

USE OF METHYL JASMONATE TO ENHANCE THE PRODUCTION OF 6-METHOXYPODOPHYLLOTOXIN IN CELL CULTURES OF LINUM THRACICUM SSP. THRACICUM

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Abstract: Secondary metabolites, such as lignans have important ecological role for plants and at the same time are lead structures for drug design in human medicine. The aryltetralin type of lignans are strong cytotoxic agents and are found in members of the genus *Linum* (Linaceae).

Objective: *In vitro* cultures of *Linum thracicum* ssp. *thracicum* were developed to identify a medium type and an elicitation technique that favor enhanced production of the aryltetralin lignan 6-methoxypodophyllotoxin.

Method: Methyl jasmonate and salicylic acid were administered to four cell lines of *Linum thracicum* ssp. *thracicum* for 24 and 72 hours. The 6-methoxypodophyllotoxin was identified by HPLS-ESI-MS/MS in positive ion mode, and its production was determined via quantitative HPLC analysis.

Results: A cell line, called *Li-20*, which was developed in reduced sucrose environment proved to be the fastest growing and the highest producing among the tested cell lines. Within 24 hours upon MJ elicitation, the eight-day-old *Li-20* increased 2.3-fold in 6-methoxypodophyllotoxin content, reaching 4.3 mg on a dry weight basis. Negative or no effect was registered after salicylic acid application.

Conclusion: MJ elicitation is an effective strategy to improve the 6-methoxypodophyllotoxin accumulation within short periods of time, and an optimization of the cultivation medium beforehand is a prerequisite in the pipeline.

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Introduction

Secondary metabolites produced by plants are low molecular weight compounds, which constitute the organism's toolbox that enable adaptive responses to the environmental cues (Kennedy & Wightman, 2011). Plants coexist alongside a specific biota and have evolved with a range of secondary metabolites that respond to a variety of insults (Atkinson & Urwin, 2012). The occurrence of secondary metabolites varies among plant tissues (Wink, 2010), but, in general, it is less than 1% of the total carbon (Bourgaud, Gravot, Milesi & Gontier, 2001). The presence of major secondary constituents is usually accompanied by a multitude of minor compounds (Wink, 2010). They can act in synergistic or additive fashion to ensure efficacy toward a range of attackers, as well as to impede the attackers, and to evolve in resistance of plant defenses (Lila & Raskin, 2005).

The ability of plants grown *in vitro* to synthesize secondary metabolites was demonstrated in 1991 by Zenk et al. (1991) in *Morinda citrifolia* cell cultures. Following that discovery, a number of species has been introduced for aseptic growth with the aim of delivering plant systems that are valuable hyper-accumulators for the humankind secondary metabolites. Biotechnologically-cultivated plant cells are regarded as renewable sources (Mulabagal & Tsay, 2004) and are an attractive alternative to harvesting wild plants due to (i) a possibility for year-round cultivation; (ii) material that is virus-, bacteria-, herbicide-, and heavy metals-free; and (iii) issues with loss of habitat of the wild plants (Gaosheng & Jingming, 2012). Moreover, different strategies to enhance the secondary metabolite production *in vitro* proved to be successful, since the biosynthesis of these metabolites is highly inducible in response to a variety of applied stressors called *elicitors* (Namdeo, 2007).

Nearly 200 species that comprise the genus *Linum* spp. (Linaceae) are known to synthesize a class of secondary metabolites, called *lignans* (Schmidt et al., 2010). Despite the fact that the lignans are diverse class with numerous pharmacological activities, considerable attention has been drawn to the subclass

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with aryltetralin skeleton due to their cytotoxic activities and marketed application as anti-tumor drugs (Apers, Vlietnick, & Pieters, 2003). *In vitro* cultures of many *Linum* species have been developed and tested for the presence of cytotoxic lignans, and a variety of techniques have been employed to increase the flax cells' productivity, including genetic transformation, elicitation, and bioreactor studies (Ionkova, Ionkova, & Sasheva, 2012a; 2012b; Ionkova, 2009; Ionkova & Fuss, 2009; Ionkova, Sasheva, Ionkov, & Milanova, 2012; Vasilev & Ionkova, 2004).

As part of our ongoing program to study Bulgarian *Linum* species for their capacity to biosynthesize cytotoxic lignans *in vitro*, we developed aseptic cultures of the endemic *Linum thracicum* ssp. *thracicum*. We aimed to identify a medium type and an elicitation technique that favors enhanced production of the aryltetralin lignan 6-methoxypodophyllotoxin. Four cell lines, developed in mediums that differed in their nutrients' concentrations were used for the elicitation experiments. We applied methyl jasmonate (MJ) and salicylic acid (SA) in two concentrations and followed the response in the 6-methoxypodophyllotoxin production for 24 and 72 hours. We used HPLC-ESI-MS/MS to confirm the presence of 6-methoxypodophyllotoxin and used HPLC analysis for quantification of the lignan content.

Materials and Methods

Plant material

Seeds of *Linum thracicum* ssp. *thracicum* Degen were used for the initiation of *in vitro* cultures using standard procedures (Ionkova, Antonova, & Momekov, 2008). Cell suspension cultures were cultivated in full strength (Li) and half strength (HS) salt base liquid Murashige and Skoog medium (Murashige & Skoog, 1962), supplemented with 0.4 mg/L α -naphthaleneacetic acid. The media differed in their sucrose concentration as follows: Li-30 and HS-30 contained 30 g/L sucrose; Li-20 and HS-20 contained 20 g/L sucrose. The suspension cultures were grown in the dark at $23 \pm 1^\circ\text{C}$ under constant agitation on orbital shaker (90 rpm) and sub-cultured every 12 days.

Elicitor application

Methyl jasmonate (MJ) and salicylic acid (SA) were applied separately in two concentrations each: 50 μM and 100 μM , dissolved in ethanol. The ethanol and the elicitors were added to the seven-day-old suspension cultures and the plant material was harvested 24 hours and 72 hours after the administration. The dry weight was measured at each harvesting event. The experiments were carried out in 25-ml volume liquid medium.

Extraction and analysis of lignan aglycons

Lignan aglycons were isolated as previously described (Ionkova et al., 2008). The dry residue was reconstituted in methanol and used for analysis. The lignan content was determined using Waters 1825 Binary HPLC Pump equipped with 2489 UV/VIS Detector, at 290 nm. The separation was performed on Luna C18 (25 cm x 4.6 mm, 5 μm) reversed phase column (Phenomenex, USA) with injection volume 20 μl . The mobile phase consisted of 0.01% o-phosphoric acid (A) and acetonitrile (B) and a gradient elution as follows: 0-17 minutes, 60% A to 33% A; 17-18 minutes, 33% A to 60% A; 18-30 minutes, 60% A with a flow rate of 1 mL/min. For the quantitative analysis of PT ($R_t = 9.350$ min) and 6-MPT, Breeze 2 software constructed calibration curves of the reference standards were used.

Mass spectrometry

Mass spectrometric detection was carried out with LC-MS/MS System TSQ Quantum Discovery MAX (Thermo Electron Corporation). The separation was performed on Luna C18 (25 cm x 4.6 mm, 5 μm) reversed phase column (Phenomenex, USA) with injection volume of 10 μl . The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) in a gradient elution program as follows: 0-10 minutes, 45% A to 25 % A; 10-11 minutes, 25% A to 25% A; 11-11.5 minutes, 25% A to 45% A; 11.5-20

minutes, 45% A, with a flow rate of 0.5 mL/min. The electrospray ionization-tandem mass spectrometry (ESI-MS/MS) detection was done using a triple quadrupole instrument by positive ionization mode. The data were processed using Xcalibur software (Thermo Electron Corporation).

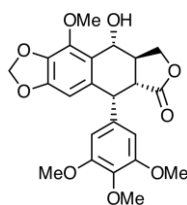
Statistical analysis

All values are mean $\pm$ SD of two independent experiments. Statistical significance was assessed using the Student's t-test, performed on the software package OriginLab 6.0.

Results and Discussion

In this study, we investigated the changes in the 6-methoxypodophyllotoxin content (Figure 1), imposed by the elicitors MJ and SA when applied in two concentrations for different periods of time to the thracian flax (*Linum thracicum* ssp. *thracicum*) cell cultures. For this purpose, we developed four cell lines that differed in their sucrose (20 g/L vs 30 g/L) and salt base (full strength, Li vs half strength, HS) content, enabling us to explore how changes in the medium alone, and in combination with the elicitors, influenced the 6-methoxypodophyllotoxin biosynthesis.

Figure 1: Structure of 6-methoxypodophyllotoxin



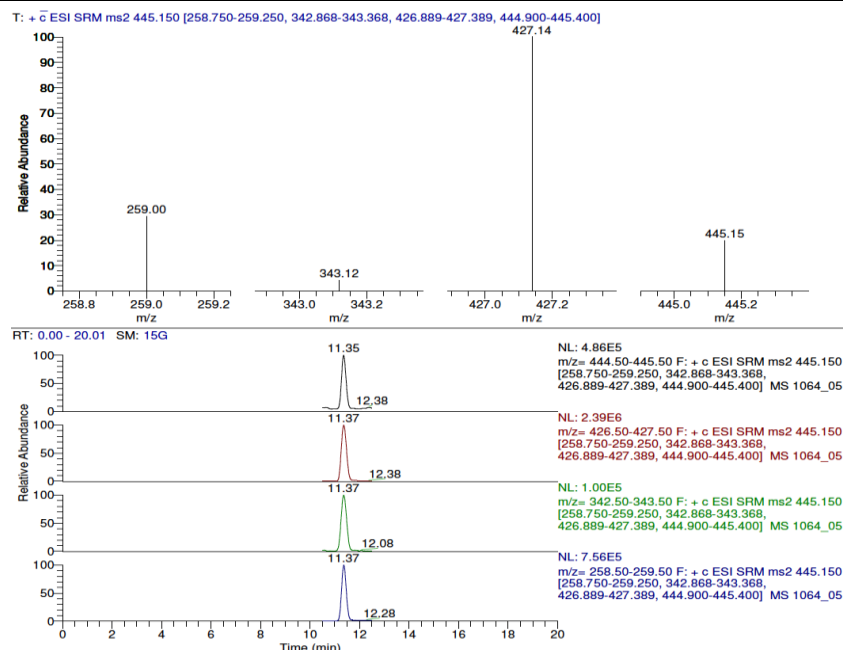
Source: Wink (2010)

We performed HPLC-ESI-MS/MS analysis in positive ion mode to identify the 6-methoxypodophyllotoxin in the thracian flax cell cultures. Figure 2 shows the Selected Reaction Monitoring (SRM) method performed on a triple quadrupole instrument.

Changes in the nutrient levels are shown to influence the secondary metabolite production in many *in vitro* cultures. While sucrose content as high as 80 g/L favors enhanced production of indole alkaloids in *Catharanthus roseus* (Misawa, 1985) and benzophenanthridine alkaloids in *Eschscholtzia californica* (Berlin, Forche, Wray, Hammer, & Hosel, 1983), the production of anthocyanins in *Aralia cordata* was optimal when the medium was supplemented with 30 g/L sucrose (Sakamoto et al., 1993). Here, we report similar to observations of Sakamoto et al. (1993); although in our experiments, better results were achieved when the applied sucrose concentration to the thracian flax cultures was even lower. Use of 20 g/L sucrose in the medium yielded a 2-3 times higher 6-methoxypodophyllotoxin content (HS-20 and Li-20) compared to the production achieved in media containing 30 g/L sucrose (HS-30 and Li-30) (Figure 3, Control variants).

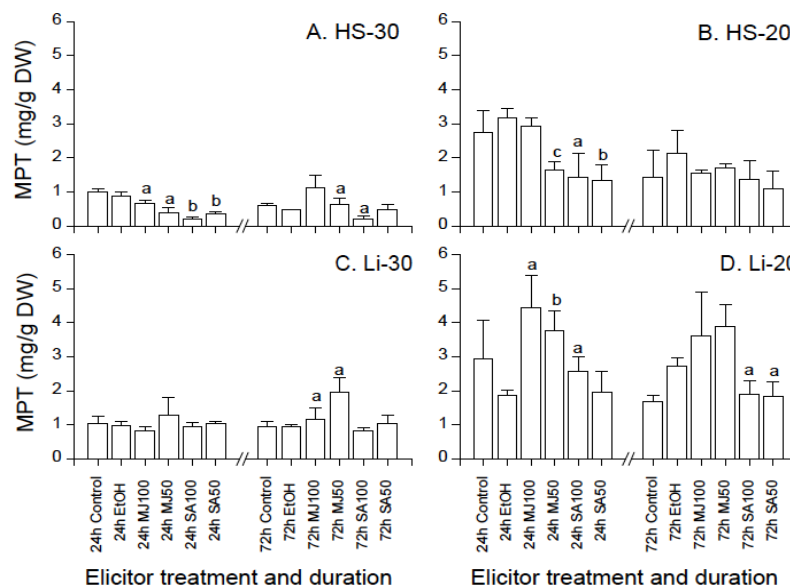
Application of MJ and SA has proven to be a successful strategy to increase the accumulation of lignans in *Linum tauricum* (Ionkova, 2009) and *Linum album* (van Furden, Humburg, & Fuss, 2005; Yousefzadi et al., 2010). MJ and SA are plant-derived hormones with prominent role in the defense responses that are triggered upon by various attackers and result in biosynthesis of defense molecules (van Verk, Gatz & Linthorst, 2009). We applied the two elicitors on day seven of the subculture cycle and harvested the plant material after 24 and 72 hours of the elicitor administration. The collected samples were used to extract the lignan aglycons, and the 6-methoxypodophyllotoxin content was determined using quantitative HPLC analysis. For the statistical analysis, we used the 6-methoxypodophyllotoxin content from ethanol-treated samples (the elicitors were dissolved in ethanol prior to the treatment).

Figure 2: Selected Reaction Monitoring (SRM) chromatogram of 6-methoxypodophyllotoxin in *Linum thracicum* ssp. *thracicum* cell cultures.



Source: Authors

Figure 3: 6-methoxypodophyllotoxin content (mg/g DW) in *Linum thracicum* ssp. *thracicum* cell lines A. HS-30, B. HS-20, C. Li-30 and D. Li-20. The cultures were elicited with methyl jasmonate (MJ) and salicylic acid (SA) for 24 hours and 72 hours. Legend: Control, no treatment; EtOH, 0.38% EtOH; MJ100, 100 μ M MJ; MJ50, 50 μ M MJ; SA100, 100 μ M SA and SA50, 50 μ M SA. The data are mean $\pm$ SD. Letters denote statistical significance as follows: (a) $P < 0.05$; (b) $P < 0.01$; (c) $P < 0.001$.



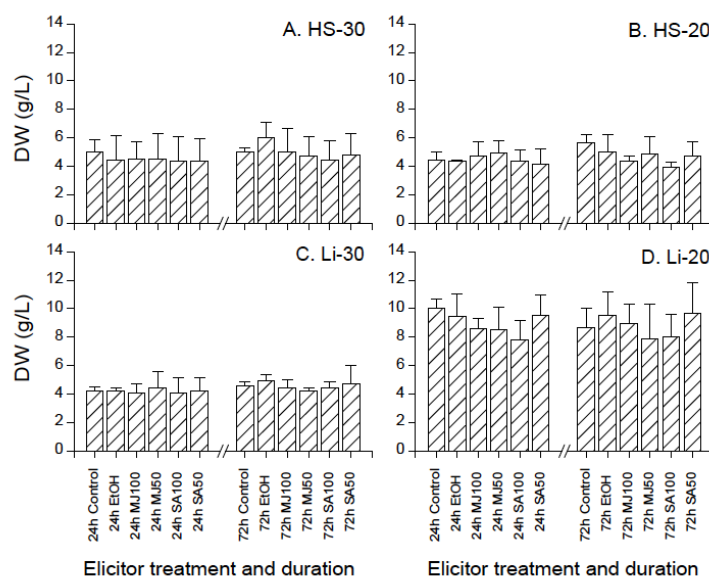
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Despite the fact that the MJ had negative effect on the 6-methoxypodophyllotoxin accumulation in the HS-20 (Figure 3B), the cell line synthesized the lignan in amounts that were higher than the respective ones found in HS-30 (Figure 3A) and Li-30 (Figure 3C). A 2-2.3-fold increase was observed in the Li-20 cell lines (Figure 3D) 24 hours after the MJ application, reaching 3.8-4.3 mg/g DW 6-methoxypodophyllotoxin. The initial lack of response in the first 24 hours of the MJ administration was followed by a two-fold increase in the 6-methoxypodophyllotoxin content in HS-30 (Figure 3A) and Li-30 (Figure 3C) cell lines.

The application of SA did not cause an increase in the 6-methoxypodophyllotoxin production in the investigated cell lines, and while no effect was observed in the Li-30, a decrease was registered in the HS-30 and HS-20 (Figure 3). Only in Li-20, the application of 100 μ M SA for 24 hours increased 1.5 times in 6-methoxypodophyllotoxin content (Figure 3D).

The cell line Li-20 (Figure 4D) showed the highest biomass accumulation, reaching 10 g/L for 8 days, thus exceeding the biomass production of the rest of the cell lines by a factor of 2 to 1. We could only detect slight changes in the biomass accumulation due to the presence of elicitors; however, these were not statistically significant (Figure 4).

Figure 4: Dry weight (g/L) of *Linum thracicum* ssp. *thracicum* cell lines A. HS-30, B. HS-20, C. Li-30 and D. Li-20. The cultures were elicited with methyl jasmonate (MJ) and salicylic acid (SA) for 24 hours and 72 hours. Legend: Control, no treatment; EtOH, 0.38% EtOH; MJ100, 100 μ M MJ; MJ50, 50 μ M MJ; SA100, 100 μ M SA and SA50, 50 μ M SA. The data are mean $\pm$ SD.



Source: Authors

Conclusion

A 2.3-fold increase in the 6-methoxypodophyllotoxin accumulation can be achieved within 24 hours upon administration of the elicitor MJ to the seven-day-old thracian flax cell line Li-20. Several factors indicated that the Li-20 is the most suitable medium for 6-methoxypodophyllotoxin production: it is the fastest growing cell line; the application of the MJ does not change the growth parameters (dry weight) within the observed time frame; and the lignan content can be boosted within a short period of time. This result demonstrated that MJ elicitation is an effective strategy to improve the 6-methoxypodophyllotoxin accumulation but only if a suitable medium was developed beforehand.

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