

The Effect of Nutritional Factors and Plant Growth Regulators on Micropropagation and Production of Phenolic Acids and Saponins from Plantlets and Adventitious Root Cultures of *Eryngium maritimum* L.

Małgorzata Kikowska · Barbara Thiem · Elwira Sliwiska ·
Monika Rewers · Mariusz Kowalczyk · Anna Stochmal ·
Wiesław Oleszek

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Abstract *Eryngium maritimum* L. is a valuable medicinal species, but since it is protected plant, collection from natural populations is forbidden. Therefore, establishing an efficient system for micropropagation of this species is desirable. To determine the optimal nutritional factors needed for shoot multiplication, root development and secondary metabolites accumulation, different media and plant growth regulators were tested. The highest plant regeneration efficiency (over 96 %), with 4.4 shoots per explant was induced on Murashige and Skoog (MS) medium supplemented with 1.0 mg L⁻¹ benzyladenine (BA) and 0.1 mg L⁻¹ indole-3-acetic acid (IAA). The in vitro-regenerated shoots were rooted (83.3–100 %) and transferred to an experimental plot with 62 % efficiency. Flow cytometric analysis revealed no variation in nuclear DNA content in field- and in vitro-delivered plant material. Ultra high performance liquid chromatography (UHPLC)

indicated that multiple shoots and roots from in vitro-regenerated plantlets and adventitious root cultures maintained the production of rosmarinic (RA) and chlorogenic (CGA) acids and triterpenoid saponins found in the rosette leaves and roots of *E. maritimum* intact plants. UHPLC revealed a 12-fold increase of RA and CGA and 3.2-fold higher accumulation of triterpenoid saponins in roots of in vitro-derived plantlets in comparison to roots from field-grown plants. Adventitious root cultures allowed continuous growth of excised root in liquid media with or without exogenous auxins. The roots grown in liquid medium supplemented with 0.1 mg L⁻¹ IAA showed higher (227-fold) phenolic acids accumulation than those without auxin. Obtained results confirmed that micropropagation is a useful strategy in the protection of endangered species and a renewable source of a high quality plant material for secondary metabolites production.

M. Kikowska · B. Thiem (✉)
Department of Pharmaceutical Botany and Plant Biotechnology,
K. Marcinkowski University of Medical Sciences, 14 Św. Marii
Magdaleny Str., 61-861 Poznań, Poland
e-mail: bthiem@ump.edu.pl

M. Kikowska
e-mail: kikowska@ump.edu.pl

E. Sliwiska · M. Rewers
Laboratory of Molecular Biology and Cytometry, Department of
Plant Genetics, Physiology and Biotechnology, University of
Technology and Life Sciences, Prof. S. Kaliskiego Ave. 7,
85-789 Bydgoszcz, Poland
e-mail: elwira@utp.edu.pl

M. Kowalczyk · A. Stochmal · W. Oleszek
Department of Biochemistry and Crop Quality, Institute of Soil
Science and Plant Cultivation, State Research Institute, 8
Czartoryskich Str., 24-100 Puławy, Poland
e-mail: asf@iung.pulawy.pl

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Introduction

Eryngium L. (Sea Holly, Eryngo) comprises about 230–250 species and it is therefore the largest genus of the Sani-culoideae subfamily from the Apiaceae family. This taxon is widespread in Central Asia, America, Central and Southeast Europe, North Africa, and Australia (Calvino and others 2008; Wörz and Diekmann 2010). Four of the 26 species described in Flora Europaea (Tutin and others 1968), including *Eryngium maritimum* L., grow in restricted regions in Poland as rare or protected plants (Piękoś-Mirkowa and Mirek 2003). *E. maritimum* is a rare

perennial under strict low protection in Poland and some European countries. It grows on coastal dunes of the Baltic Sea, Mediterranean basin, and Black Sea (Tutin and others 1968). This halophyte disappears at an alarming rate due to intensified anthropopressure particularly tourism, indiscriminate collection, transformation of the seashore and storms, overplanting dunes with willows (Piękoś-Mirkowa and Mirek 2003). In this regard, *E. maritimum* got the status of a vulnerable species in Western Pomerania (Żukowski and Jackowiak 1995).

The pharmacological activity of *Eryngium* species depends mainly on their saponin content, but the presence of phenolic acids, polyacetylenes, flavonoids, and betalains is also important for their usage in traditional medicine (reviewed in Kikowska and others 2012; Thiem and others 2013). *E. maritimum* has long been used in traditional medicine and as a culinary herb in Europe. Furthermore, this species currently has been applied for cosmetic purposes. The leaf and root extracts present antioxidant, antibacterial, and strong antifungal activities (Meot-Duros and others 2008; Thiem and others 2010; Kholkhal and others 2012). Infusion of aerial parts and roots is used in folk remedies as antitussive, diuretic, appetizer, stimulant, and aphrodisiac (Kholkhal and others 2012). Moreover, the roots are used in the treatment of flatulence, urethritis, scurvy, stone inhibitor, and removes liver and kidney obstructions (Andrew 2001).

The main phenolic acids occurring in *Eryngium* genus are caffeic acid derivatives, mostly rosmarinic and chlorogenic acids. Rosmarinic acid (RA; an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid), has been reported to have various biological activities: distinct antiseptic, antiviral, antibacterial, antiphlogistic, and anti-inflammatory (Petersen and Simmonds 2003; Le Claire and others 2005; Park and others 2008). Because of its antioxidant activity, RA has been suggested to have a beneficial effect in human health, for example, it has an inhibitory effect on acetylcholinesterase and butyrylcholinesterase enzymes, the key enzymes involved in several neurological disorders, such as Parkinson and Alzheimer's disease (Orhan and others 2008). Chlorogenic acid (CGA; an ester of CA and quinic acid) has been studied because of its biological activities, such as antioxidant, antiviral, antibacterial, blood glucose level lowering, anti-inflammatory, and anti-allergy properties (Gugliucci and Markowicz-Bastos 2009).

Triterpenoid saponins are a large class of natural compounds, which exhibit a wide variety of both structural diversity and biological activity. They are characterized by a triterpene aglycone and one or more sugar chains. They have a broad range of pharmacological applications, such as hypocholesterolemic, immunoadjuvant, antiviral, antibacterial, antifungal, anti-leishmanial, and anti-inflammatory. Saponins

also exhibit anti-HIV-1 protease activity and cytotoxicity against cancer cell lines (Sparg and others 2008; Lambert and others 2011). Recent reports suggest that saponins from *Panax ginseng* may rescue or protect neurons from insult, and may be a promising candidate to improve the cognitive deficit of Alzheimer's disease (Zhao and others 2009). The genus *Eryngium* is known to contain triterpenoid saponins as the main components. Most *Eryngium* saponins are polyhydroxylated oleanene triterpenoid saponins (Wang and others 2012). *E. maritimum* roots of intact plants contain complex of triterpenoid saponins, which were isolated, structures were evaluated and established as: 3-*O*- β -D-glucopyranosyl-(1 \rightarrow 2)- β -D-glucuronopyranosyl-22-*O*-angeloyl-R1-barrigenol (M2A), 3-*O*- β -D-glucopyranosyl-(1 \rightarrow 2)- β -D-glucuronopyranosyl-21-*O*-acetyl-22-*O*-angeloyl-R1-barrigenol (M1), 3-*O*- β -D-glucopyranosyl-(1 \rightarrow 2)- β -D-glucuronopyranosyl-22-*O*-angeloyl-A1-barrigenol (M2B) (Kowalczyk and others 2014).

In view of the increasing interest for *E. maritimum* and the difficulty of propagation: seed dormancy, high mortality of seedlings in early development stages and prohibition of plant material collection from natural populations, there is a need to develop a new approach for the propagation of this species (Atwater 1980). Differentiated organ culture, especially adventitious root culture, has been applied in many medicinal plants due to its rapid growth and stable production of secondary metabolites (Murthy and others 2008).

The aim of this study was to develop a rapid and reproducible protocol for setting up *E. maritimum* micropropagation, which guarantees the stability of the genome size of regenerated plantlets and the preservation of their ability to produce selected phenolic acids and triterpenoid saponins. To the best of our knowledge, the in vitro culture of this taxon and the capacity of regenerated plantlets to accumulate secondary metabolites have not been studied before.

Materials and Methods

Plant Material

Plant material of *Eryngium maritimum* L. (meristem fragments of 2–3-month-old plants grown in the field, rosette leaves and roots of flowering plants) were collected from the Botanical Garden of Adam Mickiewicz University, Poznan, Poland, in September 2009. The comparative voucher specimens are deposited in the Herbarium of Medicinal Plant Garden in the Institute of Natural Fibers and Medicinal Plants in Poznan (Poland). Permission for the collection of the organs from *E. maritimum* and keeping

Table 1 Effect of MS medium composition and growth regulators on shoot multiplication from shoot tip explants of *E. maritimum* after 6 weeks of in vitro culture

Medium	Growth regulator (mg L ⁻¹)		Shoot induction (%)	Shoot number per explant (± SD)	Shoot length (cm) (± SD)	Leaf number per shoot (± SD)
	BA	IAA				
1/2 MS with 1/2 vit.	0.0	0.0	53.6	2.4 ± 0.33 ^f	4.0 ± 0.17 ^b	3.1 ± 0.32 ^g
	1.0	0.0	56.6	2.0 ± 0.41 ^g	3.0 ± 0.41 ^g	3.6 ± 0.31 ^f
	1.0	1.0	72.4	2.9 ± 0.15 ^d	2.3 ± 0.28 ^h	3.5 ± 0.48 ^f
	1.0	0.1	56.9	2.5 ± 0.25 ^f	3.2 ± 0.13 ^f	5.7 ± 0.34 ^a
1/2 MS	0.0	0.0	45.6	1.6 ± 0.25 ^h	3.5 ± 0.11 ^e	4.0 ± 0.30 ^{de}
	1.0	0.0	63.3	3.4 ± 0.25 ^c	3.1 ± 0.16 ^{fg}	4.2 ± 0.32 ^d
	1.0	1.0	56.6	2.8 ± 0.20 ^{de}	3.2 ± 0.17 ^f	4.2 ± 0.34 ^d
	1.0	0.1	54.9	2.6 ± 0.25 ^{ef}	3.0 ± 0.18 ^g	5.8 ± 0.39 ^a
MS	0.0	0.0	70.1	1.5 ± 0.25 ^h	3.8 ± 0.14 ^c	4.6 ± 0.32 ^c
	1.0	0.0	71.5	2.6 ± 0.25 ^{ef}	3.6 ± 0.17 ^{de}	5.9 ± 0.40 ^a
	1.0	1.0	93.9	4.1 ± 0.15 ^b	3.1 ± 0.17 ^{fg}	3.9 ± 0.39 ^e
	1.0	0.1	96.3	4.4 ± 0.24 ^a	3.7 ± 0.14 ^{cd}	5.2 ± 0.27 ^b
	1.5	0.1	90.0	2.6 ± 0.25 ^{ef}	3.7 ± 0.18 ^{cd}	5.3 ± 0.32 ^b
	2.0	0.1	77.0	1.2 ± 0.20 ⁱ	4.2 ± 0.16 ^a	4.5 ± 0.38 ^c

Multiplication of shoots was replicated three times with 10 explants per treatment. Mean values within a column followed by the same letter are not significantly different at $P = 0.05$ (Duncan's test)

in vitro-regenerated plants at the Department of Pharmaceutical Botany and Plant Biotechnology, K. Marcinkowski University of Medical Sciences in Poznan, to use them for scientific research, was given by the Regional Director for Environmental Protection in Poznan.

For the aseptic culture initiation, the apical and axillary buds isolated from the 2–3-month-old plants, were used as explants. The isolated primary explants were washed under running tap water for 30 min, then rinsed in distilled water for 5 min and dipped in 70 % (v/v) ethanol for 30 s followed by rising in 30 % (v/v) commercial bleach (5 % solution sodium hypochloride), containing two drops of tween 80, for 8 min. They were finally rinsed three times in sterilized double-distilled water and placed on MS (Murashige and Skoog 1962) medium for axenic shoot culture establishment. In the next set of experiment, roots of in vitro-derived, multiple plantlets were used for adventitious root culture induction.

Culture Media and Incubation Conditions

All types of culture media consisted of MS medium without a gelling agent (root culture) or solidified with 0.8 % (w/v) agar (Sigma-Aldrich, St. Louis, MO, USA; shoot proliferation and rooting), supplemented with 1.5, 3, or 5 % (w/v) sucrose and plant growth regulators at various concentrations (Tables 1, 2). All plant growth regulators originated from Sigma-Aldrich (St. Louis, MO, USA). After adjusting pH to 5.8, media were autoclaved at 121 °C for 20 min at 105 kPa. Cultures were incubated in a growth

chamber under a 16/8 h photoperiod at 55 μmol m⁻² s⁻¹ light provided by cool-white fluorescent lamps and a temperature 21 ± 2 °C. Only root cultures were maintained in darkness.

Establishment of Shoot Cultures

Explants of in vitro-regenerated plantlets were used for proliferation of shoot cultures. They were placed in 250 cm³ Erlenmeyer flasks with 50 cm³ tested media. To determine the optimal nutritional factors needed for shoot multiplication, three media with different strength were used: (i) 1/2 MS + 1/2 vit.—half-strength MS medium (reduced concentrations of macro- and micronutrients) with reduced concentrations of vitamins (50.0 mg L⁻¹ inositol, 0.25 mg L⁻¹ niacin, 0.25 mg L⁻¹ pyridoxine·HCl, and 0.05 mg L⁻¹ thiamine) and 3 % sucrose; (ii) 1/2 MS with standard concentrations of vitamins (100.0 mg L⁻¹ inositol, 0.5 mg L⁻¹ niacin, 0.5 mg L⁻¹ pyridoxine·HCl, and 0.1 mg L⁻¹ thiamine) and 3 % sucrose; (iii) MS—full-strength medium with standard concentrations of vitamins and 3 % sucrose. The media were supplemented with 6-benzyladenine (BA, 1.0–2.0 mg L⁻¹) and indole-3-acetic acid (IAA, 0.1–1.0 mg L⁻¹) (Table 1).

Shoots were multiplied by repetitive transfer of original explants. Multishoots were divided into single microshoots and transferred to fresh medium every 6 weeks of subculture. Multiplication of shoots was replicated three times with 10 explants per treatment. Percentage of shoot induction, total number of shoots and leaves, and length of shoots were recorded after 6 weeks of fifth, seventh, and

Table 2 Effect of auxins on rooting of *E. maritimum* shoots after 6 weeks of in vitro culture

Medium (auxin content in mg L ⁻¹)	Root induction (%)	Root number per explant (± SD)	Root length (cm) (± SD)
1/2 MS + 1.5 % S	100.0	8.0 ± 1.2 ^{def}	5.1 ± 0.8 ^d
1/2 MS + 1.5 % S + IAA 0.1	100.0	22.2 ± 3.9 ^a	4.6 ± 0.8 ^e
1/2 MS + 1.5 % S + IBA 0.1	100.0	12.7 ± 2.5 ^c	3.4 ± 0.8 ^{gh}
1/2 MS + 1.5 % S + NAA 0.1	100.0	18.4 ± 3.6 ^b	2.0 ± 0.7 ^j
1/2 MS + 3.0 % S	83.3	6.8 ± 1.0 ^{ef}	6.0 ± 0.7 ^{bc}
1/2 MS + 3.0 % S + IAA 0.1	91.0	7.8 ± 0.5 ^{def}	4.5 ± 0.4 ^e
1/2 MS + 3.0 % S + IBA 0.1	91.0	7.6 ± 0.5 ^{ef}	3.9 ± 0.5 ^f
1/2 MS + 3.0 % S + NAA 0.1	100.0	8.3 ± 0.5 ^{def}	3.2 ± 0.4 ^h
MS + 1.5 % S	83.3	5.5 ± 0.5 ^f	5.8 ± 0.3 ^c
MS + 1.5 % S + IAA 0.1	83.3	5.8 ± 1.2 ^f	3.2 ± 0.4 ^h
MS + 1.5 % S + IBA 0.1	100.0	9.9 ± 1.2 ^{cde}	5.8 ± 0.4 ^c
MS + 1.5 % S + NAA 0.1	83.3	6.0 ± 1.1 ^f	3.8 ± 0.8 ^{fg}
MS + 3.0 % S	83.3	5.2 ± 2.0 ^f	7.1 ± 0.6 ^a
MS + 3.0 % S + IAA 0.1	100.0	12.3 ± 2.1 ^c	3.7 ± 0.8 ^{fg}
MS + 3.0 % S + IBA 0.1	100.0	11.0 ± 2.0 ^{cd}	6.1 ± 0.5 ^{bc}
MS + 3.0 % S + NAA 0.1	100.0	6.0 ± 1.1 ^f	4.9 ± 0.7 ^{de}
MS + 5.0 % S	83.3	5.8 ± 1.0 ^f	7.2 ± 0.4 ^a
MS + 5.0 % S + IAA 1.0	100.0	6.5 ± 1.1 ^f	6.5 ± 0.5 ^b
MS + 5.0 % S + IBA 1.0	100.0	5.6 ± 1.0 ^f	6.5 ± 0.5 ^b
MS + 5.0 % S + NAA 1.0	100.0	2.0 ± 0.5 ^g	2.5 ± 0.5 ⁱ

The experiment was replicated two times with 10 explants per treatment. Mean values within a column followed by the same letter are not significantly different at $P = 0.05$ (Duncan's test)

S sucrose

eighth subculture. The percentage of shoot induction was calculated as the total number of initial explants, which gave response to hormonal treatment per total number of explants multiplied by 100 %. Half of the cultured shoots were multiplied and maintained for 12 months for further phytochemical analyses. The other half of shoots were rooted and transferred into ex vitro conditions.

Rooting of Shoots and Plant Transfer into Experimental Plot

The excised shoots were separated and transferred into half or full-strength MS medium alone or with one of the three auxins: IAA, indole-3-butyric acid (IBA), or α -naphthaleneacetic acid (NAA) (0.1 or 1.0 mg L⁻¹) and different sucrose concentrations (1.5, 3.0, and 5.0 %) (Table 2). They were cultured in 250 cm³ Erlenmeyer flasks with 50 cm³ of culture medium. The experiment was replicated two times with 10 explants per treatment. Percentage of rooting, number and length of roots were recorded after 6 weeks of culture. The percentage of root induction was calculated as the total number of initial explants (shoots), which gave response to hormonal treatment per total number of explants multiplied by 100 %. Healthy plantlets with well-developed roots were

subsequently placed in plastic pots containing a mixture of sterile soil, sand, and perlite (1:1:1 v/v/v) and covered with glass beakers for 10–14 days, then transferred to the experimental plot. After hardening and acclimatization of plants, the survival frequency was recorded. Some roots excised from in vitro-derived plantlets, before potting, were collected for root culture establishment and further phytochemical analyses.

Root Cultures

For adventitious root culture initiation, root fragments with tips (2.0 cm long) obtained from 3-week-old axenic plantlets were used. The explants were transferred into full MS liquid media with auxins: IAA, IBA, or NAA (0.1–1.0 mg L⁻¹). The cultures were maintained in 300 cm³ flasks with 50 cm³ of culture medium on a rotary shaker at 100 rpm, in darkness. Root cultures were inoculated into the same liquid media and the same culture conditions as the one employed for routine subculturing. The root cultures were subcultured at 5-week intervals. To compare the effect of different types of auxin on root development, the morphological characteristics were defined. The substantial biomass from stable root culture (passages 7–9) was collected for further phytochemical quantitative analyses.

Flow Cytometry

For nuclear DNA content estimation, leaves of young intact plants and 1-year-old shoot in vitro cultures were used. Samples were prepared as previously described (Sliwiska and Thiem 2007), using Galbraith's buffer (Galbraith and others 1983), supplemented with 1 % (v/v) PVP-10, propidium iodide (PI, $50 \mu\text{g cm}^{-3}$) and ribonuclease A ($50 \mu\text{g cm}^{-3}$). *Solanum lycopersicum* cv. 'Stupicke' (1.96 pg/2C; Dolezel and others 1992) was used as an internal standard. For each sample, 5,000–8,000 nuclei were analyzed directly after preparation using a CyFlow SL (Partec GmbH, Münster, Germany) flow cytometer equipped with a high-grade solid-state laser with green light emission at 532 nm, long-pass filter RG 590 E, DM 560 A, as well as with side (SSC) and forward (FSC) scatters. Histograms were analyzed using FloMax (Partec GmbH, Münster, Germany) software. Analyses were replicated 10 times for each plant material. Coefficient of variation (CV) of G_0/G_1 peak of *E. maritimum* ranged between 3.03 and 5.38 %. Nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions *Eryngium/Solanum*, on a histogram of fluorescence intensities.

Establishment of Selected Phenolic Acid and Triterpenoid Saponin Content

Plant material from in vitro cultures: shoots and roots of in vitro-derived plantlets, roots from liquid culture as well as rosette leaves and roots of intact plants were used for chromatographic analysis. The procedure for determination of RA was adapted from the work of Krzyzanowska and others (2011) and extended to accommodate quantification of CGA and M1, M2A, M2B. Samples of plant material (approx. 100 mg) were mixed with diatomaceous earth, loaded into stainless steel extraction cells and extracted with 80 % (v/v) methanol using an accelerated solvent extraction system (ASE 200, Dionex, Sunnyvale, CA). Extractions were carried out at 10 MPa operating pressure in 40 °C. Extracts were evaporated to dryness under reduced pressure and reconstituted in 2.0 mL of methanol containing 0.05 % (w/v) ascorbic acid. Samples were then stored in –20 °C and immediately before the analyses were diluted 20 times with doubly distilled water and centrifuged at $23,000 \times g$ for 15 min. Quantitative analyses were performed on a Waters Aquity UPLC system (Waters, Milford, MA) equipped with a triple quadrupole mass spectrometer (Waters TQD). Analyses were separated on a Waters BEH C18 column ($100 \times 1 \text{ mm}$, $1.7 \mu\text{m}$) using a linear, 6 min long gradient from 5 to 80 % of acetonitrile containing 0.1 % (v/v) formic acid (solvent B) in 0.1 % formic acid (solvent A) with flow of $140 \mu\text{L min}^{-1}$.

Separations were carried out in 50 °C. The column was washed with pure solvent B for 2 min and re-equilibrated with 5 % solvent B in solvent A for 7 min prior to each injection. Injections were done in the "partial loop needle overfill" mode of a Waters Aquity autosampler. One μL was injected from each sample and analysis of each sample was repeated three times. Column's effluent was introduced into the ion source of the mass spectrometer, which operated in the negative ion mode with the following parameters of the ion source: cone voltage 130 V, capillary voltage 3.1 kV, extractor 3 V, RF lens 100 mV, source temperature 120 °C, desolvation temperature 350 °C, desolvation gas flow 500 L h^{-1} , cone gas flow 50 L h^{-1} , and collision gas flow $100 \mu\text{L min}^{-1}$. Collision cell entrance was set to –2 and exit to 0.5. Parameters of quadrupoles 1 and 3 were set to achieve unit-mass resolution: both LM and HM resolutions were set to 15, and ion energies were set to 0.9. The quantitation method was calibrated from the set of standard solution dilutions in the range of 100 pg to $16 \text{ ng } \mu\text{L}^{-1}$. RA and CGA were analyzed using the selected reaction monitoring (SRM) mode of the mass spectrometer. Precursor ions at m/z 353 (CGA) and 359 (RA) were selected and fragmented at 15 eV. Formation of product ions at, respectively, m/z 191 and m/z 161 was monitored. TS were analyzed in the single ion monitoring mode, in which deprotonated quasi-molecular ions at m/z 925 (M1), 967 (M2A), and 909 (M2B) were observed. Obtained data were processed using Waters MassLynx version 4.1 SCN 714 software.

Statistical Analysis

The collected data were subjected to a one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test. A two-sided *P* value of 0.05 was used to declare statistical significance. All analyses were conducted using STATISTICA v. 10 (StatSoft Inc. 2011).

Results and Discussion

The Effect of Culture Medium and Plant Growth Regulators on Shoot Proliferation

High quality propagation material of the selected *E. maritimum* line, rich in secondary metabolites, could be produced only by asexual methods, and therefore in vitro mass propagation is considered to be a good method for the production of true-to-type plantlets. Species belonging to the *Eryngium* genus display endogenous, morphological seed dormancy; the seeds become dormant soon after harvest (Atwater 1980). Moreover, coastal halophytes are exposed to frequent fluctuations of salinity levels, which

Fig. 1 In vitro propagation of *E. maritimum*: **a** juvenile plant from botanical garden; **b** in vitro-multiply shoots; **c** in vitro-rooted plantlet; **d** adventitious root culture; **e** hardened plantlets in plastic pots; and **f** acclimatized plants in experimental plot. Scale bar 30 mm



negatively influence seed germination and seedling establishment. The application of gibberellic acid ($0.1\text{--}2.0\text{ mg L}^{-1}\text{ GA}_3$) and/or scarification, used for other halophytes to break dormancy, were not efficient for *E. maritimum* seeds (Kikowska, unpublished). Problems with propagation by seeds were also observed for other halophytes, which are particularly salt sensitive, during seed germination and seedling emergence of *Crithmum maritimum* (Meot-Duros and Magne 2008) and *Cakile maritima* (Debez and others 2004). Chemical treatments such as potassium nitrate and thiourea had no effect on *C. maritimum* seed germination (Meot-Duros and Magne 2008). In this study, seed germination of *E. maritimum* did not improve even at optimized in vitro conditions; 95 % of seeds did not germinate, and a few seedlings that emerged had changed morphology, after a short time turned brownish and did not continue development into a healthy

plant. For these reasons, the aseptic cultures of *E. maritimum* from the apical and axillary buds isolated from the 2–3-month-old field-grown plants were cultured in vitro (Fig. 1a). Previously, there have been a few studies on the in vitro cultures of *Eryngium* species, which have been successfully micropropagated: *E. foetidum* via somatic embryogenesis and organogenesis (Arockiasamy and Ignacimuthu 1998; Arockiasamy and others 2002; Chandrika and others 2011), and *E. planum* by axillary bud proliferation (Thiem and others 2013).

For *E. maritimum* a full-strength MS medium appeared to be better than a half-strength one (Table 1). Thus, our expectation that this species would grow better on the reduced in salts and vitamins media due to poor soil conditions in which it grows naturally, was not confirmed. The full concentration of media compounds promoted higher micropropagation parameters, including shoot multiplication.

The highest efficiency of shoot induction (over 96 %) was observed on MS media supplemented with 1.0 mg L^{-1} BA and 0.1 mg L^{-1} IAA. This medium composition resulted in the highest number of new shoots (4.4 per explant) of 3.7 cm in length and relatively high leaf number (over 5). Similarly, the advantageous effect on shoot proliferation of addition of cytokinin and auxin to a medium (at the same concentrations) has been reported for *E. planum* (Thiem and others 2013). However, the number of shoots per explant for the latter species was much higher (17) than in the present experiment. It was due to the different developmental manner of those two *Eryngium* species. *E. planum*, which exhibits a natural rosette habit of growth, under in vitro culture conditions produced shoot clusters. Each shoot tip induced several (9–12) new buds and then proliferated into clusters of shoots with short-petiole leaves (Thiem and others 2013). In contrast, *E. maritimum* produced single rosettes with long-petiole leaves (Fig. 1b). The addition of BA and IAA together was more beneficial for *E. maritimum* micropropagation than of BA alone. The presence of BA and IAA in the medium has been also shown to induce organogenesis in *E. foetidum* (Arockiasamy and others 2002). Other species of the family Apiaceae such as *Thapsia garganica* (Makunga and others 2003), *Crithmum maritimum* (Grigoriadou and Maloupa 2008), *Arracacia xanthorrhiza* (Sliva and others 2010), and *Anethum graveolens* (Jana and Shekhawat 2012) responded better to lower concentrations of BA, especially when combined with NAA. For *Thymus lotocephalus*, the highest numbers of shoots were obtained on MS supplemented with BA and IAA, but the percentage of hyperhydric shoots was higher than on medium without auxin (Coelo and others 2012). Many aspects of cell growth, differentiation, and organogenesis in organ cultures have been found to be controlled by an interaction between cytokinins and auxins. The requisite concentration of each phytohormone varies depending on the species being cultured. Although both cytokinin and auxin are usually required for growth and morphogenesis.

In contrast to previous reports, in the present experiment BA alone or in combination with IAA did not favor callusing. Fortunately, even after 8–10 subcultures of *E. maritimum* mass propagation, there was no callus formation and direct organogenesis occurred, which was probably one of the factors limiting somaclonal variation.

The Effect of Culture Medium, Sucrose Content, and Plant Growth Regulators on Rooting of Shoots

Some shoots of *E. maritimum* during subcultures spontaneously formed roots in the medium for multiplication. The in vitro-regenerated healthy microshoots (2.5–3.0 cm long) formed vigorous roots with a high frequency (83.3–100 %;

Fig. 1c). On all tested rooting media, microshoots revealed direct root induction. Auxins are known to promote adventitious root formation and development, however, here the exogenous auxin addition was not essential to induce roots (Table 2). Most probably it was due to the presence of auxin in the medium used at the previous step, for multiplication of shoots. Nevertheless, the root development was improved by addition of plant growth regulators. The level of endogenous auxins depends on the rate of their metabolism, transport, and conjugation. Exogenous auxins may affect the production of endogenous auxins (Bandurski and others 1995). A similar phenomenon was observed previously for *E. planum* (Thiem and others 2013). In contrast, in *C. asiatica* and *A. xanthorrhiza*, none of the in vitro-derived shoots rooted on auxin-free MS media (Tiwari and others 2000; Sliva and others 2010). The highest root number per explant was achieved in the present experiment on half-strength MS media in the presence of IAA and 1.5 % sucrose and they were relatively long (about 5 cm). A further increase of sucrose content decreased root numbers. Also, increased content of MS salts (full-strength MS medium) was not conducive for root induction. The addition of NAA was especially unfavorable for root length.

Establishment of Adventitious Root Cultures

In approximately, 66 % of the medicinal plants used in traditional medicine roots are the main source for drug preparation. The development of a fast growing root culture system offers unique opportunities for producing root drugs in the laboratory without having to depend on field cultivation (Murthy and others 2008). As found in this study, *E. maritimum* roots are rich in secondary metabolites and therefore adventitious root cultures under in vitro conditions were established.

Morphologically, the different cultures of roots presented auxin-specific growth behavior. The roots incubated in MS liquid medium containing IAA displayed linear growth by the tip of the main axial root. They were thin and long compared to the roots growing in medium supplemented with IBA, which formed many laterals. The lateral roots were responsible for rapid growth and formation of an increased biomass. After 8 weeks, the lateral roots were elongated and formed a mat of roots. During this period, several parts of these secondary lateral roots were converted into a brown and woody form (Fig. 1d). Although roots were growing in medium with NAA, they were callus-like and fragile. Similarly, a high concentration of NAA induced callus-like roots in *Karwinskia humboldtiana* (Kollarova and others 2004).

Table 3 Nuclear DNA content in leaves of *E. maritimum* intact plants and shoot culture

Plant material	DNA content (pg/2C ± SD)
Leaf from intact plant	2.489 ± 0.046 ^{ns}
Leaf of shoot culture in vitro (MS)	2.490 ± 0.021
Leaf of shoot culture in vitro (1/2 MS)	2.467 ± 0.017

Analyses were replicated 10 times for each plant material

^{ns} No significant differences at $P = 0.05$ (Duncan's test)

Plant Transfer into Soil

The crucial step of micropropagation is acclimatization of the in vitro obtained plantlets. In vitro-regenerated plants of *E. maritimum* with no morphological abnormalities were transplanted into pots with a survival rate of 90 % (Fig. 1e). The micropropagated plantlets with well-developed roots were successfully acclimatized to ex vitro conditions; approximately 62 % of plantlets survived the transition from tissue culture to the experimental plot. The micropropagated plants were morphologically uniform and grew well in the field (Fig. 1f). In comparison, the survival rate of *E. planum* plantlets was 89 %, and that of *A. graveolens* and *A. xanthorrhiza* about 60 % (Thiem and others 2013; Sliva and others 2010; Jana and Shekhawat 2012).

Nuclear DNA Content

True-to-typeness of the plants produced in tissue cultures is especially important in large-scale production of medicinal plants. However, somaclonal variation often occurs during

in vitro culture of plant material, which is manifested as cytological abnormalities, qualitative and quantitative mutation, sequence changes and gene activation and silencing (Kaepler and others 2000). Therefore, to make the production of bioactive compounds effective and reliable, the control of genome size is recommended. Flow cytometry was used before as a fast and accurate method to establish nuclear DNA content of medicinal plants obtained in vitro (Thiem and Sliwinska 2003; Sliwinska and Thiem 2007; Makowczynska and others 2008; Thiem and others 2013). In this study, experiment flow cytometrically established genome size of *E. maritimum* was about 2.5 pg/2C, regardless of the origin of plant material (Table 3, Fig. 2). Plantlets obtained through shoot multiplication using shoot tip explants of axillary buds did not show any instability in the nuclear DNA content and their genome size was similar to this of intact plants, which confirmed the suitability of the developed micropropagation procedure for production of genetically stable plant material. The genome size of the other four *Eryngium* species, *E. coeruleum*, *E. variifolium*, *E. giganteum*, and *E. planum* has been established previously (Le Coq and others 1978; Thiem and others 2013). However, this is the first report on the genome size of *E. maritimum*.

Phenolic Acid and Triterpenoid Saponin Accumulation in Biomass Derived from In Vitro Cultures

E. maritimum intact plants produce bioactive compounds in low quantities. For this reason, an alternative means of phenolic acid and triterpenoid saponin production was undertaken. Qualitative and quantitative UHPLC analyses of methanolic extracts confirmed the presence of RA, CGA,

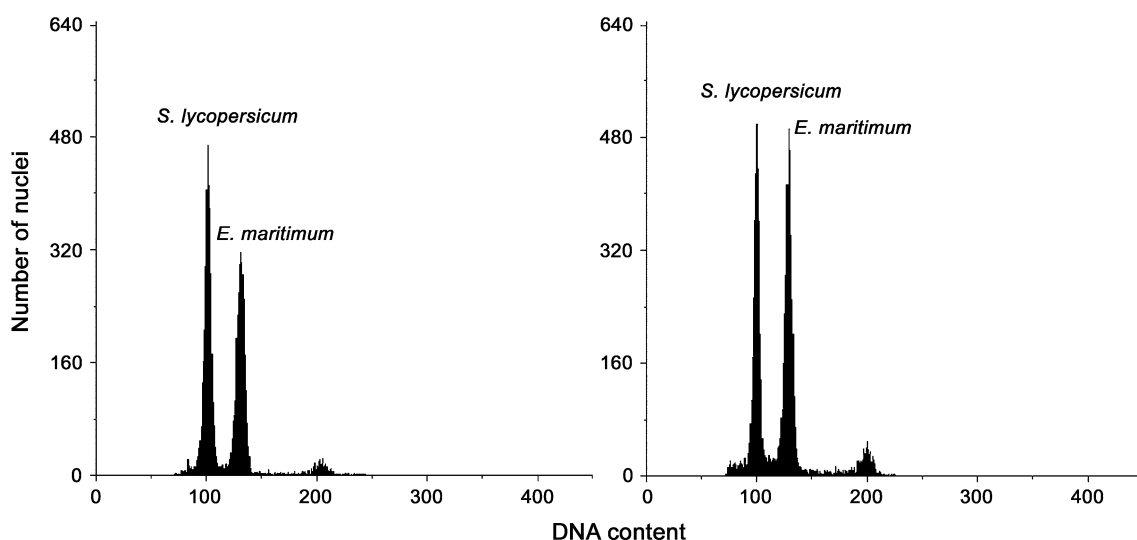


Fig. 2 Selected histograms of nuclear DNA content obtained after flow cytometric analysis of the PI-stained nuclei isolated simultaneously from the leaves of *Solanum lycopersicum* (internal standard) and *Eryngium maritimum*: (left) seedlings; (right) regenerated plantlets

Table 4 Content (mg g⁻¹ DW ± SD) of two phenolic acids: rosmarinic acid (RA), chlorogenic acid (CGA), and triterpenoid saponins (M1, M2A, and M2B) in methanolic extracts

Plant material	Phenolic acids			Triterpenoid saponins			
	RA	CGA	RA + CGA	M1	M2A	M2B	M1 + M2A + M2B
Intact plants							
Rosette leaves	0.188 ± 0.01 ^d	0.264 ± 0.02 ^c	0.452 ^d	ND	ND	0.049 ± 0.01 ^b	0.049 ^c
Roots	0.495 ± 0.01 ^c	0.227 ± 0.01 ^c	0.722 ^c	0.024 ± 0.00 ^b	0.444 ± 0.05 ^{ns}	0.199 ± 0.02 ^b	0.667 ^b
Plantlets from in vitro cultures							
Rosette leaves	0.932 ± 0.02 ^b	2.117 ± 0.04 ^b	3.049 ^b	ND	ND	0.216 ± 0.02 ^b	0.216 ^{bc}
Roots	4.309 ± 0.09 ^a	4.359 ± 0.04 ^a	8.668 ^a	0.132 ± 0.03 ^a	0.585 ± 0.1	1.407 ± 0.15 ^a	2.124 ^a

Analysis of each sample was replicated three times. Mean values within a column followed by the same letter are not significantly different at $P = 0.05$ (Duncan's test)

^{ns} No significant differences

Table 5 Content (mg g⁻¹ DW ± SD) of two phenolic acids: rosmarinic acid (RA) and chlorogenic acid (CGA), and triterpenoid saponins (M1, M2A, and M2B) in methanolic extracts of adventitious root cultures of *E. maritimum* grown in media supplemented with different auxins

Addition of auxins (mg L ⁻¹) to MS medium	Phenolic acids			Triterpenoid saponins			
	RA	CGA	RA + CGA	M1	M2A	M2B	M1 + M2A + M2B
0	0.002 ± 0.00 ^g	0.004 ± 0.00 ^f	0.006 ^g	0.004 ± 0.00 ^c	0.007 ± 0.00 ^d	0.012 ± 0.00 ^c	0.023 ^c
IAA 0.1	0.743 ± 0.00 ^a	0.620 ± 0.01 ^a	1.362 ^a	0.018 ± 0.00 ^b	0.087 ± 0.03 ^{bc}	0.245 ± 0.10 ^b	0.350 ^b
IBA 0.1	0.725 ± 0.01 ^b	0.576 ± 0.01 ^b	1.301 ^b	0.016 ± 0.00 ^b	0.064 ± 0.02 ^c	0.194 ± 0.07 ^b	0.274 ^b
NAA 0.1	0.676 ± 0.00 ^c	0.551 ± 0.01 ^c	1.227 ^c	0.020 ± 0.00 ^b	0.100 ± 0.01 ^{ab}	0.171 ± 0.02 ^b	0.291 ^b
IAA 1.0	0.439 ± 0.01 ^e	0.191 ± 0.01 ^e	0.630 ^e	0.042 ± 0.01 ^a	0.124 ± 0.03 ^a	0.359 ± 0.09 ^a	0.525 ^a
IBA 1.0	0.073 ± 0.01 ^f	0.431 ± 0.01 ^d	0.504 ^f	0.016 ± 0.00 ^b	0.107 ± 0.02 ^{ab}	0.147 ± 0.04 ^b	0.270 ^b
NAA 1.0	0.530 ± 0.01 ^d	0.423 ± 0.01 ^d	0.953 ^d	0.044 ± 0.00 ^a	0.101 ± 0.02 ^{ab}	0.254 ± 0.09 ^b	0.399 ^{ab}

Analysis of each sample was replicated three times. Mean values within a column followed by the same letter are not significantly different at $P = 0.05$ (Duncan's test)

and M2B in all examined materials from intact plants and in vitro cultures (Tables 4, 5). Two saponins from the complex (M1 and M2A) were not detected in intact plant rosette leaves and in vitro-derived shoots. In contrast to *E. planum* (Thiem and others 2013) where the aerial parts of intact plants and regenerated in vitro cultures had a higher phenolic acid content than the underground parts, *E. maritimum* roots produced significantly higher amounts of those compounds than rosette leaves, both from intact plants and in vitro cultures. RA and CGA content was almost 12-fold higher in the roots of in vitro-regenerated plantlets than in the roots of field-grown plants. In vitro culturing also resulted in increased accumulation of triterpenoid saponins, 4.4-fold in shoots and 3.2-fold in roots.

Under in vitro conditions, multiply shoots of *E. maritimum* accumulated selected phenolic acids in amounts of over 3 mg g⁻¹ (Table 4). It was higher than in shoot cultures of *Ruta graveolens*, where the production of bioactive phenolic acids reached only about 1 mg g⁻¹ (Szopa and others 2012). In shoot cultures of *Aronia melanocarpa*, the total amount of phenolic acids was affected by medium

type, the increase of bioactive compounds varied widely during the growth cycle (from 8.2 to 15.3-fold), but the maximum value was also lower than here, 1.5 mg g⁻¹ (Szopa and others 2013).

The auxins added to the adventitious root culture media, either in lower or higher concentrations, not only regulated in vitro morphogenesis processes but also increased phenolic acid and triterpenoid saponin accumulation (Table 5). It was probably due to the modification of the secondary metabolite biosynthesis pathway by those plant hormones. Roots of *E. maritimum* growing in auxin-supplemented media accumulated from 84 to 227-fold more phenolic acids and from 8 to 16-fold more triterpenoid saponins than those grown in hormone-free medium. The excised root cultures in liquid media have been successfully employed before for the production of varied secondary metabolites, and the overall phenolic acid and triterpenoid saponin content in the in vitro cultures was equivalent or higher than those found in intact plants. For example, the root biomass of different lines of six *Gypsophila* species (Gevrenova and others 2010), *Phytolacca acinosa* (Strauss

and others 1995), *Primula veris* (Okrslar and others 2007) obtained in auxin-free media produced triterpenoid saponins in amounts comparable or insignificantly higher to that in organs of intact plants. Here, *E. maritimum* in vitro root cultures produced lower amounts of triterpenoid saponins than roots from intact plants. However, due to the fact that the root collection from natural sources is forbidden because of the protected status of the plant, further studies on increasing triterpenoid saponin accumulation in the root cultures aiming at optimization of media composition and elicitation are required. It is especially important because the production of saponins in plants is still facing many problems, such as low yields and limited availability of natural resources. Therefore, much effort is being put in finding a way to enhance the production of the saponins (Lambert and others 2011).

Due to overexploitation of natural populations of *E. maritimum* and the difficulty of cultivation of this species, it is listed as endangered in Poland and some European countries. Therefore, the efficient plant in vitro propagation system developed in this study will be useful for conservation of this endangered species. In conclusion, the protocol for *E. maritimum* micropropagation allows the effective production of plant material with stable genome size and the ability to synthesize phenolic acids (RA and CGA) and triterpenoid saponins at higher contents than in intact plants. Also, in in vitro adventitious root cultures, especially after the addition of IAA to the medium, those bioactive compounds are produced at increased levels compared to field-derived plants, although not as high as in the roots of plantlets obtained from shoot tip explants.

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References

- Andrew C (2001) The encyclopedia of medicinal plants, 2nd edn. Dorling Kindersley Ltd, London
- Arockiasamy S, Ignacimuthu S (1998) Plant regeneration from mature leaves and roots of *Eryngium foetidum* L., a food flavouring agent. *Curr Sci* 75:664–666
- Arockiasamy S, Prakash S, Ignacimuthu S (2002) Direct organogenesis from mature leaf and petiole explants of *Eryngium foetidum* L. *Biol Plantarum* 45:129–132
- Atwater BR (1980) Germination, dormancy and morphology of the seeds of herbaceous ornamental plants. *Seed Sci Technol* 8: 523–573
- Bandurski RS, Cohen JD, Slovin J, Reinecke DM (1995) Auxin biosynthesis and metabolism. In: Davies PJ (ed) *Plant hormones*. Kluwer, Dordrecht, pp 39–65
- Calvino CI, Martinez SG, Downie SR (2008) The evolutionary history of *Eryngium* (Apiaceae, Saniculoideae): rapid radiations, long distance dispersals and hybridizations. *Mol Phylogenet Evol* 46:1129–1150
- Chandrika R, Vyshali P, Saraswathi KJT, Kaliwal BB (2011) Rapid multiplication of mature flowering plant of *Eryngium foetidum* L by in vitro technique. *IJBA* 3:114–117
- Coelo N, Goncalves S, Gonzalez-Benito ME, Romano A (2012) Establishment of an in vitro propagation protocol for *Thymus lotocephalus*, a rare aromatic species of the Algarve (Portugal). *Plant Growth Regul* 66:69–74
- Debez A, Ben Hamed K, Grignon C, Abdely C (2004) Salinity effects on germination, growth, and seed production of the halophyte *Cakile maritime*. *Plant Soil* 262:179–189
- Dolezel J, Sgorbati S, Lucretti S (1992) Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. *Physiol Plantarum* 85:625–631
- Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* 220:1049–1051
- Gevrenova R, Stancheva T, Voynikov Y, Laurain-Mattar D, Henry M (2010) Root in vitro cultures of six *Gypsophyla* species and their saponins contents. *Enzyme Microb Tech* 47:97–104
- Grigoriadou K, Maloupa E (2008) Micropropagation and salt tolerance of in vitro grown *Crithmum maritimum* L. *Plant Cell Tiss Organ Cult* 94:209–217
- Gugliucci A, Markowicz-Bastos DH (2009) Chlorogenic acid protects paraoxonase 1 activity in high density lipoprotein from inactivation caused by physiological concentrations of hypochlorite. *Fitoterapia* 80:138–142
- Jana S, Shekhawat GS (2012) In vitro regeneration of *Anethum graveolens*, antioxidative enzymes during organogenesis and RAPD analysis for clonal fidelity. *Biol Plant* 56:9–14
- Kaeppeler SM, Kaeppeler HF, Rhee Y (2000) Epigenetic aspects of somaclonal variation in plants. *Plant Mol Biol* 43:179–188
- Kholkhal W, Ilias F, Bekhechi Ch, Bekkara FA (2012) *Eryngium maritimum*: a rich medicinal plant of polyphenols and flavonoids compounds with antioxidant, antibacterial and antifungal activities. *Curr Res J Biol Sci* 4:437–443
- Kikowska M, Budzianowski J, Krawczyk A, Thiem M (2012) Accumulation of rosmarinic, chlorogenic and caffeic acids in in vitro cultures of *Eryngium planum* L. *Acta Physiol Plant* 34:2425–2433
- Kollarova K, Liskova D, Kakoniova D, Lux A (2004) Effect of auxins on *Karwinska humboldtiana* root cultures. *Plant Cell Tiss Organ Cult* 79:213–221
- Kowalczyk M, Masullo M, Thiem B, Piacente S, Stochmal A, Oleszek W (2014) Three new triterpene saponins from roots of *Eryngium planum*. *Nat Prod Res* 28:653–660
- Krzyzanowska J, Janda B, Pecio L, Stochmal A, Oleszek W, Czubačka A, Przybys M, Doroszevska T (2011) Determination of polyphenols in *Mentha longifolia* and *M. piperita* field-grown and in vitro plant samples using UPLC-TQ-MS. *J AOAC Int* 94:43–50
- Lambert E, Faizal A, Geelen D (2011) Modulation of triterpene saponins production: in vitro cultures, elicitation, and metabolic engineering. *Appl Biochem Biotechnol* 164:220–237
- Le Claire E, Schwaiger S, Banaigs B, Stuppner H, Gafner F (2005) Distribution of a new rosmarinic acid derivative in *Eryngium alpinum* L. and other Apiaceae. *J Agric Food Chem* 53:4367–4372
- Le Coq C, Guervin C, Hamel JL, Jolinon D (1978) La quantité d'ADN nucléaire et la garniture chromosomique chez quelques

- Ombelliferes. Actes 2e Sympos. Internat. sur Ombelliferes. Contributions pluridisciplinaires a la systematique, pp 281–291
- Makowczyńska J, Andrzejewska-Golec E, Sliwiska E (2008) Nuclear DNA content in different plant material of *Plantago asiatica* L. cultured in vitro. *Plant Cell Tiss Organ Cult* 94:65–71
- Makunga NP, Jager AK, van Staden J (2003) Micropropagation of *Thapsia garganica*—a medicinal plants. *Plant Cell Rep* 21:967–973
- Meot-Duros L, Magne Ch (2008) Effect of salinity and chemical factors on seed germination in the halophyte *Crithmum maritimum* L. *Plant Soil* 313:83–87
- Meot-Duros L, Le Floch G, Magne Ch (2008) Radical scavenging, antioxidant and antimicrobial activities of halophytic species. *J Ethnopharmacol* 116:258–262
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol Plant* 15:473–497
- Murthy HN, Hahn EJ, Paek KY (2008) Adventitious roots and secondary metabolism. *Chin J Biotech* 24(5):711–716
- Okrslar V, Plaper I, Kovac M, Erjavec A, Obermajer T, Rebec A (2007) Saponins in tissue culture of *Primula veris* L. *In Vitro Cell Dev Biol* 43:644–651
- Orhan I, Aslan S, Kartal M, Sener B, Husnu K, Baser C (2008) Inhibitory effect of Turkish *Rosmarinus officinalis* L. on acetylcholinesterase and butyrylcholinesterase enzymes. *Food Chem* 108:663–668
- Park SU, Uddin MR, Xu H, Kim YK, Lee SY (2008) Biotechnological applications for rosmarinic acid production in plant. *Afr J Biotechnol* 7:4959–4965
- Petersen M, Simmonds MSJ (2003) Rosmarinic acid. *Phytochemistry* 62:121–125
- Piękoś-Mirkowa H, Mirek Z (2003) Flora polski. Atlas roślin chronionych. Multico Oficyna, Warszawa, pp 46–47
- Sliwa S, Viehmannova I, Vitamvas J (2010) Micropropagation and morphogenesis of Arracacha (*Arracacia xanthorrhiza* Bancroft). *Agricult Trop Subtrop* 43:206–211
- Sliwiska E, Thiem B (2007) Genome size stability in six medicinal plant species propagated in vitro. *Biol Plant* 51:556–558
- Sparg SG, Light ME, van Staden J (2008) Biological activities and distribution of plant saponins. *J Ethnopharmacol* 94:219–243
- Strauss A, Spengel SM, Schaffner W (1995) Saponins from root cultures of *Phytolacca actinosa*. *Phytochemistry* 38:861–865
- Szopa A, Ekiert H, Szewczyk A, Fugas E (2012) Production of bioactive phenolic acids and furanocoumarins in in vitro cultures of *Ruta graveolens* L. and *Ruta graveolens* ssp. *Divaricata* (Tenore) Gams. under different light conditions. *Plant Cell Tiss Organ Cult* 110:329–336
- Szopa A, Ekiert H, Muszyńska B (2013) Accumulation of hydroxybenzoic acids and other biologically active phenolic acids in shoot and callus cultures of *Aronia melanocarpa* (Michx.) Elliott (black chokeberry). *Plant Cell Tiss Organ Cult* 113:323–329
- Thiem B, Sliwiska E (2003) Flow cytometric analysis of nuclear DNA content in cloudberry (*Rubus chamaemorus* L.) in vitro cultures. *Plant Sci* 164:129–134
- Thiem B, Goślińska O, Kikowska M, Budzianowski J (2010) Antimicrobial activity of three *Eryngium* L. species (*Apiaceae*). *Herba Pol* 56:52–59
- Thiem B, Kikowska M, Krawczyk A, Więckowska B, Sliwiska E (2013) Phenolic acid and DNA contents of micropropagated *Eryngium planum* L. *Plant Cell Tiss Organ Cult* 114:197–206
- Tiwari KN, Sharma NCh, Tiwari V, Singh BD (2000) Micropropagation of *Centella asiatica* (L.), a valuable medicinal herb. *Plant Cell Tiss Organ Cult* 63:179–185
- Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA (1968) Rosaceae to Umbelliferae. In: Tutin TG (ed) *Flora Europaea*, vol 2. Cambridge University Press, Cambridge
- Wang P, Su Z, Yuan W, Deng G, Li S (2012) Phytochemical constituents and pharmacological activities of *Eryngium* L. (*Apiaceae*). *Pharm Crops* 3:99–120
- Wörz A, Diekmann H (2010) Classification and evolution of the genus *Eryngium* L. (*Apiaceae* Saniculoideae): results of fruit anatomical and petal morphological studies. *Plant Div Evol* 128:387–408
- Zhao H, Li Q, Zhang Z, Pei X, Wang J, Li Y (2009) Long-term ginsenoside consumption prevents memory loss in aged SAMP8 mice by decreasing oxidative stress and up-regulating the plasticity-related proteins in hippocampus. *Brain Res* 1256:111–122
- Żukowski W, Jackowiak B (1995) Endangered and threatened vascular plants of Western Pomerania and Wielkopolska. *Bogucki Wydawnictwo Naukowe Poznań*, pp 22–25, 43