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PAN POLSKA AKADEMIA NAUK ODDZIAŁ W KRAKOWIE

MICROPROPAGATION OF *ERYNGIUM CAMPESTRE* L. VIA SHOOT CULTURE PROVIDES VALUABLE UNIFORM PLANT MATERIAL WITH ENHANCED CONTENT OF PHENOLIC ACIDS AND ANTIMICROBIAL ACTIVITY

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An efficient micropropagation protocol for production of genetically uniform clones of *Eryngium campestre* L. was developed. To determine the effect of nutritional and hormonal factors on shoot and root development and bioactive compounds production, three variants of media differing in the content of macro- and micronutrients, as well as plant growth regulators of various types and concentrations were tested. The highest regeneration (100%), with over 13 shoots per explant, was induced on Murashige and Skoog (MS) medium with 1.0 mg l^{-1} benzyladenine (BA) and 0.1 mg l^{-1} indole-3-acetic acid (IAA). The in vitro derived shoots multiplied through axillary bud formation were rooted and transferred to an experimental plot with 78% frequency of survival. Flow cytometry showed no variation in nuclear DNA between the seedlings and micropropagated plants. Preliminary thin layer chromatography (TLC) analysis indicated that phenolic acids, saponins, flavonoids and acetylenes were present in plant biomass. Ultra high performance liquid chromatography (UHPLC) analysis revealed that shoots and roots from in vitro derived plants and root cultures maintained the ability to produce rosmarinic acid (RA), rosmarinic acid hexoside (RA-HEX) and chlorogenic acid (CGA). The highest phenolic acid content was detected in roots of in vitro regenerated plants. The extract from those roots expressed the highest inhibitory effect against bacteria *Staphylococcus aureus*, as well as dermatophytes *Trichophyton mentagrophytes* and *T. rubrum*.

Keywords: chlorogenic acid, field eryngo, flow cytometry, micropropagation, rosmarinic acid

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INTRODUCTION

Field eryngo (*Eryngium campestre*), a member of the Saniculoideae subfamily from the Apiaceae family, one of 26 *Eryngium* species described in Flora Europaea, grows on steppes in restricted regions of Poland and some other European countries as a rare taxon (Tutin et al., 1968). *E. campestre* (also instead of *E. maritimum*) has been used in traditional medicine and as a culinary herb. The medicinal parts are dried roots, leaves and flowers (PDR 2000). In folk medicine infusions, extracts, decoctions, liquids and tinctures from the roots are used as a diuretic, antitussive, stimulant, appetizer and aphrodisiac (PDR, 2000; Thiem and Wiatrowska, 2007; Wang et al., 2012). Leaf and root extracts present strong antifungal and moderate antibacterial activity (Thiem et al., 2010). Moreover, the herb of *E. campestre* is administered as an extract and in homeopathic dilutions in treatment of urinary tract infections and as adjuvant to treat inflammation (PDR, 2000; Küpeli et al., 2006).

E. campestre produces various secondary metabolites. In a chemotaxonomic study, R-(+)-rosmarinic acid, its glucoside R-(+)-3'-*O*-*β*-D-glucopyranosyl rosmarinic acid and chlorogenic acid were detected (Le Claire et al., 2005). Saponins identified in *E. campestre* are interesting because of the acylation by a β,β-dimethylacrylic acid or angeloyl at the C-22 position, which is rare among triterpenoid saponins (Kartal et al., 2005; Kartal et al., 2006; Wang et al., 2012). A phytochemical analysis of the above-ground parts resulted in isolation and structural determination of flavonoids: quercetin, isoquercitrin, rutin, luteolin 7-*O*-*β*-D-glucopyranoside, astragalin, kaempferol 7-*O*-*α*-L-rhamnopyranoside, kaempferol 3,7-di-*O*-*α*-L-rhamnopyranoside, kaempferol-3-*O*-*β*-D-(2'-*p*-*E*-hydroxycinnamoyl)-glucopyranoside, kaempferol-3-*O*-*β*-D-(2'-*p*-Z-hydroxycinnamoyl)-glucopyranoside (Kartnig and Wolf, 1993; Hohmann et al., 1997; Wang et al., 2012). Simple coumarins: aegelinol benzoate, agasyllin, aegelinol and grandivittin occurred in the roots (Erdelmeier and Sticher, 1985; Wang et al., 2012). Essential oil characterized by the presence of monosesqiterpene hydrocarbons, myrcene, germacren and *α*-pinene was found in the aerial parts of the species (Erdelmeier and Sticher, 1986; Wang et al., 2012).

The dominant phenolic acids present in *Eryngium* are caffeic acid derivatives, mainly rosmarinic and chlorogenic acids. Rosmarinic acid (RA; a caffeic acid ester of 3.4-dihydroxyphenyllactic acid) has many biological and pharmacological activities, e.g., antiviral, antibacterial, antiseptic, antiphlogistic and anti-inflammatory (Petersen and Simmonds, 2003; Le Claire et al., 2005; Park et al., 2008; Orhan et al., 2008, Bulgakov et al., 2012; Kikowska et al., 2014). Chlorogenic acid (CGA) is an ester of caffeic acid and quinic acid. The research reported various biological and pharmacological activities such as: antiviral, antibacterial, anti-inflammatory, anti-allergy, antioxidant, and lowering blood glucose level (Gugliucci and Markowicz-Bastos, 2009).

The aim of the present investigation was to establish and optimize culture conditions for developing an effective protocol for *E. campestre* micropropagation through axillary branching. In vitro propagated plantlets were characterized by genome size stability and maintained the ability to accumulate selected phenolic acids. Antimicrobial activity of root extract from in vitro regenerated plants was also tested.

In the previous years, the authors conducted simultaneous research on the three Polish *Eryngium* species (*E. planum*, *E. maritimum* and *E. campestre*). The research aimed at developing productive micropropagation systems with cytogenetic evaluation of in vitro regenerated plants, and stabilizing cell (callus, cell suspension cultures) and organ (shoots, roots) cultures of three *Eryngium* species. Moreover, the potential of the systems for production of the selected secondary metabolites and the assessment of selected biological activities were tested (Thiem at al., 2010; Thiem et al., 2011; Kikowska et al., 2012; Thiem et al., 2013; Kikowska et al., 2014a,b; Kikowska et al., 2015).

MATERIALS AND METHODS

COLLECTION OF PLANTS AND SURFACE DISINFECTION OF FRUITS

The field-grown plants of *Eryngium campestre* L. (fruits, basal leaves, and roots) were collected from the steppe reserve Owczary (Poland) in August 2009. The voucher specimens (No. 6731) are deposited in the Herbarium of Medicinal Plant Garden in the Institute of Natural Fibers and Medicinal Plants in Poznan, Poland. The fruits were washed under running tap water for 30 min to remove any adherent particles, then in sterilized double-distilled water for 5 min and dipped in 70% (v/v) ethanol for 30 s followed by rinsing in 60% (v/v) commercial bleach – Clorox with two drops of Tween 80, for 10 min. They were rinsed in sterilized double-distilled water to remove the chemicals completely. All the procedures of surface disinfection were performed under aseptic conditions in a laminar air cabin. The disinfected fruits were placed in culture tubes or flask con-

taining 15 ml or 50 ml of MS (Murashige and Skoog 1962) nutrient medium with 1.0 mg l^{-1} gibberellic acid (GA_3) to provide aseptic seedlings. 20-day-old seedlings were aseptically cut into explants and shoot tips were then inoculated on an appropriate medium.

MEDIA PREPARATION AND CULTURE CONDITIONS

MS basal media without a gelling agent or solidified with 0.8% (w/v) agar (Sigma-Aldrich; St. Louis; MO; USA) were used throughout the experiments. They were supplemented with 30 or 50 mg l^{-1} sucrose (Suc) and phytohormons of various concentrations (Table 1–2). All the plant growth regulators originated from Sigma-Aldrich (St. Louis; MO; USA). pH was adjusted to 5.8 using 1N HCl or 1N NaOH and then the media were autoclaved at 121°C for 20 min at 105 kPa. The cultures were incubated in a growth room under a 16:8h photoperiod at 55 μmol m^{-2} s^{-1} light and temperature 21 ± 2°C. The root cultures were maintained in darkness.

CULTURE ESTABLISHMENT AND MULTIPLICATION OF AXILLARY SHOOTS

Epicotyls were used for induction of shoot cultures. The explants were placed in 250 cm^3 flasks with 50 cm^3 of medium: (i) ½ MS + ½ vit. – half-strength MS (reduced concentrations of macronutritiens, micronutrients and vitamins) and 30 g l^{-1} Suc, (ii) ½ MS – half-strength MS (reduced concentrations of macro- and micronutrients) and 30 g l^{-1} Suc, (iii) MS – full-strength MS and 30 g l^{-1} Suc. The media were enriched with 6-benzyladenine (BA; 1.0–2.0 mg·l^{-1}) and indole-3-acetic acid (IAA; 0.1–1.0 mg l^{-1}). In order to improve the physiological condition of the plants, adenine sulfate (AS 100 mg l^{-1}) was used (Table 1).

The shoots were multiplied through an axillary branching method by repetitive transfer of suitable explants. Multishoots were divided into single shoots and transferred to a new medium with the same supplementation every 6 weeks. Multiplication of shoots was replicated three times for each treatment using 10 explants per repetition. The percentage of explants that proliferated buds, the total number of shoots per explant and

TABLE 1. Effects of BA and IAA on shoot proliferation of *Eryngium campestre* after 6 weeks of in vitro culture

Medium	Growth regulator (mg l^{-1})		Explants that proliferated buds (%)	Shoot number per explant (± SD)	Shoot length (cm ± SD)	Leaf number per shoot (± SD)
	BA	IAA				
½ MS + ½ VIT.	0.0	0.0	50	3.90 ± 1.02^{h}	3.16 ± 0.91^{b}	3.15 ± 0.99^{d}
	1.0	0.0	78	5.80 ± 2.02^{fg}	3.02 ± 0.98^{bc}	4.00 ± 0.97^{c}
	1.0	0.1	100	9.10 ± 2.69^{bc}	1.75 ± 0.75^{fg}	5.10 ± 0.91^{b}
½ MS	0.0	0.0	70	4.10 ± 2.66^{h}	2.66 ± 0.68^{d}	2.95 ± 0.69^{d}
	1.0	0.0	78	6.20 ± 2.17^{efg}	2.37 ± 0.73^{e}	4.10 ± 0.79^{c}
	1.0	0.1	100	10.15 ± 3.13^{b}	1.56 ± 0.67^{g}	5.30 ± 0.98^{b}
MS	0.0	0.0	64	5.15 ± 1.46^{gh}	3.84 ± 1.29^{a}	4.40 ± 1.10^{c}
	1.0	0.0	83	7.50 ± 1.54^{de}	3.82 ± 1.08^{a}	3.90 ± 0.64^{c}
	1.0	0.1	100	13.30 ± 3.73^{a}	1.80 ± 0.92^{f}	6.85 ± 1.27^{a}
	1.0	1.0	100	8.30 ± 1.87^{cd}	2.45 ± 0.61^{de}	5.15 ± 0.67^{b}
MS + AS	1.0	0.1	100	6.50 ± 1.70^{efg}	2.86 ± 0.64^{c}	4.05 ± 1.10^{c}
	1.0	1.0	100	6.70 ± 1.56^{ef}	2.50 ± 0.45^{de}	3.90 ± 0.85^{c}

The results are presented as the mean ± SD of three independent experiments with 10 explants per treatment. Mean values within a column followed by the same letter are not significantly different at p = 0.05 using Duncan's Multiple Range test.

TABLE 2. Nuclear DNA content in leaves of *Eryngium campestre* obtained from seedlings and in vitro shoot culture

Plant material	DNA content (pg/2C ± SD)
Leaves from field-derived seedlings	3.337 ± 0.102[ab]
Leaves from in vitro seedlings	3.404 ± 0.098[a]
Leaves of shoot culture in vitro	3.292 ± 0.028[b]

Analyses were replicated 10 times for each plant material. Mean values within a column followed by the same letter are not significantly different at p = 0.05 using Duncan's Multiple Range test.

the length of shoots were recorded after 6 weeks of the fifth, seventh and eight subculture. A 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.

GENOME SIZE ESTIMATION

30-day-old seedlings and shoots from one-year-old shootlets from in vitro cultures were used for nuclear DNA content estimation. The samples were prepared as previously described (Sliwinska and Thiem, 2007), using Galbraith's buffer (Galbraith et al., 1983) with 1% (v/v) PVP-10, propidium iodide (PI; 50 μg cm^{-3}) and ribonuclease A (50 μg cm^{-3}). *Petunia hybrida* P × Pc6 (2.85 pg/2C; Marie and Brown, 1993) was used as an internal standard. For each sample, 5000-8000 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. The histograms were analyzed using a FloMax (Partec GmbH, Münster, Germany) software. The analyses were replicated 10 times for each plant material. The coefficient of variation (CV) of G_0/G_1 peak of *E. campestre* ranged between 2.8 and 4.7. The nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions *Eryngium*/*Petunia* on a histogram of fluorescence intensities.

ROOT INDUCTION AND ACCLIMATIZATION OF CLONED PLANTLETS

The excised shoots (2–2.5 cm) were transferred into MS alone or augmented with one of the three auxins: IAA, indole-3-butyric acid (IBA) or α-naphtaleneacetic acid (NAA) (0.1 or 1.0 mg l^{-1}) and different Suc concentration (30, 50 g l^{-1}) (Table 2). They were cultured in 250 cm^3 flasks with 50 cm^3 of a rooting medium. The percentage of rooting and number and length of roots were recorded after 6 weeks of culture. Rooting of shoots was replicated three times per treatment using 10 explants per replication. Healthy plantlets with well-formed roots were removed from adhering gel, washed gently in sterile water, placed in plastic pots containing a mixture of sterile garden soil, sand and perlite (1:2:1 v/v/v) and covered with glass beakers. After 14 days, the glass cover was removed and the plantlets were transferred to an experimental plot. After hardening and acclimatization of the plants, the survival frequency was recorded.

ADVENTITIOUS ROOT CULTURES

Root fragments with tips (2.0 cm long) obtained from plantlets were used for adventitious root cultures initiation. The explants were transferred into MS liquid media with one of auxins: IAA, IBA or NAA (1.0 mg l^{-1}) and 30 or 50 g l^{-1} Suc. The roots were cultured in 300 cm^3 flasks with 50 cm^3 of a medium on a rotary shaker at 100 rpm, in darkness. The same culture conditions as the ones employed for routine subculturing were applied. The root cultures were subcultured at 5-week intervals for a period of 25 days. The experiment was replicated three times using 5 explants per replication. For fresh biomass (FW), roots were collected from liquid media. The roots were gently pressed on filter paper and weighed. For dry biomass (DW), the roots were dried in an oven at 40ºC and weighed. The substantial biomass from stable root culture (passages 5-6) was collected for further phytochemical quantitative analyses.

EXTRACTION AND TLC ANALYSIS

The organs from the field-grown plants and biomass from in vitro cultures were oven-dried at 40ºC for 24h. 1.0 g of plant material was extracted three times with 30 ml 70% (v/v) ethanol for 1h at

the boiling point temperature under reflux. The combined 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. For phenolic acids and flavonoids detection, 5 µl aliquots of each methanolic extract was applied as 1.0 cm streaks to the cellulose and HPTLC silica gel plates (10 × 20 cm, Merck, Germany). The plates were developed in chambers with ethyl acetate – acetic acid – water (8:1:1 v/v/v) mixture, dried and viewed under UV_{366} nm or day light, before and after spraying with the following reagents: (i) 2-diphenylboranyloxy ethanamine (NA; Roth) 0.1% solution in ethanol for detection of phenolic acids (blue bands under UV) and flavonoids (yellow bands under UV), (ii) $AlCl_3$ 1% solution in ethanol (followed by heating) for detection of flavonoids. Coumarins were detected by strong blue fluorescence under UV_{366} nm light. For saponins detection, 5 µl aliquots of each methanolic extract was applied as 1.0 cm streaks to the HPTLC silica gel plates (10 × 20 cm, Merck, Germany). The plates were developed with 1-buthanol-acetic acid-water 4:1:5 (v/v/v) mixture and viewed under day light after spraying with the vanillin-sulphuric acid reagent. The spots with violet-pink color in daylight were considered as those of saponins. TLC analyses for polyacetylenes were carried out on silica gel 60 F_{254} (Merck), eluted with toluene-ethyl acetate (9:1) mixture. The chromatograms were sprayed with 0.38% $KMnO_4$ solution. The acetylenes were recognized as yellow spots against pink background in daylight.

MEASURMENT OF SELECTED PHENOLIC ACIDS CONTENT

Leaves and roots of plants from natural sites and biomass from in vitro derived plantlets (shoots and roots) were used for chromatographic analysis. Phenolic acids were determined as previously described (Krzyzanowska et al., 2011; Kikowska et al., 2014).

MICROORGANISMS AND MEDIA

The in vitro antimicrobial activity of the *E. campestre* extract was measured using (i) bacteria strains *Staphylococcus aureus* ATCC 4163, *Pseudomonas aeruginosa* ATCC 6749, (ii) yeast *Candida albicans* ATCC 10231, (iii) mould *Aspergillus niger* ATCC 16404 and dermathophytes *Trichophyton mentagrophytes* ATCC 9533 and *T. rubrum* ATCC 28188. The microorganisms were purchased from American Type Culture Collection (ATCC).

The bacterial and yeast strains were stored in Microbank cryogenic vials (ProLab Diagnostics, Canada) at -70°C ± 10°C. The filamentous fungi were maintained on Sabouraud dextrose agar (SDA; Merck) slants at 10°C. The bacteria cultures were grown in Brain Heart Infusion broth (BHI, BioMerieux, France) at 34°C for 18 h, and the yeast cultures were grown in Sabouraud dextrose broth (SDB, Merck) at 34°C for 18 h. After incubation each culture was diluted in a suitable liquid medium (bacteria – Mueller–Hinton broth (MHB; Oxoid, UK); *C. albicans* – Sabouraud dextrose broth (SDB, Merck, Germany) to obtain a final suspension containing about 10^6 CFU ml^{-1}. The filamentous fungi were inoculated on Sabouraud dextrose agar (Merck, Germany) and incubated at 34°C for 5 to 8 days (*A. niger*) and for 2 to 3 weeks (dermatophytes) for adequate sporulation. After incubation, the cultures were covered with sterile 0.9% NaCl solution with 0.1% Tween 80, carefully rubbed with a sterile cotton swab and transferred to a sterile flask. The suspensions were homogenized and filtered. The number of spores in the suspension was determined using a serial dilution method. Before using, the suspension was diluted in Sabouraud dextrose broth to obtain the final suspension containing 2–5 × 10^5 spores per ml.

DETERMINATION OF MIC, MBC/MFC

Antimicrobial activity of the examined extracts was studied by employing a broth microdilution method in accordance with EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines with modifications, using: Mueller–Hinton broth (MHB) – bacteria and Sabouraud dextrose broth (SDB) – fungi (Arendrup, 2012; Rodriguez-Tudela, 2012). The extracts were dissolved in methanol. A solution of each extract was two-fold serially diluted in culture broth. Aliquots of 100 µl of each dilution were distributed in 96-well plates (Kartell, Italy), as well as a sterility control and a growth control (containing culture broth plus solvent, without an antimicrobial substance). Each test and growth control well was inoculated with 100 µl of a microbial suspension. The microdilution trays were incubated at 34°C for: 18 h – bacteria and *C. albicans*; 48h – *A. niger*, 72 h – dermatophytes. The MIC was defined as the lowest concentration at which visible growth was inhibited.

After performing MIC test and recording the MIC end point, every well that demonstrated no growth (concentration equal to and greater than MIC) was subcultured onto an agar medium: Typcase soy agar (TSA; BioMerieux) – bacteria; Sabouraud dextrose agar (SDA; Merck, Germany)

– fungi. The plates were incubated at 34°C for: 18 h – bacteria and *C. albicans*; 48–72 h – *A. niger*, 72–96 h – dermatophytes. The MBC/MFC was defined as the lowest concentration at which no growth was observed. Amikacin for bacteria and nystatin for fungi were used as the reference positive controls. Amikacin showed MIC = 0.01 mg ml^{-1} against *S. aureus*, MIC = 0.02 mg ml^{-1} against *P. aeruginosa*. MIC for nystatin was determined to be 0.32 mg ml^{-1} against *A. niger* and 0.08 mg ml^{-1} against *C. albicans*, *T. mentagrophytes* and *T. rubrum*. All the tests were performed in duplicate and the antimicrobial activity was expressed as the mean values.

STATISTICAL ANALYSIS

The data were analyzed statistically using a one-way analysis of variance (ANOVA) and the significant differences between means were assessed by Duncan's POST-HOC test ($P = 0.05$). The analyses were conducted using STATISTICA v. 10 (StatSoft. Inc. 2011). The data were represented as the mean ± standard deviation.

RESULTS

THE EFFECT OF CULTURE MEDIUM AND PLANT GROWTH REGULATORS ON SHOOT MULTIPLICATION

The micropropagation rates for *E. campestre* differed significantly among media composition and growth regulator treatments (Table 1). Adventitious bud formation and shoot proliferation occurred on all the media; however, with different efficiency. After 6 weeks of culture, the efficiency of shoot induction ranged from 50 to 70% on the hormone-free media. Addition of the hormones increased it to 78–100%. The supplementation of media with plant growth regulators also caused an increase in the percentage of explants that proliferated buds and the number of shoots. The highest number of new shoots (13 per explant), which were short (1.8 cm) and produced high leaf number (7), were observed on MS with 1.0 mg l^{-1} of BA and 0.1 mg l^{-1} of IAA (Table 1; Fig. 1). In our study, the addition of BA and IAA together had better potential for micropropagation than of BA alone, and the ratio of BA

Fig. 1. In vitro propagation of *Eryngium campestre*: (**a**) a seedling; (**b**) in vitro multiplied shoots cultured on MS with 1.0 mg l^{-1} BA + 0.1 mg l^{-1} IAA; (**c**) adventitious root culture in liquid media MS + 30 g l^{-1} Suc + 1.0 mg l^{-1} IAA; (**d**) in vitro rooted plantlet on solid media: (**d1**) MS + 30 g l^{-1} Suc; (**d2**) MS + 30 g l^{-1} Suc + 1.0 mg l^{-1} IAA; (**d3**) MS + 30 g l^{-1} Suc + 1.0 mg l^{-1} NAA; (**e**) hardened plantlets in plastic pots; (**f**) acclimatized plants in experimental plot. Scale bar = 1 cm.

to IAA 1:0.1 over 1:1. BA alone or with IAA did not cause callusing, even short after culture initiation. There were no vitreous shoots, but long-term storage (after 8 passage) of the plant material under in vitro conditions caused loss of turgor and slightly yellow color of shoots. MS medium enriched in adenine sulphate (AS), on the one hand improved the morphology and physiological condition, but on the other hand decreased the shoot multiplication ratio (Table 1).

NUCLEAR DNA CONTENT

In the present experiment flow cytometrically established genome size of *E. campestre* produced in vitro did not differ significantly from this in plants grown in the field (about 3.3 pg/2C; Table 2; Fig. 2). However, flow cytometry showed about 3% more DNA in seedlings produced in vitro than in plantlets produced in a shoot culture.

THE EFFECT OF CULTURE MEDIUM, SOURCE CONCENTRATION AND AUXINS ON ROOTING

The in vitro derived healthy shoots spontaneously formed roots with varied frequency on all the tested rooting media (45–95%; Table 3; Fig. 1). The first roots began to appear after 3 weeks. Here the addition of exogenous auxin was not essential for the rooting itself; however, it increased the root number per explant. The root development was improved by addition of plant growth regulators. A higher number of roots were formed on media with IAA, although more laterals were induced by IBA. The increased concentration of Suc (50 g l^{-1}) had a positive effect on both the efficiency of root induction and the average root number per shoot. The highest root number was achieved on MS media in the presence of Suc 50 g l^{-1} and IAA 1.0 mg l^{-1} (8). In the present study, the roots of *E. campestre* were whitish-cream.

ESTABLISHMENT OF ADVENTITIOUS ROOT CULTURES

In the present study, *E. campestre* root cultures in MS liquid media were initiated and established (Table 4, Fig. 1). The roots maintained in a medium enriched with IAA were thin and long, showed a linear growth but those growing in a medium supplemented with IBA were characterized by a large number of laterals. On the other hand, the roots growing in a medium with NAA were fragile and surrounded by a callus-like sheath. The supplementation with IAA and NAA was superior over IBA in initiation of adventitious roots and produced the maximum biomass of roots. A higher sucrose concentration affected callusing of root explants and consequently the root biomass reached higher fresh and dry weight (Table 4). Therefore, for further phytochemical analysis roots obtained in MS with 30 g l^{-1} Suc and 1.0 mg l^{-1} IAA were selected.

ACCLIMATIZATION OF PLANTLETS

In vitro derived plants of *E. campestre* with no visible morphological changes were transplanted into a field plot (Fig. 1). The clonally propagated plants with a well-developed root system successfully accli-

TABLE 3. Effects of auxins and sucrose concentration on rooting of *Eryngium campestre* shoots after 6 weeks of in vitro culture

Medium (auxins conc. 1.0 mg l^{-1} sucrose conc. g l^{-1})	Rooting of shoots (%)	Root number per explant (± SD)	Root length (cm ± SD)
MS + 30 Suc	45	2.0 ± 0.05^{d}	5.4 ± 1.15^{b}
MS + 30 Suc + IAA	76	5.3 ± 1.75^{c}	2.4 ± 0.84^{f}
MS + 30 Suc + IBA	85	5.0 ± 1.41^{c}	3.5 ± 0.94^{d}
MS + 30 Suc + NAA	70	4.5 ± 0.89^{c}	3.8 ± 0.87^{c}
MS + 50 Suc	50	2.4 ± 1.00^{d}	6.1 ± 1.10^{a}
MS + 50 Suc + IAA	80	8.2 ± 1.84^{a}	2.8 ± 0.99^{e}
MS + 50 Suc + IBA	95	6.7 ± 1.66^{b}	4.0 ± 0.92^{cd}
MS + 50 Suc + NAA	85	5.0 ± 1.17^{c}	3.0 ± 0.77^{e}

The results are presented as the mean ± SD of three independent experiments with 10 explants per treatment. Mean values within a column followed by the same letter are not significantly different at p = 0.05 using Duncan's Multiple Range test.

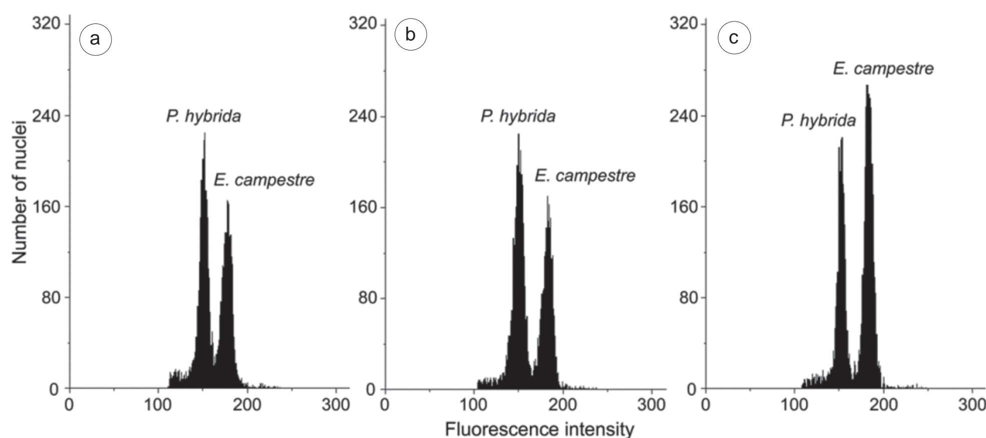

Fig. 2. Selected histograms of nuclear DNA content obtained after flow cytometric analysis of the PI-stained nuclei isolated simultaneously from leaves of *Petunia hybrida* (internal standard) and *Eryngium campestre* **(a)** leaves of in vitro shoot cultures; **(b)** leaves from seedlings; **(c)** leaves from in vitro seedlings.

matized to external conditions with approximately 80% of survival rate. The micropropagated plants were morphologically uniform (Fig. 1).

SECONDARY METABOLITES IN BIOMASS DERIVED FROM INTACT PLANTS AND IN VITRO CULTURES

Preliminary thin layer chromatography was conducted to confirm that *E. campestre* from both field and in vitro conditions, is a valuable plant rich in secondary metabolites. TLC analysis of leaf and root extracts showed that phenolic acids, saponins, flavonoids and acetylenes were present in the studied plant material. In comparison to roots, leaves of *E. campestre* were characterized by the presence of flavonoids, phenolic acids and acetylenes at different concentrations, and by different profiles of saponins. Moreover, the analysis indicated that in vitro proliferated shoots, adventitious roots from in vitro derived plants and root cultures maintained the ability to produce those compounds.

TABLE 4. Effects of auxins and sucrose concentration on *Eryngium campestre* root biomass (g) in MS liquid medium after 24 weeks of in vitro culture

Medium (auxins conc. 1.0 mg l^{-1} sucrose conc. g l^{-1})	Root biomass (g)	
	FW	DW
MS + 30 Suc	14.8 ± 0.3^{f}	4.1 ± 0.1^{c}
MS + 30 Suc + IAA	31.6 ± 0,9^{g}	6.8 ± 0.1^{d}
MS + 30 Suc + IBA	27.1 ± 0.6^{d}	5.8 ± 0.2^{e}
MS + 30 Suc + NAA	31.7 ± 0.7^{g}	6.2 ± 0.1^{e}
MS + 50 Suc	18.0 ± 0.1^{e}	5.0 ± 0.1^{b}
MS + 50 Suc + IAA	35.0 ± 0.6^{b}	7.0 ± 0.1^{d}
MS + 50 Suc + IBA	28.7 ± 0.4^{c}	6.0 ± 0.0^{e}
MS + 50 Suc + NAA	37.0 ± 0.6^{a}	8.2 ± 0.1^{a}

The results are presented as the mean ± SD of three independent experiments with 5 explants per treatment. Mean values within a column followed by the same letter are not significantly different at p = 0.05 using Duncan's Multiple Range test.

PHENOLIC ACID CONTENT IN BIOMASS DERIVED FROM INTACT PLANTS AND IN VITRO CULTURES

Qualitative UHPLC analyses of methanolic extracts confirmed the presence of phenolic acids, such as RA, RA-HEX and CGA in all the tested materials from field-grown plants and in biomass from in vitro cultures (Table 5). Qualitative analyses indicated that the main phenolic compound was RA. At its highest, RA content in in vitro derived roots was almost 6-fold higher than in the roots of intact plants (15.5 mg g^{-1} and 2.6 mg g^{-1}, respectively). A satisfactory RA-HEX content was found in the roots of in vitro regenerated plantlets and field-grown plants. CGA content in *E. campestre* was higher in basal leaves of intact plants than in in vitro regenerated plantlets, but in roots the opposite tendency was observed (Table 5). Due to the fact that the roots possess a higher content of phenolic acids than the shoots, they were selected for analysis of biological activity.

ANTIMICROBIAL ACTIVITY OF METHANOLIC EXTRACTS FROM ROOTS OF PLANTLETS CULTURED IN VITRO

Methanolic extracts from roots of intact plants and in vitro derived plantlets of *E. campestre* showed varying degrees of antimicrobial activity depending on the tested microorganism (Table 6). The highest inhibitory effect of extracts was found against Gram-positive bacteria *S. aureus* and dermathophytes: *T. metagrophytes* and *T. rubrum*, a common cause of skin infections. In addition, the extracts showed moderate antimicrobial activity toward *P. aeruginosa* and *C. albicans*. Methanolic extracts from roots of in vitro derived plantlets showed higher antibacterial (*S. aureus*, *P. aeruginosa*) and antifungal (*T. metagrophytes* and *T. rubrum*) activity than extracts from roots from intact plants.

DISSCUSION

High quality propagation material of *E. campestre* could be produced only by asexual methods. One of them, i.e., in vitro clonal propagation, is considered to be suitable for production of uniform medicinal plants. Some of *Eryngium* species have been previously micropropagated: *E. foetidum* via somatic embryogenesis and organogenesis (Arockiasamy and Ignacimuthu, 1998; Arockiasamy et al., 2002; Chandrika et al., 2011), *E. planum* and *E. maritimum* by axillary bud proliferation (Thiem et al., 2013; Kikowska et al., 2014).

Production of *E. campestre* through seeds in natural conditions is inefficient due to poor germination (Atwater, 1980). It was necessary to develop an effective method of clonal propagation. Here, different media were tested to develop an efficient protocol of micropropagation of this species via shoot culture. The micropropagation rates for *E. campestre* differed significantly among media composition and growth regulator treatments. The highest number of new shoots (13 per explant) was gained on MS enriched with 1.0 mg l^{-1} of BA and 0.1 mg l^{-1} of IAA. Similarly, the advantageous effect of cytokinin and auxin at the same concentrations on shoot proliferation has been reported for *E. planum* (17 per explant) and *E. maritimum* (4 per explant) (Thiem et al., 2013; Kikowska et al., 2014). In another experiment, BA and IAA induced organogenesis

TABLE 5. Content (mg g^{-1} DW) of rosmarinic acid (RA), rosmarinic acid hexoside (RA-HEX) and chlorogenic (CGA) in methanolic extracts from intact plants and plantlets from in vitro cultures of *Eryngium campestre*

Plant material	Phenolic acids			Sum of RA, RA-HEX, CGA
	RA (± SD)	RA-HEX (± SD)	CGA (± SD)	
Intact plants				
Basal leaves	4.229 ± 0.16^{b}	$< 0.003 \pm 0.00$	1.093 ± 0.09^{b}	5.322
Roots	2.644 ± 0.12^{d}	0.874 ± 0.001^{b}	1.041 ± 0.06^{b}	4.559
Plantlets from in vitro cultures				
Basal leaves	3.839 ± 0.14^{c}	$< 0.009 \pm 0.00$	0.989 ± 0.05^{c}	4.828
Roots	15.516 ± 0.37^{a}	1.010 ± 0.03^{a}	1.367 ± 0.10^{a}	17.893
Root culture	0.173 ± 0.05^{e}	$< 0.003 \pm 0.00$	0.059 ± 0.01^{d}	0.232

Analysis of each sample was replicated three times. Mean values within a column with the same letter are not significantly different at $p = 0.05$ using Duncan's Multiple Range test.

TABLE 6. Determination of MIC and MBC/MFC (mg ml^{-1}) of *Eryngium campestre* methanolic extract from roots of in vitro derived plantlets against different microorganisms

Microorganism	MIC		MIC control	MBC/MFC		MBC/MFC control
	Roots of intact plants	Roots of in vitro plantlets		Roots of intact plants	Roots of in vitro plantlets	
Bacterial strains			Amikacin			
Staphylococcus aureus	50	1.6	0.01	50	6.3	
Pseudomonas aeruginosa	50	25	0.02	50	50	
Microbial strains			Nystatin			Nystatin
Candida albicans	50	50	0.08	>50	>50	0.16
Aspergillus niger	>50	>50	0.32	>50	>50	10.24
Trichophyton mentagrophytes	12.5	3.1	0.08	25	6.3	0.64
Trichophyton rubrum	12.5	6.3	0.08	25	6.3	0.32

MIC – Minimal Inhibitory Concentration, MBC/MFC – Minimal Bactericidal/Fungicidal Concentration.

in *E. foetidum* (Arockiasamy et al., 2002). In contrast to previous reports on *E. planum* and *E. maritimum* micropropagation (Thiem et al., 2013; Kikowska et al., 2014), BA alone or with IAA did not cause callusing. It is extremely important due to the relatively high probability of genetic changes that may occur in organs regenerated from callus.

Flow cytometry was used as a fast and accurate method to establish nuclear DNA content of in vitro derived medicinal plants (Thiem and Sliwinska, 2003; Sliwinska and Thiem, 2007; Makowczyńska et al., 2008; Thiem et al., 2013; Kikowska et al., 2014). In the present experiment, the flow cytometrically established genome size of *E. campestre* produced in vitro did not differ significantly from this in plants grown in the field (about 3.3 pg/2C; Table 2; Fig. 2). However, flow cytometry showed about 3% more DNA in seedlings produced in vitro than in plantlets produced in shoot culture. This difference might have been due to different amounts of phenolic compounds in those two plant materials. Polyphenols are known inhibitors of PI staining; they bind to DNA, and therefore compete with this intercalating fluorochrome and consequently bias flow cytometric genome size estimation (Noirot et al., 2000; Bennett et al., 2008; Greilhuber, 2008). Addition of antioxidants, such as PVP-10 used here, to the nuclei isolation buffer, reduces the inhibitory effect of those compounds, but in various degrees depending on the concentration of polyphenols in tissues/organs (Jedrzejczyk and Sliwinska, 2010). Therefore, it can be assumed that shoot multiplication using shoot tip explants of axillary buds did not induce genome size instability. The genome size of five other *Eryngium* species: *E. coeruleum*, *E. giganteum*, *E. planum*, *E. maritimum*, and *E. variifolium* has been established (Le Coq et al., 1978; Thiem et al., 2013; Kikowska et al., 2014). This is the first report on the genome size of *E. campestre*.

The in vitro derived healthy shoots spontaneously formed vigorous roots on all the tested rooting media. Microshoots revealed direct root induction. Auxins are known to promote adventitious root formation and development (Gaspar et al., 1996). Here the addition of auxin was not essential for the rooting itself, however, it increased the root number. A higher number of roots were formed on media enriched with IAA, although more laterals were induced by IBA. A similar result was reported previously for *E. maritimum* (Kikowska et al., 2014). In contrast, in *E. planum*, in vitro derived shoots rooted on NAA supplemented media with the highest root number per explant (Thiem et al., 2013). The increased concentration of Suc (50 g l^{-1}) had a positive effect on both the efficiency of root induction and the average root number per shoot. In contrast, the highest root number in rooting process of *E. maritimum*, was achieved on ½ MS in the presence of IAA and 15 g l^{-1} Suc. Further increase of sucrose content caused a decrease of root number for this taxon (Kikowska et al., 2014). It was reported that an increased sugar content in the rooting media affected induction of the development of roots and laterals (Kusakari et al., 2000; Karam et al., 2003; Wu et al., 2006). In the present study, the roots of *E. campestre* were whitish-cream, but subsequently changed to dark brown. Similar respons-

es were reported for *E. planum* and *E. maritimum* (Thiem et al., 2013; Kikowska et al., 2014).

In the present study, *E. campestre* adventitious root cultures were established. The roots incubated in MS liquid medium with IAA were thin and long and showed a linear growth. The roots growing in a medium supplemented with IBA formed many laterals. Lateral roots are preferred in the production of secondary metabolites (Murthy et al., 2008). A high concentration of NAA induced callus-like and fragile roots not only in *E. campestre* but in some other species: *Aloe vera*, *E. maritimum*, and *Karwinskia humboldtiana* (Kollarova et al., 2004; Lee et al., 2011; Kikowska et al., 2014). In the present study, the supplementation with IAA and NAA was superior over IBA in initiation of adventitious roots and produced the maximum biomass of roots. However, when NAA was present, part of fresh and dry mass was callus. However, callus is not preferred because of high genetic instability and possibility of metabolic profile and ploidy changes.

The micropropagated plantlets with well-developed root system were acclimatized to ex vitro conditions with approximately 80% survival rate. In comparison, the survival rate of *E. planum* plantlets was 89% and *E. maritimum* 62% (Thiem et al., 2013; Kikowska et al., 2014).

Preliminary thin layer chromatography was conducted to confirm that *E. campestre*, both from field and in vitro conditions, is a valuable plant rich in secondary metabolites. TLC analysis of leaf and root extracts showed that phenolic acids, saponins, flavonoids and acetylenes were present in the studied plant material. Moreover, the analysis indicated that in vitro derived shoots, adventitious roots from plantlets and root cultures maintained the ability to produce those compounds. Our previous phytochemical analyses on *E. planum* indicated that organ cultures (adventitious root cultures), shoots and roots of micropropagated plantlets as well as undifferentiated cultures, were able to produce rosmarinic, chlorogenic and caffeic acids (Kikowska et al., 2012, 2014; Thiem et al., 2013).

Field-grown (intact) plants of *E. campestre* accumulate active compounds in low quantities (Table 5; Fig. 3). For this reason, attempts to find an alternative source of phenolic acids production were taken. Qualitative UHPLC analyses confirmed the presence of phenolic acids, such as RA, RA-HEX and CGA in all the tested extracts from field-grown plants and in vitro cultures. Qualitative UHPLC analyses indicated that the main phenolic compound was RA. At its highest, RA content in in vitro derived roots was almost 6-fold higher than in the roots of field-grown plants. Similarly, in *E. maritimum*, RA content was almost 8-fold higher in the roots of in vitro regenerated plantlets than in the roots of intact plants (Kikowska et al., 2014). Generally, the aerial parts of *Eryngium* intact plants and in vitro cultures had a higher RA content than the under-ground parts (Kikowska et al., 2012, 2014; Thiem et al., 2013). A satisfactory content of RA-HEX was indicat-

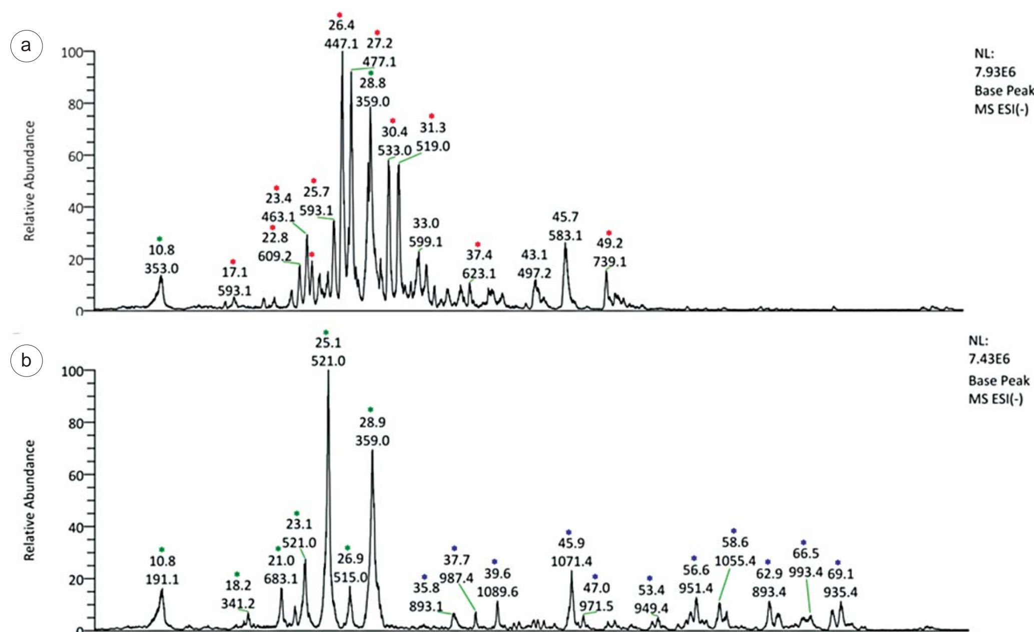

Fig. 3. Chromatograms LC-MS of methanolic extracts from (**a**) rosette leaves and (**b**) roots from intact plants of *E. campestre* • phenolic acids • flavonoids • triterpenoid saponins (values: upper – retention time, lower – *m/z* of the [M-H]- ion).

ed in the roots of in vitro regenerated plantlets and field-grown plants. CGA content in *E. campestre* was higher in basal leaves of intact plants than in in vitro regenerated plantlets, but in roots the opposite tendency was observed. *E. campestre* roots are characterized by a higher phenolic acid accumulation than roots of *E. planum* and *E. maritimum* (Thiem et al., 2013; Kikowska et al., 2014).

Phenolic acids present in *E. campestre* possess microbial activity. Therefore, for quality of the extract from roots of in vitro derived plantlets, in comparison to the analogous extract from roots of intact plants, was confirmed by antibacterial and antifungal activity studies. Methanolic extracts, rich in phenolic acids mostly rosmarinic acid, showed varying degrees of antimicrobial activity depending on the tested microorganism. The highest inhibitory effect was found against Gram-positive bacteria *S. aureus* and dermathophytes: *T. metagrophytes* and *T. rubrum*, a common cause of skin infections. In addition, the extracts showed moderate antimicrobial activity toward *P. aeruginosa* and *C. albicans*. Methanolic extracts from roots of in vitro derived plantlets showed higher antibacterial (*S. aureus*, *P. aeruginosa*) and antifungal (*T. metagrophytes* and *T. rubrum*) activity than extracts from roots of intact plants, which is justified by the quantitative content of phenolic acids in those organs.

The study on antibacterial activity of *E. campestre* was already carried out on *S. aureus*. The anti-methicillin-resistant *S. aureus* activity of the essential oil, which contained predominantly aldehydes and oxygenated monoterpenes, was tested by the agar disc diffusion method. The results showed slight activity of the essential oil against nine different strains of MRSA (Celik et al., 2011). So far antimicrobial activity has previously been reported for different *Eryngium* species: *E. planum*, *E. maritimum*, *E. campestre*, *E. creticum*, *E. palmatum*, *E. thorifolium*. The reports confirmed the capacity of *Eryngium* to produce active compounds and showed antibacterial activity (Meot-Duros et al., 2008; Thiem et al., 2010; Celik et al., 2011; Kholkhal et al., 2012; Marcetic et al., 2014).

In conclusion, the protocol we established for effective micropropagation of *E. campestre* allows for production of valuable plant biomass with a stable genome. The biomass possesses the ability to accumulate phenolic acids (RA, RA-HEX and CGA) at a higher content and with higher antimicrobial activity than field-grown plants.

AUTHORS' CONTRIBUTIONS

M. Kikowska and B. Thiem designed the experiments and contributed to all the experimental processes, data analysis and paper preparing. M. Kowalczyk and A. Stochmal took charge of UHPLC analysis. M. Rewers and E. Sliwinska estimated DNA content in plant material. J. Długaszewska examined antimicrobial activity. E. Sliwinska also provided the critical comments on the manuscript.

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