_3$ concentration (mol/L)	Time after elicitation (hours)	Taxifolin	Silychristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B
5.887x 10^{-3} (C_1)	6	0.6±0.009	0.1±0.005	0	0	0	0	0
	12	0.3±0.002	0.1±0.001	0	0	0	0	0
	24	0.5±0.002	0.1±0.002	0	0	0	0	0
	48	1.9±0.05	0	0	0	0	0	0
	72	1.2±0.08	0	0	0	0	0	0
	168	0	0	0	0	0	0	0
5.887x 10^{-4} (C_2)	6	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0
	24	0	0	0	0	0	0	0
	48	0	0	0	0	0	0	0
	72	0	0	0	0	0.6±0.002	0	0
	168	0	1.7±0.005	0	0	0.5±0.002	0	0
5.887x 10^{-5} (C_3)	6	1.6±0.05	1.3±0.005	0.4±0.002	0.3±0.002	0	0	0
	12	1.3±0.002	0	3.5±0.05	1.8±0.05	1.2±0.003	0	0
	24	0.4±0.001	0.6±0.05	0.7±0.001	0	0	0	0
	48	0.2±0.009	0.2±0.002	0.5±0.003	0	0	0	0
	72	0	0	0.2±0.001	0	0	0	0
	168	0	0	0	0	0	0	0
Control	0	0.1±0.002	0	0	0	0	0	0
	168	0	0.1±0.001	0.3±0.003	0	0	0	0

Note: Values are mean of 3 replicates ± SD.

IV. DISCUSSION

As mentioned in Section I, a successful elicitation treatment depends not only on the type of abiotic or biotic elicitor but also on the time of elicitation, on a concentration of elicitor used and on the specific character of a plant tissue. It means that there are many factors which are able to effect secondary metabolite production in *in vitro* cultures. Therefore, our experiments are focused to evaluate the effect of the concentration and the time duration of the elicitor treatment on the flavonolignans content.

The published results show of positive influence of a lower concentration of the elicitor to a production of secondary metabolites [15], [16]. Although other results demonstrated increase of accumulation of taxifolin after elicitation with the highest concentration (100µM) of methyl jasmonate and 48 h of application [17]. In other scientific survey there was published positive influence of high concentration of Ag^+ to sylimarin content (1.2mg.g^{-1}DW), particularly the content of silybin, silychristin and silydianin were 0.069, 0.388 and 0.024 mg.g^{-1}DW after 2mM Ag^+ elicitation and 96h of sampling [18]. The increased production of secondary metabolites after the silver ions elicitation was demonstrated also at other medicinal plants [19]-[21].

V. CONCLUSION

The study has shown that the silver nitrate is a potent abiotic elicitor which can induce defense responses of plant cells and stimulate flavonolignans accumulation in cell suspension cultures of *S. marianum*. The results refer that silver nitrate in lower intense concentration is more effective

for higher accumulation flavonolignans such as silychristin, silydianin, silybin A and silybin B from *S. marianum* Our findings did not confirm the influence of time of $AgNO_3$ treatment to increase flavonolignans production. Our results show that the secondary metabolites could be released from *S. marianum* cells into the nutrient medium by changed permeability of cell wall. The next research should be done in further study of this type of stress elicitation.

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REFERENCES

[1] N.C. Kim, T.N. Graf, C.M. Sparacino, M.E. Wani, M.E. Wall Complete isolation and characterization of silybins and isosilybins from Milk thistle (Silybum marianum). Organic Biomolecular Chemistry, 2003, 1:1684-1689.

[2] K. Flora, M. Hahn, K. Brenner, Milk thistle (Silybum marianum) for the therapy of liver disease. American Journal of Gastroenterology, 1998, 93:139-143.

[3] V. Kren, D. Walterova, Silybinin and silymarin new effects and applications. Biomedical Papers, 2005, 149:29-41.

[4] P. Ferreiro, M.S.S. Pais, J.M.S. Cabral, Production of silybin-like compound in cell suspension cultures of Silybum marianum. PlantaMedica, 1991, suppl. 2:2-3.

[5] T. Nürnberger, D. Scheel, D Signal transmission in the plant immune response. Trends in Plant Science, 2001, 8:372-379.

[6] T.M., Chong, M.A. Abdullah, Q.M. Lai, F.M. Nor´Aini, N.H. Lajis, Effective elicitation factors in Morindaelliptica cell suspension culture. Process Biochemistry, 2005, 40:3397-3405.

[7] I. Smetanska, Production of secondary metabolites using plant cell cultures. Advances in Biochemical Engineering Biotechnology, 2008,11:187-228.

[8] D. Kim, H. Pederson, C. Chin, Stimulation of berberine production of Thalictrumrugosum suspension cultures in response to addition of cupric sulfate. Biotechnology Letters, 1991, 13:213-216.

[9] N.N. Solichatun, E. Anggarwulan, The resperine production and callus growth of indian snake root (Rauwolfia serpentine L.) Benth. Ex Kurz culture by addition of Cu 2+. Biodiversitas, 2008, 9:177-179.

[10] K.D. Pawar, S.R. Thengane, Influence of abiotic elicitation on production of dipyranocoumarins in suspension cultures of Calophylluminophyllum L. ActaPhysiologiaePlantarum, 2011, 33:2149-2158.

[11] J.L. Zhao, L.G. Zhou, J.Y. Wu, Effect of biotic and abiotic elicitators on cell growth and tanshinone accumulation in Salvia miltiorrhiza cell cultures. Applied Microbiology and Biotechnology , 2010, 87:137-144.

[12] De Debjani, De Bratati, Elicitation of diosgenin production in Trigonellafoenum-graecum L. seedlings by heavy metals and signaling molecules. ActaPhysiologiaePlantarum , 2011, 33:1585-1590.

[13] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco cultures. PhysiologiaPlantarum, 1962, 15:473-479.

[14] Czech Pharmacopeia; Ph.B. Silybimarianifructus, in Czech Pharmacopeia, Grada Publishing, Ltd. Prague, 2009b, 3146-3147.

[15] L. Tůmová, J. Tůma, Ovlivněníprodukcesekundárníchmetabolitů v buněčnékultuřeSilybummarianumpřídavkemelicitoruParaquat. Chemickélisty, 2009, 103:503-510.

[16] F. Karimi, E. Khataee, Aluminum elicits tropane alkaloids production and antioxidant system activity in micropropagatedDaturainnoxia plantlets. ActaPhysiologiaePlantarum, 2012, 34:1035-1041.

[17] Elwekeel , A. Elfishway, S. AbouZid, Enhanced accumulation of flavonolignans in Silybummarianum cultured roots by methyl jasmonate. PhytochemistryLetters , 2012, 5:393-396.

[18] M. Khalili, T. Hasanloo, S.K.K. Tabar Ag+ enhanced silymarin production in hairy root cultures of Silybum marianum (L.) Gaertn. Plant OMICS: Journal of Plant Biology &Omics, 2010, 3:109-114.

[19] H.K. Choi, J.H. Yoon, Metabolomic profiling of Vitisvinifera cell suspension culture elicited with silver nitrate by 1H NMR spectrometry and principal components analysis. Process Biochemistry, 2007, 42:271-274.

[20] Q. Yan, Z.D. Hu, J.Y.Wu, Synergistic effects of biotic and abiotic elicitors on the production of tanshinones in Salvia miltiorrhiza hairy root culture. ZhongguoZhonggyaoZazhi, 2006, 31:188-191.

[21] Y. Xiao, S. Gao, P. Di, J. Chen, W. Chen, L. Zhang (2010) Lithospermic acid B is more responsive to silver ion (Ag+) than rosmarinic acid in Salvia miltiorrhiza hairy root cultures Biosciences Reports 30:33-40.