Phytochemical variability during vegetation of *Chamerion angustifolium* (L.) Holub genotypes derived from in vitro cultures

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Abstract

The purpose of the study was to evaluate *Chamerion angustifolium* (L.) Holub genotypes for preliminary selection and further breeding programs aimed at obtaining a suitable industrial form for the pharmaceutical applications. Clonally propagated plants representing 10 genotypes of *Ch. angustifolium* were regenerated under in vitro conditions, hardened and planted in the field. Studies included an evaluation of shoot proliferation, phytochemical assessment of in vitro and ex vitro plants as well as investigations of intraspecies variability regarding four phenological stages: vegetative, beginning of blooming, full blooming, and green fruit phases. Quantitative and qualitative analyses of bioactive compounds were performed using high-performance liquid chromatography coupled with diode array detector and tandem mass spectrometer (HPLC–DAD–MS/MS) and high-performance liquid chromatography (HPLC) methods. The efficiency of shoot multiplication varied between genotypes from 8.12 to 21.48 shoots per explant. A high reproduction rate (> 20 shoots per explant) was recorded for four lines (PL_45, PL_44, PL_58, DE_2). Plants grown in vitro synthesized oenothien B (11.2–22.3 mg g⁻¹ DW) and caffeic acid derivatives. Plants harvested from field contained the full spectrum of polyphenols characteristic for this species, and oenothien B and quercetin 3-*O*-glucuronide were the most abundant. The maximal content of oenothien B was determined in the vegetative phase of fireweed, while some flavonoids were found in the highest amount in full blooming phase. The results of analysis of variance indicated significant differences among genotypes in oenothien B, 3-*O*-caffeoylquinic acid and flavonoids accumulation in four phenological phases. PL_44 plants were characterized by high content of oenothien B and quercetin 3-*O*-glucuronide as well as a relatively high level of other flavonoids. Based on our phytochemical and micro-propagation studies, PL_44 genotype was the best candidate for early selection and further breeding programs.

Key Message

Epilobium angustifolium genotypes differ in the shoot multiplication efficiency. Significant genotypic variation among tested genotypes was found in flavonoids, oenothien B and chlorogenic acid level in four developmental phases.

Keywords *Epilobium angustifolium* · Fireweed · HPLC–DAD–MS/MS · Oenothien B · Genotypes · Phenological phases

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Introduction

Fireweed (*Chamerion angustifolium* [L.] Holub, syn. *Epilobium angustifolium* L.) is an important medicinal plant from the *Onagraceae* family. The species is native to the Northern Hemisphere and common to most areas of Europe, Asia and North America (Wagner et al. 2007). It usually grows up to 2 m high and develops inflorescences with pink flowers from June to September. Fireweed herb has been used in European folk medicine to treat wounds, skin diseases, stomach disorders, liver and prostate gland inflammation as well as kidney and urinary tract diseases (Adamczak et al. 2019; Söukand et al. 2020). In Russia and in some countries of Eastern Europe, an infusion prepared from its fermented leaves (Koporye tea or Ivan Chai) is consumed as tea (Kalle et al. 2020).

In the last decade, species from the *Epilobium* genus have received great interest in research, which documented the anti-androgenic, analgesic, antioxidant, antimicrobial, anti-inflammatory and anticancer effects of extracts in numerous preclinical studies, reviewed by Granica et al. (2014), Schepetkin et al. (2016), Vitalone and Allkanjari (2018). The European Medicines Agency (EMA) stated that *E. angustifolium* and *E. parviflorum* can be used as traditional herbal medicinal products for treatment and alleviating symptoms related to benign prostatic hyperplasia, BPH (European Medicines Agency 2015). Though almost all authors emphasize the need for evaluating safety and efficacy in the clinical studies, they also agree that *Ch. angustifolium* extracts have a positive effect on BPH (Vitalone and Allkanjari 2018; Deng et al. 2019). Recently published results of a double-blind, placebo-controlled clinical trial confirmed beneficial effect of fireweed extract in alleviating BPH symptoms (Esposito et al. 2021).

Medicinal properties of *Ch. angustifolium* have been attributed to the synergic effect of polyphenols. Over 200 constituents have been identified in fireweed plants: flavonoids, phenolic acids, tannins and other minor constituents such as sterols, triterpenoids, fatty acids, and essential oil (Adamczak et al. 2019). Oenothin B is the most abundant ellagitannin, whose content ranges up to 50% of the total mass of oligomeric ellagitannins (Baert et al. 2015). This compound is considered to be the main bioactive phytochemical, which showed anti-androgenic, apoptotic, antioxidant and anti-inflammatory properties documented in in vitro and in vivo tests (Yoshida et al. 2018). Apart from ellagitannins, more than 50 flavonoids and their derivatives were identified in *Ch. angustifolium* (Adamczak et al. 2019). Fireweed has a flavonoid content of about 1–2%, mainly the aglycones of quercetin, myricetin and kaempferol (Hevesi Tóth et al. 2009; Monschein et al. 2015). Antioxidant and anti-inflammatory properties of fireweed

are closely related to this group of compounds. Myricetin 3-*O*-glucuronide was more potent than indomethacin in the rat paw edema test (Hiermann et al. 1998) and showed an inhibitory effect on prostaglandin biosynthesis (Hiermann et al. 1991). Quercetin 3-*O*-glucuronide (miquelianin) is the most abundant flavonoid of *Ch. angustifolium* (Hevesi Tóth et al. 2009; Schepetkin et al. 2016), which has therapeutic potential in neurodegenerative diseases (Ishisaka et al. 2014; Baral et al. 2017). Oenothin B and quercetin 3-*O*-glucuronide were proposed for use as markers for a raw material standardization (Kiss et al. 2011). A great variety of phenolic acids, including 26 substances (e.g., caffeic, ellagic, ferulic, gallic, protocatechuic acids and caffeoylquinic acid isomers), was found in *Ch. angustifolium* plants (Deng et al. 2018; Gryszczyńska et al. 2018). Phenolic acids and their derivatives are significant contributors to the antioxidant and therapeutic potential of fireweed against BPH (Deng et al. 2019).

Although fireweed is a popular medicinal plant in Central and Eastern Europe, no forms or varieties have been yet developed for pharmaceutical purposes. Obtaining a new variety using traditional methods is a time-consuming process that lasts even decades. In vitro cultures and micropropagation techniques give many advantages over conventional propagation such as: high efficiency, rapid multiplication, large scale production, lack of environmental restrictions (e.g., exposure to pathogens and pests). Hence, they can be useful in reproduction of valuable genotypes for breeding purposes.

Diversity among germplasm collections is essential for selection of superior genotypes and success of any improvement in breeding programs. Despite strong interest in fireweed's polyphenols, little is known about intraspecies variability of *Ch. angustifolium*. Seasonal variation (Jürgenson et al. 2012; Maruška et al. 2014) as well as inter-population and inter-organ distribution of some polyphenols of this species (Baert et al. 2017) were reported earlier, but there are no information about the phytochemical variability between individual genotypes of fireweed. In addition, there are only a few works reporting in vitro culture of *Ch. angustifolium* (Turker et al. 2008; Dreger et al. 2016, 2020).

In our study, genetically uniform specimens of *Ch. angustifolium* were regenerated under in vitro conditions, hardened and planted in the field. Micropropagated plants were used as the reproductive material providing homogeneous and sufficient raw material from each of 10 accessions for the study on genotypic and developmental variation. The assessment of the multiplication efficiency allowed the selection of genotypes suitable for mass production. In turn, evaluation of phytochemical composition of plant raw material provided insight into the seasonal accumulation of the main phenolics of fireweed and was helpful in the selection of genotypes with the high content of bioactive

compounds. To our best knowledge, this is the first report to describe the variation between individual genotypes of *Ch. angustifolium*. The results of this study are part of a project in which in vitro cultures were used for the first time to obtain fireweed raw material for the pilot production of a dietary supplement used in BPH prevention. The results of the current study will be used in further breeding programs aimed to obtain an industrial form of fireweed suitable for pharmaceutical and food applications.

The aim of the present study was an evaluation of the micropropagation efficiency of *Ch. angustifolium* genotypes, qualitative and quantitative analysis of polyphenolic compounds in plants cultivated under in vitro and ex vitro conditions, as well as determination of the effect of genotype and phenological stages on accumulation of the main bioactive compounds.

Material and methods

Establishment of in vitro cultures and shoot proliferation

Seeds of *Ch. angustifolium* were obtained from Rieger-Hofmann GmbH (Germany) and from the Garden of Medicinal Plants in Plewiska (Institute of Natural Fibres and Medicinal Plants National Research Institute, Poland, INF&MP-NRI). The voucher specimen was deposited in the INF&MP-NRI. In vitro cultures of fireweed were initiated as described previously (Dreger et al. 2016). The seeds were sterilized by soaking in 70% ethanol for 1 min and then in ACE® solution (2:1) with a drop of Tween 20 (Sigma Aldrich) for 4 min and washed three times in sterile water. Sterilized seeds were placed on Petri dishes with MS medium (Murashige and Skoog 1962) supplemented with 30 g L⁻¹ sucrose and 8 g L⁻¹ Difco Bactoagar, without plant growth regulators, and pH set to 5.7 prior to autoclaving at 121 °C, 105 kPa for 20 min. After 6 weeks of incubation at 25 °C under the 16/8 h photoperiod (cool white fluorescent lights 25–30 µmol m⁻² s⁻¹) the seedlings were a source of primary explants—roots. Roots were cut for explants (± 1.0 cm) and placed on an induction medium consisted of MS medium supplemented with 6-benzylaminopurine, BAP (0.1 mg L⁻¹), indole-3-acetic acid, IAA (0.5 mg L⁻¹), vitamin C (0.1 g L⁻¹), casein hydrolysate (0.5 g L⁻¹), sucrose (30 g L⁻¹) and agar (8 g L⁻¹), and pH of 5.7. After four weeks multiple-shoots were obtained which were further subcultured every four weeks on the same induction medium for 15 months. Multiplication and rooting proceeded at 25 °C ± 2 °C and with a 16/8 h photoperiod under a photosynthetic flux of 50–60 µmol m⁻² s⁻¹ (daylight fluorescent tubes). To phytochemical analyses, five-week-old rooted shoots of fireweed

on rooting media (bulk sample) were collected and dried at room temperature.

For the determination of genotype effect on shoot proliferation, multi-shoot cultures (Fig. 1a) derived from individual seedlings were used. The number of shoots per explant (multi-shoot) was calculated by counting individually separated shoots (≥ 1 cm length) for every 10 multi-shoots for each genotype. The experiment was repeated five times, which corresponded to five successive passages. The seeds and seedlings of fireweed were treated separately and marked for clear identification: a number of genotypes followed by DE for German accessions (DE_2, DE_3, DE_5, DE_6, DE_11) or PL for Polish genotypes (PL_37, PL_38, PL_44, PL_45, PL_58).

Rooting, acclimatization and hardening of plants

Shoots of *Ch. angustifolium* separated from multi-shoots were transferred into the vessels (Magenta GA-7 Sigma Aldrich) with a rooting medium consisting of half-strength MS medium supplemented with IAA (0.5 mg L⁻¹), vitamin C (0.1 g L⁻¹), and agar (8 g L⁻¹). Individual shoots 1 cm in length and with a minimum of three leaves were qualified as well-developed ones. The percentage of rooted plants was recorded after four weeks for 100 specimens and the counting was repeated three times. The rooted plants at age 4–7 weeks were transferred into sterile soil and perlite substrate (2:1 v/v, Kekkila Paperpot) and then acclimatized in closed tunnels in the greenhouse conditions (21 °C) for two weeks. After 4–5 days of transplanting, the tunnels were gradually opened and the seedlings were accustomed to lower humidity. The plants were hardening in the open tunnels at a temperature of 16 °C for two weeks and the next two weeks in field conditions.

Field cultivation

Ch. angustifolium obtained from in vitro cultures was planted in the experimental field in Plewiska near Poznań (INF&MP-NRI, Poland) in 2015, on a loamy soil of medium fertility. The vegetation period started in early April and lasted until the end of October. In April, greenhouse acclimatized specimens of 10 fireweed genotypes (± 15 cm high) were planted out in the field in 0.45 × 0.25 m spacing. Each plot was 8.6 m² and had about 75 plants. For each genotype, 10 plants were collected randomly at the four following phenological stages: vegetative, beginning of blooming (about 10–20% of flowers in bloom), full blooming (about 70% of flowers in bloom), and green fruit phases (Fig. 1c–f). The above-ground plant parts were dried at room temperature and used for phytochemical analyses.

Fig. 1 *Ch. angustifolium* **a** Multi-shoots cultures, **b** In vitro rooted plants, **c** Plants in vegetative phase, **d** Beginning of blooming phase, **e** Full blooming phase, **f** Green fruit phase

HPLC–DAD–MS/MS analysis

Qualitative analysis was performed using an HPLC system with a diode array detector, DAD (Dionex, Idstein, Germany) and an ion trap mass spectrometer (Bruker Daltonic GmbH, Bremen, Germany). Powdered plant material (200 mg) was extracted (15 min) twice with 50% methanol (v/v) at 40 °C using an ultrasonic water bath. Obtained extracts were filtered through a 0.45 µm Chromafil membrane (Chromafil Xtra PET-45/25). To identify the components of the plant material, 200 µL of mobile phase A was added to 800 µL of the filtered extracts. The sample was mixed and qualitatively analyzed.

Separation was performed on a Zorbax SB C₁₈ column (150 × 2.1 mm, 1.9 µm) (Agilent, USA). The mobile phase consisted of deionized water/formic acid (0.1% HCOOH) (A) and acetonitrile/formic acid (0.1% HCOOH; 100:0.001; v/v) (B). The gradient was: 0 min—6% B, 60 min—28% B. The column temperature was 25 °C, the flow rate was 0.2 mL min^{−1}. The eluate was entered into the electrospray ionization (ESI) interface, without splitting, and compounds were analyzed in negative ion mode with the following settings: nebulizer pressure 2.8 bar; drying gas flow rate 9 L min^{−1}, nitrogen gas temperature 300 °C; capillary voltage 4.5 kV. UV spectra were recorded in the range 200–450 nm. The presence of substances in extracts was confirmed by comparison of retention times, and UV spectra and m/z signals with corresponding standards. Identification of oenothien B and quercetin 3-*O*-glucuronide was complemented by applying commercially available standards.

HPLC–DAD analysis

Quantitative analysis was performed using the UHPLC RS system (Dionex, Idstein, Germany) with DAD detection. Powdered plant material which corresponded to above-ground parts of plants collected from the field in four phenological phases (100 mg) was extracted for 15 min with 50% methanol (1.5 mL) using an ultrasonic water bath at room temperature. Extracts were centrifuged (6 min; 2000 rpm) and supernatants were added to the conical flasks (10 mL). Extraction was repeated twice, then 50% methanol was added to each flask. Samples (2 mL) were filtered using a syringe filter (0.45 µm, PVDF) and were analyzed using the HPLC method. Column, mobile phase, flow rate, gradient as well as column temperature were the same as described above. UV spectra (260 nm—oenothien B; 325 nm—chlorogenic acid; 350 nm—quercetin 3-*O*-glucuronide) were recorded.

Statistical analysis

Data collected from the in vitro experiment were statistically analyzed using one-way analysis of variance (ANOVA) and the statistical significance was determined by applying Duncan's test with p value of 0.05 (Fig. 2). Phytochemical measurements were repeated four times for each tested sample and compound. These data were analyzed using ANOVA and the statistical significance was determined by applying Tukey's test with p value of 0.05 (Fig. 3, Tables 3–7). Dunnett's test (p value of 0.05) was applied to compare the content of individual metabolites in four phenological phases calculated as mean for all genotypes tested, and the average amount of bioactive compounds in the full blooming

Fig. 2 Mean shoot number per explant (multi-shoot) calculated for genotypes. Error bars express SD. Data followed by different letters are significantly different at $p \leq 0.05$ according to Duncan's test (one-way ANOVA)

Fig. 3 Content of oenothien B (mg g^{-1} DW) in *Ch. angustifolium* shoots cultivated in vitro. Values are means of four repetitions for chemical analysis. Error bars express SD. Data followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's test

phase was set as a control (Table 8). All the statistical analyses were performed using STATISTICA 12 (StatSoft Inc., 2013).

Results

Micropropagation and acclimatization

In vitro cultures of *Ch. angustifolium* were initiated from root explants and clonally propagated via multi-shoots (Fig. 1a). The efficiency of shoot multiplication varied between genotypes (lines) and ranged from 8.12 to 21.48 shoots per explant (Fig. 2). The highest numbers per shoots (21.5–20.3) were recorded for DE_2, PL_58, PL_44 and PL_45 lines. In turn, the lowest number of shoots per

explants (8.1) was noted for genotype DE_5. The rest of the lines showed moderate efficiency of shoot organogenesis, with values between 17.8 and 13.4 shoots per explant. Isolated shoots rooted easily within 4 weeks and a high rooting rate was recorded, i.e. 98.5% (Fig. 1b). Plantlets after transplanting into the soil grew vigorously and 95.5% of them successfully survived the hardening process. The percentage of plants that survived in the field cultivation ranged from 80 to 92%.

Phytochemical characteristics of plants from in vitro cultures

Fourteen constituents were identified in the in vitro cultured plants using the HPLC–DAD–MS/MS method (Table 1). Identified polyphenols can be divided into three

Table 1 Retention time, UV and MS/MS data of the compounds present in *Ch. angustifolium* plants (shoots) cultivated in vitro

No	Compound	Rt (min)	UV (nm)	[M–H] MS ¹	Product ions' main peaks MS ²
1	Unknown	2.6	257	611	–
2	Galloyl glucose	2.8	278	331	169, 271
3	Digalloyl glucose	4.1	272	483	331
4	Caffeic acid pentoside (I)	9.4	265	311	149, 179
5	3- <i>O</i> -caffeoylquinic acid	10.2	325	353	191, 179
6	Caffeic acid pentoside (II)	11.1	327	311	179
7	Oenothien B ^a	16.5	263	1567/783*	
8	5- <i>O</i> -caffeoylquinic acid	17.1	323	353	191
9	Digalloyl-HHDP-glucose (I)	17.6	273	785	633, 483, 300
10	Ferulic acid pentoside	19.6	326	325	193
11	Bis-HHDP-glucose	21.5	260	783	765
12	Digalloyl-HHDP-glucose (III)	23.5	272	785	633, 483, 301
13	Unknown ellagitannin	24.3	265	–	–
14	Quercetin 3- <i>O</i> -glucuronide ^a	37.7	260,355	477	301

*[M–2H]^{2–}, ^aComparison with standards

main groups: hydrolysable tannins, flavone derivatives, and phenolic acids. Absence of flavonoids (except of quercetin 3-*O*-glucuronide, 14) as well as relatively high levels of oenothien B (compound 7) and caffeic acid pentoside (6) were the most characteristic for in vitro material (Table 1). It is worth noting that identification of oenothien B was complemented by applying an external standard. 3-*O*-caffeoylquinic acid (5), ferulic acid pentoside (10) and hydrolysable tannins (compounds 2, 3 and 11) were detected in all in vitro samples. Among hydrolysable tannins, monomeric (e.g., bis-HHDP-glucose, 11) and dimeric (oenothien B, 7) ones were identified. The HPLC chromatograms of identified compounds are presented in Fig. 4a (Supplementary Information). Oenothien B was determined as a dominant compound. Content of this substance ranged from 11.21 to 22.32 mg g⁻¹ dry weight (DW) depending on

genotype (Fig. 3). The differences between samples were statistically significant, and the highest level of oenothien B (22.32 mg g⁻¹ DW) was recorded for line PL_38.

Phytochemical characteristics of plants cultivated in field

Comparing to the in vitro cultures, fireweed harvested from the field had a wider spectrum of constituents, particularly phenolic acids and flavonoids. In the above-ground parts of these plants 31 phytochemicals were determined (Table 2). Oenothien B (9) and quercetin 3-*O*-glucuronide (26) were the dominant compounds (Fig. 4b-c, Supplementary Information). Twenty-two compounds including hydrolysable tannins (compounds 4–9 and 14–19), phenolic acids (1, 3, 7, 10, 24), and flavone derivatives (21, 22, 25–30) were

Table 2 Retention time, UV and MS/MS data of the compounds present in *Ch. angustifolium* plants (aerial parts) grown in field

No	Compound	Rt (min)	UV (nm)	[M–H] MS ¹	Product ions' main peaks MS ²
1	Quinic acid	2.6	280	191	111
2	Galloyl glucose	2.8	278	331	169, 271
3	Gallic acid	3.9	270	169	
4	Galloyl-HHDP-glucose (I)	4.0	270	633	301, 481
5	Digalloyl glucose	4.1	272	483	331
6	Galloyl-HHDP-glucose (III)	5.6	265	633	301, 481
7	3- <i>O</i> -caffeoylquinic acid	10.2	325	353	191, 179
8	Unknown ellagitannin	13.9	260	–	–
9	Oenothien B ^a	16.4	263	1567/783*	–
10	5- <i>O</i> -caffeoylquinic acid	17.1	323	353	191
11	Digalloyl-HHDP-glucose (II)	17.6	273	785	633, 483, 301
12	3- <i>O</i> -feruloylquinic acid	17.9	315	367	193, 134
13	4- <i>O</i> -caffeoylquinic acid	18.5	325	353	179, 173
14	Unknown ellagitannin	19.7	265	–	–
15	Unknown ellagitannin	20.0	262	–	–
16	Bis-HHDP-glucose	21.5	260	783	765
17	Digalloyl-HHDP-glucose (III)	23.5	272	785	633, 483, 301
18	Trimeric ellagitannin	24.5	265	1175*	765, 301
19	Trimeric ellagitannin	25.0	262	1175*	765, 301
20	Myricetin hexuronide	31.0	358, 260	479	317
21	Myricetin glucuronide	31.6	357, 262	493	317
22	Quercetin <i>O</i> -galloyl-hexoside	33.9	263, 354	615	463, 301
23	Quercetin <i>O</i> -galloyl-hexoside	35.3	263, 355	615	463, 301
24	Ellagic acid	36.4	253, 365	301	257, 229
25	Quercetin <i>O</i> -hexoside	37.0	260, 355	463	301
26	Quercetin 3- <i>O</i> -glucuronide ^a	37.7	260, 355	477	301
27	Quercetin <i>O</i> -pentoside	41.1	260, 353	433	301
28	Quercetin <i>O</i> -rhamnoside	43.5	262, 350	447	301
29	Kaempferol <i>O</i> -hexuronide	43.4	265, 345	461	285
30	Kaempferol <i>O</i> -rhamnoside	49.8	265, 340	431	285
31	Quercetin <i>O</i> -p-coumaroyl-hexoside	56.6	265, 315	609	463, 301

*[M-2H]²⁻, ^aComparison with standards

detected in all samples. Among hydrolysable tannins, monomeric (e.g., bis-HHDP-glucose, 16), dimeric (oenothien B, 9) and trimeric (unknown trimeric ellagitannins, 18, 19) were identified.

Content of oenothien B, 3-*O*-caffeoylquinic acid and flavonoids in four phenological phases of plants

Oenothien B

Significant variation in content of oenothien B was noted regarding the phenological phases with respect to tested genotypes (Table 3). The highest content of oenothien B (86.51 mg g⁻¹DW) was recorded for the PL_44 line. It was 20% higher than mean values for all genotypes in this

phase. The result was statistically significant and clearly indicated the distinction between the rest of the accessions in this phase. Most of genotypes reached the highest yield of oenothien B during the vegetative phase. Only PL_45 and PL_37 genotypes showed different patterns of oenothien B accumulation. It should be noted that coefficient of variation (CV) values calculated for DE_6, PL_37 and PL_45 lines were the lowest (7.35%, 7.49% and 8.16% relatively), which means that the changes in the oenothien B content were relatively small during the season compared to other genotypes.

3-*O*-caffeoylquinic acid (chlorogenic acid)

Among tested genotypes, the mean content of 3-*O*-caffeoylquinic acid (chlorogenic acid) ranged from 2.14 (DE_2) to 3.51 mg g⁻¹

Table 3 Variation in oenothien B content [mg g⁻¹ DW] between *Ch. angustifolium* genotypes in four phenological phases

Genotype	Vegetative phase	Beginning of blooming	Full blooming	Green fruit phase	All phases	CV (%)
DE_2	57.06 ± 1.79 ^{cd}	48.37 ± 3.17 ^a	38.14 ± 2.26 ^e	50.50 ± 2.72 ^{ae}	48.52 ± 7.37 ^c	15.20
DE_3	70.14 ± 2.04 ^{ab}	67.37 ± 3.55 ^{cd}	69.96 ± 2.02 ^d	45.36 ± 2.16 ^e	63.21 ± 10.93 ^{ab}	17.30
DE_5	74.16 ± 3.38 ^{bf}	74.33 ± 1.22 ^{df}	66.81 ± 4.97 ^{cd}	58.17 ± 3.27 ^{a-d}	68.37 ± 7.51 ^a	10.99
DE_6	61.67 ± 1.35 ^{de}	53.70 ± 2.07 ^{ab}	56.48 ± 4.23 ^{ab}	53.61 ± 2.21 ^{abc}	56.36 ± 4.14 ^{bcd}	7.35
DE_11	66.61 ± 3.01 ^{ae}	55.00 ± 2.39 ^{ab}	51.76 ± 0.70 ^a	50.46 ± 4.37 ^{ae}	55.96 ± 7.08 ^{bcd}	12.66
PL_37	54.56 ± 2.89 ^c	48.95 ± 3.38 ^a	53.23 ± 1.33 ^a	57.62 ± 2.69 ^{a-d}	53.59 ± 4.01 ^{cd}	7.49
PL_38	78.61 ± 3.74 ^f	56.49 ± 2.42 ^{be}	57.33 ± 1.11 ^{ab}	59.27 ± 3.72 ^{bcd}	62.92 ± 9.77 ^{ab}	15.53
PL_44	86.51 ± 1.68 ^g	67.89 ± 3.30 ^{cd}	61.95 ± 2.40 ^{bc}	61.11 ± 3.51 ^{cd}	69.37 ± 10.87 ^a	15.67
PL_45	71.05 ± 2.37 ^{ab}	76.76 ± 5.53 ^f	67.61 ± 3.55 ^{cd}	64.99 ± 3.36 ^d	70.10 ± 5.72 ^a	8.16
PL_58	71.35 ± 3.16 ^{ab}	62.66 ± 1.25 ^{ce}	61.76 ± 3.17 ^{bc}	52.33 ± 3.64 ^{abe}	62.03 ± 7.44 ^{abd}	11.99
All genotypes	69.17 ± 9.61	61.15 ± 10.13	58.50 ± 9.40	55.34 ± 6.34	61.04 ± 10.29	12.23
CV (%)	13.89	16.57	16.07	11.46	16.85	30.08

Values are mean ± SD of four repetitions for chemical analysis. Mean values in column with the same letter are not significantly different at p=0.05 (Tukey's multiple range test), CV - coefficient of variation

Table 4 Variation in 3-*O*-caffeoylquinic acid content [mg g⁻¹ DW] between *Ch. angustifolium* genotypes in four phenological phases

Genotype	Vegetative phase	Beginning of blooming	Full blooming	Green fruit phase	All phases	CV (%)
DE_2	2.54 ± 0.03 ^f	2.63 ± 0.11 ^b	2.16 ± 0.15 ^{de}	1.23 ± 0.07 ^c	2.14 ± 0.58 ^e	27.06
DE_3	3.12 ± 0.15 ^{ab}	2.70 ± 0.10 ^{bc}	2.40 ± 0.06 ^{ef}	1.94 ± 0.06 ^{ad}	2.54 ± 0.45 ^{abe}	17.77
DE_5	3.30 ± 0.09 ^{bc}	3.55 ± 0.09 ^{ad}	2.77 ± 0.23 ^{ab}	1.68 ± 0.05 ^{ab}	2.83 ± 0.75 ^{abc}	26.58
DE_6	2.84 ± 0.07 ^{af}	3.00 ± 0.13 ^{ce}	2.56 ± 0.09 ^{af}	1.79 ± 0.05 ^a	2.55 ± 0.49 ^{abe}	19.21
DE_11	3.05 ± 0.12 ^{ab}	2.74 ± 0.15 ^{bc}	2.02 ± 0.05 ^d	1.42 ± 0.07 ^{bc}	2.31 ± 0.66 ^{ae}	28.62
PL_37	3.17 ± 0.17 ^{abc}	3.28 ± 0.17 ^{de}	3.01 ± 0.13 ^{bg}	2.17 ± 0.21 ^d	2.91 ± 0.48 ^{a-d}	16.44
PL_38	3.84 ± 0.24 ^e	3.38 ± 0.22 ^{ad}	3.31 ± 0.16 ^{cg}	2.58 ± 0.22 ^e	3.28 ± 0.51 ^{cd}	15.41
PL_44	3.80 ± 0.15 ^{de}	3.69 ± 0.09 ^a	3.37 ± 0.16 ^c	3.18 ± 0.17 ^f	3.51 ± 0.29 ^d	8.21
PL_45	3.66 ± 0.13 ^{de}	3.65 ± 0.10 ^a	2.82 ± 0.12 ^{ab}	2.62 ± 0.11 ^e	3.19 ± 0.50 ^{cd}	15.68
PL_58	3.47 ± 0.17 ^{cd}	3.64 ± 0.06 ^a	3.38 ± 0.13 ^c	1.70 ± 0.11 ^{ab}	3.05 ± 0.82 ^{bcd}	26.74
All genotypes	3.28 ± 0.43	3.23 ± 0.42	2.78 ± 0.49	2.03 ± 0.59	2.83 ± 0.70	20.17
CV (%)	12.98	13.13	17.55	29.13	24.60	33.44

Values are mean ± SD of four repetitions for chemical analysis. Mean values in column with the same letter are not significantly different at p=0.05 (Tukey's multiple range test), CV - coefficient of variation

DW (PL_44) (Table 4). The highest content (3.84 mg g^{-1} DW) was recorded for the PL_38 line in the vegetative phase. PL_44 genotype was characterized by a relatively high (from 3.18 to 3.80 mg g^{-1} DW) and stable ($\text{CV}=8.21\%$) level of 3-*O*-caffeoylquinic acid during the whole season. The highest level of 3-*O*-caffeoylquinic acid was noted in the first two phases, i.e. in the vegetative and beginning of blooming ones, whereas a gradual decrease was observed from the full blooming phase to the green fruit phase.

Flavonoids

Fluctuation of flavonoids level was the highest among tested polyphenols and genotypes. The highest contents

of quercetin 3-*O*-glucuronide (21.44 mg g^{-1} DW and 20.29 mg g^{-1} DW) were recorded for PL_44 and DE_5 genotypes, respectively, at the beginning of the blooming phase (Table 5). Content of this flavonoid recorded for PL_44, PL_38 and PL_37 lines was relatively high and stable ($\text{CV}=6.48\%$, 7.84% and 7.95% , respectively) during the season.

Mean content of the sum of kaempferol 3-*O*-glucuronide and quercetin 3-*O*-rhamnoside ranged from 2.05 (DE_2) to 4.39 mg g^{-1} DW (PL_38) (Table 6). The highest level of these flavonoids ($5.84 \pm 0.28 \text{ mg g}^{-1}$ DW) was recorded for PL_38 genotype in the full blooming phase. The result was statistically significant and clearly indicated the distinction between the rest of the lines in this

Table 5 Variation in quercetin 3-*O*-glucuronide content [mg g^{-1} DW] between *Ch. angustifolium* genotypes in four phenological phases

Genotype	Vegetative phase	Beginning of blooming	Full blooming	Green fruit phase	All phases	CV (%)
DE_2	10.43 ± 0.31^c	11.83 ± 0.90^g	8.95 ± 0.54^b	8.97 ± 0.97^b	10.04 ± 1.39^e	13.88
DE_3	17.41 ± 0.64^a	9.84 ± 0.66^f	9.70 ± 0.56^b	18.19 ± 0.56^{de}	13.78 ± 4.19^a	30.43
DE_5	18.12 ± 0.73^a	20.29 ± 0.46^{de}	18.20 ± 0.44^{ef}	12.90 ± 0.86^a	17.38 ± 2.88^{bcd}	16.55
DE_6	15.26 ± 0.81^b	18.26 ± 1.00^{bc}	15.05 ± 0.86^{ad}	11.03 ± 0.19^{ab}	14.90 ± 2.74^{abc}	18.41
DE_11	18.20 ± 0.72^a	16.09 ± 0.76^a	13.18 ± 0.10^c	10.22 ± 0.72^b	14.42 ± 3.16^a	21.94
PL_37	15.29 ± 0.77^b	17.54 ± 1.10^{abc}	16.26 ± 0.56^a	15.62 ± 1.48^c	16.18 ± 1.29^{abc}	7.95
PL_38	18.74 ± 1.07^a	16.53 ± 1.10^{ab}	18.05 ± 0.74^e	16.63 ± 1.33^{cd}	17.49 ± 1.37^{cd}	7.84
PL_44	19.04 ± 0.66^a	21.44 ± 0.98^e	19.71 ± 1.02^f	19.37 ± 1.13^e	19.89 ± 1.29^d	6.48
PL_45	13.54 ± 0.50^b	18.57 ± 0.58^{cd}	14.22 ± 0.58^{cd}	12.49 ± 0.45^a	14.70 ± 2.44^{ab}	16.57
PL_58	15.08 ± 0.81^b	15.95 ± 0.15^a	16.27 ± 0.42^a	12.47 ± 0.84^a	14.94 ± 1.64^{abc}	10.99
All genotypes	16.11 ± 2.70	16.63 ± 3.49	14.96 ± 3.46	13.79 ± 3.45	15.37 ± 3.44	15.10
CV (%)	16.74	20.99	23.12	25.04	22.38	49.03

Values are mean $\pm$ SD of four repetitions for chemical analysis. Mean values in column with the same letter are not significantly different at $p=0.05$ (Tukey's multiple range test), CV - coefficient of variation

Table 6 Variation in content of sum of kaempferol 3-*O*-glucuronide and quercetin 3-*O*-rhamnoside [mg g^{-1} DW] between *Ch. angustifolium* genotypes in four phenological phases

Genotype	Vegetative phase	Beginning of blooming	Full blooming	Green fruit phase	All phases	CV (%)
DE_2	1.10 ± 0.04^g	2.15 ± 0.14^b	2.08 ± 0.16^f	2.85 ± 0.22^{ab}	2.05 ± 0.66^a	32.17
DE_3	1.79 ± 0.07^{bc}	2.80 ± 0.18^a	5.07 ± 0.42^e	2.79 ± 0.04^{ab}	$3.11 \pm 1.26^{b-e}$	40.40
DE_5	1.51 ± 0.06^a	1.74 ± 0.05^b	3.08 ± 0.17^{ab}	3.17 ± 0.17^a	2.38 ± 0.79^{ab}	33.20
DE_6	1.55 ± 0.09^{ab}	1.98 ± 0.12^b	3.35 ± 0.09^b	3.24 ± 0.11^{ac}	2.53 ± 0.81^{abc}	32.12
DE_11	1.40 ± 0.05^a	2.96 ± 0.16^a	4.09 ± 0.18^c	2.67 ± 0.08^b	$2.78 \pm 1.00^{a-e}$	35.80
PL_37	2.04 ± 0.10^{cd}	2.80 ± 0.22^a	2.72 ± 0.07^a	3.20 ± 0.28^{ac}	$2.69 \pm 0.46^{a-d}$	17.13
PL_38	2.89 ± 0.18^{ef}	4.35 ± 0.29^d	5.84 ± 0.28^g	4.46 ± 0.31^f	4.39 ± 1.10^f	25.12
PL_44	2.96 ± 0.12^f	4.09 ± 0.28^{cd}	3.16 ± 0.13^{ab}	3.73 ± 0.24^{de}	3.49 ± 0.50^{def}	14.40
PL_45	2.15 ± 0.09^d	3.71 ± 0.10^c	4.60 ± 0.22^{de}	4.22 ± 0.13^{ef}	3.67 ± 0.97^{ef}	26.53
PL_58	2.67 ± 0.15^e	3.18 ± 0.05^a	4.16 ± 0.09^{cd}	3.69 ± 0.27^{cd}	3.42 ± 0.59^{cde}	17.33
All genotypes	2.01 ± 0.63	2.98 ± 0.86	3.81 ± 1.12	3.40 ± 0.61	3.05 ± 1.06	27.42
CV (%)	31.64	28.86	29.29	17.92	34.87	32.17

Values are mean $\pm$ SD of four repetitions for chemical analysis. Mean values in column with the same letter (a...g) are not significantly different at $p=0.05$ (Tukey's multiple range test), CV - coefficient of variation

Table 7 Variation in kaempferol 3-*O*-rhamnoside content [mg g⁻¹ DW] between *Ch. angustifolium* genotypes in four phenological phases

Genotype	Vegetative phase	Beginning of blooming	Full blooming	Green fruit phase	All phases	CV (%)
DE_2	0.78 ± 0.03 ^e	1.66 ± 0.13 ^{ab}	1.84 ± 0.13 ^{ab}	1.38 ± 0.13 ^{ac}	1.42 ± 0.43 ^a	30.35
DE_3	0.25 ± 0.01 ^d	1.55 ± 0.08 ^a	2.74 ± 0.20 ^{cd}	1.15 ± 0.03 ^{ab}	1.42 ± 0.93 ^a	65.33
DE_5	0.57 ± 0.02 ^b	0.94 ± 0.07 ^c	2.09 ± 0.11 ^b	1.79 ± 0.16 ^d	1.35 ± 0.64 ^a	47.59
DE_6	0.32 ± 0.03 ^d	0.89 ± 0.07 ^c	4.00 ± 0.14 ^e	1.68 ± 0.10 ^{cd}	1.72 ± 1.45 ^{ab}	84.35
DE_11	0.68 ± 0.03 ^{ac}	2.08 ± 0.12 ^d	3.02 ± 0.13 ^c	0.85 ± 0.05 ^b	1.66 ± 0.99 ^{ab}	59.78
PL_37	0.64 ± 0.04 ^{ab}	1.62 ± 0.09 ^{ab}	2.12 ± 0.08 ^b	2.15 ± 0.15 ^e	1.63 ± 0.64 ^{ab}	38.94
PL_38	0.73 ± 0.04 ^{ce}	1.41 ± 0.06 ^a	1.66 ± 0.07 ^a	1.19 ± 0.06 ^a	1.25 ± 0.36 ^a	28.73
PL_44	0.88 ± 0.03 ^f	3.57 ± 0.20 ^e	2.60 ± 0.17 ^c	2.88 ± 0.21 ^f	2.48 ± 1.03 ^b	41.60
PL_45	0.67 ± 0.03 ^{ac}	1.85 ± 0.09 ^{bd}	1.69 ± 0.06 ^a	1.36 ± 0.04 ^a	1.39 ± 0.47 ^a	33.87
PL_58	0.62 ± 0.03 ^{ab}	1.50 ± 0.04 ^a	2.46 ± 0.04 ^c	2.22 ± 0.16 ^e	1.70 ± 0.74 ^{ab}	43.69
All genotypes	0.61 ± 0.19	1.71 ± 0.73	2.42 ± 0.70	1.67 ± 0.60	1.60 ± 0.87	47.42
CV (%)	30.82	42.50	28.87	35.89	54.57	37.06

Values are mean ± SD of four repetitions for chemical analysis. Mean values in column with the same letter are not significantly different at $p=0.05$ (Tukey's multiple range test), CV - coefficient of variation

phase. Most genotypes reached the highest peak in the full blooming phase.

Mean content of kaempferol 3-*O*-rhamnoside ranged from 1.25 (PL_38) to 2.48 mg g⁻¹ DW (PL_44) (Table 7). The highest content of kaempferol 3-*O*-rhamnoside (4.00 ± 0.14 mg g⁻¹ DW) was recorded for the DE_6 line in the full blooming phase, and this result reached statistical significance. Only two genotypes (PL_44 and PL_45) expressed a different pattern of kaempferol 3-*O*-rhamnoside accumulation. PL_44 and PL_45 plants achieved the highest level earlier, at the beginning of blooming. The large increase in the content of kaempferol 3-*O*-rhamnoside content (12-fold and 10-fold, respectively) during the season was noted for DE_6 and DE_3 genotypes.

Phytochemical variability during vegetation

To verify hypothesis that full blooming phase is the best harvest time, the content of the tested metabolites was compared in four phenological phases, and full blooming phase was set as a control (Table 8). Significantly higher levels of flavonoids: kaempferol 3-*O*-rhamnoside and sum of kaempferol 3-*O*-glucuronide and quercetin- 3-*O*-rhamnoside were

noted in full blooming phase, whereas oenothien B and chlorogenic acid contents were lower. The mean content of quercetin 3-*O*-glucuronide did not show any variation in relation to developmental phase.

Discussion

Genotypes or chemotypes which are stable regarding the expression of secondary metabolites during the season are important for developing new forms or cultivars. Knowledge of metabolite fluctuation during physiological development allows the determination of optimum harvesting time and is essential for the success of each commercial agricultural undertaking. Therefore, studies on intraspecies variability in metabolite production and on the relationship with the phenological phases significantly contribute to a solid basis for genotype selection aimed at better food or higher quality of pharmaceuticals. In the present study, in vitro cultures were used for multiplication of plants providing homogenous and sufficient material from each accession for the study on genotypic and developmental variation in ex vitro conditions.

Table 8 Variation in content of oenothien B, 3-*O*-caffeoylquinic acid and flavonoids [mg g⁻¹ DW] in four phenological phases

Phase	OB	CA	QG	KG + QR	KR
Control	58.50 ± 9.40 ^a	2.78 ± 0.49 ^a	14.96 ± 3.46 ^a	3.81 ± 1.12 ^a	2.42 ± 0.70 ^a
Vegetative phase	69.17 ± 9.61 ^b	3.28 ± 0.43 ^b	16.11 ± 2.70 ^a	2.01 ± 0.63 ^b	0.61 ± 0.19 ^b
Beginning of blooming	61.15 ± 10.13 ^a	3.23 ± 0.42 ^b	16.63 ± 3.49 ^a	2.98 ± 0.86 ^b	1.71 ± 0.73 ^b
Green fruit phase	55.34 ± 6.34 ^a	2.03 ± 0.59 ^c	13.79 ± 3.45 ^a	3.40 ± 0.61 ^a	1.67 ± 0.60 ^b

Content of metabolites in full blooming phase was set as a control. OB oenothien B, CA 3-*O*-caffeoylquinic acid, QG quercetin 3-*O*-glucuronide, KG + QR sum of kaempferol 3-*O*-glucuronide and quercetin 3-*O*-rhamnoside, KR kaempferol 3-*O*-rhamnoside. Mean values (calculated for all genotypes) in column with the same letter are not significantly different at $p=0.05$ (Dunnett's post hoc test)

In our research, ten genotypes (lines) were subjected to micropropagation and phytochemical studies. Tested genotypes significantly differed in a number of proliferated shoots per explants (Fig. 2). A high reproduction rate (> 20 shoots per explant) was recorded for four lines (PL_45, PL_44, PL_58, DE_2). The micropropagation efficiency depends on culture conditions, selection of plant growth regulators (PGRs), micropropagation technique, type of explant, age of donors' plants and genotype. Organogenesis from root explants (Turker et al. 2008; Dreger et al. 2016) and nodal explants of fireweed (Dreger et al. 2020) was reported previously. A higher regeneration rate (> 30 shoots per explants) was noted for root explants (Turker et al. 2008; Dreger et al. 2016), but without respect to the genotype effect. Type of explant and PGRs play a key role in the regeneration process, but its efficiency depends on genotype (Zimik and Arumugam 2017; Pandey et al. 2020). Genotypes differ in their levels of endogenous phytohormones. In vitro shoot regeneration is controlled by the exogenous cytokinin and auxin balances, therefore the different levels of endogenous phytohormones is assumed to be the main cause of variation between genotypes with different degrees of competence (Kumari et al. 2018). The high regeneration rate as well as high survival rate of acclimatized plants (95.5%) confirmed the appropriate selection of the PGRs as well as cultivation and micropropagation technique. Genotypes characterized by high regeneration rate should be considered as good candidates for large scale multiplication and production of the plantlets.

Phytochemical screening of in vitro regenerated shoots of *Ch. angustifolium* revealed the presence of 14 compounds in contrast to 31 constituents identified in samples collected from the field (Tables 1 and 2). First of all, absence of flavonoids (except of quercetin 3-*O*-glucuronide) was characteristic for in vitro material (Table 1). Generally, plants cultivated in vitro synthesize fewer compounds than those grown in the field due to completely different environmental conditions (Cardoso et al. 2019). Moreover, in vitro growing plants are kept in the juvenile stage and do not go through the full development phases, so specific to tissue and organ compounds are absent or minor. Presence of some flavonoids such as quercetin 3-*O*-rhamnoside, myricetin 3-*O*-rhamnoside and kaempferol- 3-*O*-rhamnoside is closely related to flowers and their development (Baert et al. 2017). Tissue-specific regulation of flavonoid biosynthesis has been intensively studied and confirmed in others species (He et al. 2020; Yuan et al. 2020). Therefore, the absence of some flavonoids in in vitro cultured shoots can be explained by specific tissue and developmental regulation of flavonoid biosynthesis. Although in vitro plants contained less phenolic compounds than those cultivated in field, but two bioactive compounds: oenothain B and caffeic acid pentoside

were abundant in shoot cultures (Table 1). Comparable oenothain B content ($24.6 \text{ mg g}^{-1} \text{ DW}$) in *Ch. angustifolium* shoot cultures was determined previously (Dreger et al. 2020). Presence of caffeic acid pentoside is reported for the first time in in vitro cultures of fireweed, although this compound and other caffeic acid derivatives are commonly found in medicinal plants with strong antioxidant action (Tajner-Czopek et al. 2020).

Contrary to in vitro cultures, plants harvested from the field contained the full spectrum of polyphenols, including flavonoids: quercetin 3-*O*-glucuronide, kaempferol 3-*O*-rhamnoside, kaempferol 3-*O*-glucuronide and quercetin 3-*O*-rhamnoside. Oenothain B ($38.1\text{--}86.5 \text{ mg g}^{-1} \text{ DW}$) and quercetin 3-*O*-glucuronide ($8.95\text{--}21.44 \text{ mg g}^{-1} \text{ DW}$) were the most abundant compounds. Total flavonoid content varied among accessions from 13.51 to $25.86 \text{ mg g}^{-1} \text{ DW}$. This is consistent with other reports (Hevesi Tóth et al. 2009; Kiss et al. 2011; Stolarczyk et al. 2013). Oenothain B is the predominant polyphenol in this species (Granica et al. 2012; Baert 2017), while total flavonoid content is about 2% (Ducrey et al. 1997; Kiss et al. 2011; Kaškonienė et al. 2015).

The important part of our study was analysis of genotypic variability in polyphenols accumulation. Quantitative analysis of oenothain B with respect to genotypes showed relatively high variation of its content under in vitro conditions confirming that biosynthesis of this compound is under control of genetic factors. It should be stressed that all accessions were kept on the same medium and treated in the same way. Oenothain B content as well as its variation among genotypes did not simply extrapolate to field conditions (Fig. 3, Table 3). Generally, content of this ellagitannin was three-fold to five-fold higher in ex vitro conditions, but did not reflect the variation among genotypes observed in in vitro cultures. Low oenothain B-producing genotypes (PL_58, DE_11, DE_5) exhibited nearly five-fold increased content of this metabolite when they were transferred to the field. On the other hand, PL_38 plants, distinguished by the highest oenothain B level ($22.32 \text{ mg g}^{-1} \text{ DW}$) under in vitro conditions, showed only a 2.8-fold increase of oenothain B content when grown in the field. Genotypic variation in oenothain B level under ex vitro conditions was strongly affected by environmental factors. The highest content of oenothain B ($86.51 \text{ mg g}^{-1} \text{ DW}$) was recorded for PL_44 genotype in vegetative phase and it was 20% higher than mean values for all genotypes in this phase. Differences among genotypes in other phenological phases were not so distinct or statistically significant. This is with line with Baert et al. (2017), who found relatively small inter-individual and moderate inter-population variation in oenothain B concentration. In the mentioned study, plant material was harvested at blooming phase therefore the differences were small or moderate.

In the present study, most genotypes showed a similar pattern of oenothien B accumulation, and the highest values of this compound were recorded in the vegetative phase, whereas the lowest concentration was observed in the green fruit phase. This is consistent with the observations of Jürge­nson et al. (2012), who recorded the highest tannin content in young plants in May, whereas in older ones it declined. Enhanced production of ellagitannins might be a part of the defense strategy against herbivores aimed at protecting young plants in order to increase reproductive success. The defensive role of ellagitannins in plants against herbivores and pathogens is well documented (Mamaní et al. 2012; Moilanen et al. 2016; Martos et al. 2020). Also oenothien B showed significant anti-herbivore (Anstett et al. 2019) and antimicrobial properties (Hatano et al. 2005).

Fluctuations of 3-*O*-caffeoylquinic acid level between genotypes of fireweed were relatively small or moderate (Table 4). Among accessions tested, PL_44 plants accumulated high and stable ($CV = 8.21\%$) content of this substance during vegetation in contrast to the other genotypes. In our study, the highest content of this phenolic acid was recorded in the vegetative and beginning of blooming phases, then the level was gradually declined (Tables 4 and 8). A similar pattern of 3-*O*-caffeoylquinic acid accumulation during the season in Polish climatic conditions was observed for *Sorbus domestica* leaves (Rutkowska et al. 2020). The highest content of this compound was recorded in May ($1.68 \text{ mg g}^{-1} \text{ DW}$), whereas the lowest was recorded in October ($0.36 \text{ mg g}^{-1} \text{ DW}$). Gradually declining level of phenolic acids might be explain by their protective role against UV radiation during foliar development. Biosynthesis of phenolic acids reflects an early response to UV radiation among other stressors (Petrulová et al. 2014). Phenolic acids act as UV filters and potent radical scavengers, which attenuate harmful UV radiation during foliar development (Mondolot et al. 2006; Li et al. 2019), whereas in the full blooming phase this role is shared by many others polyphenols i.a. flavonoids.

Genotypic variability in the content of flavonoids was the greatest among tested polyphenols (Tables 5–7). Variation in individual flavonoid level was heterogeneous and ranged from moderate (quercetin 3-*O*-glucuronide $CV = 22.38\%$) to high (kaempferol 3-*O*-rhamnoside, $CV = 54.57\%$). Genotype of PL_44 was relatively abundant in both flavonoids quercetin 3-*O*-glucuronide and kaempferol 3-*O*-rhamnoside, whereas PL_38 plants were the most rich in kaempferol 3-*O*-glucuronide and quercetin 3-*O*-rhamnoside. Quercetin 3-*O*-glucuronide (miquelianin) was the most abundant flavonoid, which was consistent with previous reports (Stolarczyk et al. 2013; Granica et al. 2014). The maximal mean value recorded for miquelianin was $19.89 \text{ mg g}^{-1} \text{ DW}$ (PL_44), which was nearly two-fold higher than the lowest recorded value of $10.04 \text{ mg g}^{-1} \text{ DW}$ (DE_2). It is worth

noting that it was two-fold higher and 3.5-fold higher than reported by Monschein et al. (2015) and Baert et al. (2017), respectively. The PL_44 genotype accumulated the highest miquelianin content, and its level was very stable during the season ($CV = 6.48\%$), which makes it a good candidate for early selection. Seasonal variation for this compound was moderate, without a clearly distinguished peak (Table 8). Miquelianin is definitely accumulated in leaves (Monschein et al. 2015; Baert et al. 2017), which are the target organs for this flavonoid, which could explain the lack of peak during blooming. In contrast to miquelianin, quercetin 3-*O*-rhamnoside (quercitrin) and kaempferol 3-*O*-rhamnoside (afzelin) are almost exclusively accumulated in flowers (Baert et al. 2017), whereas kaempferol 3-*O*-glucuronide accumulates in both types of organ equally (Monschein et al. 2015; Baert et al. 2017). Fluctuation of afzelin level during vegetation was the highest among flavonoids tested (Tables 7 and 8). The highest content of this substance was recorded in full blooming phase ($2.42 \text{ mg g}^{-1} \text{ DW}$, on average). Similarly, the highest amount of sum of kaempferol 3-*O*-glucuronide and quercetin 3-*O*-rhamnoside was detected in the full blooming phase (Tables 6 and 8). This trend in seasonal variation of these flavonoids was also reported by Maruška et al. (2014), who concluded that the most valuable plant raw material of fireweed was collected during the massive blooming phase. To verify this hypothesis, the mean content of tested metabolites was compared in four developmental phases and full blooming phase was set as the control (Table 8). The results confirmed this hypothesis—that raw material contained the richest polyphenols composition in full blooming phase, but the maximal concentrations of oenothien B and chlorogenic acid were recorded in vegetative phase.

Breeding programs are based on the recommendations for standardization of the plant raw material and take into account its potential industrial use. Oenothien B and quercetin 3-*O*-glucuronide have been proposed as the marker compounds for the identification and standardization of fireweed raw material (Bazyłko et al. 2007; Kiss et al. 2011; Monschein et al. 2015). Among *Ch. angustifolium* accessions, genotype of PL_44 was characterized by high and stable content of oenothien B and quercetin 3-*O*-glucuronide as well as a relatively high level of other flavonoids, therefore, it is a good candidate for further breeding process. Oenothien B is a main bioactive compound with therapeutic potential in BPH due to complex mechanism of action involving the regulation of androgen levels, inhibition of prostate-specific antigen (PSA) as well as anti-proliferative and apoptotic properties (Hiermann et al. 1998; Vitalone et al. 2001; Stolarczyk et al. 2013). The results of clinical trial (Esposito et al. 2021) and numerous preclinical studies indicated that *Ch. angustifolium* extracts can be used as drugs or food supplements. Accumulating evidence suggests

that regular consumption of ellagitannin-rich products can help reduce the risk of some chronic diseases, and may be useful in cancer prevention (Garcia-Muñoz & Vaillant 2014). Ellagitannins are classified as nutraceuticals due to an array of biological activities, including antioxidant, anti-inflammatory, anticancer, antidiabetic, antiaging, and antimicrobial. Currently, attention has also been paid to their metabolites—urolithins, which are produced by the intestinal microbiota (García-Villalba et al. 2020). There is a great interest in the application of urolithins as active forms of ellagitannins in the prevention of inflammatory bowel diseases, cardiovascular, metabolic syndrome, as well as neurodegenerative diseases. Flavonoids also have great therapeutic potential as they exhibit strong anti-inflammatory activity (Hiermann et al. 1998; Baral et al. 2017). *Ch. angustifolium* extract can be used in cosmetics manufacture. Skin photoprotection and anti-aging effect of fireweed extract were demonstrated in *in vitro* and *in vivo* tests (Ruszová et al. 2013). It should be noticed that the beneficial properties of fireweed are attributed to the synergic effect of polyphenols like ellagitannins, flavonoids and phenolic acids. Therefore, future breeding programs should be focused on plants characterized by the richest composition of polyphenols.

Conclusion

The micropropagation study of *Ch. angustifolium* showed relatively high variation in shoot proliferation of this species depending on the genotype. Hence, the evaluation of the multiplication efficiency was necessary to select accessions suitable for mass production of a high quality plant material. In our research, several lines (DE_2, PL_58, PL_44, PL_45) were characterized by a high reproductive rate (> 20 shoots per explants), which makes them good candidates for early selection.

The results of the phytochemical analyses exhibited that *in vitro* grown plants of *Ch. angustifolium* are relatively good source of some bioactive compounds, mainly oenothien B and caffeic acid pentoside. This finding may inspire new research on the ellagitannins production in *in vitro* cultures. On the other hand, our study confirmed that fireweed plants harvested from the field cultivations contain the full spectrum of polyphenols characteristic for this species, and oenothien B and quercetin 3-*O*-glucuronide are the most abundant. The results of analysis of variance indicated slight or moderate differences between tested genotypes, but some of them accumulated a higher and more stable level of bioactive compounds during the growing season.

In our study, the maximal content of oenothien B was recorded in the vegetative phase of fireweed, while some flavonoids (sum of kaempferol 3-*O*-glucuronide and quercetin 3-*O*-rhamnoside, kaempferol 3-*O*-rhamnoside) were

found in the highest amount in full blooming phase, which imply different optimal harvest time. Generally, harvest time should be adjusted to the content of active compounds and to the intended use of the raw material. In the full blooming phase of fireweed, the highest concentration of some flavonoids and still a relatively great level of oenothien B were determined. Regarding that the beneficial properties of fireweed are attributed to the synergic effect of polyphenols, therefore full blooming phase is the optimal harvest time.

Ch. angustifolium genotype of PL_44 was characterized by high content of oenothien B and quercetin 3-*O*-glucuronide as well as a relatively high level of other flavonoids. Based on the results of phytochemical and micropropagation studies, we can conclude that PL_44 genotype is the best candidate for early selection and further breeding programs aimed at obtaining a suitable industrial form for the pharmaceutical applications.

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Declarations

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

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