Plant Cell, Tissue and Organ Culture (PCTOC) (2020) 143:653–663
https://doi.org/10.1007/s11240-020-01949-5

ORIGINAL ARTICLE

Micropropagation and HPLC-DAD, UPLC MS/MS analysis of oenothein B and phenolic acids in shoot cultures and in regenerated plants of fireweed (*Chamerion angustifolium* (L.) Holub)

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Received: 4 May 2020 / Accepted: 12 October 2020 / Published online: 19 October 2020

Abstract

In this study, a micropropagation protocol using nodal explants from in vitro grown plants of *Chamerion angustifolium* (L.) Holub was developed and analysis of oenothein B and selected phenolic acids in shoot cultures was performed for the first time. For shoot induction and multiplication Murashige and Skoog's (MS) basal medium supplemented with 2-isopentenyladenine (2iP), zeatin (Z) and 6-benzyloaminopurine (BAP) was used. 2iP was the most responsive in terms of promoting shoots per explant with the maximum (6.57 ± 1.14) recorded at a concentration of 2.0 mg L^{-1} after 6 weeks of culture. After two subcultures the multiplication rate was increased up to 19 shoots per explant on medium with 2iP (1.0 mg L^{-1}). To prevent tissue browning, ascorbic acid and casein hydrolysate were added to the induction medium, resulting in a reduction of browning by 30%. The rooted plantlets were successfully transferred to soil and acclimatized with 97% frequency. Quantitative and qualitative assessments of oenothein B and phenolic acid contents in in vitro regenerated shoots as well as in ex vitro plants were performed using high-performance liquid chromatography with a diode-array detector (HPLC-DAD) and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC MS/MS) methods. Oenothein B (1.62−4.55 g 100 g^{-1} DW), ellagic acid, gallic and caffeic acids were identified in in vitro regenerated plants. The results of this study confirm that the oenothein B-producing plantlets can be obtained using the micropropagation method with axillary shoots being a valuable source of oenothein B and phenolic acids.

Key message

An efficient regeneration protocol from nodal explants of fireweed (*Chamerion angustifolium* (L.) Holub) was developed for the first time. The presence of oenothein B and phenolic acids in regenerated shoots and ex vitro cultivated plants was confirmed using HPLC-DAD and UPLC analysis.

Keywords *Chamerion angustifolium* (L.) Holub · Nodal explants · Shoot cultures · HPLC-DAD · UPLC MS/MS · Oenothein B

Introduction

Fireweed (*Chamerion angustifolium* (L.) Holub, *Chamaenerion angustifolium* (L.) Scop. syn. *Epilobium angustifolium* L.) is an herbaceous perennial plant from the Onagraceae family. The genus *Chamerion* is a separate monophyletic group with eight species restricted to the northern hemisphere (Wagner et al. 2007). Fireweed plants usually grow up to 2 m high and develop inflorescences with pink flowers from June to September. The species is widely distributed throughout the temperate zone of North America and Eurasia (Wagner et al. 2007).

Communicated by Ali R. Alan.

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C. angustifolium has different ecological cytotypes: diploid (2n = 2× = 36 chromosomes), autotetraploid and also triploid and hexaploid (Husband and Schemske 1998, 2000; Guo et al. 2016). Cytotypes are associated with climatic and environmental factors such as adaptation to higher elevation (Martin and Husband 2013), microclimate conditions (Guo et al. 2016) or soil nitrogen supply (Bales and Hersch-Green 2019). Fireweed cytotypes have been used as a model system for studying cytotype polymorphism. Generally, diploid cytotype occurs at higher elevations and higher latitudes (Martin and Husband 2013), tetraploids are more common in the temperate zone (Husband and Schemske 1998; Thompson et al. 2014), whereas triploids appear in mixed-ploidy populations of border areas (Husband and Schemske 1998) and hexaploids are distributed in warmer climate zones (Guo et al. 2016).

Chamerion angustifolium it is known for its medicinal properties and it is utilized as a component of drugs, nutraceuticals and cosmetic products. Extracts obtained from the underground part are recommended in the treatment of benign prostatic hyperplasia (BPH) and prostatitis (EMEA 2015). Raw material (*Epilobii herba*) is a rich source of bioactive compounds: hydrolysable tannins, flavonoids, phenolic acids, sterols and others (Adamczak et al. 2019). Ellagitannins represent around 15% of the dry mass of the herb (Baert et al. 2015) and oenothein B is the most abundant ellagitannin, constituting 6 to 8% of the dried herb (Granica et al. 2012). Oenothein B is considered to be the main active compound, displaying anti-androgenic, antiproliferative, anticancer, antioxidant, anti-inflammatory, and immunomodulatory properties (Yoshida et al. 2018). Other characteristics of fireweed extracts are: also antimicrobial (Kosalec et al. 2013), analgesic (Tita et al. 2001), photoprotective and antiaging (Ruszova et al. 2013). Medicinal properties of *C. angustifolium* extracts have been attributed to the synergic effect of polyphenols. More than 50 flavonoids and their derivatives were identified in the fireweed herb (Adamczak et al. 2019). Quercetin-3-*O*-glucuronide is the characteristic flavonoid of fireweed, contrary to myricetin-3-*O*-rhamnoside (myricitrin), which is the main flavonoid of the *Epilobium* species (Hevesi et al. 2009; Schepetkin et al. 2016). Phenolic acids and their derivatives are important contributors to the antioxidant and therapeutic potential against BPH of this species (Deng et al. 2019). Oenothein B, and quercetin-3-*O*-glucuronide levels are recommended as markers for raw material standardization (Kiss et al. 2011). *C. angustifolium* is a widely distributed plant but due to frequent interspecific hybridization is often misidentified and the marketed products are wrongly assigned (Kiss et al. 2011). Cultivation under controlled conditions can improve the yield of active compounds and production stability, providing high quality and homogeneous raw material. Conventional plant-breeding is based on cultivars which are well adapted to field conditions and mass production. However, development of suitable cultivars, characterized by high content of active compounds and stress tolerant, is a time-consuming process, lasting even decades. Harvesting raw material from the natural habitat involves the problems with: variable content of active compounds, misidentification of botanical origin, toxins and contaminants etc. Moreover, it contributes to loss of genetic diversity and habitat destruction. Fireweed is a melliferous plant and plays an important role for insects, especially wild bees and others pollinators. Consumption of medicinal plants and demand for the raw material are still growing. For the sustainable use of medicinal plant resources biotechnological approaches including micropropagation and cultivation practice are recommended (Chen et al. 2016). In vitro cultures and micropropagation techniques offers the opportunity to significantly shorten the process of reproduction, and allow large scale multiplication of true-type plants to be achieved. Micropropagated plants can be used as the reproductive material to produce disease-free clones without any seasonal variation and rich in bioactive compounds. There are only two reports on the in vitro cultures of *C. angustifolium* (Turker et al. 2008; Dreger et al. 2016). Both describe shoot regeneration from the seedling's root explants. Nevertheless, a reproducible regeneration protocol from the adult explants was missing. Such a protocol could be of potential use in germplasm conservation and for large scale multiplication of the selected high-yield genotypes of fireweed. The objective of the present study was to develop a regeneration protocol for nodal explants derived from shoots propagated in vitro and analyse the quantity and quality of phenolic compounds in plant material cultivated under in vitro and ex vitro conditions. The protocol allows for multiplication of oenothein B-producing plantlets and harvesting high–quality raw material. The results of this study are part of a project in which in vitro cultures and micropropagation technique were used for the first time in order to obtain fireweed raw material for the pilot production of a dietary supplement used in BPH prevention.

Materials and methods

Source of plant material

Chamerion angustifolium seeds, obtained from Rieger-Hofmann GmbH (Germany) and from the Garden of Medicinal Plants in Plewiska (Institute of Natural Fibres & Medicinal Plants, INF&MP Poland), were used for induction of in vitro cultures and micropropagation. Voucher specimens were identified by the authors, checked by Dr. Artur Adamczak (INF&MP Poznań, Poland) and deposited in the INF&MP. Seeds were surface-sterilized by soaking in 70% ethanol (1 min) and then in commercial bleach

(ACE® solution 2:1) with a drop of Tween 20 (3–4 min), and afterwards washed three times in sterile water. Sterilized seeds were germinated on Petri dishes containing full strength MS basal medium (Murashige and Skoog 1962) with no plant growth regulators (PGRs). Shoot tips (0.5 cm) and nodal segments (0.5–1.0 cm) including 1–2 axillary buds from the 7-week old plants were used to initiate the cultures of shoot tips and nodal cuttings. Individual shoots cut for shoot tips and nodal segments were subcultured every 7 weeks on ½ MS medium with half the macro- and micro- nutrients supplemented with 0.5 mg L^{-1} of indole-*3*-acetic acid (IAA) and sucrose (3%). Plant regeneration proceeded at 20 °C ± 2 °C with a 16 h photoperiod under a photosynthetic flux of 50–60 µmol m^2 s^{-1} (daylight fluorescent tubes).

Shoot induction, multiplication and elongation

Nodal explants (1.0 cm) with two axillary buds were taken from shoots grown in vitro for 7 weeks (Fig. 1a). Individual explants were transferred to separate glass jars (50 mL) containing MS medium supplemented with 3% sucrose, vitamin C (0.1 g L^{-1}) and PGRs, namely 6-benzylaminopurine (BAP), 2-isopentenyladenine (2iP) and zeatin (Z) at concentrations: 0.1 mg L^{-1}, 0.5 mg L^{-1}, 1.0 mg L^{-1}, 2.0 mg L^{-1} and 3.0 mg L^{-1}. MS medium without PGRs was used as control. After 6 weeks, the following data were recorded: percentage of explants producing axillary shoots and buds, and their mean number per explant and percentage of callusing explants. In the separate experiment, the proliferated explants were subcultured twice on MS medium

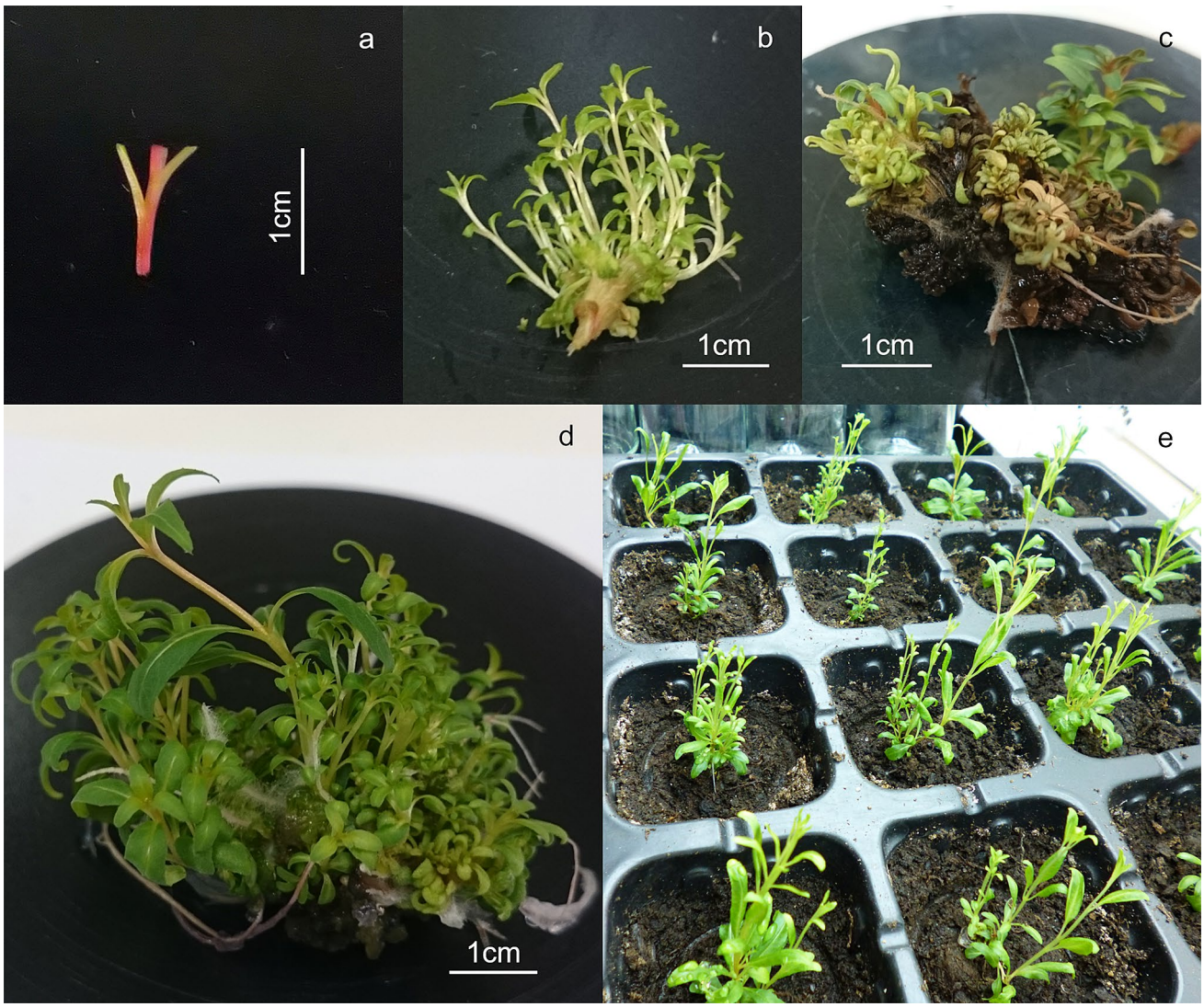

Fig. 1 *C. angustifolium* regeneration and multiplication **a** nodal explant, **b** shoots after 6 weeks of culture on MS medium with 2iP (2.0 mg L^{-1}) and vitamin C (0.1 g L^{-1}), **c** browning multi-shoot on medium with zeatin (3.0 mg L^{-1}) after second subculture, **d** shoot multiplication on MS medium with 2iP (2.0 mg L^{-1}) and vitamin C (0.1 g L^{-1}), **e** acclimatization

supplemented with 2iP (1.0 and 2.0 mg L^{-1}) and zeatin (3.0 mg L^{-1}), and after 8 weeks transferred to elongation MS medium without PGRs. After 2 weeks, the number of shoots (> 0.5 cm) and the mean number of shoots per explant were recorded and the length of shoots was measured. Multiplication of shoots was replicated three times for each treatment using at last 10 explants.

Evaluation of anti-browning agents

Ascorbic acid (0.1 g L^{-1}), alone and combined with polyvinylpyrrolidone (PVP) at concentrations of 0.25 g L^{-1} and 0.5 g L^{-1} as well as with the same concentration of casein hydrolysate, was added to medium containing 2iP (1.0 mg L^{-1}) to test their effect on suppression of browning tissue. Medium with 2iP but without anti-browning agents was a control. The number of explants that became brown was counted after 4 weeks, and the percentage of browning explants was calculated for two subcultures. The experiment was replicated three times for each treatment using 15 explants.

Rooting and acclimatization

Shoots (> 1 cm) were isolated from multi-shoots and transferred to half-strength MS medium (½ MS) with ascorbic acid (control) and ½ MS media supplemented with IAA and indole-*3*-butyric acid (IBA), both at concentration 0.5 mg L^{-1}. After 4 weeks, data were documented on percentage of rooting plants, number of roots per shoot and root length (cm). Each experiment was repeated three times with 24 shoots per treatment. Well-rooted 4-week-old plantlets were washed with sterile water to remove adherent medium from roots and planted in a plastic block tray (5 × 6) with autoclaved soil and perlite (2:1 v/v) and covered with glass cups. After 2 weeks the cups were removed. The percentage of acclimatized plants was calculated after 4 weeks of acclimatization.

Media and culture conditions

All media contained sucrose (3%), vitamin C (0.1 g L^{-1}) and were solidified with Bacto Agar (0.8%). The pH was adjusted to 5.7 before autoclaving at 121 °C at 0.1 MPa for 20 min. All chemicals were supplied by Sigma-Aldrich Ltd. Co. (St. Louis, US), except Bacto Agar (Becton, Dickinson and Company, USA) and vitamin C (POCH S.A., Poland). Plant regeneration, rooting and acclimatization proceeded at 25 °C ± 2 °C and with a 16 h photoperiod under a photosynthetic flux of 60–80 µmol m^2 s^{-1} (daylight fluorescent tubes).

Phytochemical analysis

Five-week-old axillary shoots obtained on media supplemented with 2iP and zeatin (bulk sample) as well shoots harvested from the soil-grown plants were collected and dried at room temperature. Additionally, axillary shoots derived from different media were collected separately and subjected to the phytochemical analysis of phenolic compounds: oenothein B and phenolic acids (Table 6). Content of oenothein B was determined using HPLC-DAD method. Content of selected phenolic acids (ellagic, caffeic, gallic and rosmarinic acids) was determined using the UPLC method (Tables 5 and 6).

HPLC-DAD analysis of oenothein B

The method of extraction of oenothein B developed by Bazylko et al. (2007) was adapted to this research. Approximately 50 mg of plant material was placed in a 50 mL volumetric flask, 35 mL of water was added to the sample and the mixture was extracted in an ultrasonic bath for 60 min at 40 °C. The sample was cooled down and 5.0 mL of acetonitrile was added. Subsequently, the sample was filled up with water and the solution was passed through the 0.45 µm filters. The oenothein B detection method was adapted to this study (Kiss et al. 2011). The LiChrospher 100 RP-18e, 250 mm × 4 mm × 5 µm (Merck, Germany) was used as a stationary phase. The mixture of 2 mobile phases eluted this compound: 2.5% CH_3COOH (phase A) and 2.5% CH_3COOH: acetonitrile (2:8 V/V) (phase B). The separation was performed in the following conditions: 0 min – 7% phase B, 30 min—20% phase B, 60 min—40% phase B. The column temperature was 25 °C, the flow rate was 1.0 mL/min and the detection was at λ = 263 nm. The peaks were identified comparing the retention time and UV-VIS spectra with those of standard solution.

UPLC-MS/MS analysis of phenolic acids

The extraction method described by Gryszczyńska et al. (2018) was used for phenolic acids extraction. Approximately 0.1 g of herb was placed in a 10 mL volumetric flask, 9 mL of methanol was added to the sample and the solution was extracted in an ultrasonic bath for 30 min. After cooling down, 100 µL of an internal standard (quercetin-D3, c = 0.100 mg mL^{-1}) was added to the sample and filled up with methanol to 10 mL. The sample was filtered with 0.20 µm filters and analysed with UPLC-MS/MS. A chromatographic analysis was obtained on an Acquity UPLC BEH C18 column, 50 mm × 2.1 mm × 1.7 µm (Waters, USA). The mobile phases were as follows: acetonitrile:methanol 80:20 (V/V) (phase A), 0.1% (V/V) formic acid solution in water (phase B). The flow rate was 0.15 mL/min. The assay was

performed in a gradient elution procedure: 0.0 min—95% of phase B, 1 min—95% of phase B, 1.5 min—50% of phase B, 2.5 min—25% of phase B, 4 min—0% of phase B, 6 min—0% of phase B, 9 min—90% of phase B, 10 min—95% of phase B. The column temperature was 25 °C; the ion source temperature was 100 °C; the desolvation temperature was 325 °C. The sample injection had a volume 7 µL, and the desolvation gas flow rate was 500 l h^{-1}. The analysis was performed in negative ion charge, using multiple reaction monitoring (MRM) for qualitative and quantitative analyses.

Statistical analysis

The data were statistically analysed using one-way analysis of variance (ANOVA) and the statistical significance was determined applying Duncan's POST–HOC test as well as Fisher's protected least significance differences (LSD) and Student's t test (p value of 0.05). All the statistical analysis was performed using STATISTICA 10 (StatSoft Inc., 2011).

Results

Effect of cytokinins on shoot induction and multiplication

For shoot induction BAP, 2iP and zeatin were used (Table 1). Explants cultured on control medium (MS without PGRs) did not show any bud proliferation. However, shoot bud induction was recorded in all tested treatments of 2iP, zeatin and at a lower concentration of BAP (0.1 and 0.5 mg L^{-1}). 2iP was the most responsive in terms of promoting shoots per explant, with the maximum (6.57 ± 1.14) recorded at 2.0 mg L^{-1} of 2iP after 6 weeks of culture. However, the frequency of bud proliferation was lower (maximum 73%) than that recorded for zeatin (93%). An increasing concentration of 2iP in the media significantly stimulated the number of shoots per explant up to the optimal level of 2iP, beyond which a decrease in growth parameters was noted. The shoots obtained on media with the tested 2iP concentration were vigorous and well developed and did not show any morphological aberrations or callus formation. Browning of explants was observed within 4 weeks of culture for BAP and zeatin treatments despite the addition of ascorbic acid (0.1 g L^{-1}). Most explants put on media supplemented with BAP at the highest concentration (> 0.5 mg L^{-1}) completely died out. Less intense browning of explants was observed for zeatin treatment. However, increasing the zeatin concentration (2.0 and 3.0 mg L^{-1}) resulted in darkening of multi-shoots and occasional callus formation. For shoot multiplication and elongation, the proliferated explants were subcultured twice on the same induction medium with 2iP (1.0 and 2.0 mg L^{-1}) and zeatin (3.0 mg L^{-1}) in two subcultures in 4-week intervals, and then were transferred to elongation MS medium without PGRs (2 weeks). A significant increase of shoot number per explant, up to 15.5 and 19.5 was noted on the medium with 2iP (Table 2; Fig. 1d). Shoot growth was also superior for 2iP treatment and the average length of shoots was 2.03 (± 0.26) cm and 1.04 (± 0.14) cm, corresponding inversely proportionally to 2iP concentration. The explants exposed to repeated zeatin treatment became browned and up to 45% died. In addition, unfavourable effects such as hyperhydricity and malformation were noted.

Table 1 Effect of PGRs (BAP, 2iP and zeatin) on shoot proliferation and multiplication from nodal explants after 4 weeks of cultivation on MS medium supplemented with PGRs and ascorbic acid (0.1 g L^{-1})

PGR (mg L^{-1})			Response rate (%)	Mean no. of shoots per explant	Explant forming callus (%)
BAP	2iP	Z			
0.0			0.0	1.00 ± 0.00^{ab}	0.00
0.1			10.0	1.13 ± 0.07^{ab}	0.00
0.5			13.0	1.03 ± 0.08^{ab}	0.00
1.0			10.0	0.00 ± 0.00^{a}	0.00
2.0			0.0	0.00 ± 0.00^{a}	0.00
3.0			0.0	0.00 ± 0.00^{a}	0.00
	0.1		37.7	1.87 ± 0.24^{bc}	0.00
	0.5		70.0	4.43 ± 0.73^{de}	0.00
	1.0		70.0	6.37 ± 0.82^{fg}	0.00
	2.0		73.0	6.57 ± 1.14^{g}	3.33
	3.0		66.7	4.07 ± 1.02^{de}	3.33
		0.1	46.7	1.43 ± 0.10^{abc}	0.00
		0.5	80.0	1.80 ± 0.41^{bc}	0.00
		1.0	83.0	1.87 ± 0.53^{bc}	0.00
		2.0	93.0	3.00 ± 0.51^{cd}	0.00
		3.0	90.0	5.07 ± 0.97^{ef}	3.33

Values are mean ± standard error of 10 replicates per treatment in three repeated experiments. Mean values with column with the same letter are not significantly different at p = 0.05 (Duncan's multiple range test)

Table 2 Effect of 2iP and zeatin on shoot multiplication after 2 subcultures (10 weeks of culture)

PGR (mg L^{-1})		Mean no. of shoots per explant	Shoot length (cm)	Percent of malformed or hyperhydrous shoots
2iP	Zeatin			
1.0		19.52 ± 3.33^{a}	2.03 ± 0.26^{a}	0.0
2.0		15.46 ± 2.14^{b}	1.04 ± 0.14^{b}	6.0
	3.0	2.86 ± 1.47^{c}	0.90 ± 0.60^{c}	3.0

Values are mean ± standard error of 10 replicates per treatment in three repeated experiments. Mean values in a column with the same letter are not significantly different at p = 0.05 (Duncan's multiple range test)

Table 3 Effect of combination of anti-browning agents on reducing browning on induction medium with 2iP (1.0 mgL^{-1}) and different additives in two subcultures

Anti-browning agent (g L^{-1})			Subculture 1	Subculture 2
Ascorbic acid	Casein hydrolysate	PVP	Percentage of browning explants (%)	
0.0			71.88^{a}	100.00^{a}
0.1			61.29^{ab}	77.42^{c}
0.1	0.25		43.33^{b}	83.33^{bc}
0.1	0.5		64.56^{ab}	90.32^{abc}
0.1		0.25	44.45^{b}	96.97^{ab}
0.1		0.5	44.45^{b}	81.81^{bc}

Values are the percentage of 15 replicates in three repeated experiments. Mean values in a column with the same letter are not significantly different at p = 0.05 (Fisher's test)

Effect of adsorbent and antioxidant combination on tissue browning

To prevent browning of explants the combination of ascorbic acid (0.1 g L^{-1}) with casein hydrolysate and PVP both at concentration 0.25 and 0.5 g L^{-1} were tested in a separate experiment including two subcultures (Table 3). After first subculture the most effective inhibition of browning was recorded for the combination of vitamin C with casein hydrolysate (0.25 g L^{-1}) and PVP. Less than 50% of explants put on these media showed tissue browning after 4 weeks of culture. Addition of casein hydrolysate, but only at lower concentration (0.25 g L^{-1}) led to the most effective reduction of browning (43.3%), but similar results (44.5%) were achieved for the combination of vitamin C with PVP irrespective of its concentration. The browning rate recorded for control medium (without any anti-browning agent) was 72% and for medium with vitamin C was only slightly lower (61%). After the second subculture, the significant majority of explants showed browning of tissue (100–77%) irrespectively of the treatment.

Rooting and acclimatization

The separated shoots (> 1 cm) derived from multi-shoots were rooted on media with IAA and IBA (Table 4). The shoots rooted easily within 4 weeks even on a control medium without added auxins. The highest rooting, up to 98.6%, was noted for medium enriched with IBA. However, the rooting rates for all treatments were in a similar range (97% vs. 96% vs. 99%). The number of roots per shoot was similar for auxin treatments (9.9 and 9.65 per shoot) and was significantly higher than that observed for the control medium (5.24). Shoots cultured in media with auxins produced almost two-fold more roots than in the control medium, but the root length was shorter (6.63 cm vs. 4.58 cm vs. 5.35 cm). Plantlets, after transplanting into soil, grew vigorously (Fig. 1e) and 96.7% of them successfully survived the hardening process.

Table 4 Effect of auxin (IAA/IBA) on root induction after 4 weeks of culture on rooting medium

Auxin (mg L^{-1})	% of rooted shoots	Mean no. of root per shoot	Mean root length (cm)
0.0	97.20^{a}	5.24 ± 0.24^{a}	6.63 ± 0.27^{a}
IAA (0.5)	95.80^{a}	9.90 ± 0.48^{b}	4.58 ± 0.20^{b}
IBA (0.5)	98.60^{a}	9.65 ± 0.40^{b}	5.35 ± 0.20^{c}

Values are mean ± standard error of 25 replicates per treatment in three repeated experiments. Mean values in a column with the same letter are not significantly different at p = 0.05 (Fisher's test (%) and Duncan's multiple range test)

Phytochemical analysis

The harvested shoots from in vitro culture as well as the soil-grown plants were subjected to phytochemical analysis by the HPLC-DAD and UPLC MS/MS methods (Tables 5 and 6). Relatively high contents of oenothein B (1.62–4.55 g 100 g^{-1} DW) and ellagic acid (85.50–222.41 mg 100 g^{-1} DW) were determined. No flavonoids were found in the tested material. The highest content of oenothein B (4.55 mg 100 g^{-1} DW) was found in the acclimatized plantlets (Table 5). Content of oenothein B varied in shoot cultures (1.62–3.05 g 100 g^{-1}) dependently on induction media (Table 6). The highest concentration of this compound was determined in shoots cultivated in medium with 2iP (0.5 mg L^{-1}). Among phenolic acids, ellagic acid was the most abundant, especially in axillary shoots (Table 6). The presence of small amounts of gallic acid (2.79–11.34 mg 100 g^{-1} DW), caffeic acid (1.19–4.76 mg 100 g^{-1} DW) and rosmarinic acid (0.57–1.07 mg 100 g^{-1} DW) was confirmed in all samples. The contents of phenolic acids varied depending on the induction media. The highest content of ellagic acid (221.30–222.41 mg 100 g^{-1} DW), gallic acid (11.34–11.23 mg 100 g^{-1} DW) as well rosmarinic acid (1.07–0.97 mg 100 g^{-1} DW) was determined in the shoots cultivated in media at lower concentration of 2iP (0.5 and 1.0 mg L^{-1}). Shoots cultivated on medium with zeatin (2.0 mg L^{-1}) or higher 2iP concentration (2.0 mg L^{-1}) accumulated a significantly lower level of ellagic acid as well as gallic and rosmarinic acids.

Discussion

To our best knowledge, there are only two reports concerning in vitro culture of *Chamerion angustifolium* (L.) Holub (Turker et al. 2008; Dreger et al. 2016). Both reports

Table 5 Content of oenothein B and phenolic acids in *C. angustifolium* shoot cultures cultivated on different induction media (bulk sample) and in the acclimatized plantlets

Sample	Oenothein B g 100 g^{-1} DW	mg 100 g^{-1} DW			
		Caffeic acid	Gallic acid	Ellagic acid	Rosmarinic acid
Shoot culture	2.46 ± 0.08^{a}	1.53 ± 0.02^{a}	4.17 ± 0.06^{a}	88.02 ± 0.58^{a}	0.63 ± 0.02^{a}
Plantlets	4.55 ± 0.05^{b}	1.19 ± 0.02^{b}	2.79 ± 0.07^{b}	85.50 ± 0.28^{a}	0.77 ± 0.02^{b}

Values are mean ± standard deviation of two replicates. Mean values in a column with the same letter are not significantly different at p = 0.05 (Student's t-test)

Table 6 Content of oenothein B and phenolic acids in axillary shoots of *C. angustifolium* cultivated on different induction media for 5 weeks

Induction medium	Oenothein B g 100 g^{-1} DW	mg 100 g^{-1} DW			
		Caffeic acid	Gallic acid	Ellagic acid	Rosmarinic acid
MS + Z (3.0 mg L^{-1})	2.31 ± 0.10^{a}	3.02 ± 0.48^{a}	3.55 ± 0.57^{a}	97.59 ± 14.21^{a}	0.57 ± 0.00^{a}
MS + 2iP (0.5 mg L^{-1})	3.05 ± 0.09^{b}	4.59 ± 0.02^{b}	11.34 ± 0.53^{b}	222.41 ± 5.24^{b}	1.07 ± 0.04^{b}
MS + 2iP (1.0 mg L^{-1})	1.62 ± 0.03^{c}	4.76 ± 0.04^{b}	11.23 ± 0.14^{b}	221.30 ± 1.08^{b}	0.97 ± 0.04^{b}
MS + 2iP (2.0 mg L^{-1})	2.72 ± 0.11^{d}	2.78 ± 0.00^{a}	7.35 ± 0.13^{c}	145.70 ± 0.40^{c}	0.60 ± 0.03^{c}

Values are mean ± standard deviation of two replicates. Mean values in a column with the same letter are not significantly different at p = 0.05 (Duncan's test)

describe an efficient micropropagation method through direct organogenesis using root explants derived from seedlings. The best shoot proliferation was obtained on media with BAP (0.1 mg L^{-1}) and IAA (0.5 mg L^{-1}). Stem explants did not form shoots (Turker et al. 2008) or rarely, often forming roots and callus (Dreger et al. 2016). No papers concerning shoot induction from stem explants have been published yet. A shoot culture method for micropropagation of *C. angustifolium* from nodal explants was developed in this study. Nodal explants used in all the experiments were derived from rooted shoots propagated in vitro using tip and nodal cutting technique. In vitro propagation through nodal segments has been widely used for plant regeneration and germplasm conservation of medicinal value plants (Hammond et al. 2019; Joo et al. 2019) due to its advantages related to high rate of multiplication and genetically stability of the regenerated plants.

For shoot induction BAP, 2iP and zeatin were used (Table 1). Of the tested cytokinins, 2iP was the most effective in terms of promoting shoots per explant, with the maximum (6.57 ± 1.14) recorded at 2.0 mg L^{-1}, whereas zeatin was less effective, with the maximum of 5.07 ± 0.97. Moreover, a significant increase of shoot number per explant, up to 15.5 and 19.5, was noted on the medium with 2iP when the proliferated explants were subcultured twice on the same induction medium (Table 2; Fig. 1d). 2iP is usually used in the micropropagation of plants from the Ericaceae family (Norton and Norton 1985) and in other species, e.g. *Genista* sp. (Łuczkiewicz and Piotrowski 2005), *Epilobium parviflorum* and *E. hirsutum*—both species related to *C. angustifolium* from the Onagraceae family (Deliu et al. 2013). Of the tested cytokinins, BAP induced the weakest response and at higher concentration (above 0.5 mg L^{-1}) and caused browning of explants and necrosis up to 50%. Necrosis of explants occurred in response to BAP, whereas 2iP was reported to have a favorable effect on shoot induction in some *Rhododendron* species (Norton and Norton 1985) and *Genista* sp. cultivars (Łuczkiewicz and Piotrowski 2005). Differences in the activity of cytokinins may result from their affinity to cytokinin oxidase/dehydrogenase enzymes (CKX). It has been documented that CKX enzymes are involved in one of the key mechanisms in the control of the cytokinin content in plants (Werner et al. 2006; Niemann et al. 2018). Because CKX recognizes the double bond of the isoprenoid side chain, it is highly specific for isoprenoid cytokinins, e.g. trans-zeatin and 2iP. BAP is an aromatic cytokinin and it is practically resistant to CKX enzymes. Individual CKX isoforms are expressed in different tissues and differ partially in their substrate specificities (Werner et al. 2006). Different affinity to CKX enzymes did not explain browning of explants and necrosis. However, it has been suggested that cytokinin degradation by CKXs in the apoplast might be functionally linked to quinones (Galuszka et al. 2005), which are highly reactive and might lead to lethal browning of explants (Murata et al. 2002; Ko et al. 2009). Moreover, a high concentration of BAP is able to induce programmed cell death in plant cultured cells (Kunikowska et al. 2013).

This study also documents that the key obstacle for in vitro culture of *C. angustifolium* is the browning of explants. This physiological reaction is often a challenge for successful establishing of in vitro cultures, especially in the case of phenolic-abundant species. Tissue browning in *C. angustifolium* cultures was also reported by Turker et al. (2008) and Dreger et al. (2016). In our previous study, about 20% of shoot tips died due to tissue browning or necrosis

(Dreger et al. 2016). In general, tissue browning is a result of polyphenols' oxidation by different oxidase enzymes (peroxidase, polyphenol oxidase, and L-phenylalanine ammonialyase) (Xu et al. 2007; Leng et al. 2009). When tissues are wounded, which is inevitable during cutting or subculture of explants, enzymes and polyphenol substrates (*O*-hydroxypolyphenols, tyrosine etc.) are released and quinones are produced as a result of polyphenols' oxidation (Murata et al. 2002; Leng et al. 2009). Further, polymerization of quinones is manifested by brown or black pigment (Murata et al. 2002). Tissue browning might lead to necrosis of explants; therefore different antioxidants such as ascorbic acid (Turker et al. 2008) or adsorbents such as PVP or activated carbon (Li et al. 2015) are added to the media, to prevent browning. In this study ascorbic acid (0.1 g L^{-1}) was added to all media, but the effect was not satisfactory. In the first experiment, most of the formed multi-shoots showed browning of tissue with varying degrees of severity (Fig. 1c). Ascorbic acid is light- and thermo-labile, so its efficacy is limited, particularly in prolonged culture. Therefore, combinations of vitamin C with PVP and with casein hydrolysate were tested in a separate experiments including two subcultures (Table 3). Testing showed that the combination of vitamin C with casein hydrolysate (0.25 g L^{-1}) as well as with PVP significantly limited browning of explants, contrary to control medium and medium supplemented only with ascorbic acid. Less than 50% of explants put on these media showed tissue browning after the first subculture. Addition of casein hydrolysate, but only at lower concentration (0.25 g L^{-1}) led to the most effective reduction of browning (browning rate 43%), but similar results (45%) were achieved for the combination of vitamin C with PVP irrespective its concentration. Casein hydrolysate is a mixture of peptides and amino acids with antioxidant activity and nutrient value (Irshad et al. 2015). It is a source of amino nitrogen and free amino acids, which differ in composition and exert variable influence on in vitro growth and morphogenesis (Baskaran and Jayabalan 2007; Al-Khayri 2011). The combination of ascorbic acid and casein hydrolysate might have resulted in a synergistic effect of antioxidant activity, which was shown as the reduction of browning. These results agree with the findings obtained by Bzducha and Wołosiak (2006), who documented the efficacy of the synergic effect of vitamin C and casein hydrolysate in a linoleic acid model oxidation system obtaining. The synergic effect was observed only for the lower concentration of casein hydrolysate (0.25 g L^{-1}), which may mean that the combination of the components works within a certain concentration range and should be adjusted. Combination of vitamin C with PVP was comparably effective in inhibition of browning tissue. PVP is commonly used to reduce injury and tissue browning in in vitro cultures (Figueiredo et al. 2001; Li et al. 2015). It prevents oxidative browning of wound-induced polyphenolics *via* a hydrogen bonding mechanism (Tang et al. 2004). However, the anti-browning effect was time-limited and after the second subculture most of the multi-shoots (100–77%) showed browning irrespectively of the treatment. Therefore, shortening of culture time to 3 weeks and earlier transfer of multi-shoots to elongation medium should be worth considering and recommending.

Rooting and hardening of plantlets are often critical stages for the micropropagation, but *C. angustifolium* plantlets were easily rooted (95.8–98.6%) even on the control medium without auxins (Table 4). The optimal rooting rate (98.6%) and others parameters like number of roots per shoot were achieved for the variant with IBA, although the differences between auxins and control were statistically insignificant. These findings are inconsistent with results obtained by Turker et al. (2008), who recorded the best rooting rate and number of roots for medium (MSMO) with IAA (0.5 mg L^{-1}). On the other hand, Deliu et al. (2013) obtained the optimum rooting efficiency for medium without auxins but supplemented with activated charcoal. Alosaimi et al. (2018) also noted easy rooting of *Epilobium canum garretti* shoots on medium without added auxin. The noted differences may result from different plant material originating from different populations derived from different climatic zones. Therefore, further research on various plant material (different genotypes/populations) and the effects of different auxins on shoot rooting is worth considering. Plantlets, after transplanting into soil, grew vigorously and 96.7% of them successfully survived the hardening process. Similarly high survival rates of 98% and 95% of the acclimatized plants were obtained previously by Dreger et al. (2016) and Turker et al. (2008) respectively.

Quantitative analysis of oenothein B, caffeic, gallic and rosmarinic acids in axillary shoots as well in the shoots from the soil-harvested plants were performed by applying HPLC-DAD and UPLC MS/MS (Tables 5 and 6). Relatively high contents of oenothein B (1.62–4.55 g 100 g^{-1} DW) were confirmed in all samples. The content of oenothein B in axillary shoots was comparable with field-cultivated plants (Gryszczyńska et al. 2018). Content of oenothein B varied in shoot cultures (1.62–3.05 g 100 g^{-1}) dependently on induction media (Table 6). However, the highest content of oenothein B (4.55 mg 100 g^{-1}) was found in the acclimatized plantlets (Table 5). Production of oenothein B and other ellagitannins (tellimagrandin I and II) and galloylglucoses in *O. tetraptera* shoot cultures was reported by Taniguchi et al. (2002). Changes in composition and content of ellagitannins was reported in shoots during regeneration and acclimatization. In vitro regenerated plants contained nearly two-fold higher concentration of oenothein B than seedlings or plantlets. The authors suggested a correlation between tannin accumulation with the leaves' development as an adaptation to UV light exposure (Taniguchi et al. 2002). Changes in the ellagitannin level are mainly related to their

distribution in plant organ (Baert et al. 2017), plant stage (Jürgenson et al. 2012) or population origin (Baert et al. 2017). On the other hand, ellagitannins play a defensive role in plants against herbivores (Moilanen and Salminen 2008; Moilanen et al. 2016). Antiherbivore (Anstett et al. 2019) and antimicrobial (Hatano et al. 2005) properties of oenothein B have been documented. Therefore, increasing accumulation of oenothein B in plants may be influenced by environmental factors such as stress conditions during ex vitro hardening. Further studies are needed to determine the factors and their role in ellagitannin biosynthesis and their influence on the content of ellagitannins in plants.

Fireweed plants synthesise nearly 50 different flavonoids and their derivatives (Adamczak et al. 2019), but the presence of flavonoids was not detected in shoot cultures or the plantlets. The absence of flavonoids in shoot cultures of the related species—*E. parviflorum* and *E. hirsutum*—was reported earlier (Deliu et al. 2013). Presence and abundance of flavonoids in this species are mainly correlated with the blooming phase (Jürgenson et al. 2012; Baert et al. 2017). Therefore, the reason for the lack of flavonoids may be that the development phase of the tested plant material was too early.

Phenolic acids and their derivatives are one of the dominant groups of phenolics found in *C. angustifolium* plants, and are important contributors to the antioxidant and therapeutic potential of this species (Deng et al. 2019). In this study, relatively high contents of ellagic acid (85.50–222.41 mg 100 g^{-1} DW) were determined (Table 6). The presence of small amounts of gallic acid (2.79–11.34 mg 100 g^{-1}), caffeic acid (1.19–4.76 mg 100 g^{-1} DW) and rosmarinic acid (0.53–1.07 mg 100 g^{-1} DW) was confirmed in all samples. Among phenolic acids, ellagic acid was the most abundant, especially in axillary shoots. The contents of gallic acid and ellagic acids were variable dependently on induction media. The highest content of ellagic acid (221.30–222.41 mg 100 g^{-1} DW) was determined in shoots cultivated on media with lower concentration of 2iP (0.5 and 1.0 mg L^{-1}). Shoots cultivated on medium with zeatin (2.0 mg L^{-1}) or higher 2iP concentration (2.0 mg L^{-1}) accumulated a significantly lower level of ellagic acid as well as gallic and rosmarinic acids. The effect of PGRs in culture media on the accumulation of different groups of secondary metabolites is well documented. Variable content of phenolic acids depending on a medium hormonal variant was recorded in different species, e.g.: *Ocimum americanum* (Rady and Naagla 2005), *Aronia melanocarpa* (Szopa and Ekiert 2014) or *Eryngium alpinum* (Kikowska et al. 2019). This is the first report on the quantitative analysis of oenothein B and phenolic acids in shoot cultures of *C. angustifolium*. Based on the results of this study, it may be concluded that the developed micropropagation protocol allows one to obtain oenothein B-producing plantlets and harvest high-quality raw material.

Conclusions

In this study, a micropropagation protocol from nodal explants of *C. angustifolium* was developed and analysis of oenothein B and selected phenolic acids in shoot cultures was performed for the first time. This method is efficient since multiplication occurred within 6 weeks, producing more than 6 axillary shoots per explant or more than 19 shoots per explant within 10 weeks. The developed protocol may be used in mass propagation of the selected high-yield genotypes of fireweed, with axillary shoots being a valuable source of oenothein B and phenolic acids.

Acknowledgements The authors would like to express their thanks to Dr. Artur Adamczak for identification of the species and to Professor of Institute of Natural Fibres and Medicinal Plants Waldemar Buchwald for providing *Chamerion angustifolium* seeds from the collection of the Garden of Medicinal Plants in Plewiska.

Author contributions All authors contributed to the study concept and design. Material preparation and analysis were performed by MD and AG. Statistical analysis was performed by MS. The first draft of the manuscript was written by MD with a critical input from all authors. The draft of the manuscript was reviewed and edited by KW. All authors read and approved the final manuscript.

Funding This work was funded by the Polish National Centre of Research and Development (Grant No. PBS2/A8/23/2013).

Compliance with ethical standards

Conflict of interest The authors declare that they had no conflict of interest.

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