www.czasopisma.pan.pl PAN POLSKA AKADEMIA NAUK www.journals.pan.pl

ACTA BIOLOGICA CRACOVIENSIA Series Botanica 61/1: 75–86, 2019
DOI: 10.24425/118060

PAN POLSKA AKADEMIA NAUK ODDZIAŁ W KRAKOWIE

PHENYLETHANOID GLYCOSIDES IN *PLANTAGO MEDIA* L. ORGANS OBTAINED IN *IN VITRO* CULTURES

ANNA BUDZIANOWSKA*, MAŁGORZATA KIKOWSKA, MAGDALENA MAŁKIEWICZ, INGA KAROLAK AND JAROMIR BUDZIANOWSKI

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

Received October 16, 2018; revision accepted December 3, 2018

Micropropagation of *Plantago media* L. and the presence of phenolic compounds in organs of multiplied plants were investigated for the first time. Multiplication of plant material was achieved in shoot-tip cultures and via direct organogenesis on Murashige and Skoog (MS) medium with four variants of plant growth regulators (M1–M4). The best multiplication coefficient – 9.2 was obtained in seedling shoot-tip cultures on MS medium M3 with BA 0.2 mg/L and IAA 1.0 mg/L. Methanol extracts prepared separately from shoots and roots of *in vitro*-derived plantlets were found to contain typical of the genus *Plantago* L. phenylethanoid glycosides as the only phenolics. Acteoside and plantamajoside were the major compounds – both known to possess a wide range of promising biological activities applicable for medicinal (therapeutic) and cosmetic uses. Martynoside, as a trace constituent, was also found for the first time in the studied species. The quantitative screening of the extracts by TLC video densitometric method showed a higher content of acteoside in shoots (range 62.43–93.03 mg/g, dry weight) and plantamajoside in roots (range 22.45–44.08 mg/g); the highest recorded values – 93.03 mg/g and 44.08 mg/g, respectively, were found in the organs obtained on MS medium M4 with BA 2.0 mg/L.

Keywords: acteoside, direct and indirect organogenesis, hoary plantain, micropropagation, phenylethanoids, plantamajoside, TLC densitometry

INTRODUCTION

Plantago media L. (Plantaginaceae) is a cosmopolitan wild perennial, which can be found in the Polish flora together with nine other species (Tacik, 1967). According to Flora Europea, it has one or a few rosettes consisting of elliptical to ovate-elliptical hairy leaves gradually narrowed into a petiole, usually less than half as long as the lamina (Chater and Cartier, 1967). *P. media* is a species with medicinal properties related to *P. lanceolata* and *P. major* and like them having similar usage (Jędrzejko, 1997). Plants from the genus *Plantago* exhibit many medicinal activities, which are assumed to be affected by the occurrence of polysaccharides, iridoids, phenylethanoid glycosides, triterpenes, flavonoids and phenolic acids (Gonçalves and Romano, 2016). In *P. media* the following chemical constituents were found: iridoid glucosides (aucubin, 10-*O*-acetylaucubin, melittoside, monomelittoside, 10-aetylmonomelittoside), polysaccharides, phenylpropanoids (caffeic acid, chlorogenic acid, including phenylethanoid glycosides – acetoside, plantamajoside (Fig. 1)) (Rønsted et al., 2003; Olennikov et al., 2011), flavonoids (apigenin, apigenin 7-*O*-glucoside, rutin) (Beara et al., 2009), phenolic acids (p-hydroxybenzoic acid, vanilic acid, gentyzinic acid, syringic acid, p-coumaric acid, ferulic acid) (Świątek, 1977).

The major phenolics in the genus *Plantago* are phenylethanoid glycosides represented mainly by acteoside and plantamajoside (Rønsted et al., 2000; Rønsted et al., 2003). Acteoside (verbascoside), which occurs in over 20 plant families of Asteridae (Schlauer et al., 2004; Alipieva et al., 2014), exhibits a wide range of activities: antioxidant, antiproliferative, cytotoxic, anticancer, antimetastatic, anti-inflammatory, and immunomodulatory (Alipieva et al., 2014).

* Corresponding author, email: abudzian@ump.edu.pl

PL ISSN 0001-5296

© Polish Academy of Sciences and Jagiellonian University, Cracow 2019

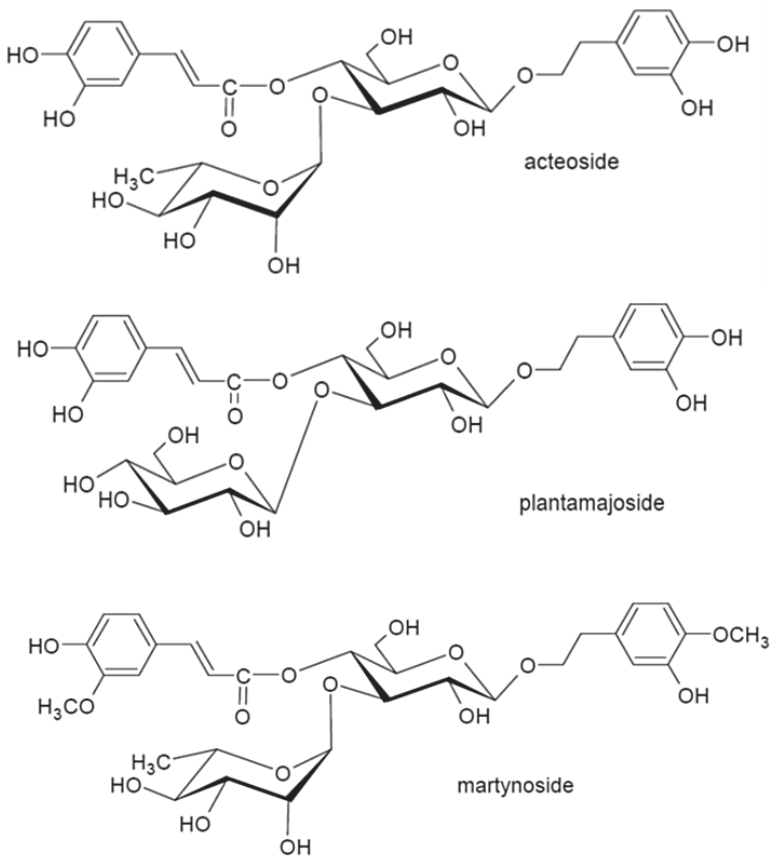

Fig. 1. Chemical structures of acteoside, plantamajoside and martynoside.

Moreover, acteoside, as an antioxidant compound, may be used in dermocosmetic and pharmaceutical topical formulations (Vertuani et al., 2011). Acteoside and martynoside exhibit antiestrogenic effects (Papoutsi et al., 2006). Plantamajoside, which was found in three plant families (Ravn et al., 2015), has numerous biological and pharmacological activities: antioxidant, antibiotic, antifungal, anti-inflammatory, cytotoxic, and enzyme inhibitory (Ravn et al., 2015).

So far, *in vitro* propagation of *Plantago* species was investigated for *P. asiatica* (Makowczyńska and Andrzejewska-Golec, 2003; Zeng, 2008), *P. camtschatica* (Andrzejewska-Golec and Makowczyńska, 2008), *P. lanceolata* (Fons et al., 1998; Fons et al., 1999; Budzianowska et al., 2004; Khawar et al., 2005), *P. major* (Mederos et al., 1997/1998; Saker and Kawashity, 1998), *P. maritima* (Makowczyńska and Andrzejewska-Golec, 2009) and *P. ovata* (Barna and Wakhlu, 1998; Pramanik et al., 1995).

The strong influence of environmental conditions in the natural habitats of plants on the presence of bioactive compounds in *Plantago* inclined scientists to use the biotechnological methods to obtain a high and constant content of desired metabolites in the biomass from shoots and roots of the micropropagated plantlets. *In vitro* techniques provide a perfect opportunity to produce a large amount of *Plantago media* biomass, rich in bioactive compounds, without environmental restrictions.

The aim of the current study was to develop an efficient method for *in vitro* propagation of *Plantago media* L. by comparison of the three techniques: development from shoot tips, regeneration via direct and indirect organogenesis. Moreover, the biomass obtained from *in vitro* cultures – shoots and roots of the micropropagated plantlets from selected MS media was analyzed for the presence of phenolic compounds to find phenylethanoids – acteoside and plantamajoside. To the best of our knowledge, this is the first report on *P. media* micropropagation and qualitative and quantitative estimation of these phenylethanoids in *in vitro* cultures of this species.

MATERIAL AND METHODS

PLANT MATERIAL, MEDIA COMPOSITION, AND SURFACE STERILIZATION

Plant material. The voucher specimens of *P. media* (no. PM001/2012) is deposited in the Herbarium of Department of Pharmaceutical Botany and Plant Biotechnology, Poznan University of Medical Sciences, Poland. Mature seeds of *P. media* were collected from the botanical garden of Department of Medicinal and Cosmetic Products, Poznan University of Medical Sciences (52°25'30.7" N, 16°53'50.8" E), in July 2012. Botanical identity was confirmed by nuclear DNA content using flow cytometry with comparison to the reference standard (Thiem et al., 2013).

Media composition. Culture media consisted of MS (Murashige and Skoog, 1962) media solidified with 0.8% (w/v) agar (Sigma-Aldrich) with 30 g/L (w/v) sucrose and appropriate plant growth regulators (PGRs) at various concentrations (Tables 1, 2, 3). The PGRs originated from Sigma-Aldrich. After adjusting pH to 5.8, the media were autoclaved. The cultures were incubated in an air-conditioned growth chamber (16/8 h photoperiod, 55 μmol/m^2s^1 light, temp. 21 ± 2°C).

Surface sterilization of seeds. For aseptic culture initiation, the seeds were surface decontaminated. They were washed under running tap water and then rinsed in distilled water followed by submerging in 70% (v/v) EtOH for 30 s to defat the seeds. The disinfection step was to treat the

TABLE 1. Influence of PGRs on shoot induction (%) in *Plantago media* shoot-tip cultures in three subcultures.

Abbrev.	MS medium		No. of subculture		
	Cytokinin (mg/L)	Auxin (mg/L)	1st(%)	2nd(%)	3rd(%)
	BA	NAA			
M1	2.0	0.1	100	94.6	83.2
	BA	IAA			
M2	2.0	0.1	100	91.3	81.6
M3	2.0	1.0	100	75.0	73.3
M4	2.0	–	100	100	100

BA – 6-benzyladenine; **IAA** – indole-3-acetic acid; **MS** – Murashige and Skoog medium; **NAA** – α-naphthaleneacetic acid; 56, 174, 183 explants per 1st, 2nd and 3rd subculture, respectively.

TABLE 2. Influence of PGRs on shoot formation (MC ± SE) originating from *Plantago media* seedling shoot tips.

Abbrev.	MS medium		No. of passage		
	Cytokinin (mg/L)	Auxin (mg/L)	Development from seedling tips	Shoot proliferation	
			1st	2nd	3rd
	BA	NAA			
M1	2.0	0.1	5.1 ± 0.1b	2.9 ± 0.1b	2.4 ± 01a
	BA	IAA			
M2	2.0	0.1	4.9 ± 0.1b	1.3 ± 0.1c	3.0 ± 0.2a
M3	2.0	1.0	9.2 ± 0.3a	3.6 ± 0.2a	2.8 ± 0.2a
M4	2.0	–	2.1 ± 0.1c	4.0 ± 0.2a	2.6 ± 0.1a

BA – 6-benzyladenine, **IAA** – indole-3-acetic acid, **MS** – Murashige and Skoog medium; **NAA** – α-naphthaleneacetic acid; Mean values within a column with the same letter are not significantly different at P = 0.05 (one-way analysis variance ANOVA followed by Duncan's Multiple Range test /STATISTICA v.13; StatSoft, Inc. 2015).

TABLE 3. Influence of PGRs and type of explant on shoot direct organogenesis and shoot proliferation (MC) in *Plantago media*.

MS medium	PGRs		Type of seedling expl. for 1st passage (1.) / rosettes for 2nd (2.) – 3rd (3.) passage											
			Organogenesis				Proliferation							
Abbrev.	Cytokinin (mg/L)	Auxin (mg/L)	Leaves	Cotyledons	Roots	Hypocotyls	Leaf-derived shoots		Cotyledon-derived shoots		Root-derived shoots		Hypocotyl-derived shoots	
			1.	1.	1.	1.	2.	3.	2.	3.	2.	3.	2.	3.
	BA	NAA												
M1	2.0	0.1	1.5	–	4.2	2.3	2.4	2.5	–	–	1.4	2.6	1.7	3.0
	BA	IAA												
M2	2.0	0.1	2.2	4.6	4.6	8.5	3.1	4.8	4.2	2.6	5.2	2.7	1.1	1.8
M3	2.0	1.0	0.8	2.8	3.4	6.8	2.6	2.9	0.7	1.9	1.8	2.9	0.9	2.3
M4	2.0	–	0.5	2.3	8.0	2.8	0.9	0.1	1.1	2.4	1.8	1.6	1.6	1.3

BA – 6-benzyladenine, **IAA** – indole-3-acetic acid, **MS** – Murashige and Skoog medium; **NAA** – α-naphthaleneacetic acid.

seeds with commercial bleach at a concentration of 25% for 20 min. They were finally rinsed five times in sterilized distilled water and transferred to ½ MS (reduced concentration of macro- and micronutrients) medium to obtain aseptic seedlings. After 1–8 weeks of incubation the *in vitro* germinated seedlings, were the source of the primary explants (shoot tips, leaves, cotyledons, roots, hypocotyls) (Figs. 2 and 3).

Seedling shoot-tip cultures and shoot proliferation. The shoot tips of seedlings in the first subculture and the single shoots from plantlets in the second and third subcultures were placed in 250–300 cm^3 Erlenmeyer flasks with 50–70 cm^3 MS media. The media were supplemented with selected PGRs: 6-benzyladenine (BA) and indole-3-acetic acid (IAA) or α-naphthaleneacetic acid (NAA) to give four media variants (Tables 1 and 2).

For this experiment, four variants of MS medium (M1–M4) were used with 14 explants per one variant. The shoots were multiplied by transfer of single shoots to a freshly prepared medium every 6 weeks of subculture. Multiplication of the shoots was replicated twice. The coefficient of shoot proliferation was calculated by the formula MC = DSN/EN (MC – multiplication coefficient; DSN – number of developed shoots; EN – number of explants). After the third subculture, some vigorous and healthy shoots were transferred to a rooting medium with a selected auxin.

Direct organogenesis and shoot proliferation. Fragments of aseptic seedlings (leaves, cotyledons, roots and hypocotyls) were placed in 250–300 cm^3 Erlenmeyer flasks with 50–70 cm^3 MS media for direct shoot formation (without callus stadium). The media were supplemented with PGRs: 6-benzyladenine (BA) and indole-3-acetic acid (IAA) or α-naphthaleneacetic acid (NAA) to give four media variants (Table 3). The regenerated and multiplied shoots were separated. The single shoots were transferred to a freshly prepared medium with the PGRs for the second and third subculture.

Callus induction and indirect organogenesis. Fragments of aseptic seedlings (leaves, cotyledons, roots and hypocotyls) were placed in 250–300 cm^3 Erlenmeyer flasks with 50–70 cm^3 MS media for callus induction. The media were supplemented with selected PGRs: 2,4-dichlorophenoxyacetic acid (2,4-D; 1.0–2.0 mg/L), 6-benzyladenine (BA; 1.0–2.0 mg/L), and kinetin (Kin; 0.5 mg/L). In this experiment the best explant and medium variants for callus induction were selected and the number of regenerated shoots was counted.

Rooting of shoots and acclimatization of in vitro-propagated plants. The healthy shoots, which were not selected for phytochemical analyses, were separated and transferred into 250–300 cm^3 Erlenmeyer flasks with 50–70 cm^3 MS medium alone or with IAA 1.0 mg/L. The percentage of

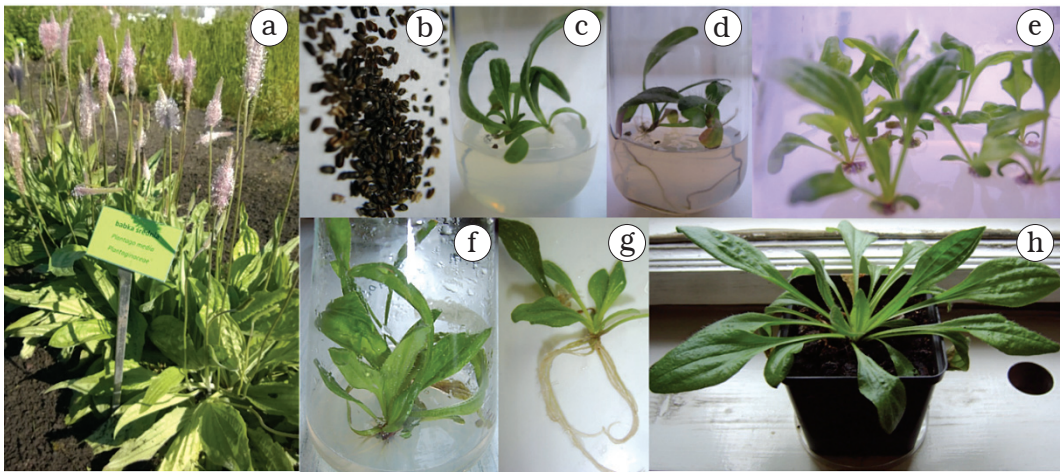

Fig. 2. *Plantago media* L. micropropagation via shoot tip explants. (**a**) Flowering plant from garden of medicinal plants. (**b**) Seeds. (**c**) 15-days-old seedling. (**d**) 50-days-old seedling. (**e**) *In vitro*-multiplied shoots. (**f**) Plantlets with developed roots. (**g**) Rooted plantlet. (**h**) Hardened plant in plastic pot.

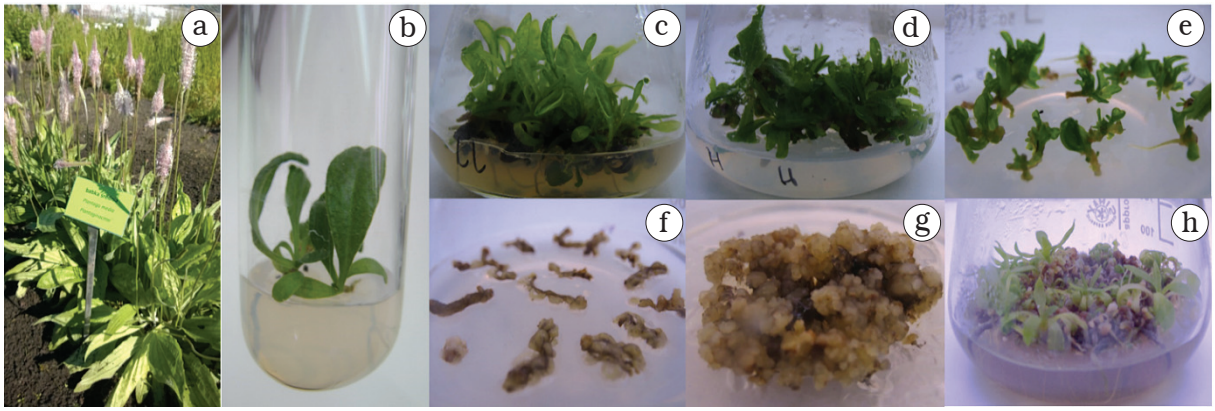

Fig. 3. *Plantago media* L. shoot regeneration via direct and indirect organogenesis. (**a**) Mature flowering plant from garden of medicinal plants. (**b**) 15-days-old seedling. (**c-e**) Shoots regenerated from: cotyledons (**c**), hypocotyls **(d)** and roots (**e**). (**f**) Callus initiation on root explants, (**g**) callus, (**h**) shoots and roots regenerated from callus.

rooting, and the number and length of roots were recorded. The plants with well-developed roots were placed in pots containing sterile soil and covered with glass beakers for 14 days.

PHYTOCHEMICAL SCREENING

Preparation of extracts. 2.000 g portions of dried shoots and roots (from four media variants M1–M4) were each extracted three times with boiling methanol (50–60 mL) for 1h each time. The combined extracts were rotary evaporated under reduced pressure at 40°C to dryness and weighted (Table 2).

Analysis of extracts. Solutions of extracts in 70% ethanol (100 mg/mL), along with standard solutions of phenylethanoid glycosides (1 mg/mL) were applied (5 mL) to TLC (thin-layer chromatography) plates. The following stationary and mobile phases were used: silica gel (pre-coated, Merck) developed with ethyl acetate-acetic acid-water 4:1:1 (v/v/v), cellulose (pre-coated, Merck) developed two-dimensionally with 1-butanol-acetic acid-water 4:1:5 (v/v/v, organic layer) followed by 15% acetic acid, polyamide DC6 (Macherey-Nagel, home-made) developed with water-1-butanol-acetone 16:3:3 (v/v/v) or ethyl acetate-ethanol-water 20:3:2 (v/v/v). The compounds on chromatograms

were visualized under UV 254 nm and 366 nm light before and after spraying with 0.1% NA (2-aminoethanol diphenylborate, Roth) in ethanol (for general detection of phenolics) or 1% aluminum chloride in ethanol followed by heating (for detection of flavonoids) (Budzianowski et al., 1999; Schlauer et al., 2004).

Fractionation of extracts from shoots and roots from medium M3. Dry methanol extracts were each suspended in water (10 mL), extracted with dichloromethane 3 × 10 mL and the obtained fractions were evaporated to dryness. Portions of water fractions (each 0.1 g) were separated on polyamide SC6 (50–160 mm, Macherey-Nagel) columns (1 × 10 cm) by sequential elution with water, methanol, 0.1% conc. ammonia in methanol (v/v) and 0.1% formic acid in methanol (v/v). The collected fractions were concentrated under reduced pressure, re-dissolved in 70% ethanol and examined by TLC. Only the methanol fractions (shoots – 24.6 mg, roots – 15.9 mg) contained phenolic compounds, which were phenylethanoid glycosides, mainly acteoside and plantamajoside by comparison with the reference samples isolated from *Plantago lanceolata* (Budzianowska et al., 2004).

A 0.1 g sample of the water fraction from the shoots was separated on a polyamide column (1.5 × 15 cm) eluted with ethyl acetate-ethanol-water 30:3:2 mixture, followed by the preparative TLC on polyamide DC-6 (Macherey-Nagel) using an ethyl acetate-ethanol-water 20:3:2 mixture as a mobile phase (Budzianowski and Skrzypczak, 1995). Four bands of phenylethanoids were eluted with methanol. The major compounds were acteoside and plantamajoside. Two further compounds were minor components of which one was tentatively identified as martynoside by TLC comparison with the sample isolated from *P. lanceolata* (Budzianowska et al., 2004) and UV spectra recorded as described previously (Schlauer et al., 2004), which were in accord with literature data (Kim et al., 2001) and showed a bathochromic shift with sodium hydroxide (free phenolic group) but no shift with aluminum chloride indicative of absence of a free phenolic *ortho*-dihydroxy group.

Densitometry. Solutions of extracts in 70% ethanol (50 mg/mL) (e.g., 2.5 mL, i.e., 125 mg) and solutions of a standard compound (1 mg/mL) (7.5 mL), each in triplicate, were applied bandwise to the TLC silica gel 60 F254, 10 × 20 cm glass-backed plates (Merck), by using a Linomat 5 automatic TLC sampler (Camag, Mutenz). The band length was 7.0 mm, distance from the lower edge – 8 mm, distance from the left edge – 15 mm, track distance – 12.1 mm, track number per plate – 15 (Fig. 4). The standard samples of acteoside and plantamajoside were isolated from *Plantago lanceolata* and their purity was 98% according to NMR and HPLC analyses (Budzianowska et al., 2004). The samples were stored in tightly stoppered containers, protected from light.

For the preparation of calibration curves the solutions of standards were applied in volumes of 2.5, 5.0, 7.5, 10.0 and 15.0 mL (i.e., 2.5, 5, 7.5, 10 and 15 mg per band), each in triplicate. The plates were developed in a vertical TLC chamber for 20 × 20 cm plates (Camag, Mutenz, Switzerland) with an ethyl acetate-acetic acid-water 4:1:1 mixture to a distance of 9 cm. The chromatograms were air-dried and their images were captured under UV light with Camag Reprostar 3 device

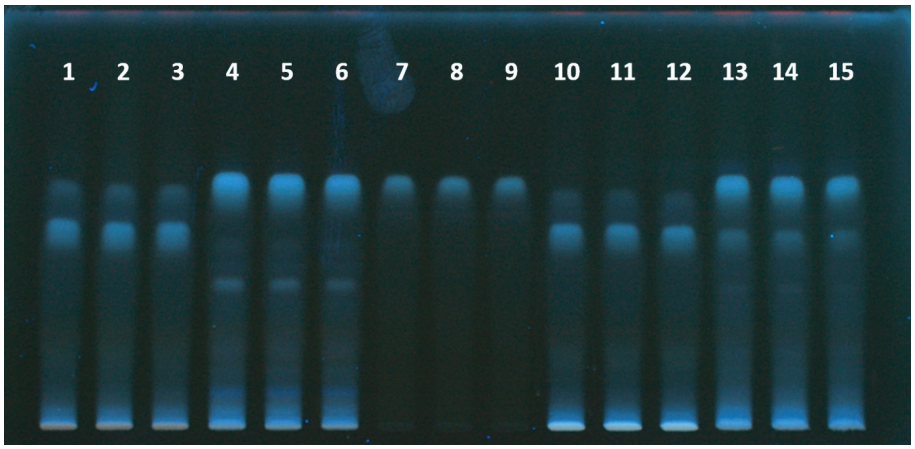

Fig. 4. Exemplary TLC chromatogram captured in 366 nm and applied for video densitometric screening of phenylethanoid glycosides content in extracts (each 125 mg) from roots and shoots of *Plantago media* from *in vitro* cultures. Tracks: 1–3 roots M2, 4–6 shoots M2, 7–9 acteoside standard 7.5 mg, 10–12 roots M1, 13–15 shoots M1.

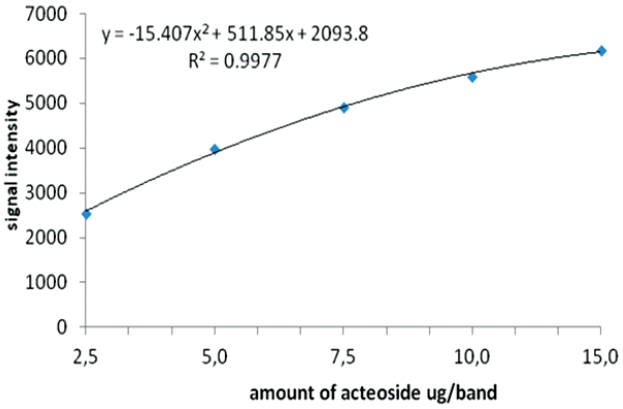

Fig. 5. Calibration plot for acteoside.

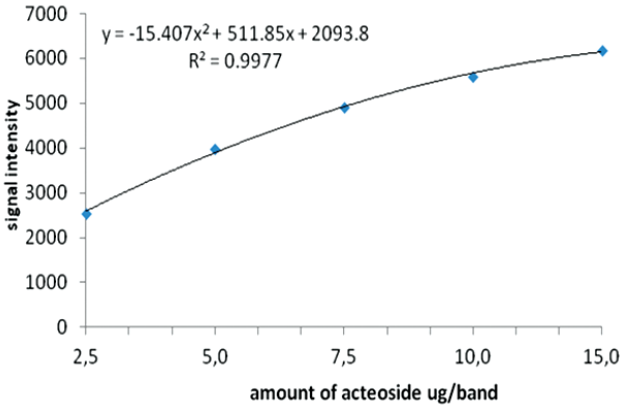

Fig. 6. Calibration plot for plantamajoside.

using Videostore software (Camag), and then evaluated by a VideoScan program (Camag) in a fluorescence mode at 366 nm. The integration parameters were the following: minimum peak width 5, minimum peak height 400, minimum peak area 1000, filter width 7. The five-point calibration curves were prepared by plotting the average height of the bands against the amount per band (Figs. 5 and 6). Because the qualitative analysis suggested large differences in the content of phenylethanoid glycosides in the extracts, the wide range of standards concentrations was chosen (2.5–15.0 μg). Hence, the obtained calibration data best fitted with polynomial regression, which was applied for quantification. The polynomial regression equation for acteoside was $y = -15.407x^2 + 511.85x + 2093.8$ with a correlation coefficient $R^2 = 0.9977$, and for plantamajoside it was $y = -26.504x^2 + 761.65x + 665.53$, $R^2 = 0.9992$.

STATISTICAL ANALYSIS

All experiments were carried out in triplicate and the data were expressed as mean values ± SD. Statistical analyses were performed by the one-way analysis of variance (ANOVA) test followed by Duncan's multiple range test at $P = 0.05$ significant level (Statistica 10 software, StatSoft Inc., Tulsa, OK).

RESULTS

Due to the problems with proper identification of *P. media* in natural sites, the morphology of the leaves of the donor plants was intensively studied and compared with the monograph of the species (Lukova et al., 2017) and also cytogenetically analyzed in order to avoid adulteration with similar species, e.g., *P. major* or *P. lanceolata*. Flow cytometry analysis of nuclear DNA conducted by dr. Monika Rewers from the Laboratory of Molecular Biology and Cytometry of the University of Technology and Life Sciences in Bydgoszcz, Poland, revealed that the genome size of the leaves from the donor plants was 5.2 pg/2C, the same value as that mentioned in the DNA Bank of Kew Botanic Gardens. At the time, the results of these cytometric analyses pointed out that the studied species was *P. media*, from which the seeds were collected.

SEED GERMINATION

The efficiency of the germination process was 89%. The seeds started germinating after 3–4 days, but the first leaves appeared after 14 days. In the morphological terms, the seedlings exhibited proper organ formation (Figs. 2 and 3).

COMPARISON OF TWO PROPAGATION METHODS

Two methods of shoots regeneration – from the shoot tips and other explants of seedlings were applied (Figs. 2 and 3). The efficiency of shoot formation from the seedling shoot tips in the first subculture was 100%, regardless of the tested medium composition. The induction of the shoot formation differed in the range of 75.0%–100%, depending on the medium after the second subculture, and 73.3%–100% after the third subculture (Table 1).

Regardless of the seedlings age, the explants were able to form shoots by developing new axillary buds. The highest number of shoots was obtained from the shoot tips of seedlings (MC = 9.2), and then from the hypocotyl explants (MC = 8.5), and from the roots (MC = 8.0) (Tables 2 and 3). Individual results were obtained on different media

– M3, M2, M4, respectively. Satisfactory results were also obtained for the shoot tips of seedlings placed on the medium with BAP 2.0 mg/L and NAA 0.1 mg/L (5.1 shoots/explant) (M1) (Table 2). However, the rosettes were difficult to separate, and the callus was formed at the base of the shoot. The highest results of organogenesis induction for leaf, cotyledon and hypocotyl explants, as well as moderate results for root explants and shoot tips of seedling were achieved on the medium with BAP 2.0 mg/L and IAA 0.1 mg/L (M2). This allows M2 to be considered the best medium for micropropagation. The weakest results were obtained for the leaf explants (MC = 0.5–2.2). Exceptionally, the roots showed high MC (8.0) on MS with BAP 2.0 mg/L (M4), which may indicate that shoot induction from root explants does not require an exogenous auxin (Table 3). Taking into account the potential of achieving a large number of progeny plants, it is more advantageous to regenerate them from the root explants because of their availability, multiplicity, and the possibility of division into smaller fragments.

Proliferation of the shoots obtained from shoot tips of seedlings and organogenesis was at a similar level in the second and third passages. The three highest results were observed on M2 medium (MC = 5.2, 4.8, 4.2) for both methods. Some shoots spontaneously formed roots (Tables 2 and 3; Figs. 2 and 3).

CALLUS INDUCTION AND INDIRECT ORGANOGENESIS

Callus induction was observed on every type of the medium after about 14 days. The roots were the most callusing explants, in contrast to cotyledons and leaves, which weakly induced callus. Better quality callus appeared on the youngest explants. In all cases the biomass of calli turned into organogenic tissue. The regenerated shoots were often fragile and morphologically unstable (Fig. 3). Therefore, those plants were not analyzed phytochemically.

ROOTING OF SHOOTS AND ACCLIMATIZATION OF *IN VITRO*-PROPAGATED PLANTS

Some shoots spontaneously formed roots on the media for shoot multiplication. However, exogenous addition of IAA was used to increase the efficiency of the process. The shoots growing on MS with IAA revealed direct root induction with a 100% frequency. The mean number of roots was 12.5 ± 2.6 with the mean length of 9.0 cm ± 3.2. The roots from this medium were characterized by a significant length and a larger number of laterals. The *in vitro*-propagated plants were transplanted into pots with a survival rate of 100% (Fig. 2).

PHYTOCHEMICAL SCREENING

Chromatographic TLC examination of methanolic extracts exhibited a similar pattern of phenolic compounds in shoots and roots of the micropropagated plantlets from all media (M1–M4). Phenylethanoid glycosides like acteoside and plantamajoside were present and no flavonoids could be detected. The phenylethanoids appeared under UV light as blue bands exhibiting strong blue fluorescence after spraying with NA. In turn, no yellow or brown bands changing color to brilliant yellow fluorescence under UV upon derivatization with aluminium chloride (Budzianowski and Skrzypczak, 1995), expected for flavonoids, were observed. Those observations were substantiated by column chromatography separation of water fractions from roots and shoots M3. Polyamide was chosen as a chromatography adsorbent, which not only allows for the removal of highly polar compounds by elution with water, but specifically separates neutral phenolics (elution with methanol) from acidic derivatives (e.g., those containing carboxylic groups), which can be desorbed without the risk of decomposition with weak ammonia solution in methanol, or other strongly retained compounds (elution with methanol containing an organic acid) (Budzianowski et al., 1999). An additional preparative chromatography of water fraction from the shoots M3 led to isolation of acteoside and plantamajoside and two trace phenylethanoids of which one was tentatively identified as martynoside.

Quantitative analysis of methanol extracts by means of TLC video densitometry screening showed a generally high content of acteoside in shoots (62.43–93.03 mg/g) and that of plantamajoside in roots (32.99–44.08 mg/g) (Table 4). Roots always contained more plantamajoside than acteoside, while more acteoside than plantamajoside was always observed for shoots (Fig. 7). The highest content of acteoside was found in the shoots (93.03 mg/g), while that of plantamajoside was recorded in the roots (44.08 mg/g), both harvested from the same medium M4. In turn, the lowest content of acteoside was found in the roots M1 (3.63 mg/g) and that of plantamajoside – in the shoots M2 (10.13 mg/g).

DISCUSSION

For the purpose of micropropagation of *Plantago media* those medium and growth regulators were chosen which were previously successfully applied for other members of the genus *Plantago*, i.e., MS supplemented with BAP as a cytokinin and IAA or NAA as an auxin (Andrzejewska-Golec

TABLE 4. Content of acteoside and plantamajoside in organs of *in vitro* cultured *Plantago media*.

Medium	Plant organ (2.000g) DW	Extract (mg) DW	Acteoside		Plantamajoside	
			(mg/g) DW	SD	(mg/g) DW	SD
M1	Roots	738.9	**3.63**[a]	0.54	**35.18**[d]	2.56
	Shoots	814.2	**62.43**[c]	5.65	**15.93**[b]	1.22
M2	Roots	742.4	**7.09**[b]	0.53	**32.99**[d]	0.87
	Shoots	902.9	**91.56**[e]	9.40	**10.13**[a]	0.04
M3	Roots	653.5	**6.73**[b]	0.86	**22.45**[c]	1.68
	Shoots	908.0	**89.05**[d]	3.37	**13.51**[b]	1.21
M4	Roots	757.0	**5.73**[b]	0.30	**44.08**[e]	0.44
	Shoots	886.4	**93.03**[e]	1.16	**20.65**[c]	1.27

Mean values within a column with the same letter are not significantly different at P = 0.05 using one-way analysis variance ANOVA (Duncan's Multiple Range Test).

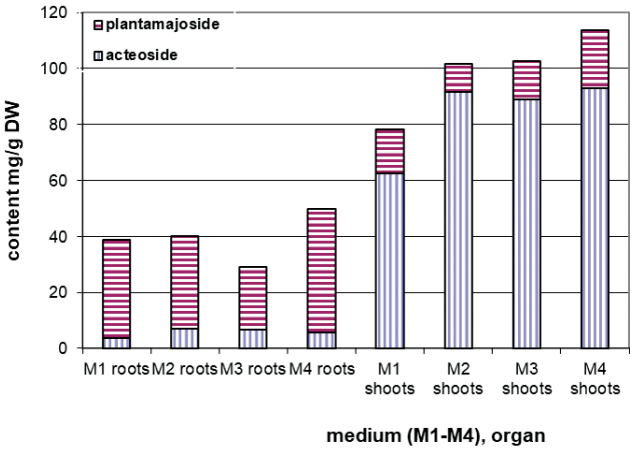

Fig. 7. Content of acteoside and plantamajoside in organs of *in vitro* cultured *Plantago media*.

and Makowczyńska, 2008; Makowczyńska and Andrzejewska-Golec, 2003, 2009; Zeng, 2008; Fons et al., 2008). As a result of our own studies, the media selected for micropropagation through shoot-tip explants were assessed as suitable. Induction and multiplication of shoots were observed in each hormonal variant of the medium. The age of the seedlings, from which the explants were taken, was not significant for shoot induction. The same result was observed for *P. asiatica* micropropagation using 14–28 days-old-seedlings (Makowczyńska and Andrzejewska-Golec, 2003). The multiplication coefficient in seedling shoot-tip cultures using MS + BAP 2.0 mg/L + IAA 0.1 mg/L was 2.4 for *P. asiatica* (Makowczyńska and Andrzejewska-Golec, 2003), 4.41 for *P. camtschatica* (Andrzejewska-Golec and Makowczyńska, 2008), 3.8 for *P. maritima* (Makowczyńska and Andrzejewska-Golec, 2009), and 4.9 for *P. media* in our experiment. As we saw in our studies, the addition of auxin and its concentration had a beneficial effect on the multiplication coefficient in *P. media* propagation. No such relationship was observed for *P. asiatica* (Makowczyńska and Andrzejewska-Golec, 2003). The applied media were good for both induction and multiplication of shoots via shoot-tips and direct organogenesis. Shoot induction from hypocotyls cultured on MS media with BAP 2.0 mg/L was 2.8 rosettes for *P. media* (our studies) and 7–8 shoots for *P. asiatica* (Zeng, 2008). The most efficient shoot induction process, applying MS medium with BAP 2.0 mg/L + IAA 0.1 mg/L, was achieved on hypocotyl explants (13.2) from *P. camtschatica*, which is consistent with the experiment carried out for *P. media* (8.5). The weakest results of organogenesis induction in *P. camtschatica* cultures were obtained for leaf explants (Andrzejewska-Golec and Makowczyńska, 2008), which was also confirmed in our study.

Micropropagated *Plantago media* has never been investigated for the presence of secondary metabolites. The methanol extracts prepared from the shoots and roots were analyzed for the phenolic compounds and were found to contain phenylethanoids acteoside and plantamajoside previously reported for *P. media* plants from the natural sites (Rønsted et al., 2000; Rønsted et al., 2003; Olennikov et al., 2011). The studied extracts showed absence of flavonoids, as it was previously observed in the case of *in vitro* cultures of *P. lanceolata* (Budzianowska et al., 2004). In addition to the previously reported acteoside

(Olennikov et al., 2011) and plantamajoside from *P. media* (Andary et al., 1988; Olennikov et al., 2011), in *P. media* from *in vitro* cultures we could also detect a trace amount of martynoside, a phenylethanoid glycoside previously reported in *P. asiatica* (Miyase et al., 1991) and in *P. lanceolata* (Budzianowska et al., 2004). The quantitative content of extractable acteoside and plantamajoside was determined by TLC video densitometry, which was established as an efficient tool for the quantitative analysis comparable to the more frequently used slit scanning densitometry (Mustoe and McCrossen, 2001; Popovic and Sherma, 2014) including quantification of phenylethanoid glycosides (Agatonovic-Kustrin et al., 2013), which was earlier performed by slit densitometry (Umek et al., 2005). The contents of acteoside in shoots and plantamajoside in roots or shoots of *in vitro* cultured *P. media* were significantly higher in comparison with those in the leaves of *P. media* collected from natural habitats (acteoside 0.64 mg/g, plantamajoside 7.69 mg/g) (Olennikov et al., 2011). Studies conducted on *P. lanceolata* cultured *in vitro* on MS without PGRs showed that roots accumulated more acteoside (26 mg/g) and plantamajoside (30–100 mg/g) than leaves – 8.8 mg/g and 2.4 mg/g, respectively (Fons et al., 1998; Fons et al., 2008). In our earlier investigations of micropropagated *P. lanceolata* the content of acteoside and plantamajoside in shoots was 17.85 mg/g and 0.56 mg/g, respectively (Budzianowska et al., 2004). In untransformed and transformed roots of *P. lanceolata* cultured on MS, a similar content of acteoside (6–12 mg/g) and plantamajoside (30–80 mg/g) was found, which was not influenced by supplementation of the culture medium with cinnamic acid – a compound considered to be a biosynthesis precursor of phenylethanoids (Fons et al., 1998). Due to the importance of the biotechnological production of acteoside (Alipieva et al., 2014) and also plantamajoside (Ravn et al., 2015), the tissue and cell cultures of different plant species were investigated for such an ability.

The content of acteoside and plantamajoside in callus cultures of *P. lanceolata* performed on MS without NH_4NO_3 (nitrogen total content 20 mM, NH_4/NO_3 molar ratio 0:1) supplemented with 2,4-D and Kin (each 1 mg/L) was 6.3 mg/g and 28.4 mg/g, respectively (Budzianowska et al., 2004). Those yields were increased to 13.0 mg/g (acteoside) and 35.4 mg/g (plantamajoside) on MS with nitrogen total content 40 and 10 mM, respectively and NH_4/NO_3 ratio 1:2 (Gonda et al., 2014). In turn, acteoside contents in callus of *Rehmania glutinosa* (Orobanchaceae) (cultured on MS with IAA 0.1 mg/L and BA 1.0 mg/L) (Piątczak et al., 2015) and *Verbena officinalis* (Verbenaceae) (cultured on MS with BA and IBA, 1.0 mg/L each) (Kubica et al., 2017) were 12.5 mg/g and 24.5 mg/g, respectively. Cell cultures of *Scrophularia stritata* (Scrophulariaceae) produced acteoside 0.46 mg/g when performed in shake-flasks and 1.40 mg/g after upgrade to bioreactor (Ahmadi-Sakka et al., 2016). In cell cultures of *Cistanche tubulosa* (Orobanchaceae) performed on B5 medium with sucrose 30 g/L the initial level of acteoside 21.7 mg/g, was increased after supplementation of the medium with tyrosine (29.2 mg/g) and methyl jasmonate (MeJ, 200 mM) (52.4 mg/g) (Liu et al., 2018). In turn, cell suspension culture of *Buddleja cordata* Kunth. (Loganiaceae) produced acteoside 116 mg/g DW, more than callus – 86 mg/g DW (Estrada-Zuniga et al., 2009). Hairy roots, established from the stems of *Paulownia tomentosa* Steud. (Bignoniaceae) and performed on half-strength Gamborg's B5 medium, produced acteoside 95 mg/g DW (Wysokińska and Rózga, 1998).

In comparison with the above-mentioned approaches, *in vitro* cultures of *Plantago media* appear to contain significant amounts of acteoside (93.0 mg/g in shoots) and plantamajoside (44.1 mg/g in roots) and hence may be considered as another attractive source of pharmacologically important phenylethanoids.

CONCLUSION

In conclusion, our research clearly shows that the content of phenylethanoids in *in vitro* cultures of *Plantago media* L. is organ-specific. Acteoside dominates in the shoots and plantamajoside in the roots of the studied species. In addition, the effect of the hormonal supplementation of the culture medium on the content of the tested compounds was observed. The addition of exogenous auxin was not essential to increase accumulation of phenylethanoids in the cultured organs.

AUTHORS' CONTRIBUTIONS

AB: concept of investigations, designed experiments, provided plant material, collected articles, revised manuscript; MK: prepared and revised manuscript, analyzed and interpreted biotechnological and phytochemical data; MM: conducted in vitro experiments; IK: conducted phytochemical experiments; JB: prepared results of densitometry, collected data from literature, prepared discussion. The Authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

This study was supported by the Department of Botany and Plant Biotechnology, Poznań University of Medical Sciences, Poland.

REFERENCES

Agatonovic-Kustrin S, Loescher CM, and Singh R. 2013. Quantification of phenylpropanoids in commercial *Echinacea* products using TLC with video densitometry as detection technique and ANN for data modelling. *Phytochemical Analysis* 24: 303–308.

Ahmadi-Sakha S, Sharifi M, and Niknam V. 2016. Bioproduction of phenylethanoid glycosides by plant cell culture of *Scrophularia striata* Boiss.: from shake-flasks to bioreactor. *Plant Cell, Tissue and Organ Culture* 124: 275–281.

Alipieva K, Korkina L, Orhan IE, and Georgiev MI. 2014. Verbascoside – a review of its occurrence, (bio)synthesis and pharmacological significance. *Biotechnology Advances* 32: 1065–1076.

Andary C, Motte-Florac ME, Gargadennec A, Wylde R, and Heitz A. 1988. Les esters cafeiques du genre *Plantago*. Identication et valeur chimiotaxinomique. *Plantes Medicinales et Phytotherapie* 22: 17–22.

Andrzejewska-Golec E, and Makowczyńska J. 2008. Micropropagation of *Plantago camtschatica* Link. *Acta Societatis Botanicorum Poloniae* 77: 269–273.

Barna KS, and Wakhlu AK. 1998. Axillary shoot induction and plant regeneration in *Plantago ovata* Forssk. *Plant Cell, Tissue and Organ Culture* 15(2): 169–173.

Beara IN, Lesjak MM, Jovin ED, Balog KJ, Anačkov GT, Orčić DZ, and Mimica-Dukić NM. 2009. Plantain (*Plantago* L.) species as novel sources of flavonoid antioxidants. *Journal of Agriculture and Food Chemistry* 57: 9268–9273.

Budzianowska A, Skrzypczak L, and Budzianowski J. 2004. Phenylethanoid glucosides from *in vitro* propagated plants and callus cultures of *Plantago lanceolata*. *Planta Medica* 70: 834–840.

Budzianowski J, Korzeniowska K, Chmara E, and Mrozikiewicz A. 1999. Microvascular protective activity of flavonoid glucuronides fraction from *Tulipa gesneriana*. *Phytotherapy Research* 13(2): 166–168.

Budzianowski J, and Skrzypczak L. 1995. Phenylpropanoid esters from *Lamium album* flowers. *Phytochemistry* 38: 997–1001.

Chater AO, and Cartier D. 1967. *Plantago* L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, and Webb DA [eds.], *Flora Europea*. vol. 4, 39–41. Cambridge University. Cambridge, London, New York, Melbourne.

Estrada-Zúñiga ME, Cruz-Sosa F, Rodríguez-Monroy M, Verde-Calvo JR, and Vernon-Carter EJ. 2009. Phenylpropanoid production in callus and cell suspension cultures of *Buddleja cordata* Kunth. *Plant Cell, Tissue and Organ Culture* 97: 39–47.

Fons F, Gargadennec A, Gueiffier A, Roussel JL, and Andary C. 1998. Effects of cinnamic acid on polyphenol production in *Plantago lanceolata*. *Phytochemistry* 49: 697–702.

Fons F, Gargadennec A, and Rapior S. 2008. Culture of *Plantago* species as bioactive components resources: a 20-year review and recent applications. *Acta Botanica Gallica* 155: 277–300.

Fons F, Tousch D, Rapoir S, Gueiffier A, Roussel JL, Gargadennec A, and Andary C. 1999. Phenolic profiles of untransformed and hairy root cultures of *Plantago lanceolata*. *Plant Physiology and Biochemistry* 37: 291–296.

Gonçalves S, and Romano A. 2016. The medicinal potential of plants from the genus *Plantago* (Plantaginaceae). *Industrial Crops and Products* 83: 213–226.

Gonda S, Kiss-Szikszai A, Szucs Z, Máthé C, and Vasas G. 2014. Effects of N source concentration and $NH4^+/NO3^-$ ratio on phenylethanoid glycoside pattern in tissue cultures of *Plantago lanceolata* L.: A metabolomics driven full-factorial experiment with LC–ESI–MS^3. *Phytochemistry* 106: 44–54.

Jędrzejko K. 1997. *Zarys wiedzy o roślinach leczniczych*. 269, 285. Śląska Akademia Medyczna, Katowice.

Khawar KM, Sarhin EO, Sevimay CS, Cocu S, Parmaksiz J, Kranbey S, Ipek A, Kaya MP, Sancak C, and Ozcan S. 2005. Adventitious shoot regeneration and micropropagation of *Plantago lanceolata* L. *Periodicum Biologorum* 107: 113–116.

Kim HJ, Woo ER, Shin CG, Hwang DJ, Park H, and Lee YS. 2001. HIV-1 integrase inhibitory phenylpropanoid glycosides from *Clerodendron trichotomum*. *Archives of Pharmacal Research* 24(4): 286–291.

Kubica P, Szopa A, and Ekiert H. 2017. Production of verbascoside and phenolic acids in biomass of *Verbena officinalis* L. (vervain) cultured under different *in vitro* conditions. *Natural Product Research* 31(14): 1–6.

Liu X, Yan Y, Liu Y, Mo1T, Wang X, Song Y, Chen Q, Zhao Y, Shi Sh, and Tu P. Cell culture establishment and regulation of two phenylethanoid glycosides accumulation in cell suspension culture of desert plant *Cistanche tubulosa*. *Plant Cell, Tissue and Organ Culture* 134(1): 107–118.

Lukova P, Dimitrova-Dyulgerova I, Karcheva-Bahchevanska D, Mladenov R, Iliev I, and Nikolova M. 2017. Comparative morphological and qualitative phytochemical analysis of *Plantago media* L. leaves with *P. major* L. and *P. lanceolata* L. leaves. *International Journal of Medical Research and Pharmaceutical Sciences* 4: 20–26.

Makowczyńska J, and Andrzejewska-Golec E. 2003. Micropropagation of *Plantago asiatica* L. through culture of shoot-tips. *Acta Societatis Botanicorum Poloniae* 72: 191–194.

Makowczyńska J, and Andrzejewska-Golec E. 2009. Micropropagation of *Plantago maritima* L. – a vanishing species in Poland. *Acta Societatis Botanicorum Poloniae* 78: 13–18.

Mederos S, Martin C, Navorro F, and Ayuso MJ. 1997/1998. Micropropagation of a medicinal plant, *Plantago major* L. *Biologia Plantarum* 40(3): 465–468.

Miyase T, Ishino M, Akahori C, Ueno A, Okhawa Y, and Tanizawa H. 1991. Phenylethanoid glycosides from *Plantago asiatica*. *Phytochemistry* 30: 2015.

Murashige T, and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473–497.

Mustoe SP, and McCrossen SD. 2001. A comparison between slit densitometry and video densitometry for quantitation in thin layer chromatography. *Chromatographia* Suppl. 53: S-474.

Olennikov DN, Tankhaeva LM, Stolbikova AV, and Petrov EV. 2011. Phenylpropanoids and polysaccharides from *Plantago depressa* and *P. media* growing in Buryatia. *Chemistry of Natural Compounds* 47: 165.

Papoutsi Z, Kassi E, Mitakou S, Aligiannis N, Tsiapara A, Chrousos GP, and Moutsatsou P. 2006. Acteoside and martynoside exhibit estrogenic / antiestrogenic properties. *The Journal of Steroid Biochemistry and Molecular Biology* 98: 63–71.

Piątczak E, Kuźma Ł, Sitarek P, and Wysokińska H. 2015. Shoot organogenesis, molecular analysis and secondary metabolite production of micropropagated *Rehmannia glutinosa* Libosch. *Plant Cell, Tissue and Organ Culture* 120: 539–549.

Popovic N, and Sherma J. 2014. Comparative study of the quantification of thin-layer chromatograms of a model dye using three types of commercial densitometers and image analysis with ImageJ. *Trends in Chromatography* 9: 21–28.

Pramanik S, Chakraborty S, and Raychaudhuri SS. 1995. *In vitro* propagation and characterization of clonal regenerants of *Plantago ovata* Forssk by isozyme analysis. *Cytobios* 82: 123–130.

Ravn HW, Mondolot L, Kelly MT, and Lykke AM. 2015. Plantamajoside – a current review. *Phytochemistry Letters* 12: 42–53.

Rønsted N, Göbel E, Franzyk H, Jensen SR, and Olsen CE. 2000. Chemotaxonomy of *Plantago*. Iridoid glucosides and caffeoyl phenylethanoid glycosides. *Phytochemistry* 55: 337–348.

Rønsted N, Franzyk H, Mølgaard P, Jaroszewski JW, and Jensen SR. 2003. Chemotaxonomy and evolution of *Plantago* L. *Plant Systematics and Evolution* 242: 63–82.

Saker MM, and Kawashity SA. 1998. Tissue culture and flavonoid content of *Nepeta* and *Plantago* species endemic in Egypt. *Fitoterapia* 69: 358–364.

Schlauer J, Budzianowski J, Kukułczanka K, and Ratajczak L. 2004. Acteoside and related phenylethanoid glycosides from *Byblis liniflora* Salisb. plants propagated *in vitro* and its systematic significance. *Acta Societatis Botanicorum Poloniae* 73: 9–15.

Świątek L. 1977. Kwasy fenolowe i glukozydy irydoidowe w niektórych krajowych gatunkach leczniczych z rodzaju *Plantago*. *Herba Polonica* 23: 201–209.

Tacik T. 1967. Plantaginaceae. In: Pawłowski B [ed.], *Flora Polska. Rośliny naczyniowe Polski i ziem ościennych*, 237–257. PWN, Kraków.

Thiem B, Kikowska M, Krawczyk A, Więckowska B, and Sliwinska E. 2013. Phenolic acid and DNA contents of micropropagated *Eryngium planum* L. *Plant Cell Tissue and Organ Culture* 14(2): 196–207.

Umek A, Rupert A, Mlinarič A, and Kac J. 2005. HPTLC method for determination of acteoside in Ribwort plantain (*Plantago lanceolata* L.). *Journal of Planar Chromatography* 18(102): 147-150.

Wysokińska H, and Rózga M. 1998. Establishment of transformed root cultures of *Paulownia tomentosa* for verbascoside production. *Journal of Plant Physiology* 152: 78–83.

Vertuani S, Beghelli E, Scalambra E, Malisardi G, Copetti S, Dal Toso R, Baldisserotto A, and Manfredini S. 2011. Activity and stability studies of verbascoside, a novel antioxidant, in dermo-cosmetic and pharmaceutical topical formulations. *Molecules* 16: 7068–7080.

Zeng J. 2008. Direct induction of adventitious buds and establishment of regeneration system in *Plantago asiatica* L. *Journal of Anhui Agricultural Sciences* 36: 1810–1811.