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ORIGINAL ARTICLE

Various in vitro systems of Ragged Robin (*Lychnis flos-cuculi* L.): a new potential source of phytoecdysteroids?

Michał P. Maliński[1] · Małgorzata Kikowska[1] · Dariusz Kruszka[2] · Marta Napierała[3] · Ewa Florek[3] · Elwira Sliwinska[4] · Barbara Thiem[1]

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Abstract

Lychnis flos-cuculi L. is a species containing ecdysteroids, triterpenoid saponins, flavonoids, and phenolic acids, and therefore is a plant of potential medicinal value. In the presented research, diverse in vitro cultures of this taxon were developed to obtain the uniform material capable of producing ecdysteroids, including micropropagated plantlets, shoot cultures, liquid agitated whole plant cultures with fast-growing roots, and callus. A protocol of micropropagation through axillary bud formation was established using plant growth regulators at different concentrations and combinations. All the variants of plant growth regulator supplementation significantly affected a shoot proliferation rate ranging from 8 to 16 plantlets per explant, depending on the medium; DNA content of all the studied plant materials was similar. The thin-layer chromatography analysis of the extracts revealed the presence of ecdysteroids in every plant material apart from callus. The content of 20-hydroxyecdysone and polypodine B was evaluated by high-performance liquid chromatography. Agitated plantlets were in vitro cultures that efficiently formed abundant root biomass with significant concentrations of ecdysteroids. In vitro-derived adventitious roots contained twofold higher content of ecdysteroids than those of intact plants. The organs of flowering in vitro-propagated plants, transferred to experimental plot, contained twice as much ecdysteroids when compared to the organs of plants from the natural site, among which flowers were the richest in ecdysteroids. The results revealed that adventitious roots from *L. flos-cuculi* agitated cultures can be considered as an alternative biotechnological source of biomass rich in pharmaceutically active ecdysteroids.

Key message

Diverse types of *Lychnis flos-cuculi* in vitro cultures have been developed. HPLC analysis demonstrated much higher ecdysteroid content in cultures and regenerated plants when compared to natural site plants.

Keywords Micropropagation · Callus · 20-Hydroxyecdysone · Polypodine B · Agitated plant cultures · Genome size

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✉ Michał P. Maliński
mpmalinski@ump.edu.pl

[1] Department of Pharmaceutical Botany and Plant Biotechnology, Poznan University of Medical Sciences, 14 Św. Marii Magdaleny St., 61-861 Poznań, Poland

[2] Institute of Plant Genetics, Polish Academy of Sciences, 34 Strzeszyńska St., 60-479 Poznań, Poland

[3] Laboratory of Environmental Research, Department of Toxicology, Poznan University of Medical Sciences, 30 Dojazd St., 60-631 Poznań, Poland

[4] Laboratory of Molecular Biology and Cytometry, Department of Agricultural Biotechnology, UTP University of Science and Technology, 7 Prof. Kaliskiego Ave., 85-789 Bydgoszcz, Poland

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
20HE	20-Hydroxyecdysone
BAP	N^6-benzylaminopurine
Dic	Dicamba (3,6-dichloro-2-methoxybenzoic acid)
IAA	Indole-3-acetic acid
Kin	Kinetin (N^6-furfuryladenine)
MS	Murashige & Skoog medium
NAA	1-Naphthaleneacetic acid
PGRs	Plant growth regulators
Pic	Picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid)
polB	Polypodine B (5β,20-dihydroxyecdysone)
TDZ	Thidiazuron (1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea
TLC	Thin-layer chromatography

Introduction

Lychnis flos-cuculi L. [*Silene flos-cuculi* (L.) Greuter & Burdet, *Coronaria flos-cuculi* (L.) A. Braun], commonly known as Ragged Robin, is a herbaceous perennial from the family Caryophyllaceae, native to Europe and Northwestern Asia. It grows in moist and sunny open habitats, the most often wet meadows. Reproduction occurs by seeds as well as vegetatively, either through underground stolons or by developing secondary daughter rosettes from axillary buds. The rosettes grow remaining attached to the mother plant, forming an interconnected clone (Chaloupecká and Lepš 2004).

The knowledge regarding secondary metabolites of *L. flos-cuculi* is largely incomplete, although several classes of compounds have been already described. Apart from phenolic acids, flavonoids, and complex triterpenoid saponins, the plant contains ecdysteroids, namely polar steroid compounds related to triterpenoids, based on C27 cholest-7-en-6-one backbone (Fig. 1). The ecdysteroids such as 20-hydroxyecdysone (20HE), being the most ubiquitous ecdysteroid in the plant kingdom, have been proven to exert multidirectional beneficial activity in humans such as adaptogenic, antioxidant, neuroprotective, and wound-healing effects as well as increased metabolism of carbohydrates, fats, and proteins (Baltaev 2000; Lafont and Dinan 2003; Dinan and Lafont 2006). A total of 11 ecdysteroids were reported in *L. flos-cuculi*, with 20HE and polypodine B (polB) as main constituents, and the remaining compounds in minor or trace amounts (Báthori et al. 2001). Apart from ecdysteroids, the herb contains phenolic acids (*p*-OH-benzoic, protocatechuic, vanillic, *p*-coumaric, caffeic, and ferulic acids) and flavonoids (apigenin and luteolin) and their C-glycosyl derivatives (vitexin and orientin) (Tomczyk 2008) as well as apigenin-*O*-glucoside and rutin (Costea et al. 2017). So far, only two oleanane-type triterpenoid saponins have been isolated from *L. flos-cuculi*, namely coronosides A and B (Bucharow et al. 1974), while the rest of saponin complex remains unidentified. The secondary metabolites present in *L. flos-cuculi* were described in a review by Maliński et al. (2014).

Nowadays, plant in vitro cultures are commonly considered to be a useful source of phytochemicals because of their numerous advantages. The clonal propagation of medicinal plants from existing meristems can occur under controlled conditions. It provides large quantities of high-quality genetically uniform plant material for phytochemical studies. This excludes the need to harvest plants from their natural habitat, which, at times, can be difficult because of availability, changing seasons and natural genetic heterogeneity. The efficient, rapid plant propagation in in vitro cultures under optimized conditions is still of interest as a source of biomass for the phytochemical and biological activity analyses (Smetanska 2008; Filova 2014; Chandana et al. 2018).

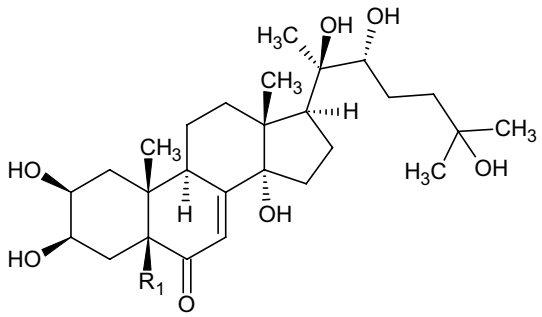

Fig. 1 Structural formulae of ecdysteroids present in *L. flos-cuculi* in major quantities: R=H–20-hydroxyecdysone, R=OH–polypodine B

Ecdysteroids are primarily obtained from the plant material harvested from the natural site or from cultivated plants (Lafont and Dinan 2003; Głazowska et al. 2018). They are, however, also produced employing plant in vitro cultures, a source that is often richer in those compounds and where numerous methods increasing ecdysteroid content can be applied under controlled conditions (Thiem et al. 2017).

The aim of this study was to evaluate the capacity of diverse types of *L. flos-cuculi* in vitro cultures for the biosynthesis of ecdysteroids and to initiate and maintain diverse in vitro systems. Therefore, various types of cultures such as micropropagated plantlets, shoot cultures, liquid agitated whole plantlet cultures, and callus cultures were established. The phytochemical analysis was employed to evaluate the presence of ecdysteroids and to compare the content of 20-hydroxyecdysone and polypodine B in the plant material of different origin. To our knowledge, in vitro cultures of this species and the analysis of ecdysteroid content in biomass of various in vitro cultures have not been previously reported.

Materials and methods

Plant material and growth conditions

Flowering herb and roots of *L. flos-cuculi* intact plants were collected from their natural habitat in Poland (a wetland meadow near Kuźnica Trzcińska, Greater Poland Voivodeship; 51°09′21″N 18°03′24″E) in May/June 2016. The voucher specimens (No. CP-Lfc-2016-0601) were deposited in the Herbarium of Department of Pharmaceutical Botany and Plant Biotechnology of Poznan University of Medical Sciences. The seeds used as primary explant to establish in vitro cultures were acquired from the same location.

For aseptic culture initiation, the seeds were dipped in 70% (v/v) ethanol for 30 s, left to soak in distilled water for an hour, dipped in ethanol again, and rinsed in 20% (v/v) solution of commercial bleach (Ace, Procter & Gamble) equivalent to 5% sodium hypochlorite solution, containing a droplet of Tween 80, for 15 min. Finally, they were rinsed five times in autoclaved double-distilled water under the laminar flow hood and placed on solid Murashige and Skoog (MS) (Murashige and Skoog 1962) basal medium for axenic seedling establishment.

All the types of culture media based on MS medium, liquid or solidified with 0.72% (w/v) agar (Sigma-Aldrich, St. Louis, MO, USA), were supplemented with 3% (w/v) sucrose and different combinations of plant growth regulators (PGRs; Sigma-Aldrich) at various concentrations. The pH of media was adjusted to 5.8. The cultures were kept in a growth chamber equipped with cool-white fluorescent lamps emitting light of 55 µmol m^{-2} s^{-1} intensity, under a 16:8 h photoperiod, at a temperature of 23 ± 2 °C. The callus cultures were incubated at similar conditions but kept in darkness.

Establishment of shoot cultures

Shoot tips of 30-day-old seedlings (up to 1 cm long) were used for establishment of shoot cultures. The explants were placed in 150 ml Erlenmeyer flasks filled with 30 ml of MS medium supplemented with N^6-benzylaminopurine (BAP; 0.5–3.0 mg l^{-1}) alone or with either indole-3-acetic acid (IAA; 0.1–1.0 mg l^{-1}) or 1-naphthaleneacetic acid (NAA; 0.1–1.0 mg l^{-1}). Shoots were multiplied by repetitive subcultures of original explants, using shoot tips, and by stimulating axillary bud formation. After proliferation, every 6 weeks, clusters of shoots were divided into single shoots and transferred to the fresh medium. The total number of shoots and their length were recorded from 3rd to 6th subculture, using 50–100 explants, depending on the medium. A batch of shoots was further multiplied via axillary buds on the same media until enough material for the phytochemical analyses was collected. Another portion of shoots, after rooting, was transferred to ex vitro conditions.

Rooting of shoots and plant transfer into soil

Single shoots from shoot cultures (minimum 2 cm long) were transferred for rooting to the half-strength basal medium (1/2 MS; halved concentration of macro- and micronutrients) or the full-strength MS medium, with or without one of the following three auxins: IAA, NAA or indole-3-butyric acid (IBA) at a concentration of 0.1 mg l^{-1}. They were cultured in test tubes containing 20 ml of culture medium. After 4 weeks, fresh and dry weight of roots were recorded. The root number was difficult to count because they were numerous, delicate, and very fragile. Rooted plantlets were transferred to plastic pots with a mixture of sterile soil and sand (5:4, v/v) with 10% Perlite. The pots were covered with glass beakers for 14 days to harden and acclimatize the plants for lower humidity. After that, survival frequency was recorded and the plants were transferred to the experimental plot, where they were monitored for two vegetation seasons. The micropropagation was repeated twice between 2016 and 2018.

Establishment of liquid agitated whole plantlet culture for production of adventitious root biomass

Agitated plantlet cultures were used as an efficient method to obtain root biomass basing on an observation that the shoot growing in the liquid medium induces abundant adventitious roots at a quick rate, faster than in the solid medium. Moreover, the attempts to establish adventitious root cultures from root tips in the liquid MS medium (with or without auxins) failed. Liquid agitated whole plantlet cultures were established by transferring 2–3 cm long shoots to 150 ml Erlenmeyer flasks containing 25 ml of liquid MS medium enriched with 0.2 mg l^{-1} IAA. The cultures were maintained on a rotary shaker at 110 rpm, at growth chamber conditions as described above. On day 42 of the growth cycle the plant material was harvested and fresh and dry weight of roots were recorded. The experiment was performed three times, using at least 40 explants (1 explant per flask) each time.

Establishment of callus cultures

To induce callus formation, hypocotyls, cotyledons, and roots of seedlings were isolated from 10- to 14-day-old seedlings and placed in 30 ml test tubes with 10 ml of MS medium. The media were supplemented with auxins, including 2,4-dichlorophenoxyacetic acid (2,4-D), NAA, dicamba (Dic), and picloram (Pic) as well as cytokinins, namely kinetin (Kin), BAP, and thidiazuron (TDZ), at different concentrations. The cultures were incubated for 30 days in darkness. The obtained tissues were evaluated in terms of three criteria, namely response, rate of proliferation, and type of morphology. The most viable of callus lines were chosen for the next part of experiment.

Five fragments of callus sized ca. 1 cm in diameter were placed in 200 ml Erlenmeyer flasks with 25 ml of solid MS medium containing the same PGRs combinations and were subcultured every 42 days. The growth index [GI] was calculated after three passages during three consecutive subcultures from the following formula:

$$GI = \left[\left(FW_X - FW_0\right)/FW_0\right] \times 100$$

where FW_X is the fresh weight of callus at the last day of culture (42 days), FW_0 is the fresh weight of callus at the first day of culture.

Flow cytometric estimation of nuclear DNA content

For genome size estimation, the following plant materials were subjected to the flow cytometric analysis: (1) leaves of 30-day-old seedlings (control); (2) leaves of shoot cultures in 6th passage grown on the solid MS media: (2a) BAP 1.0 mg l^{-1}, (2b) BAP 1.0 mg l^{-1} + IAA 0.1 mg l^{-1}, and (2c) BAP mg l^{-1} + NAA 0.2 mg l^{-1}; (3) roots of micropropagated plantlets grown on the solid MS basal medium; (4) roots from agitated plantlet culture grown in the liquid MS medium with IAA 0.2 mg l^{-1}; (5) seeds collected from natural habitats; (6) seeds obtained from ex vitro grown plants. The samples were prepared as previously described by Tomiczak et al. (2016). *Pisum sativum* cv. Set (2C = 9.11 pg; Sliwinska et al. 2005) was used as an internal standard. The samples were analyzed directly after preparation by a Partec CyFlow flow cytometer (Partec GmbH, Münster, Germany), using linear amplification. The analysis was replicated five times for each plant material, and DNA content for each sample was established in 3000–5000 nuclei. Histograms were evaluated using FloMax software (Partec GmbH, Münster, Germany). CV of the G_0/G_1 peak of *L. flos-cuculi* ranged between 3.5 and 5.8%. Nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions of *Lychnis*/*Pisum* on a histogram of fluorescence intensities.

Preliminary thin layer chromatography (TLC) analysis for detection of ecdysteroids

The following material was subjected to the preliminary phytochemical analysis: (1) inflorescences, (2) flowering herb and (3) roots of plants from the natural site; (4) shoot cultures—MS + BAP 1.0 mg l^{-1} + IAA 0.1 mg l^{-1} and (5) roots on the solid MS + IAA 0.1 mg l^{-1}; (6) shoots and (7) roots of liquid agitated whole plantlet cultures—MS + IAA 0.2 mg l^{-1}; along with (8) callus cultured on MS + 2,4-D 1.0 mg l^{-1} + Kin 0.1 mg l^{-1} and (9) MS + 2,4-D 1.0 mg l^{-1} + NAA 0.1 mg l^{-1}. Plants propagated in vitro and transferred to soil (ex vitro) were also included, namely (10) flowering herb, (11) roots, and (12) seeds.

The dried material was ground and extracted three times with methanol (POCh, Gliwice, Poland) under reflux at 85 °C. The aliquots were filtered through a cotton filter, gathered, and evaporated to dryness under a rotary evaporator. The procedure for the preliminary thin-layer chromatography (TLC) analysis to confirm the presence of ecdysteroids was carried out as described by Nowak et al. (2013).

The samples of the extracts (0.1 g ml^{-1}) along with the standards of 20HE and polB, were applied to a 20 × 10 cm silica gel coated glass plate with fluorescence indicator (Merck, Darmstadt, Germany) and developed in a mixture of dichloromethane-methanol (5:1, v/v). The chromatogram was observed under UV light (λ = 254 nm), where ecdysteroid spots were dark due to absorption of UV light. To further confirm the presence of ecdysteroids, the plate was sprayed with an anisaldehyde reagent [*p*-anisaldehyde 0.5 ml (Merck, Darmstadt, Germany); glacial acetic acid 10 ml; concentrated sulfuric acid 4.5 ml; methanol 85 ml (POCh, Gliwice, Poland)]. After heating at 103 °C for about 1 min, the spots of ecdysteroids (under visible light) turned bluish-purple, and after 24 h they changed the color to yellowish-green, which is typical for ecdysteroids (Nowak et al. 2013; Głazowska et al. 2018).

Quantitative analysis by high performance liquid chromatography (HPLC) analysis for detection of ecdysteroids

The same plant material and its preparation as for TLC were used. The samples of 5.0 g were ground and extracted three times in 50 ml of methanol (POCh, Gliwice, Poland) at 85 °C under reflux. Aliquots were filtered through a cotton filter, gathered, and evaporated to dryness. The seed extract, due to presence of non-volatile oil, was inundated in water and extracted with hexane (POCh, Gliwice, Poland) five times. Aqueous phase was evaporated to dryness, yielding the defatted seed extract that was used for the analysis. All the extracts were dissolved in HPLC grade methanol (POCh, Gliwice, Poland).

Both 20HE and polB were used as analytical standards after isolation from *Serratula wolffii*, purification and identification by spectral methods, including NMR (Nowak et al. 2013). All HPLC solvents were of gradient grade: methanol (Merck, Darmstadt, Germany) and water purified with Merck Direct-Q 3UV.

The chromatography analyses were performed on Agilent 1200 SL HPLC equipped with a quaternary pump, a degasser, a diode array detector, an auto-sampler, and a thermostat column compartment. Analytical separation and guard columns were RP Select B Lichrospher 60, LiChroCART 125-4 5 µm (Merck, Darmstadt, Germany). The dimension of the separation column was 125 × 4 mm I.D. (internal diameter) with 5 µm particle size. The guard column cartridge was 4 × 4 mm I.D. with 5 µm particles of identical chemistry to the separation column. Between the sample injections, the injection needle was washed with 5% aqueous methanol. All the experiments were carried out at 20 °C and the flow-rate of the mobile phase was 1 ml min^{-1}. The autosampler injected 15 µl of each sample in triplicate. The UV absorption was measured at

λ_{max} = 242 nm in methanol, characteristic for ecdysteroid cholest-7-ene-6-one chromophore (Głazowska et al. 2018). The mobile phase contained water and methanol; the chromatography step was conducted using gradient elution. A gradient elution scheme, used for analysing active substances of *L. flos-cuculi*, was as follows: t_0 [min]—5% MeOH, t_{10}—30% MeOH, t_{27}—30% MeOH, t_{32}—5% MeOH; post time was 5 min. The analyses were performed using an external standard. Standard solutions of compounds in methanol (1.5 mg ml^{-1}) were used as the baseline to prepare the calibration curves. Eight calibrators of each ecdysteroids for DAD detection (100, 300, 500, 600, 700, 800, 1000, and 1500 µg ml^{-1}) were prepared by making serial dilutions from stock solutions.

The extracts from various plant material, prepared as described above, were dissolved in methanol, at a concentration of 10 mg ml^{-1}. The concentrations of polB and 20HE were determined based on the daily standard curve. Linearity of the HPLC detection was evaluated, and the calibration curves for both compounds were linear in the range of 100–1500 µg ml^{-1}. Parameters characterizing reliability, reproducibility, and accuracy of the HPLC analysis have been presented in Table 1.

Statistical analysis

Statistica 11 (StatSoft) software was used for performing the statistical analyses. The gathered data were subjected to a one-way analysis of variance (ANOVA) as well as Duncan's post hoc test. To determine statistical significance, a two-sided *P* value of 0.05 was applied.

Results

Shoot multiplication

The micropropagation of *L. flos-cuculi* was conducted by stimulating the growth through axillary bud formation. In contrast to the control medium, the multiplication rate was relatively high on the media containing PGRs, ranging from 8 on MS + BAP 2.0 mg l^{-1} to 16 shoots per explant on MS + BAP 1.0 mg l^{-1} + IAA 0.1 mg l^{-1} (Table 2; Fig. 2a–c). The resulting clusters of vivid green shoots were easy to separate into single shoots.

It was observed that even a low concentration of cytokinin added to the culture medium results in the increased number of produced axillary shoots, while additional enrichment of medium with auxins in some cases enhanced this effect (Table 2). Any supplementation with cytokinins yields better results than when no PGRs were added. While the addition of PGRs decreased shoot length, the resulting microshoots were still large enough (ca. 2 cm long) to be used as an explant (Table 2).

For further multiplication, MS medium containing BAP 1.0 mg l^{-1} and IAA 0.1 mg l^{-1} was used due to the highest number (16) of obtained shoots per explant. Medium containing NAA 0.1 mg l^{-1} instead of IAA, despite the high multiplication rate, often caused forming of undesirable organogenic callus at the base of the shoot.

Rooting of shoots and plant transfer into soil

The process of rooting was very similar for shoots maintained on all the tested media. The roots—very delicate and numerous in all the cases, forming dense root hairs, started to grow about 7–10 days after transfer of the single

Table 1 Reliability, reproducibility, and accuracy of the HPLC analysis

Parameter	Polypodine B	20-Hydroxyecdysone
LOD (limit of detection)	5 µg ml^{-1}	10 µg ml^{-1}
LOQ (limit of quantification)	15 µg ml^{-1}	30 µg ml^{-1}
Intra-day RSD	0.58–1.05%	0.61–1.16%
Intra-day accuracy	95–106%	96–104%
Inter-day RSD	0.78–1.13%	0.51–1.22%
Inter-day accuracy	96–108%	95–106%

LOD limit of detection, *LOQ* limit of quantification, *RSD* relative standard deviation

Table 2 Effect of plant growth regulators (PGRs) on shoot proliferation from shoot explants of *L. flos-cuculi*

MS + PGRs (mg l^{-1})		Shoot no. per explant ± SD	Shoot length (cm) ± SD
Cytokinin	Auxin		
0	0	1.23 ± 0.12^{e}	5.38 ± 0.53^{a}
BAP			
0.5	0	10.00 ± 0.69^{cd}	2.53 ± 0.13^{cd}
1.0	0	11.91 ± 0.89^{c}	2.82 ± 0.12^{c}
2.0	0	8.00 ± 0.76^{d}	2.68 ± 0.16^{bc}
3.0	0	10.57 ± 0.65^{cd}	1.68 ± 0.13^{f}
BAP	IAA		
1.0	0.1	16.35 ± 0.66^{a}	1.97 ± 0.06^{ef}
1.0	1.0	10.00 ± 0.93^{cd}	3.16 ± 0.20^{b}
2.0	0.2	13.20 ± 1.28^{bc}	2.42 ± 0.16^{cde}
BAP	NAA		
1.0	0.1	15.31 ± 1.22^{ab}	2.20 ± 0.07^{de}
1.0	1.0	12.14 ± 1.09^{c}	2.17 ± 0.09^{de}
2.0	0.2	13.18 ± 0.64^{bc}	2.44 ± 0.11^{cd}

Mean values within a column with the same letter are not significantly different at $P = 0.05$ using Duncan's Multiple Range test

BAP benzylaminopurine, *IAA* indole-3-acetic acid, *MS* Murashige & Skoog medium, *NAA* 1-naphthaleneacetic acid

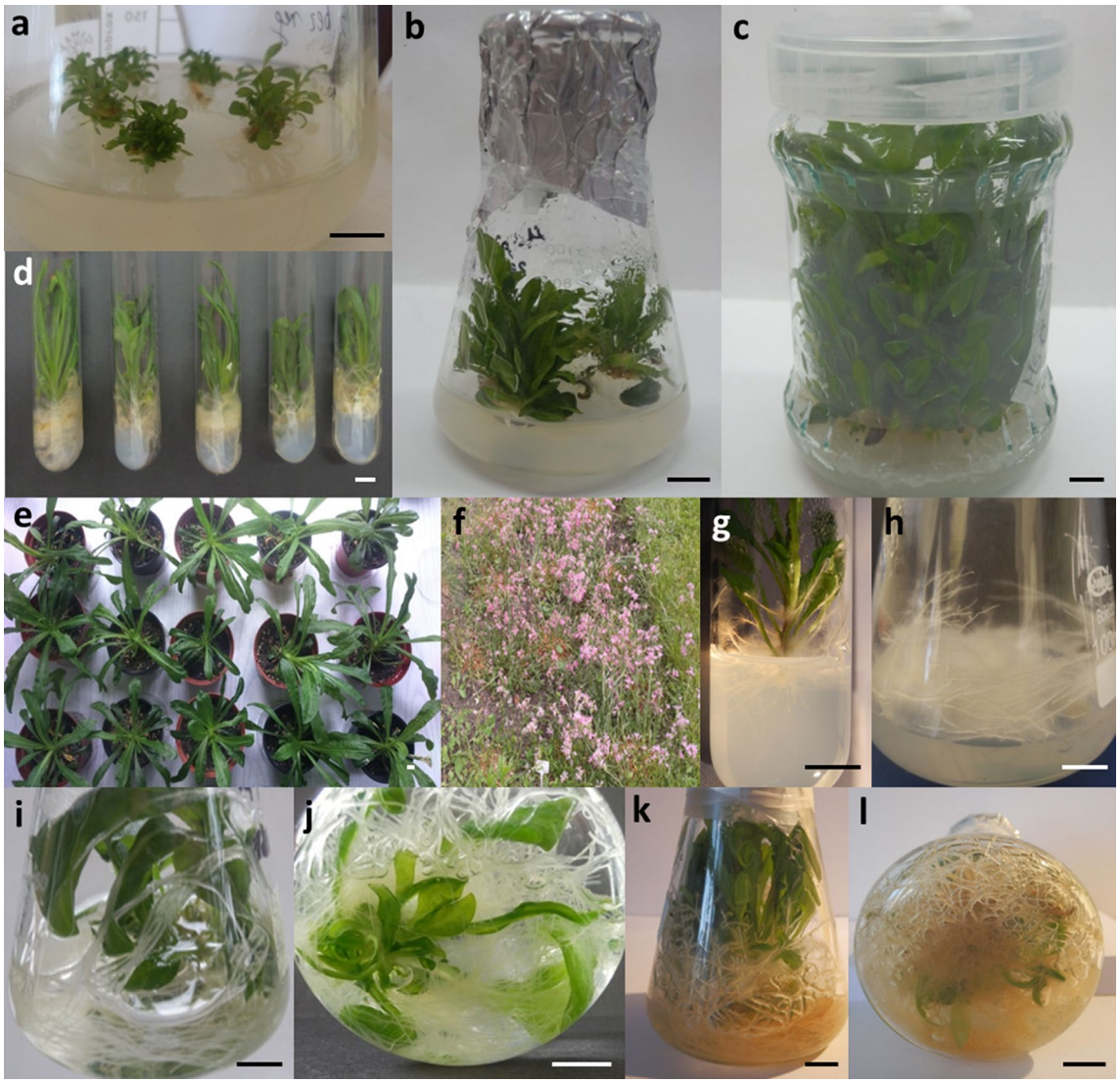

Fig. 2 Micropropagation and in vitro cultures of *L. flos-cuculi*: **a** young shoot cultures of *L. flos-cuculi*; **b** shoots on MS + BAP 1.0 mg l^{-1} + IAA 0.1 mg l^{-1} medium; **c** multiplied shoots on basal MS medium; **d** rooted shoots; **e** plantlets transferred to pots (ex vitro); **f** flowering plants on experimental plot (ex vitro); **g** plantlet with numerous roots growing above the medium and developing from the stem; **h** adventitious root culture on solid medium; **i**, **j** agitated whole plantlet culture; **k**, **l** mature agitated whole plantlet culture. The bars shown are 1 cm in width

shoot into the rooting medium. A tendency of roots to grow partially above the surface of the agar medium was observed (Fig. 2d, g); this effect was more pronounced on the auxin-supplemented media. Although the roots growing on the media supplemented with auxins, abundantly covered with root hairs, seemed denser than those growing on the basal media without supplementation of PGRs, their mass was in fact significantly lower (Table 2). The spontaneous rooting of shoots was also observed in most shoot cultures on MS or ½ MS basal medium. Overall, the tendency of *L. flos-cuculi* to regenerate roots is exceptionally strong, roots growing from the stem nodes and internodes were observed in almost all the types of shoot cultures, even on the micropropagation media containing cytokinins (Fig. 2g, h).

After the transfer of plantlets into pots and hardening by gradual exposure to non-aseptic conditions with lower humidity, the survival frequency during acclimatization in pots was high, namely 91%. The plantlets were then transferred in three batches of 25 plants to the experimental plot, with 98% survival rate during the vegetative season.

Liquid agitated whole plantlet cultures

Adventitious root tips as well as fragments of roots (1–3 cm long) transferred as explants to the liquid medium, regardless of the presence of auxins or light conditions, did not elongate and had a continuous tendency to produce callus. On the other hand, shoots placed into the liquid medium rapidly developed numerous adventitious roots. Therefore, agitated whole plantlet cultures in the liquid MS were used as an in vitro system being an abundant source of adventitious roots (Fig. 2i–l).

Based on earlier observations, the full-strength MS enriched with 0.2 mg l^{-1} IAA medium was chosen as most suitable for root formation; the roots appeared after 7 days. In a few cases, the shoots exhibited moderate hyperhydricity after about 2 weeks of culture, however, it did not seem to affect negatively the growth of roots. Agitated plantlet cultures provided large amounts of root biomass, however, their fresh weight was not higher than this produced in vitro on the half-strength solid MS without PGRs. Nevertheless, dry weight of the roots was the highest when they developed in the liquid medium (Table 3).

Callus culture

Hypocotyl fragments were the most effective explant to induce callus, with root tips being slightly less effective and cotyledons exhibiting the least capacity for callus induction. Callus was successfully induced on MS medium with different auxins, but the best induction along with the highest growth index (over 550%) was on medium supplemented with 1.0 mg l^{-1} 2,4-D, and 0.1 mg l^{-1} Kin (Table 4). Stabilized callus growing on this medium was friable and of white color (Fig. 3).

Table 3 Biomass of adventitious roots developed from single shoots of *L. flos-cuculi* after 42 days of culture

In vitro-developed roots (Medium + PGRs, mg l^{-1})	Fresh weight per plant (g) ± SD	Dry weight per plant (g) ± SD
Solid MS		
Basal (no PGRs)	0.489 ± 0.030^{b}	0.057 ± 0.004^{b}
Half-strength (no PGRs)	0.631 ± 0.030^{a}	0.057 ± 0.004^{b}
IAA 0.1	0.330 ± 0.031^{c}	0.045 ± 0.004^{bc}
IBA 0.1	0.310 ± 0.030^{c}	0.038 ± 0.004^{c}
NAA 0.1	0.308 ± 0.030^{c}	0.044 ± 0.004^{bc}
Liquid MS		
IAA 0.2	0.639 ± 0.196^{a}	0.099 ± 0.025^{a}

Mean values within a column with the same letter are not significantly different at $P = 0.05$ using Duncan's Multiple Range test

IAA indole-3-acetic acid, *IBA* indole-3-butyric acid, *MS* Murashige & Skoog medium, *NAA* 1-naphthaleneacetic acid, *PGRs* plant growth regulators

DNA content in different plant materials

The flow cytometric analysis revealed that nuclear DNA content in different plant material ranged from 5.69 to 5.87 pg/2C (Table 5; Fig. 4). However, the differences were not statistically significant (ANOVA, at $P < 0.05$). All the in vitro-derived plant material, including shoot cultures and roots as well as seeds from both ex vitro and intact plants, contained the same amount of DNA as seedlings.

TLC analysis of ecdysteroids in different plant materials

TLC was used for preliminary confirmation of the presence of ecdysteroids in different plant material. The fingerprinting with the use of ecdysteroid reference standards allowed for quick evaluation of presence of 20HE and polB in the studied extracts. The dark spots quenching fluorescence induced by 254 nm UV light, which turned bluish-purple under visible light after derivatization with anisaldehyde reagent and then gradually turned yellowish-green, confirmed the presence of ecdysteroids, namely 20HE (Rf = 0.40) and polB (Rf = 0.48). The results of preliminary TLC screening indicate that most of the analyzed extracts reveal intense spots of ecdysteroids, with exception of the agitated plant shoot extract with only pale spots and the callus extracts with no spots visible (Fig. 5).

HPLC analysis of ecdysteroids in different plant materials

To elucidate tissue specificity of ecdysteroid accumulation, 20HE and polB content in the plant material originating from each tissue and organ cultures as well as from intact

Table 4 The influence of plant growth regulators (PGRs) on *L. flos-cuculi* callus growth

MS medium (mg l^{-1})		Growth index
Auxin	Cytokinin	Mean ± SE
Pic 1.0	–	367.88 ± 28.25^{bc}
Pic 1.0	TDZ 0.1	412.79 ± 13.41^{bc}
Dic 1.0	–	391.27 ± 34.95^{bc}
Dic 1.0	TDZ 0.1	440.88 ± 14.59^{b}
2,4-D 1.0	Kin 0.1	552.17 ± 28.43^{a}
2,4-D 1.0 NAA 0.1		344.46 ± 18.46^{c}

Mean values within a column with the same letter are not significantly different at $P = 0.05$ using Duncan's Multiple Range test

2,4-D 2,4-dichlorophenoxyacetic acid, *Dic* dicamba, *Kin* kinetin, *MS* Murashige & Skoog medium, *NAA* 1-naphthaleneacetic acid, *Pic* picloram, *TDZ* thidiazuron

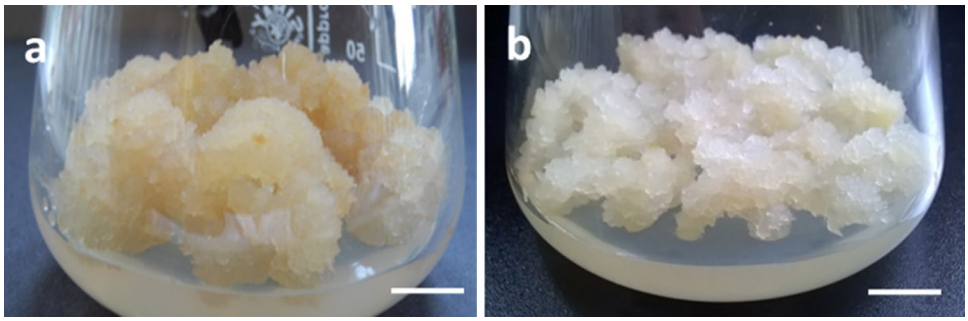

Fig. 3 Callus cultures on solid MS medium with PGRs (mg l^{-1}): **a** 2,4-D 1.0 + Kin 0.1; **b** 2,4-D 1.0 + NAA 0.1. The bars shown are 1 cm in width

Table 5 Nuclear DNA content of selected *L. flos-cuculi* plant material samples

Plant material, medium + PGRs (mg l^{-1})	Nuclear DNA content (pg) ± SD
30-day old leaves of seedlings (control)	5.775 ± 0.086^{NS}
Leaves of shoot cultures (6th passage)	
MS + BAP 1.0	5.874 ± 0.085
MS + BAP 1.0 + IAA 0.1	5.692 ± 0.087
MS + BAP 1.0 + NAA 0.1	5.833 ± 0.029
Roots of micropropagated plantlets (solid MS + IAA 0.2)	5.767 ± 0.187
Roots from liquid agitated plantlets (liquid MS + IAA 0.2)	5.792 ± 0.118
Seeds from intact plants	5.725 ± 0.022
Seeds from ex vitro plants	5.824 ± 0.019

BAP benzylaminopurine, *IAA* indole-3-acetic acid, *MS* Murashige & Skoog medium, *NAA* 1-naphthaleneacetic acid, *PGRs* plant growth regulators; ^{NS} no statistically significant difference between the values at $P = 0.05$

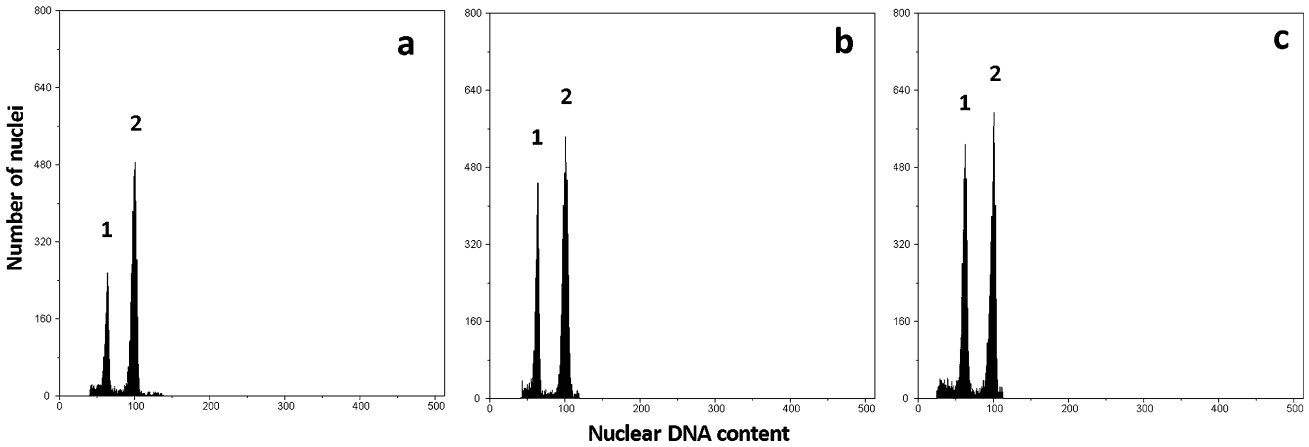

Fig. 4 Histograms demonstrating nuclear DNA content in samples of live *L. flos-cuculi* plant material from various sources: **a** leaves of 30-day old seedlings (control); **b** leaves from shoot cultures on MS + BAP 1.0 mg l^{-1} + IAA 0.1 mg l^{-1}; **c** adventitious roots induced on MS + IAA 0.2 mg l^{-1}; 1—*L. flos-cuculi*; 2—*Pisum sativum* internal standard

(natural site) and ex vitro (micropropagated) plants was estimated.

The highest accumulation of the sum of the main ecdysteroids, expressed as mg g^{-1} of dry weight by in vitro systems, was demonstrated in roots from the solid medium (6.7 mg g^{-1}) and agitated plantlets (6.1 mg g^{-1}). A similar level of ecdysteroids was found in roots of ex vitro plants (6.9 mg g^{-1}), surpassing the level in roots of intact plants (3.6 mg g^{-1}). Though the highest accumulation of main ecdysteroids was observed in the flowering herb of ex vitro plants (8.7 mg g^{-1}) and the inflorescence of intact plants (8.1 mg g^{-1}), it is the aforementioned in vitro systems that can be an alternative, renewable, and continuous source of

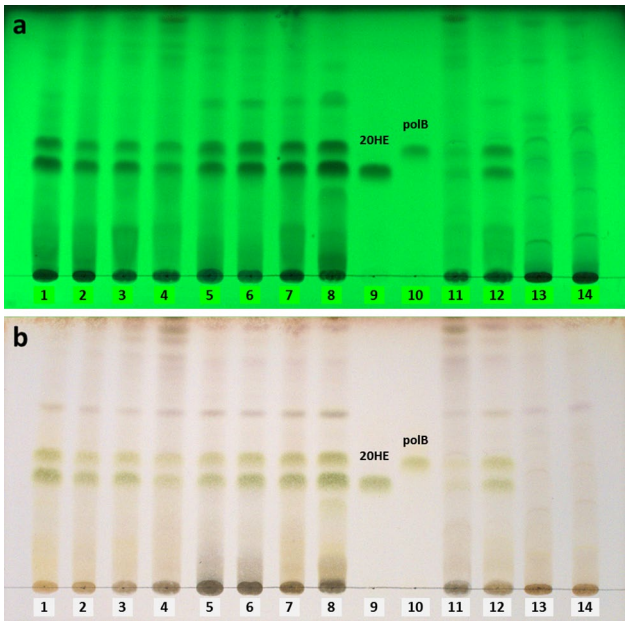

Fig. 5 Thin-layer chromatograms indicating presence of 20-hydroxyecdysone and polypodine B in extracts from various plant material of *L. flos-cuculi*. Silica gel plate with fluorescence indicator, developed in dichloromethane-methanol (5:1)—**a** under UV 254 nm and **b** 24 h after derivatization with anisaldehyde reagent (visible light). Samples: extracts from [1] inflorescence (natural site); flowering herb: [2] natural site, [3] ex vitro; [4] shoot cultures; roots: [5] natural site, [6] ex vitro, [7] in vitro cultures; [8] seeds ex vitro; standards of [9] 20-hydroxyecdysone and [10] polypodine B; extracts from agitated plantlet cultures: [11] shoots and [12] roots; callus cultured on [13] MS + 2,4-D 1.0 mg l^{-1} + Kin 0.1 mg l^{-1} and [14] MS + 2,4-D 1.0 mg l^{-1} + NAA 0.1 mg l^{-1}

ecdysteroids, with a possibility to apply further biotechnological techniques (Table 6).

The content of ecdysteroids in various plant material depends on a culture type. Generally, higher levels in roots than in shoots of in vitro cultures were observed. The levels of ecdysteroids in roots from plantlet in vitro cultures on the solid MS (3.5 mg g^{-1} 20HE, 3.2 mg g^{-1} polB) also surpass the level in roots of intact plants almost twice, while levels in roots from agitated whole plantlet cultures (2.5 mg g^{-1} 20HE, 3.6 mg g^{-1} polB) are higher by 1.3- and 2.1-fold, respectively. In shoot cultures from the solid medium, only the content of polB is higher than in herb of intact plants. The shoots of agitated plant cultures, however, have the lowest levels of both ecdysteroids. Almost all of the investigated materials contain more 20HE than polB, apart from shoot cultures from the solid medium and shoots and roots of plantlets from agitated cultures.

Ex vitro plants maintain the high biosynthetic capability of producing ecdysteroids, and the content of both ecdysteroids in ex vitro flowering herb (4.8 mg g^{-1} 20HE, 3.9 mg g^{-1} polB) and roots (3.7 mg g^{-1} 20HE, 3.2 mg g^{-1} polB) is among the highest, it is twofold higher than the content in respective organs of a plant from the natural site. Inflorescence is the organ of intact plants most abundant in ecdysteroids, containing 4.6 mg g^{-1} 20HE and 3.5 mg g^{-1} polB (Table 6).

HPLC analyses did not confirm the presence of ecdysteroids in hypocotyl-derived callus cultures of *L. flos-cuculi* on MS + 2,4-D 1.0 mg l^{-1} + Kin 0.1 mg l^{-1} and MS + 2,4-D 1.0 mg l^{-1} + NAA 0.1 mg l^{-1} (Table 6). Example HPLC chromatograms have been presented in Fig. 6.

Discussion

The natural habitat of *L. flos-cuculi* is mainly wet meadows, floodplains, marshes, and ditches. These environments are nowadays subjected to man-made changes as they are meliorated and transformed into arable lands. Though the species is not rare, its population is on decline. Moreover, the fragmentation of populations of *L. flos-cuculi* leads to the loss of genetic diversity and inbreeding (reviewed by Maliński et al. 2014). The above factors encouraged the authors to introduce this species into in vitro cultures and to multiply its biomass under controlled conditions for the phytochemical analyses.

A few related species from the genus *Lychnis* have been already successfully introduced to in vitro cultures: *L. senno* (Chen et al. 2006a, b), *L. wilfordii* (Bae et al. 2014), *L. fulgens*, and *L. cognata* (Bae and Yoon 2015). In this study, different cultures of *L. flos-cuculi* have been obtained for the first time. Compared to the other species, the multiplication rate for *L. flos-cuculi* is significantly higher. The mean number of shoots per explants was over 16 growing on MS medium enriched with BAP 1.0 mg l^{-1} and IAA 0.1 mg l^{-1}. In contrast, a single *L. senno* shoot tip explant resulted in formation of at most three axillary shoots on MS medium containing BAP 5.0 mg l^{-1} with NAA 0.5 mg l^{-1}. The shoot cultures of the other species, namely *L. cognata*, *L. fulgens*, and *L. wilfordii,* were all established through indirect organogenesis, therefore the efficiency of multiplication coefficient is difficult to compare (Bae et al. 2014; Bae and Yoon 2015). For *L. flos-cuculi*, the presence of auxins in the media was not crucial in the process of rhizogenesis. Roots formed spontaneously on all the types of used media. The shoots of *L. senno* required the addition of at least NAA 0.5 mg l^{-1} for rooting (Chen et al. 2006a, b). There have been no reports on accumulation of ecdysteroids in obtained in vitro tissues of those *Lychnis* species.

Plant tissue cultures offer a possibility to propagate the species in a strictly controlled environment and the micropropagated plants from explant with existing meristems usually retain the genetic stability and uniformity (Sliwinska and Thiem 2007; Kikowska et al. 2014). It is especially important in research of potential medicinal plants and the

Table 6 Content of 20-hydroxyecdysone (20HE) and polypodine B (polB) in *L. flos-cuculi* plant material from various sources, quantified by HPLC

Plant material	Main ecdysteroid content in dry mass		
	20HE (mg g^{-1}) ± SE	polB (mg g^{-1}) ± SE	Sum (mg g^{-1})
Intact plants			
Inflorescences	4.6 ± 0.09^{b}	3.5 ± 0.09^{b}	8.1
Flowering herb	2.3 ± 0.05^{e}	1.6 ± 0.06^{e}	3.9
Roots	1.9 ± 0.07^{f}	1.7 ± 0.06^{e}	3.6
In vitro cultures (solid MS)			
Shoots	1.8 ± 0.02^{f}	2.3 ± 0.14^{d}	4.1
Roots	3.5 ± 0.07^{c}	3.2 ± 0.06^{c}	6.7
Liquid agitated plantlet cultures			
Shoots	0.4 ± 0.01^{g}	1.0 ± 0.02^{f}	1.4
Roots	2.5 ± 0.05^{e}	3.6 ± 0.03^{b}	6.1
Ex vitro plants			
Flowering herb	4.8 ± 0.05^{a}	3.9 ± 0.08^{a}	8.7
Roots	3.7 ± 0.06^{c}	3.2 ± 0.06^{c}	6.9
Seeds	2.8 ± 0.03^{d}	2.1 ± 0.04^{d}	4.9
Calli (MS + PGRs [mg l^{-1}])			
2,4-D 1.0 + Kin 0.1	Not detected	Not detected	–
2,4-D 1.0 + NAA 0.1	Not detected	Not detected	–

Mean values within a column with the same letter are not significantly different at $P = 0.05$ using Duncan's Multiple Range test

2,4-D 2,4-dichlorophenoxyacetic acid, *Kin* kinetin, *MS* Murashige & Skoog medium, *NAA* 1-naphthaleneacetic acid

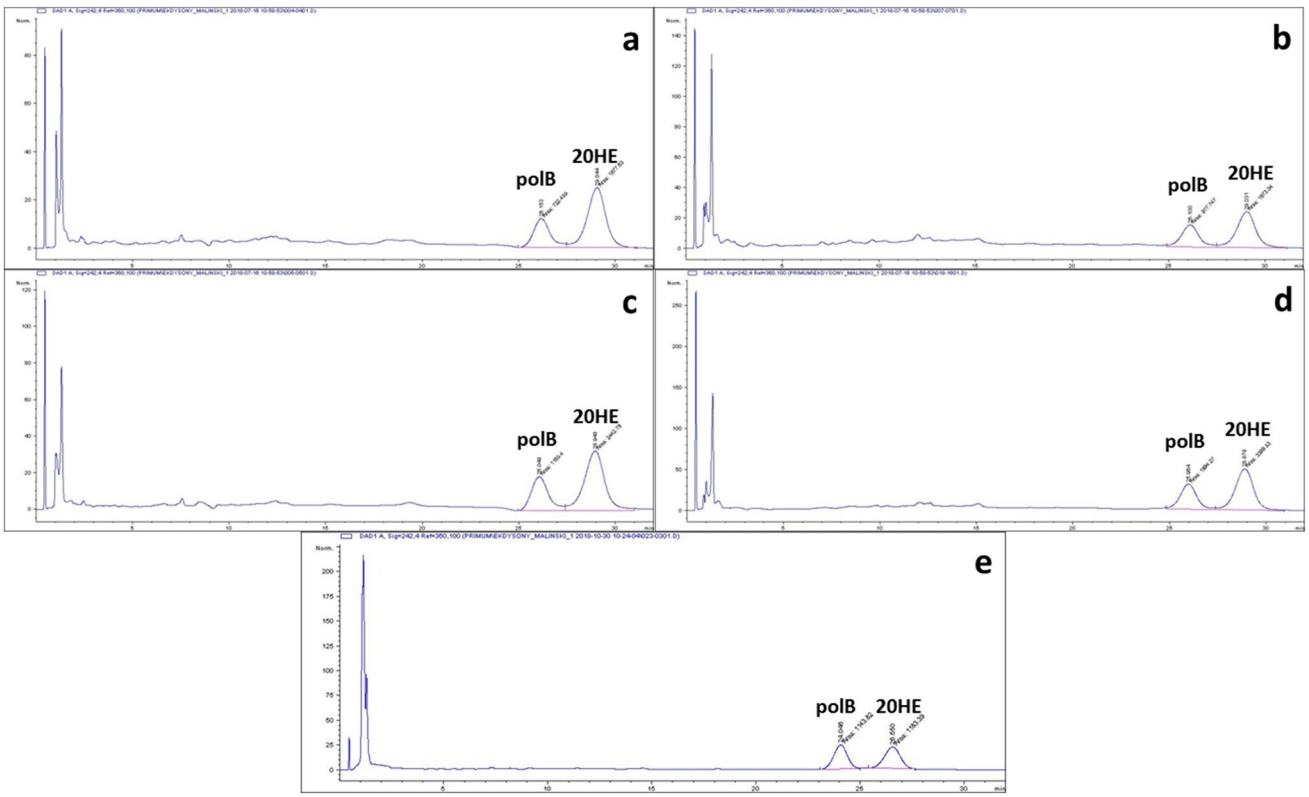

Fig. 6 HPLC chromatograms demonstrating content of 20-hydroxyecdysone (20HE) and polypodine B (polB) in extracts from selected *L. flos-cuculi* plant materials: **a** flowering herb and **b** roots of plant from natural site; **c** flowering herb and **d** roots of ex vitro plant; **e** roots of liquid agitated whole plantlet cultures

phytochemical analysis of biologically active constituents, as genetic uniformity in a given environment mostly determines a uniform phytochemical profile as well (Smetanska 2008; Filova 2014; Chandana et al. 2018). Methods based on the flow cytometry are applied to ascertain that in vitro culture conditions do not affect the cytogenetic stability of a plant as well as the occurrence of somaclonal variations. DNA content in several micropropagated species was evaluated after growing in in vitro conditions (Thiem and Sliwinska 2003; Thiem et al. 2013; Sliwinska 2018). In the presented studies, the stability of genome size (about 5.7–5.8 pg/2C) in different plant materials indicates that in vitro cultures of *L. flos-cuculi* are a viable, homogenous, alternative, and renewable source of plant material.

The model of agitated shoot cultures or liquid whole plantlet cultures, which aimed at optimizing the biosynthetic capabilities of cultured species and accumulating the desired metabolites was employed earlier by other authors, for example, in cultures of *Centella asiatica* (Kim et al. 2004), *Aronia arbutifolia*, *A.* × *prunifolia* (Szopa et al. 2018), *Hypericum perforatum* (Kwiecień et al. 2015), *Knautia sarajevensis* (Karalija et al. 2017), and *Rhododendron tomentosum* (Jesionek et al. 2017).

The advantages of this model include more efficient saturation of medium with gasses and continuous agitation of the medium leading to the equal distribution of nutrients and their better availability, associated with increased production of secondary metabolites (Jesionek et al. 2017). Additionally, in the case of *L. flos-cuculi*, it positively affects the rate of rhizogenesis. According to literature data, adventitious root cultures can exhibit active secondary metabolism and vigorous growth in the media supplemented with PGRs. Moreover, these roots are natural and genetically stable (Zakirova and Yakubova 2002; Murthy et al. 2008). Many medicinally valuable substances of diverse chemical structure are produced by adventitious root cultures with high efficiency, which is the consequence of their unique biosynthetic capabilities as an organ, as well as optimization of medium composition and enrichment with PGRs (Bais et al. 2001; Hahn et al. 2003). Biosynthesis of ginsenoside by *Panax ginseng* (Kim et al. 2007) and with anolide A by *Withania somnifera* (Praveen and Murthy 2010) adventitious root cultures are examples of efficient production of triterpenoid-related compounds. Additionally, adventitious root cultures of many valuable medicinal plants, such as *Morinda citrifolia*, *Echinacea* sp., *Hypericum perforatum* and *Panax ginseng* can be upscaled for growth in bioreactors exceeding 1000 l of volume, as described in a review by Baque et al. (2012). It is also convenient to enrich the medium with agents such as precursors or elicitors for future studies. Attempts to excise the roots and use them as explant to establish adventitious root cultures failed; it seems that efficient and stable rhizogenesis requires the photosynthesizing part of a plant.

One of biotechnological methods is the establishment of callus and cell suspension cultures, which often can be an ample source of biologically active compounds. However, biosynthesis of some secondary metabolites, including ecdysteroids, occur in organized tissues (Cheng et al. 2008). The absence of ecdysteroids in *L. flos-cuculi* callus suggests that they require more organized tissue to be produced, as undifferentiated plant cells cultures often lose their ability to accumulate certain secondary metabolites (Zakirova and Yakubova 2002). For example, callus cultures of *Rhaponticum carthamoides* (Skała et al. 2015) and *Ajuga reptans* (Tomas et al. 1992) were unable to synthesize ecdysteroids. Another example is *Chenopodium album* cell culture that produced only a fraction (10 µg g^{-1} DW) of nominal level present in roots (377 µg g^{-1}) (Corio-Costet et al. 1993). In contrast, it is known that cell suspension cultures of certain species, for example, *Ajuga turkestanica*, are able to produce ecdysteroids with high efficiency (Cheng et al. 2008) at 6.9 µg mg^{-1} in dry extract. However, Zakirova and Yakubova (2002) reported that the content of ecdysteroids in cell suspension culture of this species significantly decreased after 4 years.

In the presented research, callus of *L. flos-cuculi* did not accumulate ecdysteroids but produced triterpenoid saponins, compounds of similarly complex structure; that suggests that triterpenoid biosynthetic route, a prerequisite necessary for ecdysteroid biosynthesis, was functioning during the culture. The presence of complex, unidentified triterpenoid saponins in the callus extract and micropropagated plants was found during preliminary TLC and HPLC analysis (Stochmal, unpublished data). The TLC protocol used for the qualitative analysis of main ecdysteroids is a rapid analytical method for convenient preliminary screening before qualitative methods (HPLC) are employed.

The level of ecdysteroids in *L. flos-cuculi* from the natural site compared to the other plants is relatively high. The present comparative analysis revealed that organs of intact plants contained the sum of the main ecdysteroids, namely inflorescence—0.81%, flowering herb—0.39%, and roots—0.36%. In most plants the content varies from 0.001 to 0.1% of dry weight, with only several taxa reported surpassing 1% (equal to 10 mg g^{-1}),—for example, *Rhaponticum carthamoides* herb—ca. 1% (Głazowska et al. 2018), *Serratula inermis* inflorescence—2%, *Cyanotis arachnoidea* roots—2.9%, and *Diploclisia glaucescens* stems—3.2% (Baltaev 2000). Genus *Silene,* closely interrelated to *Lychnis*, includes numerous species also exceptionally rich in diverse complex of ecdysteroids, with 20HE as dominant constituent (Báthori et al. 2000; Mamadalieva et al. 2014). Variations in ecdysteroid content occur among different plant organs, additionally

changing during the vegetative season, mostly peaking in reproductive organs (Zibareva 2000; Zibareva et al. 2003).

In this study, roots cultured in different in vitro systems, including micropropagated plants—roots from in vitro cultures (6.7 mg g^{-1}; 0.67%, sum of 20HE and polB) and from liquid agitated plantlet cultures (6.1 mg g^{-1}; 0.61%) were the most promising source of ecdysteroids. Roots from in vitro cultures are therefore an alternative source of biomass that is difficult to obtain from a natural source due to delicate nature and fragility of roots, coexistence with other plant species, and mutual intertwining of roots and rhizomes, which is especially dense at wet meadows. There are numerous literature positions on the ecdysteroids production in in vitro systems (Thiem et al. 2017).

For *R. carthamoides*, the level of 20HE in dried leaves and roots of plantlets developed from callus tissue was reported as 0.74% and 0.304%, respectively (Skała et al. 2015). Prothalli of *Polypodium vulgare*, under stress conditions, produced 2.23% of dry weight (Reixach et al. 1997). A study on *Achyranthes aspera* showed that levels of 20HE in hairy roots vary between 0.019 and 0.04% of dry weight, compared to 0.009% in roots and 0.025% in seeds of intact plant (John et al. 2017).

Rich sources of ecdysteroids are of particular interest due to promising perspectives on the use of these compounds in many subdisciplines of medical sciences. As multi-drug resistance inhibitors, they can become a valuable adjuvant in cancer chemotherapy, while their antioxidant, neuroprotective, and anti-apoptotic activities can prove useful in prevention or treatment of neurodegenerative diseases and ischemic injuries (Thiem et al. 2017).

Conclusions

In vitro cultures of *L. flos-cuculi* have been established for the first time in order to obtain the plant material for phytochemical investigation in search of ecdysteroids with potential medicinal properties. Organs from agitated cultures, shoot cultures and micropropagated plantlets are able to biosynthesize ecdysteroids, in contrast to undifferentiated callus. High-quality, uniform in vitro-propagated plantlets accumulate more ecdysteroids compared to plants from the natural site.

Adventitious roots from *L. flos-cuculi* liquid agitated whole plantlets culture can be an alternative, renewable, and continuous biotechnological source of plant biomass rich in pharmaceutically active ecdysteroids. The liquid agitated whole plantlet cultures can constitute a reliable in vitro system for convenient application of advanced biotechnological techniques and potential up-scaling to further increase the yield of ecdysteroids.

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Author contributions BT, MM and MK conceived and designed the experiments, wrote the paper, and analyzed the data. MM and DK performed in vitro experiments and TLC analyses. MN and EF performed HPLC analyses. ES performed and evaluated the flow cytometry analyses and contributed to preparing the manuscript. All the authors participated in proofreading of the article and accepted its final version.

Compliance with ethical standards

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

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