ELICITATION OF PHENOLIC COMPOUNDS IN CELL CULTURE OF *Vitis vinifera* L. BY *Phaeomoniella chlamydospora*

MARTIN SÁK[1,2], IVANA DOKUPILOVÁ[1,2], DANIEL MIHÁLIK[3,4], JANA LAKATOŠOVÁ[1,2], MARCELA GUBIŠOVÁ[3,5], JÁN KRAIC[3,6*]

[1]*Research Institute of Viticulture and Enology, Hlavná ulica 326, SK-90041, Rovinka, Slovak Republic*
[2]*Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, SK-81237 Bratislava, Slovak Republic*
[3]*Research Institute of Plant Production, Bratislavská cesta 122, 921 68, Piešťany, Slovak Republic*
[4]*Institute of High Mountain Biology, University of Žilina, SK-05956 Tatranská Javorina 7, Slovak Republic*
[5]*Department of Botany and Genetics, Faculty of Natural Sciences, Constantine The Philosopher University, Tr. A. Hlinku 1, SK-94974 Nitra, Slovak Republic*
[6]*Department of Biotechnology, Faculty of Natural Sciences, University of SS. Cyril and Methodius, J. Herdu 2, SK-91701 Trnava, Slovak Republic (kraic@vurv.sk)*

Abstract: The *in vitro* cell cultures of *Vitis vinifera* L. cv. St. Laurent were treated with two elicitors - synthetic methyl jasmonate and natural, prepared from grapevine plant infected with the *Phaeomoniella chlamydospora*, the agent causing the Esca disease of grapevine. Efficiency of phenolic compounds production after elicitation of cell culture was analysed immediately after treatment (15 min, 30 min, 60 min) and later (after 24, 48, and 72 hours). The cell growth and content of phenolic compounds (+)-catechin, (-)-epicatechin, *p*-coumaric acid, syringaldehyde, rutin, vanillic acid, and trans-resveratrol were analysed in cultivated cells as well as in cultivation medium. *Pch*-treatment increased production of total polyphenols the most significantly 15 min after the elicitation and in optimal time was 2.86 times higher than in non-elicited culture and 1.44 times higher than in MeJa induced cell culture.

Abstract: *Vitis vinifera* L., *Phaeomoniella chlamydospora*, phenolics, cell culture, elicitation

1. Introduction

Plant cells cultivated *in vitro* could potentially be competitive systems for effective production of marketable secondary metabolites possessing biological activities which can not be produced by microorganisms or by chemical synthesis. Production of such molecules and compounds has been demonstrated in different plant species (MULABAGAL and TSAY, 2004) as well as by *in vitro* cultivation of organs, tissues, or cells (reviewed by RAO and RAVISHANKAR, 2002). An overproduction of some secondary metabolites, in the comparison with level in intact plants, was achieved in *in vitro* systems, e.g. production of rosmarinic acid in cell cultures of *Salvia officinalis* L. and *Coleus blumei* (ULBRICH *et al*., 1985; HIPPOLYTE *et al*., 1992), antraquinones in cell cultures of *Morinda citrifolia* L. (ZENK *et al*., 1975). The cell cultures *in vitro* have been used advantageously in the production of substances such as vincristine,

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vinblastin, taxol, camptothecin, ellipticine, and others (RAO and RAVISHANKAR, 2002). The most attractive compounds among them, from the commercial point of view, are plant-derived pharmaceuticals (XU *et al*., 2011; ABDULLAHIL BAQUE *et al*., 2012) and food additives (JIMÉNEZ-APARICIO *et al*., 1999; MATKOWSKI, 2008).

The plant biotechnology tools are instruments also for industrial exploitation of plant cell cultures, but the economical point of view is very important and always predominant. Moreover, efficient *in vitro* production of secondary metabolites needs high effective systems with optimized cultivation conditions, methods of extraction of secondary metabolism products as well as specific factors improving production such as biotic and abiotic elicitors (DÖRNENBURG and KNORR, 1995; ZHAO *et al*., 2005), or simulation of nutritional deficiency of cells (TAVARES *et al*., 2013).

The grapevine (*Vitis vinifera* L.), especially ripe berries, contain many phenolic compounds. LIANG *et al*. (2011) identified 36 polyphenols including anthocyanins, flavanols, flavonols, hydroxycinnamic derivatives, and hydroxybenzoic acid, and 48 different polyphenols in berries of wild *Vitis* species (LIANG *et al*., 2012). Grapevine phytochemicals consumed in berries and wine are associated with the positive health benefits and relevant dietary style (IRITI and FAORO, 2006). They contain compounds with antioxidant and antibacterial activities, compounds decreasing cholesterol level, protecting against reactive oxygen species inducing DNA damages, modulating glucose uptake, and anticancer activities (WILLIAMSON and CARUGHI, 2010; DELGADO ADÁMEZ *et al*., 2012; APOSTOLOU *et al*., 2013). Aqueous extracts from grapevine leaves also contain phenolic compounds with antioxidant activity (FERNANDES *et al*., 2013). Production of secondary metabolites in the grapevine cell cultures *in vitro* has been presented in many studies and most of these experiments have been focused to improvement of *in vitro* production by adding of precursors, by genetic transformation of plants, or by elicitation. Application of specific precursors and genetic transformations are expensive and laborious, therefore the most common approach is the induction of defence mechanisms by elicitors which are signalling triggers of secondary metabolite formation (MULABAGAL and TSAY, 2004). ZHANG *et al*. (2002) induced biosynthesis of anthocyanins by irradiation and by jasmonic acid, CAI *et al*. (2012) enhanced production of anthocyanins and resveratrol by indanoyl isoleucine, N-linolenoyl-l-glutamine, and insect saliva. SAW *et al*. (2012) stimulated synthesis of anthocyanins by ethephon and pulsed electric field. Also other elicitors were tested, well-known and frequently used are synthetic methyl jasmonate and salicylic acid.

Infections of plants by pathogens induce upregulation of plant defence mechanisms and production of anti-pathogen phenolic compounds. The fungal pathogen *Phaeomoniella chlamydospora* (*Pch*), associated with the Esca-disease, also increased accumulation of phenolic components in infected young grapevine plants (MARTIN *et al*., 2009). BRUNO and SPARAPANO (2006) applied three Esca-disease associated fungi - *Phaeomoniella chlamydospora*, *Togninia minima*, and *Fomitiporia mediterranea* as elicitors in dual *in vitro* cultivation with grapevine calluses. Each fungus reduced growth of calluses but only *T. minima* induced higher production of total phenols in callus cultures. New, effective, and especially natural elicitors could improve production of secondary metabolites *in vitro*. Therefore ESCORIAZA *et al*.

(2013) treated grapevine (cv. Chardonnay) callus and cell cultures by the fungus *Phaeoacremonium parasiticum* associated with disease known as "hoja de malvón". The main response of plant tissue and cell defensive mechanism to the pathogen attack was increased terpene synthase activity and the production of nerolidon through *de novo* synthesis. Nerolidon is able to retard the fungal growth.

The broader spectrum of elicitors, the more their combinations could be used. The species-specific elicitors are very perspective and also desired for the production of secondary metabolites used for plant protection. Natural elicitors should be also easily available for industrial (pharmaceutical, nutritional) applications. Therefore the aims of this study were to: i) study response of grapevine cell culture *in vitro* to the species-specific natural elicitor prepared from *Phaeomoniella chlamydospora*, ii) analyse time course of polyphenols production in elicited cell cultures, and iii) compare production of secondary metabolites in cell cultures treated with this elicitor and with widely used methyl jasmonate.

2. Material and methods

The callus cultures were initiated from sterile leaf segments of grapevine (*Vitis vinifera* L.) cultivar St. Laurent. The leaf segments of size 1 cm^2 were cultivated on the MURASHIGE and SKOOG medium (1962) with salt concentration reduced to one half (½ MS), containing 3 % sucrose, 0.7 % (w/v) agar, 0.1 mg/l NAA, and 0.2 mg/l BAP, pH 5.8, at 25±1°C under a photoperiod 16 h light/8 h dark and were transferred to fresh cultivation medium every 30 days. The cell cultures have been initiated from calluses in 100 ml Erlenmeyer flasks containing 40 ml of ½ MS liquid medium and cultivated with shaking (120 rpm) under the same conditions as callus cultures.

Elicitor from the *P. chlamydospora* (*Pch*) was prepared from the cortex of grapevine plant infected by this fungus according to the procedure described by YU *et al*. (2001). Carbohydrate concentration was determined by the orcinol-sulfuric acid method (FRANCOIS *et al*., 1962). One-month old cell cultures were elicited by adding either of *Pch* (0.2 g/l) or MeJa (0.18 g/l) and cultivated for 1 hour at 50°C. Three parallel experiments for each cultivation were carried.

Reagents of analytical grade were used for HPLC analysis including standards (+)-catechin, vanillic acid, (-)-epicatechin, *p*-coumaric acid, syringaldehyd, rutin, and *trans*-resveratrol (Sigma-Aldrich, St. Louis, USA). Reference standard solutions of polyphenols were prepared in 50 % aqueous methanol and stored at 4°C in the dark. Other used reagents were Evans blue, fluorescein diacetate (FDA), methyl jasmonate (MeJa), (Sigma-Aldrich, St. Louis, USA), 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP), sucrose, agar, yeast extract, bacto-peptone, magnesium sulphate heptahydrate, potassium dihydrogen phosphate, sodium dodecylsulphate (SDS) (Sigma-Aldrich, St. Louis, USA). Water was prepared by the Direct-Q® 3 UV Water Purification System (Merck Millipore, Darmstadt, Germany).

Polyphenols were extracted from the cultivation medium as well as from cells according to CAI *et al*. (2011) and LIU *et al*. (2010) and analysed by HPLC (Agilent 1200 Series system with photo-diode-array detector, operated under the Agilent ChemStation Software). The plant cell cultures were separated from cultivation

medium by vacuum filtration and fresh weight was determined. Dry cell weight was determined after freezing and lyophilisation. Homogenised dry cells were extracted with ethyl acetate and methanol (1:1, v/v) in the ratio 1:10 (w/v) overnight at the room temperature. Supernatant obtained after centrifugation (10 min at 10°C and 10 000 rpm) was collected and sediment was extracted for the second time. Pooled supernatants were evaporated by vacuum and diluted in 1.5 ml of methanol. Ten microliters of internal standard was added to samples for better identification. Samples were filtered with syringe membrane filters (0.45 μm) before HPLC analysis. Twenty microliters of sample were injected into the Supelcosil™ LC-18 column (250 mm × 4,6 mm, 5 μm particle size) warmed up to 30°C. Program of HPLC measurements was carried out according to RODRIGUEZ-BERNALDO de QUIRÓS *et al.* (2009).

Total content of selected phenolic compounds was established as a sum of all quantified phenolic compounds.

3. Results and discussion

The cell cultures of *Vitis vinifera* (cv. St. Laurent) were elicited with the commercially produced and commonly available MeJa or by elicitor prepared from grapevine plant infected by the fungus *Phaeomoniella chlamydospora*. This pathogen acts as an inducer of oxidative burst also in grapevine *in vitro* cell culture (LIMA and DIAS, 2012). Both used elicitors induced change in the colour of cell cultures from green to brown and inhibited cell growth as reported by TASSONI *et al*. (2005). The non-elicited (control) cell cultures had a higher mass of growing cells measured as fresh weight of cells. Growth of cells decreased during the first 24 hours in MeJa- and *Pch*-treated cell cultures and during the first 4 hours in non-elicited culture. After maximal decreasing of growth, i.e. 24 hours after elicitation, cell growth increased in both elicited cultures, nevertheless did not reach levels of non-elicited control culture (Fig. 1).

Fresh weight of cells in culture elicited by MeJa after 72 hours of cultivation was similar to non-elicited culture. The lower fresh weight of cells elicited by *Pch* was caused probably by polypeptides secreted by *P. chlamydospora* inhibiting plant cell activities and growth (LUINI *et al*., 2010). *P. chlamydospora* cultivated *in vitro* produced large amounts of extracellular polysaccharides containing pullulans and addition of crude extract or filtrate from culture to grapevine calluses reduced their growth by 85 % (SPARAPANO *et al*., 2000). Another group of chemical compounds produced by *P. chlamydospora* are extracellular enzymes also inhibiting growth of calluses (SANTOS *et al*., 2006).

The pH of the cultivation medium measured in samples for polyphenol analysis was almost constant in non-elicited sample (difference was ± 0.01) but decreased in average by 0.15 after each day of cultivation in elicited cultures. It can also reduce fresh cell weight as was described by TASSONI *et al*. (2005) after using of MeJa.

The total contents of polyphenols produced in cell cultures *in vitro* were determined immediately after treatment by elicitors (15 min, 1 hour, and 4 hours after) and later (1, 2, and 3 days after), respectively (Fig. 1). The total content of polyphenols in non-elicited cultures increased continuously during 3 days but only moderately. The

impact of both elicitors on polyphenols production was different. Their accumulation culminated 48 hours after MeJa addition then declined. The addition of the *Pch* elicited the highest polyphenolic production immediately (15 - 60 min) after treatment, after 4 hours were minimal, then increased again and 72 hours after treatment polyphenols production was higher than producing MeJa-elicited cells. Elicitation with the *Pch* had a biphasic mode of production with two maxima - immediately after elicitation and 3 days after. This could relate to a biphasic model of oxidative burst induced by extract of *P. chlamydospora* in grapevine cell suspension (LIMA *et al*., 2012), although the cultivar Vinhão used in their study responded slightly differently and they analysed other phenolic compounds – viniferin-type and piceid-type stilbenes. Time of accumulation and intensity of oxidative burst induced by pathogen can be very diverse, depending on plant species, used plant system, and resistance or tolerance level of specific cultivar.

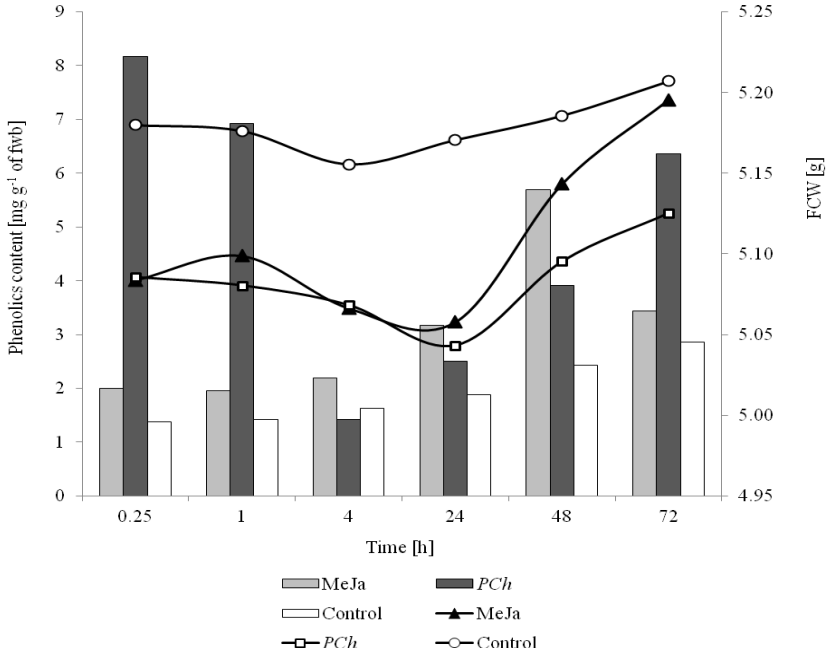

Fig. 1. The effect of *PCh* and MeJa elicitors on cell growth (curves with symbols ▲, □, ○) and the sum of detected phenolic compounds (columns).

Individual polyphenols determined in cultivation medium were: (+)-catechin, (-)-epicatechin, vanillic acid, *p*-coumaric acid, syringaldehyde, trans-resveratrol, and rutin (Table 1). Significant differences in content of generated polyphenols were among the non-elicited and MeJa-elicited culture, between non-elicited and *Pch*-elicited cultures, as well as between MeJa- and *Pch*-elicited cultures. Polyphenols in the cultivation medium in non-elicited cultures were not detected. Very low quantity of polyphenols was released by MeJa-treated (vanillic acid, *p*-coumaric acid, and trans-resveratrol,

totally 1.27 mg/l) and *Pch*-treated cells (*p*-coumaric acid and trans-resveratrol, totally 0.53 mg/l) into cultivation medium 72 hours after elicitation. The vanillic acid and trans-resveratrol were present only in the cultivation medium.

Table 1. Concentration of phenolics (±SD = standard deviation) in cultivation medium (A) (mg/l) and cultivated cells (B) (mg/g FW) (n.d. = not detected).

Time [hours]	**Vanillic acid[A]**		***p*-Coumaric acid[A]**	
	MeJa	***PCh***	**MeJa**	***PCh***
0.25	n.d.	n.d.	0.07±0.01	0.07±0.02
1	n.d.	n.d.	0.05±0.004	0.05±0.02
4	n.d.	n.d.	n.d.	0.05±0.04
24	0.33±0.02	n.d.	n.d.	0.14±0.08
48	0.49±0.03	0.32±0.19	0.06±0.005	0.15±0.05
72	0.73±0.05	0.34±0.20	0.09±0.01	0.19±0.06
Control after 72 hours	n.d.		n.d.	
Time [hours]	***trans*-Resveratrol[A]**		**(+)-Catechin[B]**	
	MeJa	***PCh***	**MeJa**	***PCh***
0.25	n.d.	n.d.	0.58±0.03	2.74±0.18
1	0.36±0.004	n.d.	0.58±0.03	2.40±0.16
4	0.41±0.01	n.d.	0.66±0.04	0.46±0.02
24	0.43±0.01	n.d.	0.83±0.05	0.79±0.05
48	0.45±0.01	n.d.	1.48±0.05	0.92±0.05
72	0.45±0.01	n.d.	0.99±0.06	1.34±0.07
Control after 72 hours	n.d.		0.82±0.05	
Time [hours]	**(-)-Epicatechin[B]**		***p*-Coumaric acid[B]**	
	MeJa	***PCh***	**MeJa**	***PCh***
0.25	0.43±0.01	0.73±0.03	n.d.	0.08±0.01
1	0.38±0.01	0.70±0.03	n.d.	0.07±0.01
4	0.43±0.01	0.26±0.01	n.d.	0.05±0.004
24	0.48±0.01	0.44±0.01	n.d.	n.d.
48	2.05±0.02	0.69±0.02	n.d.	0.04±0.004
72	0.73±0.02	1.16±0.03	n.d.	0.08±0.01
Control after 72 hours	0.37±0.012		0.06±0.004	
Time [hours]	**Rutin[B]**		**Syringaldehyde[B]**	
	MeJa	***PCh***	**MeJa**	***PCh***
0.25	0.59±0.03	3.10±0.20	0.39±0.03	1.53±0.01
1	0.69±0.04	1.99±0.13	0.30±0.02	1.76±0.12
4	0.66±0.04	0.37±0.02	0.44±0.03	0.27±0.02
24	1.11±0.07	0.54±0.03	0.73±0.05	0.72±0.05
48	1.32±0.05	0.64±0.03	0.84±0.06	1.62±0.11
72	0.72±0.04	1.01±0.05	0.96±0.07	2.77±0.19
Control after 72 hours	0.69±0.04		0.92±0.06	

The non-elicited cells contained (+)-catechin, (-)-epicatechin, *p*-coumaric acid, rutin, and syringaldehyde, totally 2.86 mg of phenolic compounds per gram of fresh weight of cell biomass (mg/g fwb). Addition of MeJa increased polyphenolic production, except the *p*-coumaric acid. The total amount of other four polyphenols at time of maximal production (48 hours after elicitation) was 5.69 mg/g fwb. The addition of *Pch* increased level of monitored polyphenols after 48 hours to 3.91 mg/g fwb, after 72 hours to 6.36 mg/g fwb, but the most significantly immediately, i.e. 15 min after elicitation, to 8.18 mg/g fwb (especially content of rutin to 3.1 mg/g fwb). Production of total polyphenols elicited by *Pch* at time of maximal production was 2.9 times higher than in non-elicited culture and 1.4 times higher than in MeJa-induced culture. Water content in cultivated cells in our cell cultures was 89.7 %. Lima et al. (2012) increased production of other types of phenolic compounds – piceid-type and viniferin-type stilbenes by MeJa and *Pch* elicitors, in comparison to non-treated control, 9-fold and 20-fold, respectively, but grapevine genotypes and evaluated phenolic compounds were different in their experiments.

Polyphenols are synthesised during the normal development of grapevine plant. The basic effect of genotype on polyphenols production documented LIANG *et al*. (2011) in European *Vitis vinifera* L. germplasm as well as in accessions of wild *Vitis* species (LIANG *et al*., 2011). The plant genotype determines this production essentially nevertheless their synthesis correlates with response to different environmental biotic and abiotic factors. Plant tissues and cells cultivated *in vitro*, moreover affected by elicitors, can respond more sensitively. Extract from the *P. chlamydospora* used in our study represented effective elicitor for polyphenols production in grapevine cell cultivation system *in vitro* and the *Vitis* cell cultures might be a practicable system for biotechnological production of valuable compounds. The advantage of the *Pch*-originated elicitor is also lower price and less health hazard during operation.

4. Conclusions

The present paper demonstrates enhancement of polyphenols production by application of synthetic and native elicitors – methyl jasmonate and extract from the grapevine pathogen *Phaeomoniella chlamydospora*, respectively. Different progress in elicitation of polyphenols production in cell culture was observed. Elicitation with MeJa culminated 48 hours after addition *Pch* elicited the highest polyphenols production immediately after treatment and again 72 hours later. Finally, it could be concluded that elicitation by *Pch* was more effective than by MeJa.

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