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Regular paper

Methyl jasmonate, yeast extract and sucrose stimulate phenolic acids accumulation in *Eryngium planum* L. shoot cultures

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Eryngium planum L. has been reported as a medicinal plant used in traditional medicine in Europe. The tissue cultures may be an alternative source of the biomass rich in desired bioactive compounds. The purpose of this study was to investigate the influence of the biotechnological techniques on the selected phenolic acids accumulation in the agitated shoot cultures of _E. planum_. Qualitative and quantitative analyses of those compounds in 50% aqueous — methanolic extracts from the biomass were conducted by applying the HPLC method. Methyl jasmonate (MeJA), yeast extract (YE) and sucrose (Suc) stimulated accumulation of the phenolic acids: rosmarinic (RA), chlorogenic (CGA) and caffeic (CA) in _in vitro_ shoot cultures. Cultivation of shoots in liquid MS media supplemented with 1.0 mg L^{-1} 6–benzyladenine and 0.1 mg L^{-1} indole–3–acetic acid in the presence of 100 µM MeJA for 48h was an optimum condition of elicitation and resulted in approximately 4.5–fold increased content of RA + CGA + CA in plant material compared to the control (19.795 mg g^{-1} DW, 4.36 mg g^{-1} DW, respectively). The results provide the first evidence that the selected phenolic acids can be synthesized in elicited shoot cultures of flat sea holly in higher amount than in untreated shoots.

Key words: chlorogenic acid, elicitation, flat sea holly, *in vitro* shoot culture, rosmarinic acid

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INTRODUCTION

Eryngium species (Apiaceae), including *Eryngium planum* L., are of great value for use in traditional European medicine because they contain phenolic acids, flavonoids, saponins, coumarins, essential oils, and acetylenes. These compounds are associated with diuretic, expectorant, spasmolytic anti-inflammatory, antinociceptive, haemolytic, and antimycotic properties (Thiem *et al*., 2011; Wang *et al*., 2012).

Phenolic compounds, such as rosmarinic acid (RA), chlorogenic acid (CGA), and caffeic acid (CA) are known for their astringent, antiviral, antibacterial, anti-inflammatory, and antimutagenic properties (Petersen & Simmonds, 2003; Gugliucci & Markowicz-Bastos, 2009). This makes them interesting targets for phytochemical research in the context of human health.

The accumulation of metabolites often occurs in plants subjected to stress, due to various elicitors or signal molecules (Zhao *et al*., 2005). The jasmonates are possibly signal compounds in the elicitation process inducing transcriptional activation of genes involved in *de novo* formation of secondary metabolites (Zhang & Memelink, 2009). For this reason, elicitation has been used to increase the accumulation of RA and other phenolic acids in plant tissue and cell cultures (Matkowski, 2008). The sucrose is not only an important carbon and energy source for plant cells but it also greatly influences the production of metabolites of the phenylpropanoid pathway (Gertlowski & Petersen, 1993).

The aim of the present experiment was to establish the shoot cultures of *E. planum* and to test their ability to synthesise selected phenolic acids–rosmarinic, chlorogenic and caffeic acids. Moreover, we evaluated the influence of methyl jasmonate, yeast extract and sucrose on the production of those phenolic acids. Until now, the accumulation of the mentioned phenolic acids in shoots in *in vitro* cultures of plants belonging to *Eryngium* species has not been studied.

MATERIAL AND METHODS

Plant material. Fruits of *Eryngium planum* L. were collected from natural habitats in Poland. To break dormancy, cleaned fruits were stratified, sterilized (see Thiem *et al*., 2013) and placed in Erlenmeyer flasks containing 50 ml of Murashige and Skoog medium. Shoot tips of 30-day-old seedlings were placed on solid MS with 1 mg L^{-1} 6-benzyladenine (BA; Sigma-Aldrich) and 0.1 mg L^{-1} indole-3-acetic acid (IAA; Sigma-Aldrich). Multishoots were divided into single microshoots and transferred to fresh medium every 5–6 weeks. After culture stabilization (see Thiem *et al*., 2013), shoots (3–5 cm long) were transferred to Erlenmeyer flasks containing 20 ml of MS liquid medium with 1.0 mg L^{-1} BA and 0.1 mg L^{-1} IAA and incubated on a rotary shaker (110 rpm) at 21±2°C under a 16/8 light/day cycle (55 µmol m^{-2} s^{-1} light provided by cool-white fluorescent lamps). The growth characteristic of the shoot cultures was recorded on the base on the dry weight at the 1st, 5th, 10th, 15th, 20th, 25th and 30th culture day. All measurements were performed in triplicate. The shoot biomass was subjected to the elicitor treatment.

Elicitor preparation and treatment. The shoot cultures were used for the biotechnological experiments – elicitation by methyl jasmonate (MeJA; Sigma-Aldrich),

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Abbreviations: BA, 6-benzyladenine; CA, caffeic acid; CGA, chlorogenic acid; DW, dry weight; HPLC, high performance liquid chromatography; IAA, indole-3-acetic acid; MeJA, methyl jasmonate; MS, Murashige & Skoog medium; RA, rosmarinic acid; Suc, sucrose; YE, yeast extract.

yeast extracts (YE; Difco) and sucrose (Suc; Chempur) in increased concentration. The filter-sterilized solution of MeJA dissolved in 96% (v/v) ethanol was added to the culture medium resulting in a final concentration of 100 μM. The autoclaved (121°C for 20 min) solution of YE in water was added to the culture medium in a final concentration of 1 g L^{-1}. The solution of MeJA and YE were added separately to the liquid medium for shoots elicitation on the 14th day of the growth cycle and exposed for 24 or 48 hours. Control experiment was run with an equivalent amount of ethanol or water only. 50g of sucrose was added to the culture medium (1 L) at the 1st day of the culture for 10, 20 and 30 days. All treatments were performed in triplicate. For the selected phenolic acid accumulation in the shoot culture, the optimal elicitor and time of exposure were investigated.

Measurement of phenolic acid content. Qualitative and quantitative analysis of phenolic acids in 50% aqueous–methanolic extracts were performed using high–performance liquid chromatography (HPLC). Exact amounts of fresh biomass were dried to a constant weight. Then, 0.2 g of dried and powdered shoot biomass was extracted three times with 15 mL 50% (v/v) methanol for 30 min at the boiling point temperature of the extractive mixture under reflux. Cooled and filtered extract was then diluted with the methanol 50% (v/v) to 50 or 100 mL. The solution was filtered through a 0.2 μm filter (Schleicher & Schuell) and 10 μL aliquots were analysed. The phenolic acid content was determined by reverse phase high performance liquid chromatography (RP HPLC), using Merck–Hitachi apparatus D+7000 coupled to photodiode array (DAD) on a LiChrospher 100 250×4 mm reversed phase column RP 18e, 5 μm (Merck). The solvent system was a linear gradient of acetonitrile and aqueous solution of phosphoric acid pH 2.2 : acetonitrile from 15 to 60% (v/v) for 40 min; 60% for 15 min; from 60 to 15% for 1 min; and 15% for 9 min. The flow rate was 1 mL min^{-1} and the effluent monitored by UV detection at 320 nm. RA, CGA and CA were identified by comparison of their retention times (RT) and UV-VIS spectra with those of authentic standards. The retention times (RT) and on-line UV spectra of detected phenolic acids were identified by comparison with standards of RA (RT 13.707 min), CGA (RT 4.600 min) and CA (RT 6.367 min). The calibration was obtained by peak areas of RA, CGA and CA against a concentrated standard solution (mg 100 mL^{-1}). The relative standard deviation of peak areas of RA, CGA and CA were 1.5%, 1.3% and 1.1%, respectively. Chemicals (methanol, acetonitrile, phosphoric acid) were obtained from Merck. Reference substances originated as follows: RA (purity 96.5%; Sigma-Aldrich), CGA (purity 96.9%; EDQM), CA (purity 99.5%; Fluka). Results are means of three separate analyses from three samples of dried plant material.

Statistical analysis. Data were subjected to a one-way ANOVA followed by Duncan's POST-HOC test (STATISTICA v.10; StatSoft, Inc. 2011). A two-sided P=0.05 was used to declare statistical significance.

RESULTS AND DISCUSSION

The growth cycle of *Eryngium planum* L. shoot culture was 30 days (Fig. 1). The growth of shoot biomass increased slightly over the first 10 days. Presumably, this phenomenon is related to adopting of the plant material to the new physicochemical conditions of liquid media. The largest increase in biomass was observed between

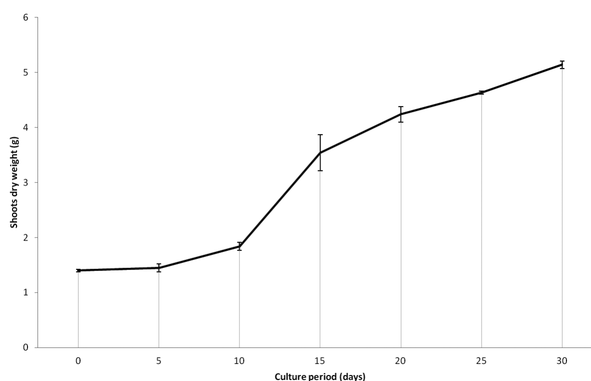

Figure 1. The growth curve of *Eryngium planum* L. shoots in liquid medium.
The values represent a mean ± S.D. of three replicates.

10 and 15 days of the culture – dry weight increased almost 2-fold. In the following days of the culture, the shoot growth decreased significantly and reached a stationary phase. Due to the observations that after 15 days the shoot biomass increased slowly and every shoot culture has a limited life, the 15th day was set to treat the cultures with elicitors.

Our previous phytochemical analyses indicated that undifferentiated cultures (cell suspension, callus), micropropagated plantlets (shoots, roots) and adventitious root cultures of *Eryngium planum* L. are able to produce selected phenolic acids (Kikowska *et al.*, 2012; Thiem *et al.*, 2013). Unfortunately, the accumulation of RA, CGA and CA in the cultured cells of flat sea holly was low. This was despite our extensive efforts to treat cell suspension and callus with elicitors. The media supplementation with 50 g L^{-1} sucrose resulted in the enhanced phenolic acids production of 8.53 mg g^{-1} in callus. Moreover, the elicitation with 100 μM MeJA of callus growing on MS with 40 g L^{-1} sucrose enhanced total phenolic acid production (16.8 mg g^{-1} DW). The content of the total phenolic acids in cell suspension culture was 2.04 mg g^{-1} DW and increased after 24 h and 48 h elicitation with 100 μM MeJA (3.37 mg g^{-1} DW, 4.24 mg g^{-1} DW, respectively) (Kikowska *et al.*, 2012). Because undifferen-

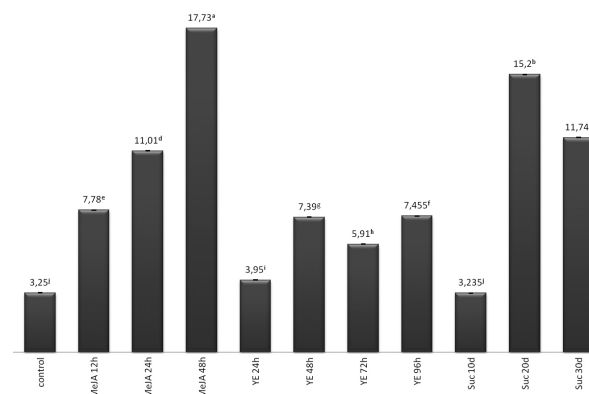

Figure 2. Effect of 100 μM methyl jasmonate (MeJA), 1.0 g L^{-1} yeast extract (YE) and 50 g L^{-1} sucrose (Suc) on rosmarinic acid (RA) accumulation (mg g^{-1} DW) in *Eryngium planum* L. shoots (h, hours; d, days).
Mean values ± S.D. of three replicates with the same letter are not significantly different at P=0.05 using Duncan's Multiple Range test.

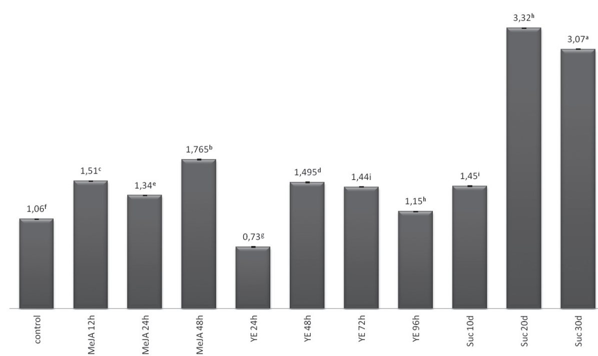

Figure 3. Effect of 100 µM methyl jasmonate (MeJA), 1.0 g L^{-1} yeast extract (YE) and 50 g L^{-1} sucrose (Suc) on chlorogenic acid (CGA) accumulation (mg g^{-1} DW) in *Eryngium planum* L. shoots (h, hours; d, days).
Mean values ±S.D. of three replicates with the same letter are not significantly different at P=0.05 using Duncan's Multiple Range test.

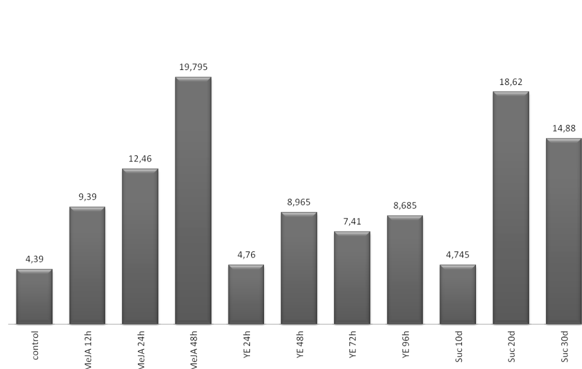

Figure 5. Effect of 100 µM methyl jasmonate (MeJA), 1.0 g L^{-1} yeast extract (YE) and 50 g L^{-1} sucrose (Suc) on selected phenolic acids (RA+CGA+CA) accumulation (mg g^{-1} DW) in *Eryngium planum* L. shoots (h, hours; d, days).

tiated cells lose their biosynthetic ability to accumulate secondary products, we switched our efforts to organs of *E. planum*.

Qualitative and quantitative HPLC analyses of 50% aqueous–methanolic extracts from the non-elicited and elicited *in vitro* shoot cultures of *E. planum* confirmed the presence of RA, CGA, and CA in all of the examined materials. The effects of methyl jasmonate, yeast extract and sucrose on rosmarinic acid, chlorogenic acid, caffeic acid and sum of those acids accumulation in the shoots were different depending on the applied elicitor type and the treatment duration (Figs. 2–5). Thus, all the treatments increased RA+CGA+CA content in the plant material. RA was the main phenolic compound of all tested cultures (Fig. 2). The longer duration of MeJA (100 µM) treatment, the higher accumulation of RA in the shoots — 12 h, 24 h and 48 h elicitation increased rosmarinic acid content (7.78 mg g^{-1} DW, 11.01 mg g^{-1} DW, 17.73 mg g^{-1} DW, respectively). The quantity of RA also increased significantly in shoots cultured for 20 and 30 days in MS with 50 g L^{-1} sucrose. Ten days of exposure to Suc suppressed RA production in shoots. In this medium variant, the amount of phenolic acid in shoots increased 4.68-fold and 3.61-fold for 20 and 30 days of elicitation (18.62 mg g^{-1} DW and 14.88 mg g^{-1} DW, respectively). The addition of 50 g L^{-1} sucrose to the medium, in which shoots were cultured for 20 and 30 d, induced a significant accumulation of CGA (3.32 mg g^{-1} DW and 3.07 mg g^{-1} DW, respectively) (Fig. 3). In all tested samples, CA content was low (from 0.06 to 1.1 mg g^{-1} DW) — just shoots treated with MeJA for 48h exhibited 3.75-fold higher accumulation of this compound (Fig. 4).

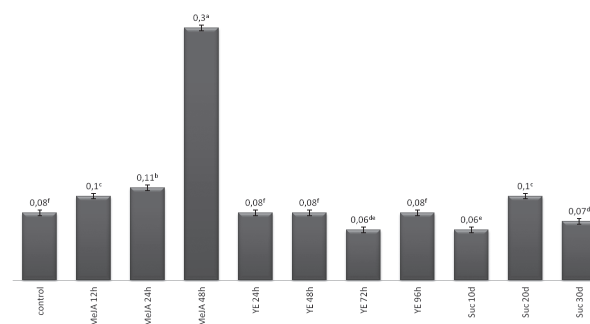

Figure 4. Effect of 100 µM methyl jasmonate (MeJA), 1.0 g L^{-1} yeast extract (YE) and 50 g L^{-1} sucrose (Suc) on caffeic acid (CA) accumulation (mg g^{-1} DW) in *Eryngium planum* L. shoots (h, hours; d, days).
Mean values ±S.D. of three replicates with the same letter are not significantly different at P=0.05 using Duncan's Multiple Range test.

E. planum shoots treated with YE for 24–96 h exhibited low phenolic acids production (Figs. 1–4), which may be associated with too short elicitation time. The response of *Drosera burmanii* shoots by YE was noted after 6 d of elicitation, but there was no significant difference in plumbagin accumulation in cultures at 3 d (Putalun *et al.*, 2010).

The results of several publications showing the important quantitative changes by biosynthetic regulation of secondary metabolites accumulation were obtained from shoot cultures of many medicinal plants. The induction mechanism of elicitors (both biotic and abiotic) is generally regarded as activating the expression of defense-related genes. The treatment with methyl jasmonate has been a useful strategy to enhance bioactive compounds production in *in vitro* shoot cultures of *Catharanthus roseus* (vindoline), *D. burmanii* (plumbagin increased 3-fold), *Mitragyna speciosa* (mitragynine increased 3-fold), *Bacopa monnieri* (bacoside A increased 1.8-fold), *Withania somnifera* (withanolide A increased 14-fold, withanolide B 11-fold, withaferin A 13-fold, withanone 12-fold) (Vazquez-Flota *et al.*, 2009; Putalun *et al.*, 2010; Wungsintaweekul *et al.*, 2012; Sharma *et al.*, 2013; Sivanandhan *et al.*, 2013). The elicitation with yeast extract has been employed for secondary metabolites enhancement in *in vitro* shoot cultures of *Centella asiatica* (asiaticoside increased 1.4-fold), *D. burmanii* (plumbagin increased 3.5-fold), *M. speciosa* (mitragynine) (Kim *et al.*, 2004; Putalun *et al.*, 2010; Wungsintaweekul *et al.*, 2012).

This is the first report on the synthesis of the selected phenolic acids by an *Eryngium planum* L. shoot culture and to the best of our knowledge, in *Eryngium* genus. Our results show that the specific metabolites can be modulated by an elicitor addition to the stabilized shoot culture. Moreover, the osmotic stress created by sucrose was found to regulate phenolic acids production. *In vitro* cultures of *E. planum* would provide a good model for examining the accumulation of the respective antioxidative compounds under controlled chemical and physical conditions. In conclusion, these *in vitro* systems are far

from being suitable for production of rosmarinic acid but they can be regarded as alternative systems for obtaining valuable biomass with bioactive compounds.

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