

The influence of L-phenylalanine, methyl jasmonate and sucrose concentration on the accumulation of phenolic acids in *Exacum affine* Balf. f. ex Regel shoot culture*

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Phenolic acids are an important group of plant secondary metabolites with various, valuable therapeutic properties. Apart from plants growing in the open air, tissue cultures can be an alternative source of the secondary metabolites. The yield of their accumulation in *in vitro* cultures can be increased by different methods, including culture medium supplementation with precursors, elicitors and changing the standard amounts of the medium components. The purpose of this study was to investigate the influence of the precursor (L-phenylalanine), the elicitor (methyl jasmonate) and a higher sucrose concentration on the phenolic acids accumulation in the agitated shoot cultures of *Exacum affine* Balf. f. ex Regel (*Gentianaceae*). Qualitative and quantitative analyses of the phenolic acids in methanolic extracts from the biomass were conducted by applying the HPLC method. Fourteen phenolic acids and cinnamic acid were found in all samples. The total content of free phenolic acids increased from approximately 0.242% to 0.635% (2.6-fold) and the total content of the whole phenolic acids (free and bound) — from 0.712% to 1.160% (1.6-fold). The studies show that the best variant for the accumulation of most of the identified phenolic acids contained 6% of sucrose (double the standard amount), L-phenylalanine 1.6g L⁻¹ of medium and methyl jasmonate 100 μM. The analysis of the results in the experiment presented here showed that it is possible to increase the accumulation of the phenolic acids in *Exacum affine* shoot cultures — by adding the precursor (L-phenylalanine), the elicitor (methyl jasmonate) and by increasing the sucrose concentration.

Key words: *Exacum affine* Balf. f. ex Regel, Persian violet, elicitation, precursor feeding, L-phenylalanine, methyl jasmonate, sucrose concentration

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INTRODUCTION

Exacum affine Balf. f. ex Regel belongs to the *Gentianaceae*. There are 65 *Exacum* species spreading from westernmost Africa to Madagascar and Socotra, and from India to New Guinea. *E. affine* is endemic to Socotra (Yuan *et al.*, 2005). This species is known in Europe and North America as a beautiful potted plant (names: Persian gentian; Persian, Arabian or German violet). Of the all species of *Exacum*, only *E. affine* has been suc-

cessfully domesticated and so far gained economic importance as an ornamental crop (Riseman, 2006). Apart from the floriculture use, we might notice some interest in *E. affine*'s phytochemical constituents and its therapeutic activity. *E. affine* was mentioned by Mothana and co-researchers (Mothana & Lindequist, 2005; Mothana *et al.*, 2006; Mothana *et al.*, 2007) in their ethnopharmacobotanical studies published in recent years. *E. affine* showed strong antiviral activity (Mothana *et al.*, 2006). This species has been investigated as one of the 25 medicinal plants which are used in Yemeni traditional medicine to treat infectious illnesses (e.g. skin and respiratory tract infections). The results demonstrate that *E. affine* shows the significant antiviral activity against herpes simplex virus (HSV-1 strain KOS) and human influenza virus A/WSN/33 (H1N1) London. These results confirm the traditional use of the plant against viral infections. Mothana *et al.* (2006) also suggest that phenolic acids may be one of the chemical groups responsible for such activity.

Hegnauer (1966) mentioned *p*-coumaric acid as a constituent of *E. affine*. Skrzypczak-Pietraszek (unpublished data) analysed *E. affine* pot plants and found fourteen phenolic acids (caffeic, chlorogenic, ferulic, gallic, gentisic, *o*-coumaric, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, rosmarinic, salicylic, sinapic, syringic, vanillic). Most of the compounds were only in the bound form and were detected after acid hydrolysis. Protocatechuic acid was the main phenolic acid of *E. affine* herb. Only few information relate to the phenolic acid content in the other species of the genus *Exacum*. Daniel and Sabnis (1978) made the qualitative analysis of *E. bicolor* and *E. pedunculatum*. They found four phenolic acids in *E. bicolor* (*p*-coumaric, *p*-hydroxybenzoic, protocatechuic, vanillic) and six compounds in *E. pedunculatum* (vanillic, syringic, *p*-hydroxybenzoic, protocatechuic, *p*-coumaric, ferulic). Jeeshna and Paulsamy (2011) qualitatively determined the chlorogenic acid content in *E. bicolor*.

Phenolic acids are the significant constituents of secondary metabolites widespread in the plant kingdom, showing — apart from the antiviral action — a broad spectrum of therapeutic activities, such as antiseptic, antioxidant, antiphlogistic, cholagogic, immunostimulating,

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Abbreviations: BAP, 6-benzylaminopurine; DW, dry weight; GA₃, gibberellic acid; MeJA, methyl jasmonate; MS, Murashige and Skoog (1962) medium; NAA, *a*-naphthaleneacetic acid; Phe, L-phenylalanine; Suc, sucrose

spasmolytic and others (Khadem & Marles, 2010). Phenolic acids biosynthesis is based on a shikimate-phenylpropanoid pathway. Apart from their potential pharmacological activities, these compounds play an important role as active defence factors in the plants' resistance to diseases and other biotic and also abiotic stresses. The phenylpropanoid pathway is often induced by stress and has a specific function in plant protection (Arora, 2010).

The biotechnological production of the secondary metabolites in plant cell and organ cultures is an attractive alternative to the extraction of the whole plant material. Numerous cell and tissue cultures have been derived from many medicinal plants but they do not often produce sufficient amounts of the required compounds. For this reason, many biotechnological strategies have been prepared and experimented for the enhanced production of secondary metabolites from medicinal plants. Some of them include the screening of high yielding cultures, media modifications, the precursor feeding, elicitation and others (Namdeo, 2007; Arora, 2010).

Sucrose is one of the standard component of the culture medium and it is an important carbon and energy source for the plant cells. In addition, the sucrose content influences the production of secondary metabolites of the phenylpropanoid pathway (Arora, 2010).

Phenylalanine (Phe) is an amino acid, the precursor of the phenylpropanoid pathway leading to the formation of phenolic acids, flavonoids and other phenolic compounds. Phenylalanine has been successfully used to increase the metabolite production *in vitro* in many different plant cultures (Arora, 2010).

The methyl jasmonate (MeJA) is one of the abiotic elicitors. It activates phenylalanine ammonia lyase (the enzyme that catalyzes the first step in the shikimic acid pathway – the deamination of Phe) and thus induces the secondary metabolites production (Namdeo, 2007).

According to our knowledge, there was no literature information on the elicitation and the precursor feeding experiments in the *in vitro* cultures of the plants from the genus *Exacum*. Some publications concerned the other taxons from the family *Gentianaceae*. The influence of the methyl jasmonate, L-phenylalanine (Phe) or the sucrose concentration on the accumulation of iridoids or xanthenes in *Swertia chirata* (Keil *et al.* 2000; Kumar *et al.* 2013) *Centaurium* sp. (Abd El-Mawla *et al.* 2001; Beerhues and Berger 1995) *in vitro* cultures.

The purpose of this study was to investigate the influence of the precursor (Phe), the elicitor (MeJA) and the different sucrose concentrations on the phenolic acids accumulation in the agitated shoot cultures of *Exacum affine* Balf. f. ex Regel (*Gentianaceae*).

MATERIALS AND METHODS

Plant material. *E. affine* potted plants were bought in 2009 from the commercial specialized horticultural company (F.P.H. "Ogród Łęg" s.c. B.J.T. Gawęda, Kraków, Poland). The plants were at an early flowering phase. Plants identity was verified by Dr. Skrzypczak-Pietraszek, referring to available literature. A voucher specimen was deposited in the Department of Pharmaceutical Botany (Jagiellonian University, Collegium Medicum, Kraków, Poland) with the number 2009-EA01. Plants were used to establish *in vitro* cultures.

***In vitro* cultures. Shoot cultures induction and culture conditions.** Shoot cultures were established in 2009. Stems from potted plants (see: Plant material) were used as initial explants. Stems were surface-

sterilized with 70% (v/v) ethanol for 0.5 min, followed by 0.2% HgCl₂ treatment for 20 min. Then, they were rinsed three times with sterile distilled water, cut into 1.0–1.5 cm pieces and transferred onto previously selected (Skrzypczak-Pietraszek *et al.*, 2005) the MS (Murashige & Skoog, 1962) medium, supplemented with 1.0 mg L⁻¹ BAP (6-benzylaminopurine), 0.5 mg L⁻¹ NAA (α -naphthaleneacetic acid), 0.25 mg L⁻¹ GA₃ (gibberellic acid). The MS medium contained 3% sucrose (w/v) and was solidified with 0.8% agar (w/v). After 1–2 weeks, green callus appeared on the explants and differentiated into the shoot culture during 1–2 subcultures. Shoot cultures were grown in 100 mL Erlenmeyer flasks in a culture room at 25±2°C under constant artificial light (16 μ mol·m⁻²·s⁻¹) and were transferred onto the same fresh medium every 4–5 weeks.

Agitated shoot culture. In order to perform the experiment, 21th subculture of *E. affine* shoot cultures was used to establish an agitated shoot culture in 500 mL Erlenmeyer flasks (4 g of fresh shoots/flask) on a rotary shaker at 140 rpm and in the same other culture conditions. The MS medium was supplemented with the same growth regulators. The medium was liquid (without agar) and contained a standard content of sucrose (Suc) — 3% (w/v) (variant A' — reference) or double amount of Suc — 6% (w/v) (A-F variants). Agitated cultures were maintained for 14 days and then they were treated with Phe and MeJA.

Elicitation and precursor feeding experiment. The stock solutions of Phe (Merck) and MeJA (Sigma-Aldrich) were prepared: Phe was dissolved in water, MeJA in 96% (v/v) ethanol and both were filter-sterilised (Milligore Millex-GP, 0.22 μ m). The precursor and elicitor were added to the agitated shoot culture on the 14th day. Phe achieved a final concentration of 1.6 g L⁻¹ (\approx 10 mM) and MeJA: 100 μ M or 800 μ M. Phe and MeJA were added to the cultures independently or in a combination, according to the following scheme:

- variant B — MeJA 100 μ M
- variant C — MeJA 800 μ M
- variant D — Phe 1.6 g L⁻¹
- variant E — Phe 1.6 g L⁻¹; MeJA 100 μ M
- variant F — Phe 1.6 g L⁻¹; MeJA 800 μ M

Variants A' and A were treated as controls. Variants without Phe or/and MeJA were supplemented with sterile water or ethanol, respectively. Ethanol achieved a final concentration of 0.1% (v/v). After elicitation and precursor feeding, all the cultures were placed again on the rotary shaker. The biomass was harvested after 1, 3 and 7 days, frozen and lyophilised. The dried material was used for the phytochemical analysis.

Phytochemical analysis

General experimental procedures. Methanol and acetic acid (both of HPLC grade) were purchased from Merck (Darmstadt, Germany). The phenolic acid standards were from Fluka and Sigma. The water was purified by re-distillation followed by filtration through Millipore 0.22- μ m filters. The standard solutions or the studied samples were filtered through 0.22- μ m syringe filters (Millipore, Millex-GP) before being injected to HPLC. The qualitative and quantitative HPLC analyses of phenolic acids were conducted using VWR Hitachi apparatus (autosampler L 2200, pump L 2130, column oven L 2350, diode array detector (DAD) L 2455) at UV range 200–400 nm and Purospher[®] RP-18e column (250×4 mm, 5 μ m) thermostated at 25°C. A mobile phase was composed of methanol — 0.5% acetic acid (1:4, v/v)

as solvent A and methanol (solvent B). The following gradient elution scheme was used (A:B ratio): 100%:0% t=0–20 min; 80%:20% t=35 min; 70%:30% t=45 min; 60%:40% t=55 min; 50%:50% t=60 min; 25%:75% t=65 min; 0%:100% t=70–75 min; 100%:0% t=80–90 min.

Extraction. The extraction process was carried out as follows: 1 g of each dried powdered (in a mortar) plant material was extracted twice with 50 mL of boiling methanol (at 67.4°C) for two hours under reflux condenser. The combined extracts (100 mL) were concentrated to dryness, using a rotary vacuum evaporator (Büchi) at 40°C. The residues were dissolved in 5 mL of methanol. The extract samples contained free phenolic acids.

Hydrolysis. The hydrolysis process was carried out as follows: 1 g of each dried powdered (in a mortar) plant material was hydrolysed with 50 mL of 2 M hydrochloric acid for two hours at 100°C. The hydrolysates were extracted with ethyl acetate (5 times with 40 mL of ethyl acetate). The acetate extracts were combined (200 mL) and concentrated to dryness in a rotary vacuum evaporator (Büchi) at 40°C. The hydrolysed samples contained all phenolic acids present in the plant material: free and earlier bound with other plant metabolites. The residues were dissolved in 5 mL of methanol.

HPLC-DAD analysis. A HPLC method was applied according to the procedure developed by Ellnain-Wojtaszek & Zgorka (1999). The comparison of UV spectra and retention times with standard compounds enabled the identification of phenolic acids present in the analysed samples. The qualitative analyses were supplemented with an internal standard method. The phenolic acids identified by the analyses were quantified against their external standards (the calibration curve method).

Statistical methods. The statistical analysis usually requires data pre-processing, e.g. an outliers detection or a reduction of dimensionality through the non-parametric data-driven procedures: the cluster analysis (CA) (Everitt *et al.*, 2011) or the principle component analysis (PCA) (Jolliffe, 2010). In the research described here, the data pre-processing was not necessary, contrary to the author's previous experience (Skrzypczak-Pietraszek & Pietraszek, 2012).

The multiple comparison of means was conducted by applying a classic oneway analysis of variance (ANOVA) (Fisher, 1935; Kempthorne & Hinkelmann, 2007). The equality of variances across samples, usually called a homoscedasticity or a homogeneity of variances, is one of the basic assumptions in ANOVA. The typical test of such equality is Bartlett's test (Bartlett, 1937) but it requires the normality of general population. Such an assumption could not be reliably tested by any test of normality e.g. Shapiro-Wilk (Shapiro & Wilk, 1965) or Anderson-Darling (Anderson & Darling, 1954) due to a small size of the samples obtained. This specific situation led to the Levene's test (Levene, 1960), which does not require the normality of general population.

When the test response was negative (the critical p-Value less than the assumed significance level alpha), the Box-Cox transformation (Box & Cox, 1964) was used to achieve a better balance of variances. When the Levene's test response for transformed data was non-negative, ANOVA was evaluated. If the Levene's test was still negative, the non-parametric variant of ANOVA (Kruskal & Wallis, 1952) was evaluated for original data. The power of non-parametric ANOVA is smaller than that of classic parametric ANOVA but it does not require any equality of variances across samples. The results of the statistical analysis were illustrated by a box-plot.

RESULTS AND DISCUSSION

In the present experiment, the agitated shoot culture of *Exacum affine* was maintained in the MS medium supplemented with BAP 1 mg L⁻¹, NAA 0.5 mg L⁻¹ and GA₃ 0.25 mg L⁻¹. The medium A' contained 3% sucrose, which is the standard content, while the remaining media A, B, C, D, E and F were enriched with 6% sucrose. After 14-day culture in the media BF, the precursor (Phe) and elicitor (MeJA) were added, according to the scheme presented. The media A and A' were treated as controls. The plant material was collected from cultures 1, 3 and 7 days after the precursor and/or the elicitor had been added.

The effect of medium composition on the biomass growth

The comparison of the dry weights demonstrated that the biomass growth on the media A and A' was the lowest after the first day and the highest after 7 days, as expected (Fig. 1).

Adding the precursor (Phe) to the variant D of the medium initially stimulated the biomass growth in a culture, however a slowdown was observed after 7 days. A similar relationship was noted for the variant B (supplemented with MeJA — 100 µM) and the variant E (combined treatment with Phe and MeJA — 100 µM). Methyl jasmonate at 800 µM (variants C and F) markedly suppressed the culture growth. The slight browning of shoots was observed especially after 7 days, which can indicate even a tissue damage. Our observations, indicating the inhibitory effect of MeJA on the biomass growth, are similar to others known from the literature (Namdeo, 2007; Kikowska *et al.*, 2012).

Qualitative analysis of phenolic acids

All analyzed extracts were found to contain 14 phenolic acids, including 5 cinnamic acid derivatives and 7 benzoic acid derivatives. Cinnamic acid was identified, as well. The results of the present experiment demonstrated that Phe was present in all biomass extracts and

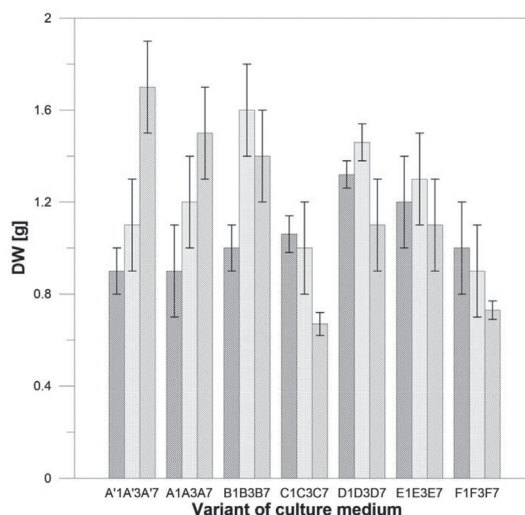


Figure 1. Dry weight of the biomass collected from *E. affine* agitated shoot cultures.

Data are expressed as mean ± standard error (n = 3). Variants of media: A' (Suc — 3%); A (Suc — 6%); B (MeJA — 100 µM; Suc — 6%); C (MeJA — 800 µM; Suc — 6%); D (Phe — 1.6 g L⁻¹; Suc — 6%); E (Phe — 1.6 g L⁻¹; MeJA — 100 µM; Suc — 6%); F (Phe — 1.6 g L⁻¹; MeJA — 800 µM; Suc — 6%).

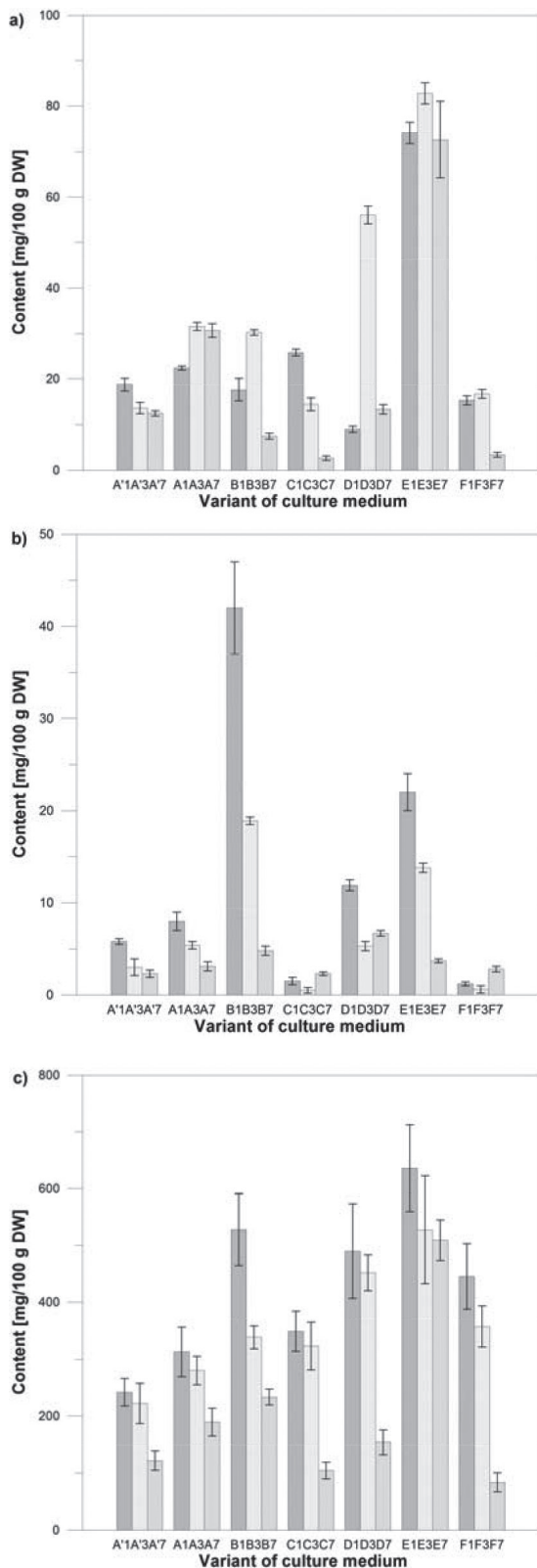


Figure 2. Content of the free phenolic acids in the biomass collected from *E. affine* shoot cultures: (a) chlorogenic acid, (b) caffeic acid, (c) total phenolic acids content (the sum of all identified compounds).

Data are expressed as mean \pm standard error (n = 3). Variants of media: A' (Suc — 3%); A (Suc — 6%); B (MeJA — 100 μ M; Suc — 6%); C (MeJA — 800 μ M; Suc — 6%); D (Phe — 1.6 g L⁻¹; Suc — 6%); E (Phe — 1.6 g L⁻¹; MeJA — 100 μ M; Suc — 6%); F (Phe — 1.6 g L⁻¹; MeJA — 800 μ M; Suc — 6%).

post culture media. Notably, none or only trace amounts of phenolic acids were detected in the media. The identity of the compounds was confirmed by comparing the retention times and UV spectra of the test substances with the respective parameters of the reference standards. The internal standard method was applied as well.

Quantitative analysis of phenolic acids

The comparison of the total contents of phenolic acids in the study plant material (Fig. 2 and Fig. 3) demonstrated a wide variation, especially among free phenolic acids. Adding Phe (variant D) or MeJA (variant B) stimulated the accumulation of the studied compounds. The treatment combined with both compounds (variant E) was very beneficial. It was important to choose correctly the MeJA concentration because this substance can be harmful for the biomass cultured *in vitro*. It was observed that a higher elicitor concentration of 800 μ M caused such a disadvantageous effect. The concentration of Phe (1.6 g L⁻¹ medium) was selected on the basis of earlier studies (Skrzypczak-Pietraszek unpublished data). MeJA was added to *E. affine* shoot cultures for the first time, so its concentrations (100 μ M and 800 μ M) were chosen on the basis of literature data, indicating that suspension cultures were more sensitive to the toxic effect of MeJA than organ (e.g. shoot) cultures (Namdeo, 2007; Kikowska *et al.*, 2012). It should also be remembered that too low elicitor concentration will not produce a stimulating effect on the accumulation of secondary metabolites.

The experiment demonstrated that the variant E containing 6% sucrose (twice higher a concentration than a standard value), the precursor Phe (1.6 g L⁻¹ medium) and the elicitor MeJA (100 μ M) was the most beneficial of the tested variants. This medium variant was especially stimulating for the accumulation of some phenolic acids (e.g. chlorogenic acid (Fig. 2a, Fig. 3a) and 4-hydroxybenzoic acid), both in the bound and free form.

Other phenolic acids (e.g. syringic acid and vanillic acid) showed a wide variability of the free form content in response to stimulation by different medium variants supplemented with the precursor and elicitor, while the total accumulation of the compound (free + bound in extracts after hydrolysis) differed much less. It can be assumed that some variants (e.g. variant E) are particularly beneficial to the accumulation of phenolic acids in the free form, while the total biosynthetic capability is not increased so much. In other medium variants, derivatives of phenolic acids were the dominating form.

The largest quantities of most of the tested phenolic acids were accumulated in shoots cultured on variant E, but there were some exemptions, e.g. variant B1 was the most beneficial to caffeic acid (Fig. 2b, Fig. 3b) and *o*-coumaric acid accumulation (both acids in the free form).

The greatest increase in the free form accumulation *vs.* control was observed for:

- syringic acid — 9.7-fold,
- *o*-coumaric acid — 9.4-fold,
- caffeic acid — 7.2-fold (Fig. 2b),
- *p*-coumaric acid — 7.2-fold,
- chlorogenic acid — 6.0-fold (Fig. 2a),
- vanillic acid — 5.8-fold,

whereas the greatest increase of the total content (free plus bound) was noted for:

- syringic acid — 7.7-fold,
- chlorogenic acid — 7.3-fold (Fig. 3a),
- 4-hydroxybenzoic acid — 3.6-fold.

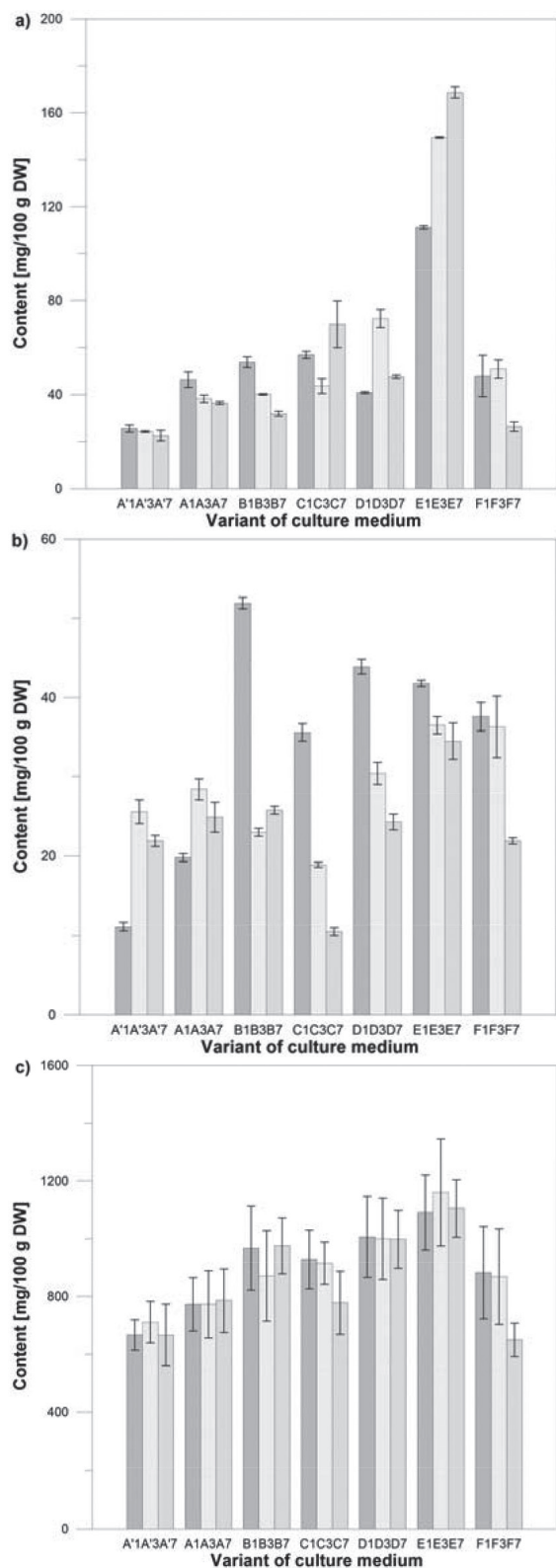


Figure 3. Content of phenolic acids (free and bound — samples after hydrolysis) in the biomass collected from *E. affine* agitated shoot cultures: (a) chlorogenic acid, (b) caffeic acid, (c) total phenolic acids content (the sum of all identified compounds). Data are expressed as mean \pm standard error ($n = 3$). Variants of media: A' (Suc — 3%); A (Suc — 6%); B (MeJA — 100 μ M; Suc — 6%); C (MeJA — 800 μ M; Suc — 6%); D (Phe — 1.6 g L⁻¹; Suc — 6%); E (Phe — 1.6 g L⁻¹; MeJA — 100 μ M; Suc — 6%); F (Phe — 1.6 g L⁻¹; MeJA — 800 μ M; Suc — 6%).

The total content of free phenolic acids increased 2.6-fold and the total content of the whole phenolic acids (free and bound) — 1.6-fold (Fig. 2c, Fig. 3c).

Not only the addition of the precursor or elicitor but also the time of shoot harvesting influenced phenolic acid accumulation. The highest content was often noted on the first day after medium supplementation, which was usually followed by a decrease in metabolite accumulation on the seventh day. Some plants, e.g. *Oryza sativa*, contain two isoforms of the enzyme. One of these isoforms — phenylalanine ammonia lyase — is involved in the elicitor-induced response (Giberti *et al.*, 2012) and it exhibits its activity twice: 6 hours and then 48–72 hours after elicitation. Perhaps, it is similar for *E. affine*. The highest phenolic acid concentrations are observed one or three days after elicitation and then their contents decrease because they can be converted into different phenolic compounds by other enzymes involved in the phenylpropanoid pathway.

Comparing the medium variants: A' (3% (w/v) of Suc) and A (6% (w/v) of Suc), it can be noticed that a higher concentration of Suc increases the phenolic acids accumulation. Similar results were mentioned by other authors, e.g. (Namdeo, 2007; Kikowska *et al.*, 2012).

On the other hand, Keil *et al.* (2000) found that the addition of Suc at 6% and 9% (w/v) resulted in the better growth rates of root cultures of *Swertia chirata* (*Gentianaceae*), but had no influence on the relative amarogentin content compared to 3% (w/v) Suc. Numerous elicitors, precursors of amarogentin biosynthesis and plant growth regulators were tested, but only chitosan increased the amarogentin accumulation. Shoot cultures of *Swertia chirata* (Kumar *et al.*, 2013) treated with 50 and 75 μ M MeJA showed a significant inhibition of the biomass production and iridoids (swertiamarin, mangiferin, amarogentin) accumulation compared to the control. The lowest MeJA concentration, 25 μ M — influenced the significant increase of the analysed compounds. The influence of elicitors and precursors on the accumulation of the other important *Gentianaceae* secondary metabolites — xanthenes — in cell cultures of *Centaurium* sp. was investigated (Beerhues & Berger, 1995; El-Mawla *et al.*, 2001). Different elicitors induced the formation of two different xanthenes: 1-hydroxy-3,5,6,7-tetramethoxyxanthone (MeJA) or 1,5-dihydroxy-3-methoxyxanthone (yeast extract) (Beerhues & Berger, 1995). In the other experiment, three radiolabelled precursors (Phe, benzoic acid, 3-hydroxybenzoic acid) were incorporated into xanthenes in *Hypericum androsaemum* cells, whereas only one of them (3-hydroxybenzoic acid) was incorporated into *Centaurium erythraea* xanthenes. MeJA can increase the accumulation of the compounds from different chemical groups: e.g. iridoids, xanthenes (earlier mentioned), diterpenoids (e.g. Grzegorzczuk & Wysokinska, 2009), phenolic acids (e.g. Grzegorzczuk & Wysokinska, 2009; Kikowska *et al.*, 2012), flavonoids (e.g. Thiem & Krawczyk, 2010), alkaloids (e.g. Jaremicz *et al.*, 2014). It is interesting to observe the MeJA influence on the accumulation of different chemical compounds in one experiment (e.g. Grzegorzczuk & Wysokinska, 2009). The maximum level of diterpenoids in shoot cultures of *Salvia officinalis* was achieved at 3 days after elicitation with 20 μ M MeJA, but the highest amount of rosmarinic acid was observed on the 5th day after elicitation (50 or 100 μ M MeJA).

Some authors suggest the beneficial effect of the simultaneous addition of the elicitor and the precursor e.g. (Krolicka *et al.*, 2008; Qu *et al.*, 2011; Riedel *et al.*, 2012). Our results with MeJA/Phe confirm these observations. On the other hand, sometimes the elicitor/precursor

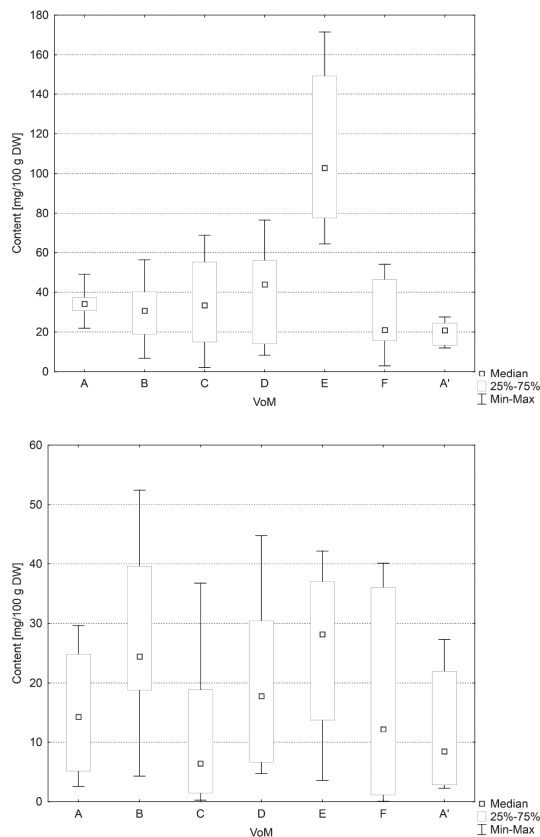


Figure 4. Non-parametric Kruskal-Wallis ANOVA (a — chlorogenic acid, b — caffeic acid)

Data are expressed as mean \pm standard error ($n = 3$). Variants of media: A' (Suc — 3%); A (Suc — 6%); B (MeJA — 100 μ M; Suc — 6%); C (MeJA — 800 μ M; Suc — 6%); D (Phe — 1.6 g L⁻¹; Suc — 6%); E (Phe — 1.6 g L⁻¹; MeJA — 100 μ M; Suc — 6%); F (Phe — 1.6 g L⁻¹; MeJA — 800 μ M; Suc — 6%).

combinations do not influence the secondary metabolites accumulation (Cousins *et al.*, 2010).

Apart from elicitors, precursors, osmotic stress (higher concentrations of Suc), there are much more factors which may influence the accumulation of phenolic acids and other secondary metabolites in cell and tissue cultures. These factors include: type of culture medium e.g. MS, 1/2 MS, B5 (Gamborg *et al.*, 1968), 1/2 B5 etc. (e.g. Weremczuk-Jeżyna *et al.*, 2013), different combinations and concentrations of plant growth regulators (e.g. Kumar *et al.*, 2013), the method of the lighting (e.g. Weremczuk-Jeżyna *et al.*, 2013).

Statistical analysis

The experiment was conducted according to the three-way ANOVA of the main effects scheme. The two responses which were analyzed were the content of caffeic and chlorogenic acids. The classification factors included: the status of the sample (STATUS: BH — before hydrolysis, AH — after hydrolysis), the variant of the medium (VoM: A', A, B, C, D, E, F) and the day of harvesting (DAY: 1, 3, 7). The whole scheme consisted of 42 groups, each containing 3 numerical measurements (triplicate) taken at the same settings of STATUS, VoM and DAY.

The first analysed variable was the content of the chlorogenic acid. The test of variance homogeneity returned a positive response for DAY (p -Value = 0.44) but negative responses for STATUS (p -Value = 0.03)

and VoM (p -Value = 0.00). The Box-Cox transformation returned the value of optimal $\lambda = 0.212$, but unfortunately the test of variance homogeneity failed again (STATUS p -Value = 0.03; VoM p -Value = 0.00; DAY p -Value = 0.03). Due to the violation of the basic ANOVA assumptions, the classical parameter ANOVA could not be processed and the alternative, non-parametric ANOVA proposed by Kruskal and Wallis was involved for each of the grouping factors separately. The results obtained for STATUS are evident (Fig. 4a): STATUS = BH differs from STATUS = AH with the significance level p -Value lower than $3 \cdot 10^9$. The results obtained for VoM revealed that VoM = E differs significantly from other analysed groups. The critical level of significance (p -Value) for multiple comparisons with E medium were lower than 0.01 for all other groups. The pairs involving other groups than E medium were not significantly different. The results obtained for DAY revealed that DAY is not a statistically significant differentiating factor. The impacts of DAY = 1 and DAY = 3 are practically indistinguishable: p -Value = 1.00 in the multiple comparisons test. The DAY = 7 is slightly different, however, it is not statistically significant: p -Value with respect to DAY = 1 is 0.44 and to DAY = 3 is 0.08.

The second analysed variable was the content of the caffeic acid. The test of variance homogeneity returned a positive response for STATUS (p -Value = 0.09) but negative responses for DAY (p -Value = 0.00) and VoM (p -Value = 0.02). The Box-Cox transformation returned the value of optimal $\lambda = 0.555$, but unfortunately the test of variance homogeneity failed again (STATUS p -Value = 0.19; VoM p -Value = 0.00; DAY p -Value = 0.15). Due to the violation of the basic ANOVA assumptions, the classical parameter ANOVA could not be processed and the alternative, non-parametric ANOVA proposed by Kruskal and Wallis was involved for each of the grouping factors separately. The results obtained for STATUS are evident (Fig. 4b): STATUS = BH differs from STATUS = AH with the significance level p -Value lower than $1 \cdot 10^{16}$. The results obtained for VoM revealed that VoM = C differs significantly in comparison to VoM = B (p -Value = 0.01) and VoM = E (p -Value = 0.02), however, other possible comparisons are not significantly different. It should be noted that the comparison between VoM = A' and VoM = B has the value of the critical significance level only slightly greater than the threshold $\alpha = 0.05$. It is 0.07, which suggests that this pair could be considered as slightly different, for the fact that the threshold is not a strictly sharp decision switch, but rather fuzzy. The results obtained for DAY revealed that one pair: DAY = 1 and DAY = 7 significantly differs. The critical significant level p -Value is equal to 0.02. Other pair comparisons (1 *vs.* 3 and 3 *vs.* 7) are not significantly different.

CONCLUSIONS

The analysis of the obtained results showed that the accumulation of phenolic acids in shoot cultures of *E. affine* could be increased by elevating the sucrose concentration in the medium and adding the precursor Phe and the elicitor MeJA. The present experiment indicated that the medium variant E, containing 6% sucrose (twice as much as the standard value), supplemented with the precursor Phe (1.6 g L⁻¹ medium) and elicitor MeJA (100 μ M) was the most efficient to increase phenolic acid accumulation.

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