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SHORT COMMUNICATION

Accumulation of rosmarinic, chlorogenic and caffeic acids in in vitro cultures of *Eryngium planum* L.

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Abstract *Eryngium planum* L. cell and organ cultures were maintained on Murashige and Skoog media (MS), supplemented with exogenous hormones of different types and various concentrations for high biomass growth. The callus and cell suspension cultures were treated with increased sucrose concentration and/or elicited by methyl jasmonate for the enhancement of selected phenolic acids accumulation. Three phenolic acids, rosmarinic acid (RA), chlorogenic acid (CGA) and caffeic acid (CA), were detected by HPLC-DAD in those cultures. The sum of their content in the dry material was found to be higher in the shoot culture (3.95 mg g^{-1}), root culture (7.05 mg g^{-1}), callus (6.20 mg g^{-1}) and cell suspension (2.04 mg g^{-1}) than in the leaves (1.87 mg g^{-1}) and roots (0.76 mg g^{-1}) of intact plants. The major compound of in vitro cultures was always rosmarinic acid. The content of RA could be increased approximately threefold (16.24 mg g^{-1}) in the callus culture and approximately twofold (3.91 mg g^{-1}) in the cell suspension culture by elicitation with 100 μM methyl jasmonate (MeJA). The higher concentration of sucrose (S) in the medium (5, 6 %) led to over a twofold increase of CGA content in the callus culture (2.54 mg g^{-1}). The three mentioned phenolic acids have been found in *E. planum* undifferentiated and differentiated in vitro cultures for the first time.

Keywords *Eryngium planum* L. · In vitro cultures · Rosmarinic acid · Sucrose · Methyl jasmonate

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	6-Benzyladenine
CA	Caffeic acid
CGA	Chlorogenic acid
KIN	Kinetin
MeJA	Methyl jasmonate
MS	Murashige and Skoog medium
NA	0.1 % 2-aminoethanol diphenylborate in ethanol
PGRs	Plant growth regulators
RA	Rosmarinic acid
RP HPLC	Reverse phase high-performance liquid chromatography
S	Sucrose
TLC	Thin layer chromatography

Introduction

Eryngium planum L. (Flat Sea Holly), a species that belongs to the *Apiaceae* family and to the *Saniculoideae* subfamily, has been reported as a medicinal plant used in traditional medicine in Europe (*E. plani herba* and *E. plani radix*). The presence of secondary metabolites, such as phenolic acids (rosmarinic, chlorogenic and caffeic acids), triterpenoid saponins, flavonoids (kaempferol

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and quercetin derivatives) and coumarins is considered to determine its multidirectional pharmacological activities: diuretic, antidiabetic, expectorant, spasmolytic, anti-inflammatory, antinociceptive, haemolytic and antimycotic (Duke et al. 2002; Küpeli et al. 2006). Triterpenoid saponins (Hiller et al. 1974; Voigt et al. 1985), flavonoids (Hiller et al. 1980), chlorogenic acid, rosmarinic acid and its glucoside (Le Claire et al. 2005), coumarins (Erdelmeier and Sticher 1985) and essential oil (Thiem et al 2011) have been found in *E. planum* organs. Rosmarinic acid (RA) and chlorogenic acid (CGA) have been described for many *Eryngium* species (Le Claire et al. 2005).

Rosmarinic acid, an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, has been found in many species of the families: *Lamiaceae*, *Boraginaceae* and *Apiaceae* (*Saniculoideae* subfamily) (Le Claire et al. 2005). This compound has been reported to have various biological activities: antioxidative (reviewed in Matkowski 2008), distinct antiseptic, antiviral, antibacterial, antiphlogistic and anti-inflammatory, which make it an important agent for health promoting effects and a valuable product for cosmetic and food industries (Le Claire et al. 2005; Park et al. 2008; Petersen et al. 2009). Chlorogenic acid has been studied because of its antioxidant (reviewed in Matkowski 2008) and other biological activities: antiviral, antibacterial, the blood glucose level lowering, anti-inflammatory and anti-allergy (Gugliucci and Markowicz-Bastos 2009).

The accumulation of valuable 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 (Yukimune et al. 1996; Zhang and Memelink 2009). For this reason, elicitation with methyl jasmonate has been used to increase the accumulation of RA and other phenolic acids in plant tissue and cell cultures (Szabo et al. 1999; Matkowski 2008). The sucrose content 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 and Petersen 1993).

The aim of the present experiment was to establish the undifferentiated (callus and cell suspension) and organ (shoot and root) cultures of *E. planum* and to test their ability to synthesise phenolic acids RA, CGA and CA. Moreover, we evaluated the influence of MeJA and high concentration of sucrose on the production of the mentioned acids. Until now, the accumulation of those phenolic acids in in vitro cultures of plants belonging to *Apiaceae* has not been studied.

Materials and methods

Plant material

Plants of *E. planum* L. (roots, leaves and fruits of intact plants) were collected from natural habitats in Poland (Kujawy region) in August 2008.

In vitro cultures

Initiation of in vitro cultures

Aseptic seedlings of *E. planum* were obtained from the seeds, which were isolated from the ripened fruits after their stratification and scarification. The isolated seeds were washed with distilled water and dipped in 70 % ethanol for 30 s followed by rinsing with 20 % Clorox (5 % sodium hypochlorite) solution containing two drops of Tween 80 for 5 min. They were finally rinsed three times in sterilised, doubly distilled water. The seed coat was removed under stereoscopic microscope under aseptic conditions.

Culture medium and conditions

The cultured media consisted of MS (Murashige and Skoog 1962) basal medium without a gelling agent (cell suspension culture, root culture) or solidified with 0.8 % agar (callus, plantlets, multiplied shoots) and supplemented with 3 % sucrose and plant growth regulators (PGRs). The cultures were incubated in a growth chamber under a 16/8 h photoperiod (55 μmol m^{-2} s^{-1} light provided) at a temperature of 23 ± 2 °C. The root cultures were maintained in darkness.

Callus culture

The callus culture was from different explants of 30-day old sterile seedlings (cotyledon, hypocotyl, leaf blade and petiole, root) and then established on solid media: MS with 0.1 mg L^{-1} 2,4-D + 1.0 mg L^{-1} KIN and MS with 1.0 mg L^{-1} 2,4-D + 1.0 mg L^{-1} BA. Subcultures were performed at 4-week intervals. The stable and homogenous root-derived callus growing on the medium with 2,4-D and BA (32nd subculture) was used for the biotechnological experiments—sucrose addition in increased concentration and elicitation by methyl jasmonate. In the first set of experiments, the media for callus cultures were supplemented with a higher concentration of sucrose (4, 5, 6 %) for osmotic stress creation. In the second experiment, the obtained callus cultured on the MS medium with 4 % S was elicited by MeJA in concentrations of 100 μM, as

described below. The elicited cultures were maintained for 30 days. Callus initiation and its development were first observed visually and then the growth callus index was calculated by the following equation: Growth index = (final dry cell weight − initial dry cell weight)/initial dry cell weight.

Cell suspension culture

A portion of 4.0 g of the stabilised friable callus from the seedling's root explants was cultured on MS medium supplemented with 1.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} BA, and was rubbed through a stainless steel sieve into 50 mL of the fresh medium (MS) containing 0.5 mg L^{-1} 2,4-D. A liquid suspension culture was established by continuous shaking at 110 rpm and maintained by subculturing at regular intervals for 8 days. The 5 mL inoculation of an old culture was added to 50 mL of a new medium to maintain the cell vitality. The experiment was set up in a completely randomised design. The growth of cell suspension was monitored by the measurement of the fresh and dry biomass on 3, 6, 9, 12, 15, 18, 21 and 24 days for a curve determination. While the fresh weight was assessed by filtering 2 cm^3 of solution from each flask on filter paper and then centrifuging at 2,000*g* for 5 min in a graduated centrifuge tube, the dry weight was obtained by oven drying for 48 h at 40 °C.

Organ in vitro culture

The shoot tips (0.8–1 cm long) of seedlings were used for the induction of shoot cultures and established on previously selected MS medium supplemented with 1.0 mg L^{-1} BA and 0.1 mg L^{-1} IAA. The obtained multishoots were divided into single microshoots and transferred to the fresh medium every 5–6 weeks of subculture in several multiplication assays. Half of the multiplied shoots were rooted in an agar medium supplemented with 0.1 mg L^{-1} IAA to obtain the axenic plantlets. Root cultures in the liquid medium (MS with 0.1 mg L^{-1} IAA) were obtained from the tips of adventitious roots (0.8 mm) of plantlets. The root cultures were grown on a rotary shaker in darkness and subcultured to a fresh medium every 6 weeks. Organ cultures were cultured in 250 cm^3 Erlenmeyer flasks with 50 mL of culture medium.

Addition of MeJA

The filter-sterilised solution of MeJA (Sigma-Aldrich) dissolved in 96 % ethanol was added to the culture medium resulting in a final concentration of 100 μM. The final concentration of ethanol in the medium was 0.1 % (v/v). The solution of MeJA was added to the solid medium for callus elicitation and to the liquid medium for cell suspension elicitation on the day of linear phase (9th day). The elicitation experiments lasted 30 days for callus and 24 or 48 h for cell suspension. A control experiment was run with ethanol only, at a final concentration of 0.1 % (v/v).

Phytochemical analysis

Biomass of callus (passage 32nd) cultured on MS with 1.0 mg L^{-1} 2,4-D + 1.0 mg L^{-1} BA and cell suspension (passage 9th) cultured on MS with 2,4-D (0.5 mg L^{-1}) were taken for phytochemical analysis. Moreover, for comparison, leaves and roots of intact plants, shoot culture (passage 9th, at the end of 5th week of culture), root culture (passage 9th, at the end of 6th week of culture) and roots (passage 9th, at the end of 5th week of culture) obtained from in vitro-derived plantlets were also analysed.

Preliminary chromatographic TLC analysis

1.0 g of the dried biomass was extracted three times with 30 mL 70 % (v/v) ethanol for 1 h at the boiling point temperature under reflux. The combined, cooled and filtered extracts were concentrated under reduced pressure below 40 °C. The equal portions of each extract (0.1 g) were dissolved in 1.0 mL 70 % (v/v) ethanol. The 5 μL aliquots of each extract were applied as 1.0 cm streaks besides solutions of the reference compounds, rosmarinic acid (RA), chlorogenic acid (CGA) and caffeic acid (CA), in ethanol (each 1 mg/1 mL) to the HPTLC silica gel plates 10 × 20 cm (Merck, No. 5628, 5641, 5642), which were developed with a solvent mixture consisting of ethyl acetate–acetic acid–water (4:1:1 v/v/v) mixture to a distance of 9 cm in a chamber for 20 × 20 cm plates (Camag, Muttenz, Switzerland) (unsaturated, temperature 21 °C, humidity 45 %). The developed plates were dried at ambient temperature and viewed under $UV_{366\ nm}$ before and after spraying with the following reagents: (1) NA (Roth) 0.1 % solution in ethanol for the detection of phenolic acids (blue bands under UV) and flavonoids (yellow or orange bands under UV), (2) $AlCl_3$ 1 % solution in ethanol (followed by heating) for the detection of flavonoids (yellow bands under UV). The acids displayed the following R_f values: 0.92 (RA), 0.54 (CGA) and 0.96 (CA). A Camag Video documentation System (Camag) was used for recording and analysing the images of the developed TLC plates.

HPLC analysis

The 0.5 g of dried biomass was extracted three times with 15 mL 50 % (v/v) methanol for 30 min under reflux. The cooled and filtered extract was next diluted with the

methanol 50 % (v/v) up to the volume of 50 or 100 mL. The solution was filtered through a 0.2 μm filter (Schleicher & Schuell) and 10 μL aliquot was analysed. The phenolic acids presence and content in the methanolic extracts were determined by RP HPLC [using Merck-Hitachi apparatus D-7000 coupled to photodiode array (DAD)] on a Li-Chrospher 100 250 × 4 mm reversed phase column (RP 18e, 5 μm, Merck). The solvent system was a linear gradient of acetonitrile/water and phosphoric acid pH = 2.2; acetonitrile from 15 to 60 % (v/v) for 40 min, 60 % for 15 min, from 60 to 15 % and 15 % for 9 min. The flow rate was 1 mL min^{-1} and the effluent was monitored by UV detection at 320 nm. The retention times (RT) and online UV spectra of detected phenolic acids were compared to those of 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 the concentration standard solution (mg/100 mL). One concentration level was analysed in seven replications. The relative standard deviation of peak areas of RA, CGA and CA are 1.5, 1.3 and 1.1 %, respectively.

Reference substances

Standards were as follows: rosmarinic acid (Sigma-Aldrich, purity 96.5 %), chlorogenic acid (EDQM, purity 96.9 %) and caffeic acid (Fluka, purity 99.5 %). In this investigation, 0.001 % methanolic solutions of these standards were used.

Statistical analysis

All results were means of three separate analyses from three samples of dried plant material for the estimation of phenolic acids. The results were expressed as mg g^{-1} dry weight (d.w.).

The results (phenolic acids content) were reported as the mean ± 95 % confidence interval [standard error (SE)·1.96 (quantile of normal distribution for probability of 95%)] and the data were analysed by one-way analysis of variance (ANOVA) to compare means of group (type of cultures and intact plant material) followed by mean separation using Tukey-Kramer multiple comparisons test as a post hoc test. Differences with $P < 0.05$ were considered to be significant.

Results and discussion

Eryngium planum callus, cell suspension and organ cultures were established to study the accumulation of chosen phenolic acids. The enhancement of an accumulation of RA, CGA and CA by manipulation of the medium sucrose content and elicitation by methyl jasmonate (MeJA) in callus and cell suspension cultures was investigated.

The in vitro cultures of *E. planum* were initiated from seeds of intact plants. Various parts of seedlings were employed for the establishment of callus and organ cultures. The induction of callus occurred on all types of explants on the previously selected media. The best explants for callus induction and proliferation were seedling hypocotyl and root, irrespective of the tested medium. For the further experiments root-derived callus was maintained on MS medium enriched with 1.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} BA. The influence of various sucrose concentrations (3, 4, 5, 6 %) and methyl jasmonate (100 μM) on the growth of *E. planum* callus was observed. The callus growth index decreased from 513 to 211 when the concentration of sugar increased. The lowest growth index was indicated after treatment with both 4 % sucrose and MeJA (Table 1). The soft and friable callus was used for cell suspension initiation. In our study, the suspension culture exhibited a typical growth curve divided into four phases (Fig. 1). The growth of a cell population inoculated into a fresh medium did not ensue immediately. The cells were taken from the exponential phase; hence, the lag phase was determined to be brief. The cells reached the linear phase, performing multiplication in geometric progression. At the end of the culture, cell suspension reached the stationary phase.

Shoot cultures were obtained from shoot tips of seedlings on the MS basal medium supplemented with 1.0 mg L^{-1} BA and 0.1 mg L^{-1} IAA. The percent of explants showed shoot regeneration ranged from 97.9 to 100 %. The efficiency of axillary bud proliferation stayed at the same level during sequential passages. The rate of multiplication was ~17 new buds per explant after shoot culture stabilisation. All shoots showed high vigour and well-expanded leaves. The regenerated shoots rooted on the agar MS medium with 0.1 mg L^{-1} IAA formed three to

Table 1 The influence of various sucrose concentration and methyl jasmonate in the medium on the growth of *Eryngium planum* L. callus on MS medium with 1.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} BA

Concentration of sucrose and methyl jasmonate	Callus growth index[a]		Mean value
	Series 1	Series 2	
3 % (control)	510.0 ± 28.9	517.0 ± 25.2	513.5
4 %	475.0 ± 32.1	502.5 ± 32.4	488.8
5 %	230.2 ± 18.8	251.1 ± 20.4	240.6
6 %	211.5 ± 20.3	211.4 ± 20.7	211.4
4 % + 100 μM MeJA	39.7 ± 3.2	45.7 ± 3.2	42.7

Two series with 15 replications

[a] Callus growth index ± SE (standard error)

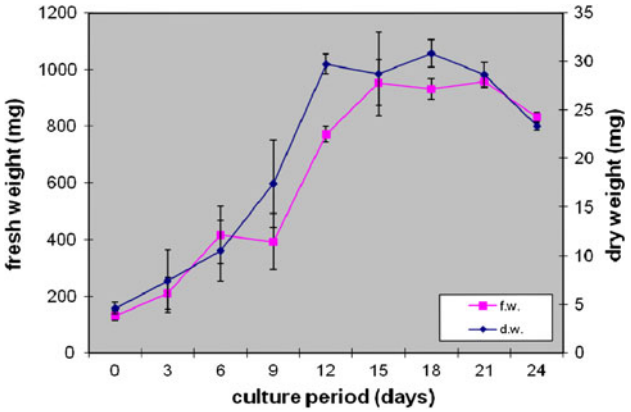

Fig. 1 *Eryngium planum* L. cell suspension curve. The values are mean of four replications

four adventitious roots, which reached ~4 cm length after 6 weeks. Other type of culture, roots in the liquid MS medium supplemented with 0.1 mg L^{-1} IAA, showed good viability and growth characteristic.

The preliminary phytochemical TLC analyses of extracts from the above-ground parts of intact plants and in vitro shoot cultures showed the presence of two phenolic group of secondary metabolites—phenolic acids and flavonoids. Phenolic acids were present in roots of intact plants, in vitro root cultures and undifferentiated (callus, cell suspension) cultures. The detection of three phenolic acids, RA, CGA and CA, in all extracts (with exception of CA in extracts from cell cultures) by TLC co-chromatography with standards was confirmed by HPLC-DAD on the basis of an agreement of retention times and online UV spectra with those of the reference compounds (Fig. 2). The quantitative determinations of RA, CGA and CA were performed by HPLC (Table 2).

Rosmarinic acid (0.55 and 0.74 mg g^{-1}) and chlorogenic acid (0.19 and 1.10 mg g^{-1}) were the main phenolic acids of roots and leaves, respectively, of *E. planum* plants from natural habitat (Table 2). It was noticed that the content of the sum of those acids RA + CGA + CA (in mg g^{-1}) was always higher in organ cultures, i.e. shoot culture (3.95), root culture (7.05) and undifferentiated cultures, callus (6.20) and cells (2.04) than in leaves (1.87) and roots (0.76) of intact *E. planum* plants (Table 2). The main phenolic acid of any cultures was always RA, the content of which varied in the range from 6.3 mg g^{-1} in the case of the root culture to 1.73 mg g^{-1} in the cell suspension culture and was apparently higher than that in the leaves (0.74 mg g^{-1}) and roots (0.55 mg g^{-1}) of intact plants. Our studies have shown that the content of rosmarinic acid in in vitro shoot cultures was 3.5 times higher than in the leaves of *E. planum* intact plants. Similar results were observed for *Salvia officinalis* L. in vitro shoot cultures, which showed levels of rosmarinic acid 2.9 times over that of the commercial samples of leaves (Santos-Gomes et al. 2006).

The absence of flavonoids in cell cultures could be explained by the lack of ability of *E. planum* undifferentiated cells to synthesise those phenolic compounds. Similar results have been observed in other callus cultures (Thiem et al. 2001; Santos-Gomes et al. 2003; Budzianowska et al. 2004). Rosmarinic and chlorogenic acids are not greatly influenced by the tissue differentiation level in comparison to flavonoids.

RA is defence response molecule, for this reason, elicitation and stress condition could be an efficient strategy for increasing production of phenolic acids in in vitro cultures (Zhao et al. 2005). The increased sucrose (S) concentration in the medium or its supplementation with methyl jasmonate (MeJA) was applied to study their impact on the phenolic acids accumulation in the callus culture. In all experiments, the content of each – RA, CGA and CA was measured by HPLC (in mg g^{-1} d.w.) and the sum of those contents expressed as a total phenolic acids content. The percent of each single phenolic acid in the total phenolic acids content was calculated (Table 2).

In the first set of experiments, the media were supplemented with higher, i.e. 4, 5 and 6 % concentration of sucrose for osmotic stress creation. Satisfactory proliferation of the callus (growth index 513) took place on MS medium containing 1.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} BA with 3 % S. The higher the sugar level in the medium, the lower was the callus growth index observed. For this reason, the medium with 4 % S content, where good callus development was still observed (growth index 488), was chosen for the second set of experiment—elicitation by MeJA at concentration of 100 μM.

The callus growth, expressed by the average callus growth index, showed a significant negative correlation with the accumulation of RA in treated cultures (Tables 1, 2). At the 4 % S concentration the sum of RA, CGA and CA content appeared to be somewhat lower (5.41 mg g^{-1}) compared to the 3 % S concentration (6.20 mg g^{-1}). However, the S concentration of 5 and 6 % resulted in the enhanced phenolic acids production of 8.53 and 8.45 mg g^{-1}, respectively. Although RA was again the major compound, the increase in its content was lower than that of CGA, which more than doubled from 0.94 mg g^{-1} (3 %) to 2.54 and 2.37 mg g^{-1} (5 % S and 6 % S, respectively). The elicitation with 100 μM MeJA of callus growing on MS medium supplemented with 4 % S enhanced 2.5-fold total phenolic acid production (from 6.2 to 16.8 mg g^{-1}) and threefold of RA (from 5.1 to 16.2 mg g^{-1}) accumulation compared to the control e.g. callus on MS with 3 % S. The higher S content (4 %) and elicitation with 100 μM MeJA seemed to be the best treatment for RA production in callus culture. Probably, the

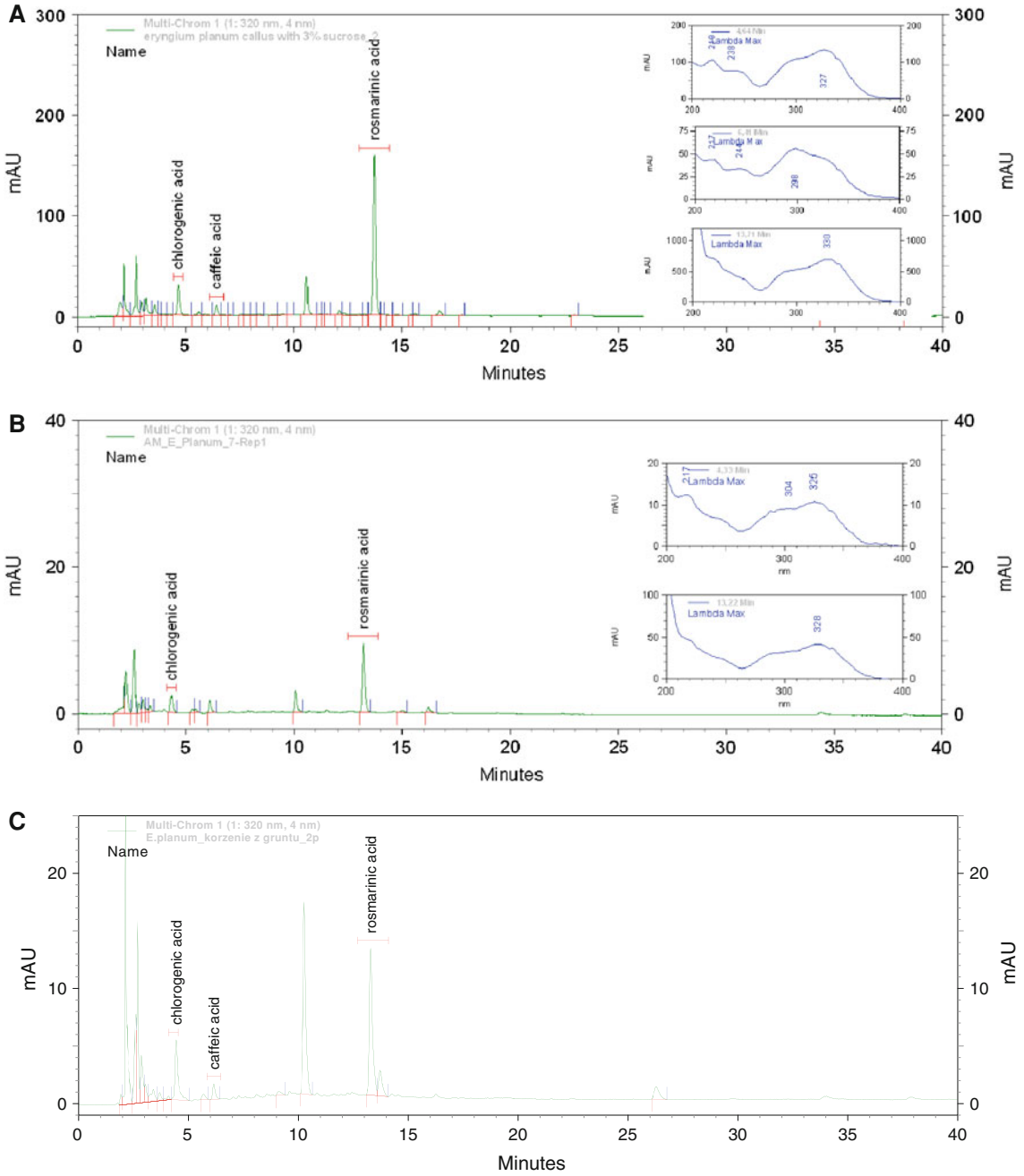

Fig. 2 HPLC chromatograms of the extracts and UV spectra of the investigated phenolic acids in the callus (**a**), cell suspension culture (**b**) and the root of *Eryngium planum* L. intact plant (**c**)

metabolism change caused by MeJA relies on the use of common intermediary precursors from phenylpropanoid pathway increasing the RA biosynthesis at CGA expense (Petersen et al. 2009). Those factors were chosen for our experiments because of its influence not only on phenolic acids, but also on saponins production (Misawa 1994). The in vitro production of saponins will be the objective of future studies.

Sucrose is the most favourable carbon source for cell growth and in most cases is used at concentrations of 3 %. Higher carbohydrate content in the culture medium generates stress on the tissue, which may result in changes in cells metabolism, growth and secondary metabolites production, mostly in the phenylpropanoid pathway (Hippolyte et al. 1992; Misawa 1994). The increased content of RA in *E. planum* callus was inversely related with the respective biomass growth. The accumulation of RA in the calli of *S. officinalis* is also inversely related with the respective growth (Santos-Gomes et al 2003). In contrast to previous reports, the 6 % S treatment produced the

Table 2 Rosmarinic acid (RA), chlorogenic acid (CGA) and caffeic acid (CA) contents in extracts of *Eryngium planum* L. intact plants and various in vitro cultures

Plant material	Compound content ± (SE)·1.96 (mg g^{-1} d.w.) (% of the total phenolic acid content)			
	RA	CGA	CA	Total phenolic acids content (RA + CGA + CA)
Intact plants				
Leaf	0.74 ± 0.03 (39.6)	1.10 ± 0.01 (58.8)	0.03 ± 0.00 (1.6)	1.87
Root	0.55 ± 0.00 (72.4)	0.19 ± 0.00 (25.0)	0.02 ± 0.00 (2.6)	0.76
Organ in vitro cultures				
Shoot culture	2.59 ± 0.01 (65.6)	0.63 ± 0.02 (15.9)	0.73 ± 0.03 (18.5)	3.95
Root from in vitro plantlet	0.56 ± 0.01 (75.7)	0.12 ± 0.01 (16.2)	0.05 ± 0.00 (8.1)	0.74
Root culture	6.3 ± 0.03 (89.3)	0.52 ± 0.02 (7.4)	0.23 ± 0.00 (3.3)	7.05
Undifferentiated cultures				
Callus: 3 % S	5.14 ± 0.03 (83.0)	0.94 ± 0.02 (15.1)	0.12 ± 0.00 (1.9)	6.20
Callus: 4 % S	4.14 ± 0.08 (76.5)	1.14 ± 0.01 (21.0)	0.13 ± 0.02 (2.4)	5.41
Callus: 5 % S	5.83 ± 0.08 (68.3)	2.54 ± 0.11 (29.8)	0.16 ± 0.02 (1.9)	8.53
Callus: 6 % S	5.97 ± 0.12 (70.6)	2.37 ± 0.02 (28.0)	0.11 ± 0.01 (1.3)	8.45
Callus: 4 % S + 100 μM MeJA	16.24 ± 2.29 (96.4)	0.48 ± 0.02 (2.9)	0.12 ± 0.01 (0.7)	16.84
Cell suspension	1.73 ± 0.03 (84.8)	0.31 ± 0.02 (15.2)	ND	2.04
Cell suspension + 100 μM MeJA/24 h	3.12 ± 0.09 (92.6)	0.25 ± 0.03 (7.4)	ND	3.37
Cell suspension + 100 μM MeJA/48 h	3.91 ± 0.05 (92.2)	0.33 ± 0.01 (7.8)	ND	4.24

The results are expressed as mean values ±95 % confidence interval

S sucrose, *MeJA* methyl jasmonate, *ND* not detected

maximum callus growth in *Zataria multiflora* tissue cultures (Bernard et al. 2007). The opposite results were stated for *Rheum ribes* callus culture; the concentration of 6 % S in the medium gave optimal growth of callus, but the maximum content of secondary metabolites was obtained for 3 % S. In this context the sugar concentration should be optimised for an individual species. Our results indicate that the phenolic acid accumulation was affected by sucrose—its higher concentration (5 %, 6 %) enhanced apparently CGA and slightly RA accumulation in callus.

In cell suspension biomass, the concentration of RA varied depending on the phases of the cycle. The highest level of RA was found on the 9th day of the linear phase of suspension culture and decreased when it reached the stationary phase (data not shown). The content of the total phenolic acids (RA + GCA + CA) in cell suspension culture on the 9th day was 2.04 mg g^{-1} and increased significantly after elicitation with 100 μM MeJA 1.65-fold and 2.08-fold after treatment for 24 h (3.37 mg g^{-1}) and for 48 h (4.24 mg g^{-1}), respectively (Table 2). The RA represented nearly 90 % in the non-elicited culture (84.8 %) and after MeJA treatment—92.6 and 92.2 % after 24 and 48 h, respectively. The amounts of RA in cell suspension culture increased significantly after elicitation with 100 μM MeJA—1.69- and 2.21-fold after treatment for 24 h (3.12 mg g^{-1}) and 48 h (3.91 mg g^{-1}) respectively, compared with the control (1.73 mg g^{-1}). The percent of RA in total phenolic acid content was enhanced to 92.6 %. In contrast, the percent of CGA decreased with the addition of MeJA (Table 2). Probably, common intermediary precursors from phenylpropanoid pathway (Petersen et al. 2009) are used for the biosynthesis of RA at CGA expense after treatment with MeJA. Rosmarinic acid production by the cell suspension culture can be correlated with the growth or begins only when growth has stopped. In our studies, RA accumulation was enhanced during the linear phase of the cell growth cycle. The first few days are required to recover from the stress of being transferred to fresh medium. The addition of MeJA (9th day) affected the RA increase in cell suspension culture compared with the control cell culture. Similarly, Ketchum et al. (1999) suggested that the greater effectiveness of the elicitor on *Taxus* cell culture was achieved when the MeJA was added 7, 8 or 14 days of inoculation, rather than at the time of inoculation.

Plant in vitro cultures are often an effective system for producing natural compounds for pharmaceutical applications. In general, the level of RA in plants of *Lamiaceae* family (*Nepetoidae* subfamily) was high and ranged from 0.01 to 9.30 mg g^{-1} (Janicsak et al. 1999). Plants from *Apiaceae*, which contain rosmarinic acid only in *Saniculoideae* subfamily, synthesise a low RA, e.g. *E. planum* root has 0.4 mg g^{-1} of RA (Le Claire et al. 2005). The results of several publications showing the important

quantitative changes by biosynthetic regulation of RA accumulation were obtained from undifferentiated cultures of *Lamiaceae* and *Boraginaceae* family*: Coleus blumei* (Gertlowski and Petersen 1993), *Lavandula vera* (Georgiev et al. 2006), *Ocimum basilicum* (Kintzios et al. 2003), *Lithospermum erythrorrhizon* (Yamamoto et al. 2002), *S. officinalis* (Hippolyte et al. 1992), *Salvia* sp. (Grzegorczyk et al. 2005) and other species (Matkowski 2008). It has been reported that elicitors influenced RA biosynthesis in numerous plant in vitro cultures and that RA may be accumulated in non-organ and organ cultures in higher concentration than in the intact plants (Petersen and Simmonds 2003; Matkowski 2008). Production of bioactive compounds in a number of plant cultures was affected by sucrose concentration (Su and Humphrey 1990; Gertlowski and Petersen 1993) or by jasmonates treatment (Zhao et al. 2005).

This is the first report on the synthesis of RA by an *E. planum* cell culture and to the best of our knowledge in *Apiaceae* family. Rosmarinic acid biosynthesis was stimulated in *E. planum* callus and cell suspension culture by MeJA. Our results show that the specific metabolites can be modulated by an elicitor addition to the stabilised cell suspension culture. The osmotic stress created by sucrose was found to regulate phenolic acids production in *E. planum* callus. Moreover, our results confirm that accumulation of RA, CGA and CA does not require a specialisation of cells of *E. planum.* Callus, cell suspension culture and root culture accumulated higher amounts of phenolic acids than intact plants.

The presence of RA and other phenolic compounds in the extracts from in vitro cultures and organ of intact plants could be responsible for the antioxidant property. This activity reported for e.g. *S. officinalis* callus and cell suspension cultures (Grzegorczyk et al. 2005) will be monitored for *E. planum* extracts. 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. This alternative way for the production of antioxidative compounds has not been studied.

Our study provides the first evidence that RA, CGA and CA can be synthesised in the undifferentiated cultures and are present in the root of in vitro-derived plantlets, root culture, callus and cell suspension cultures of *E. planum*. In conclusion, these in vitro systems are far from being suitable for the production of RA, but they can be regarded as alternative systems for obtaining valuable compounds with pharmacological properties.

Author contribution M. Kikowska and B. Thiem designed the experiments and contributed to all the experimental processes, data analysis and paper preparing. J. Budzianowski was responsible for qualitative phytochemical analysis and paper preparing. A. Krawczyk took charge of quantitative HPLC analysis.

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