South African Journal of Botany 95 (2014) 159–164

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Changes of phenylethanoid and iridoid glycoside distribution in various tissues of shoot cultures and regenerated plants of *Harpagophytum procumbens* (Burch.) DC. ex Meisn

R. Grąbkowska [a,*], W. Mielicki [b], M. Wielanek [c], H. Wysokińska [a]

[a] Department of Biology and Pharmaceutical Botany, Medical University of Lodz, Muszynskiego 1, 90-151 Lodz, Poland
[b] Department of Pharmaceutical Biochemistry, Medical University of Lodz, Muszynskiego 1, 90-151 Lodz, Poland
[c] Department of Plant Physiology and Biochemistry, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland

ARTICLE INFO

Article history:
Received 24 June 2014
Received in revised form 25 August 2014
Accepted 16 September 2014
Available online 9 October 2014

Edited by J Van Staden

Keywords:
Harpagide
Harpagoside
High-performance liquid chromatography (HPLC)
Isoverbascoside
Verbascoside

ABSTRACT

Harpagophytum procumbens is a medicinal plant containing several compounds with pharmaceutical activity. Previously, we established shoot culture and *in vitro* regenerated plants of *H. procumbens.* In this study, HPLC and LC-ESI-MS were used to identify harpagoside, harpagide, verbascoside and isoverbascoside in various tissues (stems, leaves and callus) of shoots multiplied on Schenk and Hildebrandt (SH) solid medium supplemented with 0.57 μM indole-3-acetic acid (IAA) and 8 μM 6-benzylaminopurine (BAP), as well as in stems, leaves and root tubers of *in vitro* propagated plants grown in the greenhouse for 3, 6 and 12 months. The content of the compounds was also determined by HPLC. For comparison, control *H. procumbens* plants initiated from seeds were analyzed. *H. procumbens* shoots grown under *in vitro* conditions accumulated lower amounts of iridoids and phenylethanoids than the plants derived from them. The levels of analyzed compounds were higher in the organs of 3- or 6-month-old plants than in those of 12-month-old plants. Differences in the distribution of secondary metabolites were also observed between organs. The aerial parts (stems, leaves) of 3-month-old *in vitro* regenerated plants were characterized by the highest amounts of phenylethanoids, which significantly exceeded those detected in control plants. Total iridoid content, calculated as the sum of harpagoside and harpagide, was highest in the root tubers of 6-month-old plants. In these organs the level of harpagoside was comparable to that in root tubers of 6-month-old seed-propagated plants, but the level of harpagide was much lower.
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1. Introduction

Harpagophytum procumbens (Burch.) DC. ex Meisn. (Pedaliaceae) is an important medicinal plant naturally grown in southern Africa, especially in the Kalahari region. Clinical studies have shown that the dry extract of root tubers of the plant species exhibits analgesic, antiphlogistic and antiarthritic properties (ESCOP, 2003; Mncwangi et al., 2012). Good therapeutic results have been achieved using *Harpagophytum* root extracts for loss of appetite and in therapy of degenerative diseases of the musculoskeletal system and degenerative disorders of the locomotor system (Stewart and Cole, 2005; Van Wyk and Gericke, 2000). The medicinal properties are attributed mainly to the presence of iridoid glycosides such as: harpagoside, harpagide, 8-p-coumaroylharpagide, pagoside, procumbide, and harprocumbide A and B (Boje et al., 2003; Guillerault et al., 1994; Qi et al., 2006). From a pharmacological point of view, the phenylethanoid glycosides verbascoside and isoverbascoside are also interesting constituents of *H. procumbens* roots (Boje et al., 2003; Clarkson et al., 2006).

For medical purposes, the *H. procumbens* roots are mainly harvested from natural environment and/or are collected from field-cultivated plants. Since plants propagated by cuttings fail to produce primary tubers (Kathe et al., 2003), propagation through seeds is preferred (Jordaan, 2011; Raimondo and Donaldson, 2002). This is, however, difficult due to low seed germination rate after dormancy (less than 20%) and low survival of seedlings (Ernst et al., 1988; Stewart and Cole, 2005). Therefore, it is important to develop an efficient method of *H. procumbens* micropropagation and obtain plants producing the desired metabolites. Several reports have been published on the micropropagation of *H. procumbens* in order to provide a sufficient amount of material for medicinal purposes (Bairu et al., 2009, 2011; Grąbkowska et al., 2014; Kaliamoorthy et al., 2008; Levieille and Wilson, 2002; Shushu, 2001). However, only two reports (Bairu et al., 2011; Levieille and Wilson, 2002) describe the accumulation of iridoids in *in vitro* regenerated plants of *H. procumbens.* In those reports, total iridoid content, expressed as harpagoside equivalents, in roots and leaves was measured by colorimetry. The present work uses HPLC to evaluate the content of harpagoside, harpagide, verbascoside and isoverbascoside in various tissues of *in vitro* cultivated shoots and micropropagated *H. procumbens* plants after 3, 6 and 12 months of growth in the greenhouse. For comparison, similar

* Corresponding author.
E-mail address: renata.grabkowska@umed.lodz.pl (R. Grąbkowska).

http://dx.doi.org/10.1016/j.sajb.2014.09.002
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analyses were carried out in *H. procumbens* plants propagated from seeds. This is the first study of the effects of age and organ type on the production of pharmacologically important compounds in *H. procumbens* plants.

2. Materials and methods

2.1. Plant materials and sample preparation

The seeds of *H. procumbens* used to initiate shoot cultures and *in vivo*-derived plants were obtained from the Groenvlei Farm, belonging to the Grassroots Group (Gouda, South Africa). The procedures used for the establishment of shoot cultures, plant micropropagation from shoot cultures and acclimatization of obtained plants have been described previously (Grąbkowska and Wysokińska, 2009; Grąbkowska et al., 2014). *In vitro*-derived plants were grown in the greenhouse for 3, 6 and 12 months. For comparison, plants derived directly from seeds were also grown under identical conditions as the *in vitro* regenerated plants, i.e. in the greenhouse for 3, 6 and 12 months. The plants were positively identified as *H. procumbens* according to Flora Zambesiaca (Ihlenfeldt, 1988) by R. Grąbkowska. A voucher specimen was deposited in the herbarium of the Department of Biology and Pharmaceutical Botany, Medical University of Lodz, Poland.

For analyses the following plant materials were used:

- shoot cultures grown on agar (0.7%) solidified SH medium (Schenk and Hildebrandt, 1972) supplemented with 0.57 μM indole-3-acetic acid (IAA) and 8 μM 6-benzylaminopurine (BAP). The shoots were kept in a growth chamber at 26 ± 2 °C under a 16/8 h (light/dark) photoperiod with light at an intensity of 40 μM m^{-2} s^{-1}. The cultures were subcultured 31 times at 5-week intervals, i.e. for about 3 years. The leaves, stems and callus tissue formed at the base of the shoots were used in the study. The experiments were conducted with three replicates: each replicate used leaves, stems and callus tissue from five shoots;
- *in vitro* propagated plants (called *in vitro* plants) cultured in pots containing sand, soil, peat and perlite (4:2:2:4 *v*/*v*/*v*/*v*). The plants were harvested after 3, 6 and 12 months of growth in greenhouse and three different parts were used for analysis: leaves, stems and root tubers;
- leaves, stems and root tubers of *H. procumbens* plants propagated from seeds (called *in vivo* plants) were also used. After 3, 6 and 12 months of growth in greenhouse, the growth parameters (i.e. shoot length, number of nodes, length of internodes, leaf area and number of plants forming tubers) of *in vitro*- and seed-derived plants were recorded. Each evaluation comprised 10–15 plants.

Samples of callus, leaves, stems and root tubers from shoot cultures and *in vitro*- and *in vivo*-derived plants of *H. procumbens* were extracted according to Sesterhenn et al. (2007). Briefly, lyophilised and powdered plant material (250 mg dry weight) was extracted with methanol (50 ml) for 3 min at room temperature using an ultrasonic disintegrator (type UD-20) and incubated in a water bath at 50 °C for 30 min. Then, the extraction was continued on a rotary shaker (100 rpm) overnight for about 15 h. The samples were centrifuged at 4000 rpm for 10 min. The supernatant was filtered and the residue was washed with methanol (2 × 10 ml). After filtration, the supernatants were combined and evaporated to dryness under reduced pressure. The residue was dissolved in methanol (3 ml), filtered through a 0.22 μm Ultrafree-MC nylon filter (Sigma-Aldrich) and used for HPLC analysis. The experiments were conducted with three replicates; each replicate comprised 3 plants at the different time of growth (3, 6 and 12 months).

2.2. Quantification of harpagoside, verbascoside and isoverbascoside

The analyses were carried out by HPLC using a Varian ProStar 240 apparatus equipped with a Varian ProStar 320 detector. The compounds were separated on a Supelcosil RP-18 column (4 × 250 mm, 5 μm) with a Supelguard RP-18 precolumn (4 × 20 mm, 5 μm) at a column temperature of 24 °C. The mobile phase comprised water with phosphoric acid (pH 4) (A) and acetonitrile (B). The gradient elution had the following profile: time 0–2 min 10% solvent B; 2–12 min 10 → 40% B; 12–22 min 40 → 100% B; 22–23 min 100 → 10% B; 23–26 min 10% B. The flow rate was 1.2 ml min^{-1}, and volume of extract injected was 20 μl. Detection was performed at 280 nm. Peaks were identified by comparing their retention times (t_R) with these of the reference standards (Table 1). Harpagoside and verbascoside standards were purchased from PhytoPlan (Heidelberg, Germany) and isoverbascoside from Roth (Karlsruhe, Germany). The amounts of the compounds were calculated using calibration curves constructed for standard compounds over the range of 6–500 μg ml^{-1}. The linearity of the calibration curves was verified by the correlation coefficient ($r^2 = 0.96$–0.99). The experiments were repeated three times and the results are presented as milligrams per gram of dry weight ± standard error. The recovery of the known quantities of harpagoside, verbascoside and isoverbascoside added to the biomass samples was as presented previously (Grąbkowska et al., 2010).

2.3. Quantification of harpagide

For harpagide analysis, a Dionex HPLC with photodiode array detector (DAD) and a Supelcosil RP-18 column (4 × 250 mm, 5 μm) was used with a solvent system of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The gradient elution program was as follows: 0–11 min 10 → 70% B, 11–12 min 70 → 70% B, 12–13 min 70 → 10% B, and 13–15 min 10 → 10% B. The flow rate was 1.2 ml min^{-1}. Detection was performed at 280 nm. Harpagide was identified by comparing the chromatographic peak retention time of the test solution with that of the authentic compound. Thus the t_R for harpagide was found to be 5.35 min (Table 1). The iridoid was quantified using calibration curves prepared with pure compound. Harpagide standard was purchased from Roth (Karlsruhe, Germany). The results presented as mg g^{-1} of dry weight are the mean of three replications ± the standard error. The recovery of the known quantities of harpagide added to the biomass samples was 96% efficient.

2.4. Liquid chromatography-mass spectrometry (LC-ESI-MS) analysis

Identification of harpagoside, harpagide, verbascoside and isoverbascoside was also confirmed by mass spectra. The LC-MS was performed by using the API LC/MS/MS system (Applera, USA) with an electrospray ionization (ESI) source equipped with a Dionex (Germany) HPLC system. The elution program and ESI conditions have been reported earlier (Grąbkowska et al., 2010). Detection was performed in the negative ion mode and full scan range from 100 to 700 *m*/*z* (Table 1).

2.5. Statistical analysis

The estimated values are the means of three samples for each plant material. Each sample was replicated in triplicate. Results were tested for normality using the Shapiro–Wilk test. As the tested data did not have a normal distribution, the Kruskal–Wallis nonparametric test was used to determine significant differences. STATISTICA version 10 (STATSoft, Poland) was used for statistical analyses. The results were assumed to be significant at $p \leq 0.05$.

3. Results and discussion

Iridoid and phenylethanoid glycosides in different parts of *H. procumbens* shoots cultured *in vitro* and regenerated plants grown in the greenhouse for 3, 6 and 12 months were analyzed by

Table 1
Retention times (t_R), ESI-MS fragmentation ions of the iridoids and phenylethanoid glycosides in *H. procumbens*.

Compound	Retention times (t_R) (min)	Negative ion mode (m/z)
Verbascoside	8.87	461.2 $[M - H - \text{caffeic acid}]^-$; 623.2 $[M - H]^-$; 669.6 $[M + HCOO]^-$
Isoverbascoside	9.36	461.2 $[M - H - \text{caffeic acid}]^-$; 623.2 $[M - H]^-$; 669.6 $[M + HCOO]^-$
Harpagoside	12.21	146.8 $[M - H - \text{harpagide}]^-$; 345.1 $[M - H - \text{cinnamic acid}]^-$; 492.9 $[M - H]^-$; 494.4 M^-; 538.9 $[M + HCOO]^-$
Harpagide	5.35	362.7 $[M - H]^-$; 364.3 M^-; 409.2 $[M + HCOO]^-$

Separation conditions for HPLC and LC-ESI-MS analyses are described in Materials and methods.

HPLC and LC-ESI-MS. For comparison, the ability of these compounds to accumulate in seed-derived plants of *H. procumbens* was also studied. Harpagoside, harpagide, verbascoside and isoverbascoside were identified in all tested plant materials by comparing their retention times with those of standards and additionally by the presence of pseudomolecular and fragmentary ions in ESI-MS spectra (Table 1). Quantitative HPLC analyses showed that harpagoside was the dominant iridoid in all analyzed samples and verbascoside content was always higher than isoverbascoside.

3.1. Content of iridoids and phenylethanoid glycosides in shoot cultures

The distribution of verbascoside, isoverbascoside, harpagoside and harpagide in different tissues of *in vitro* cultured shoots of *H. procumbens* is shown in Fig. 1. The levels of these compounds were evaluated separately in the leaves and stems, as well as the callus

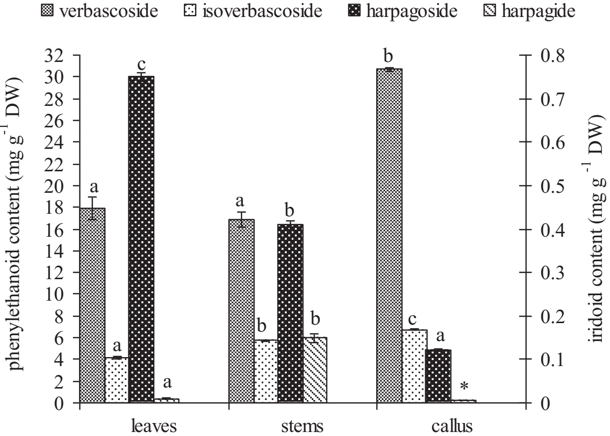

Fig. 1. Verbascoside, isoverbascoside, harpagoside and harpagide content in different tissues (callus, stems and leaves) of *in vitro* cultured shoots of *H. procumbens*. The shoots were grown on SH medium with 0.57 µM IAA and 8 µM BAP for 5 weeks. * trace (content < 0.01 mg g^{-1} DW). Results are means ± standard errors from three independent experiments; each experiment comprised five shoots ($n = 15$); for each metabolite, values with different letters are significantly different according to the Kruskal–Wallis test ($p \leq 0.05$).

formed at the base of shoots, which consisted of shoot primordia and small adventitious shoots. The highest concentration of harpagoside was identified in leaves (0.75 mg g^{-1} DW) followed by the stems (0.41 mg g^{-1} DW) and then by callus (0.12 mg g^{-1} DW). Harpagide was also detected in all parts of cultured shoots but its level was low (max. 0.15 mg g^{-1} DW in stems). In callus tissue, only traces of the iridoid were found (Fig. 1). The results were similar to those reported by Bairu et al. (2011), who showed that tissue cultured shoots produced 5 times more iridoids (about 0.7 mg g^{-1} DW) than the callus at the base of shoots (about 0.13 mg g^{-1} DW), calculated as total iridoid content (expressed in mg harpagoside equivalents per g DW). Earlier, Abou-Mandour (1977) has reported that *H. procumbens* calli were not capable of producing iridoids in measurable amounts.

HPLC examination found that callus tissue of *H. procumbens* shoot culture was a good source of phenylethanoid glycosides, accumulating 7.0 mg g^{-1} DW isoverbascoside and 31 mg g^{-1} DW verbascoside. These values were significantly higher than those in the leaves and stems of cultured shoots (Fig. 1). The verbascoside content of callus cultured shoots was higher than that achieved by other tested culture systems of *H. procumbens*, such as cell suspension (Georgiev et al., 2010; Gyurkovska et al., 2011; Stancheva et al., 2011), hairy roots grown in flasks (Grąbkowska et al., 2010; Gyurkovska et al., 2011; Homova et al., 2010) and bioreactor (Homova et al., 2010).

3.2. Morphology of in vitro- and seed-derived plants

The shoot cultures of *H. procumbens* were used to establish micropropagated plants (Grąbkowska and Wysokińska, 2009; Grąbkowska et al., 2014). Plantlets were transplanted to pots and grown in the greenhouse: the plants had an elongated stem with green, well-developed leaves. The morphology of *in vitro* raised and seed-derived (*in vivo*) plants is presented in Fig. 2. The comparative analysis of morphological characterization revealed that the growth parameters (i.e. shoot length, number of nodes, length of internodes and leaf area) of *in vitro*- and seed-derived plants were similar when they were grown in the greenhouse for 3 or 6 months. The average shoot length of the 3-month-old plants was about 18 cm with 9 nodes per shoot, the length of internodes was about 2 cm and the mean left area was about 4.4 cm^2. Around 10–12 weeks after being transplanted to the greenhouse, the plants began to produce tuberised roots: the

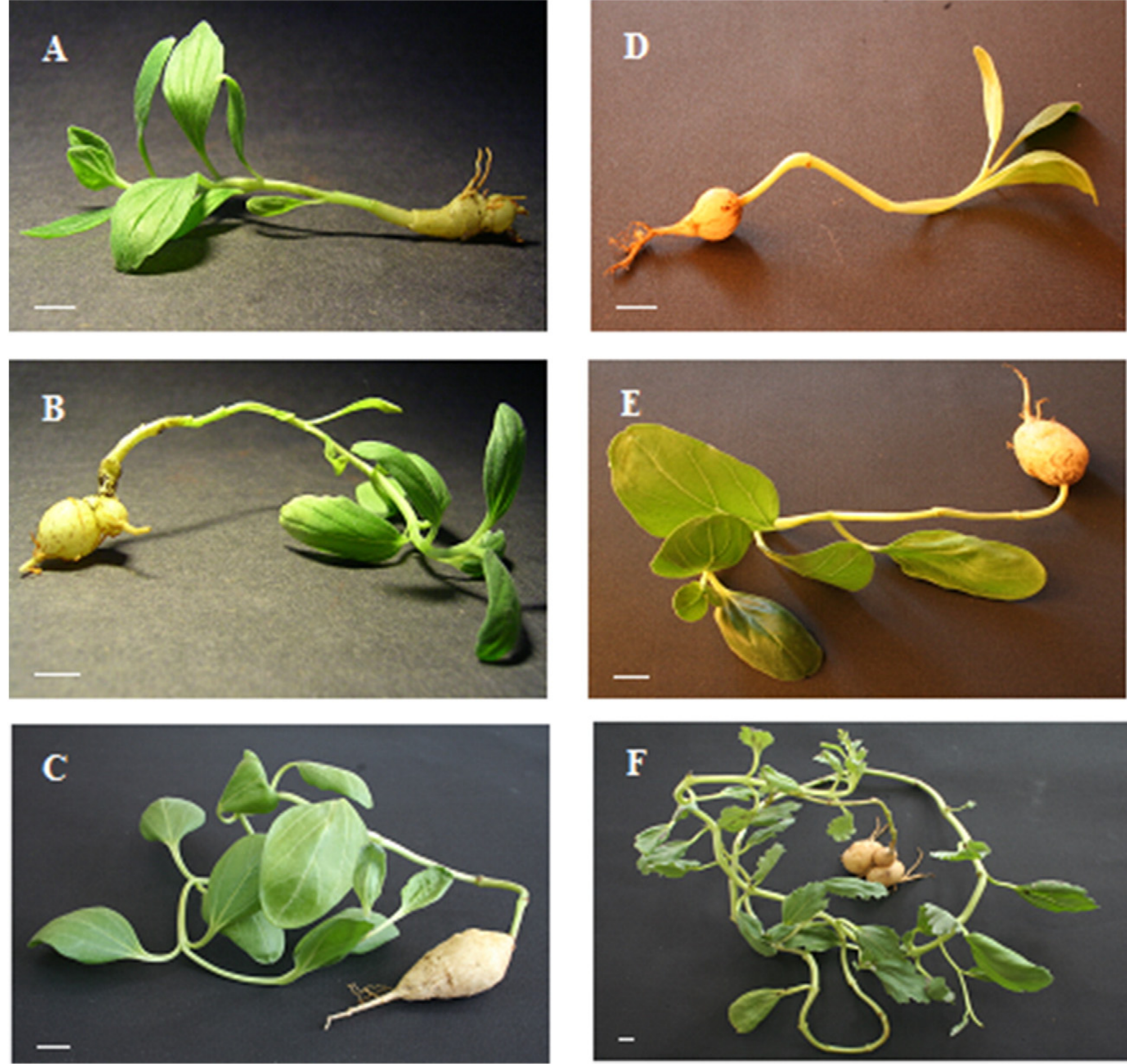

Fig. 2. *In vitro* (A, B, C) and *in vivo* (D, E, F) derived plants of *H. procumbens* after 3 (A, D), 6 (B, E) and 12 (C, F) months grown in greenhouse. Bar 1 cm.

process being completed after 24 weeks. After 6 months of growth, the mean shoot length had increased to about 25 cm (15 nodes/shoot). The mean leaf areas of *in vivo* and *in vitro* plants ranged from 6 to 7 cm^2, respectively. Further morphological observation showed that the 1-year-old seed-derived plants exhibited longer shoots than the *in vitro* plants at the same age (100 vs 60 cm) (Fig. 2). The *in vivo* plants had approximately 60 nodes per shoot, which was twice as many as that of the *in vitro* plants. The seed-derived plants were characterized by larger leaves: the leaf area of these plants was 11 cm^2, while that of the *in vitro* plants was 7.9 cm^2.

3.3. Content of secondary metabolites in regenerated plants

In the present study, the contents of iridoids (harpagoside and harpagide) and phenylethanoid glycosides (verbascoside and isoverbascoside) were determined in the leaves, stems and root tubers of *in vitro* regenerated plants grown for 3, 6 and 12 months in the greenhouse. These levels were then compared to those in different organs of corresponding seed-derived plants. The parts of young, 3- and 6-month-old, plants demonstrated considerably higher levels of both groups of secondary metabolites than older, 12-month-old, plants (Tables 2 and 3). The highest concentration of verbascoside (45–47 mg g^{-1} DW) was found in above-ground parts (leaves and stems) of 3-month-old *in vitro* regenerated plants (Table 2), which contained almost 3-fold higher amounts of verbascoside than the stems and leaves of *in vitro* multiple shoots (Fig. 1). The content of verbascoside was found to be lower (up to 23–24 mg g^{-1} DW) in the methanolic extracts of leaves and stems of 6-month-old *in vitro*-derived plants (Table 2). Further reductions (up to 13–14 mg g^{-1} DW) were observed in the leaves and stems of 12-month-old micropropagated plants. The greatest level of isoverbascoside (20.0 mg g^{-1} DW) was achieved in the leaves and stems of 6-month-old *in vitro* propagated plants. This value was about 20% higher than in above-ground parts of 3-month-old plants and exceeded twice level of the compound in the stems and leaves of 12-month-old plants (Table 2). As can be seen in Table 2, no significant differences were observed between leaves and stems in terms of verbascoside and isoverbascoside accumulation, except for the organs of 12-month-old plants (at $p \leq 0.05$). On the other hand, large differences exist in distribution of the compounds

Table 2
Quantitative analysis of phenylethanoid glycosides in *H. procumbens*.

Source of material	Plant organ	Content (mg g^{-1} DW) ± SE		
		Verbascoside	Isoverbascoside	Total*
In vitro plants				
3-month-old	Leaves	47.16 ± 1.02l	17.28 ± 0.21i	64.44 ± 5.28
	Stems	45.22 ± 0.89l	17.23 ± 0.1i	62.45 ± 4.68
	Roots	32.21 ± 0.45k	10.76 ± 0.30fgh	42.97 ± 3.39
6-month-old	Leaves	24.04 ± 1.27j	21.02 ± 0.21j	45.06 ± 0.75
	Stems	22.68 ± 0.11j	20.13 ± 0.18j	42.81 ± 0.46
	Roots	20.73 ± 0.08i	11.0 ± 0.08gh	31.73 ± 1.53
12-month-old	Leaves	13.39 ± 0.15e	9.11 ± 0.20ef	22.50 ± 0.72
	Stems	14.29 ± 0.07fg	11.62 ± 0.11gh	25.91 ± 0.45
	Roots	5.4 ± 0.11a	1.33 ± 0.03a	6.73 ± 0.64
In vivo plants				
3-month-old	Leaves	16.39 ± 0.23h	7.95 ± 0.13de	24.34 ± 1.49
	Stems	13.48 ± 0.09ef	7.47 ± 0.14d	20.95 ± 0.95
	Roots	5.12 ± 0.31a	2.89 ± 0.10b	8.01 ± 0.39
6-month-old	Leaves	14.87 ± 0.07g	12.63 ± 0.07h	27.50 ± 0.36
	Stems	14.61 ± 0.15g	10.10 ± 0.1fg	24.71 ± 0.69
	Roots	11.57 ± 0.19d	6.79 ± 0.07d	18.36 ± 0.76
12-month-old	Leaves	18.72 ± 0.22h	11.26 ± 0.12gh	29.98 ± 1.25
	Stems	10.1 ± 0.06c	8.34 ± 0.08e	18.44 ± 0.27
	Roots	7.83 ± 0.03b	5.06 ± 0.04c	12.89 ± 0.49

Results are means ± standard errors (SE) from three independent experiments; each replicate used leaves, stems and root tubers from 3 plants ($n = 9$); different letters within the column indicate significant differences according to the Kruskal–Wallis test ($p \leq 0.05$).
* Sum of verbascoside and isoverbascoside.

between aerial and underground parts of the analyzed plants, with the latter demonstrating much lower concentrations of phenylethanoid glycosides, ranging from 1.3 to 11.0 mg per g dry weight.

The results given in Table 2 confirm that *in vitro* conditions are favorable to the production of phenylethanoid glycosides, since their levels were significantly higher in the *in vitro* regenerated *H. procumbens* plants compared to those in plants propagated from seeds. The trend was particularly evident in young plants which were harvested 3 or 6 months after their transfer to *ex vitro* conditions. For example, the leaves of 3-month-old control plants accumulated 16.4 mg g^{-1} DW verbascoside and 8.0 mg g^{-1} DW isoverbascoside i.e. 2–3 times less than leaves of an *in vitro* regenerated plant of the same age. The increase of phenylethanoid glycoside content, especially verbascoside, in *in vitro* regenerated plants of *H. procumbens* could be considered a stress response to *in vitro* conditions. According to Van Staden et al. (2006) the hormonal composition of culture media can be one of the main causes of morphological and physiological modification in regenerated plants, and it could alter the capacity of the plant to produce secondary metabolites. Amoo et al. (2012) report that exogenous supply of growth regulators during *in vitro* propagation significantly alters the quantity of phenolic compounds in micropropagated plants of *Aloe aborescens*. The authors have explained this fact by the carry-over effect of growth regulators applied during micropropagation. The effect may be reduced during long-term *ex vitro* cultivation. This might be a reason for the reduction of verbascoside concentration with age of leaves, stems and roots of *H. procumbens ex vitro* raised plants (Table 2). The values in organs of older (1-year old) plants were approximately 37–74% lower than those of organs from 6-month-old plants. This result is distinctly different than that of *H. procumbens* seed-derived plants, where the leaves of 1-year-old plants produce more verbascoside (about 14 and 26%) than the leaves of younger plants (Table 2). Higher accumulation of verbascoside in *in vitro* regenerated plants has also been identified in *Plantago lanceolata* (Budzianowska et al., 2004) and *Castilleja tenuiflora* (Sanchez et al., 2013). The increased verbascoside and isoverbascoside concentrations in *H. procumbens* after *in vitro* propagation are significant, as these compounds have inter alia antioxidant, antitumor, antibacterial, anti-inflammatory and immunosuppressive effects (Jiménez and Riguera, 1994; Korkina, 2007; Pan et al., 2003).

The variations of harpagoside and harpagide contents in different parts of *in vitro*- and *in vivo*-derived plants of *H. procumbens* are presented in Table 3. Among various tissues of *in vitro*- derived plants, the highest content of total iridoids (4.15 mg g^{-1} DW) calculated as the sum of harpagoside and harpagide was found in the root tubers of 6-month-old plants. They contain a similar value of harpagoside to that seen in root tubers of plants the same age grown directly from seeds: 3.9 mg g^{-1} DW compared with 3.5 mg g^{-1} DW, respectively. However, quantitative differences were found when the harpagide contents in tubers from *in vitro*- and *in vivo*- derived plants were compared; the latter accumulated more of the iridoid (0.72 mg g^{-1} DW vs 0.26 mg g^{-1} DW). Iridoids (harpagoside and harpagide) were detected in aerial parts (leaves and stems) of all tested plants. In the aboveground parts of *in vitro* culture-derived plants, total iridoid content ranged from 2.85 mg g^{-1} DW, in the leaves of 3-month-old plants, to 0.39 mg g^{-1} DW, in the leaves of 12-month-old plants. The range for seed-propagated plants was between 3.01 mg g^{-1} DW, in the leaves of 3-month-old plants, to 1.84 mg g^{-1} DW, in the leaves of 12-month-old plants. As could be seen in root tubers, the leaves and stems of *in vivo*-derived plants produced more harpagide than the aerial parts of micropropagated plants (Table 3). The reason for such differences in harpagide levels in regenerated and seed-derived plants is not clear, because the mechanisms behind the regulation of natural product biosynthesis by plants are as yet unknown. It is possible that the iridoid biosynthetic pathway in both *in vivo*- and *in vitro*-derived *H. procumbens* plants differs only in the last step, leading to the formation of harpagoside from harpagide (Georgiev et al., 2013). It was observed that an increase in *H. procumbens* plant age usually led to a decline in iridoid content in leaves and increased their level in root tubers. For example, harpagoside level in root tubers of 12-month-old *in vitro*-derived plants was 1.6 mg g^{-1} DW compared to 0.37 mg g^{-1} DW in leaves and 1.29 mg g^{-1} DW in stems. The leaves, stems and root tubers of 3-month-old plants were found to contain 2.66 mg g^{-1} DW, 1.92 mg g^{-1} DW and 2.1 mg g^{-1} DW harpagoside, respectively. A similar trend was noticed in *in vivo*-derived plants (Table 3), which suggests a translocation of iridoids with plant age. It is possible that the compounds are synthesized in young leaves and transported via stems to roots during tuber development. However, this is only a hypothesis and further studies are required to verify it. There is no information in the literature about the site of iridoid

Table 3
Quantitative analysis of iridoid glycosides in *H. procumbens*.

Source of material	Plant organ	Content (mg g^{-1} DW) ± SE		
		Harpagoside	Harpagide	Total*
In vitro plants				
3-month-old	Leaves	2.66 ± 0.07g	0.19 ± 0.01de	2.85 ± 0.42
	Stems	1.92 ± 0.01cde	0.43 ± 0.01gh	2.35 ± 0.22
	Roots	2.10 ± 0.03def	0.20 ± 0.001e	2.30 ± 0.38
6-month-old	Leaves	2.72 ± 0.05g	0.04 ± 0.002b	2.76 ± 0.45
	Stems	2.46 ± 0.10fg	0.17 ± 0.01cde	2.63 ± 0.39
	Roots	3.89 ± 0.07h	0.26 ± 0.01f	4.15 ± 0.52
12-month-old	Leaves	0.37 ± 0.01a	0.02 ± 0.001a	0.39 ± 0.05
	Stems	1.29 ± 0.10b	0.03 ± 0.001b	1.32 ± 0.26
	Roots	1.6 ± 0.03b	0.13 ± 0.003c	1.73 ± 0.21
In vivo plants				
3-month-old	Leaves	2.8 ± 0.07g	0.21 ± 0.001ef	3.01 ± 0.43
	Stems	1.8 ± 0.01cd	0.36 ± 0.01g	2.16 ± 0.24
	Roots	2.17 ± 0.01def	0.37 ± 0.01g	2.54 ± 0.36
6-month-old	Leaves	2.28 ± 0.03ef	0.50 ± 0.00h	2.78 ± 0.26
	Stems	1.88 ± 0.03cd	0.53 ± 0.01h	2.41 ± 0.20
	Roots	3.46 ± 0.07h	0.72 ± 0.004i	4.18 ± 0.39
12-month-old	Leaves	1.68 ± 0.12bc	0.16 ± 0.004cd	1.84 ± 0.32
	Stems	1.83 ± 0.005cd	0.03 ± 0.001b	1.86 ± 0.26
	Roots	2.28 ± 0.05ef	0.13 ± 0.01c	2.41 ± 0.43

Results are means ± standard errors (SE) from three independent experiments; each replicate used leaves, stems and root tubers from 3 plants ($n = 9$); different letters within the column indicate significant differences according to the Kruskal–Wallis test ($p \leq 0.05$).
* Sum of harpagoside and harpagide.

biosynthesis in *H. procumbens*. So far, only Levieille and Wilson (2002) have investigated the iridoid concentration in aerial parts of *H. procumbens*. The authors found that the iridoid content of leaves of 18-month-old *in vitro* regenerated plants was 10-times lower than that of the underground parts of the plants. In another study, Bairu et al. (2011) found that the root tubers of *in vitro*-derived *H. procumbens* plants cultivated in a greenhouse for one year contained about 5.5 mg g^{-1} DW iridoids, expressed as harpagoside equivalents. However, it is difficult to compare these results with the yield of iridoid obtained in our experiments since different analytical methods for quantitative determination of iridoids were used, i.e. colorimetric vs HPLC.

4. Conclusion

In summary, the findings of the present study demonstrate that *H. procumbens* plants producing iridoids (harpagoside and harpagide) and phenylethanoids (verbascoside and isoverbascoside) can be obtained by *in vitro* propagation. Not only the tested organ, but also the age of plants should be taken into consideration when *H. procumbens* plant material has been chosen for extraction of pharmacologically important compounds. Young plants (3- and 6-month-old) represent most appropriate stage of development for iridoid and phenylethanoid biosynthesis. Of the *in vitro*-propagated plants, the root tubers of 6-month-old plants were found to accumulate the highest total content of iridoids (4.15 mg g^{-1} DW) similar to that in root tubers of 6-month-old *in vivo*-derived plants (4.18 mg g^{-1} DW). Another promising source of iridoids are the leaves of 3-month-old *in vitro* regenerated plants, which produced about 3.0 mg g^{-1} DW harpagoside and harpagide. The harvesting of leaves does not require the plant to be cut down and the material can be obtained within a short time of cultivation. Additionally, the leaves were characterized by high production of verbascoside and isoverbascoside. However, the pharmacological activity of *H. procumbens* leaf extracts has not yet been studied.

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