Devil's Claw Hairy Root Culture in Flasks and in a 3-L Bioreactor: Bioactive Metabolite Accumulation and Flow Cytometry[◊]

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Phenylethanoids are a group of natural water-soluble compounds with high biological value, which could potentially be commercially produced by hairy root cultures. Thus, we have examined the capacity of transformed root cultures of Devil's claw (*Harpagophytum procumbens*) to accumulate four phenylethanoid glycosides – *β*-OH-verbascoside, verbascoside, leucosceptoside A, and martynoside – in shake-flasks and a 3-L stirred tank reactor. Verbascoside was found to be the major phenylethanoid, and its maximal contents were the same (1.12 mg/g dry weight) in both kinds of culture. However, peak leucosceptoside A contents were 1.6-times higher in bioreactor cultures than in shake-flask cultures. Flow cytometry analysis revealed that G_0 + G_1-phase cells predominated throughout the growth of the cultures, which was in accordance with the very high proportion of quiescent cells in the transformed roots. The results provide the first demonstration of the potential utility of Devil's claw hairy roots as biofactories for producing high-value phenylethanoid glycosides.

Key words: Hairy Root, *Harpagophytum procumbens*, Phenylethanoids

Introduction

Plants produce vast arrays of metabolites (ca. 100,000 have been identified and structurally elucidated), many of which have been used for centuries as drugs, agrochemicals, dyes, food, and cosmetic additives (Fu *et al.*, 2008; Georgiev *et al.*, 2009). These compounds include phenylethanoid glycosides, a large class of natural water-soluble compounds that are widely distributed in the plant kingdom. Characteristic chemical features of these metabolites are a hydroxyphenylethyl moiety linked to a *β*-glucopyranose (apiose, galactose, rhamnose or xylose) via a glycosidic linkage (Dembitsky, 2005). To date, several hundred compounds of this type have been isolated from several plant families (Fu *et al.*, 2008). The present study focused on the production of four of these substances by Devil's claw (*Harpagophytum procumbens*) hairy root cultures: verbascoside (also known as acteoside), a very active phenylethanoid glycoside that has been shown to have antileukemic and cytotoxic activity against a murine cell line and anti-inflammatory activity (Pettit *et al.*, 1990; Diaz *et al.*, 2004); martynoside, which reportedly has antimicrobial activity (Dembitsky, 2005); and two compounds previously isolated from cell suspension cultures of Devil's claw – leucosceptoside A and *β*-OH-verbascoside – which have strong antioxidant activities in several *in vitro* assays (Georgiev *et al.*, 2010).

Plant *in vitro* culture offers a relatively novel and sustainable alternative for mass production of high-value plant-derived metabolites (Georgiev *et al.*, 2009). *Agrobacterium rhizogenes*-transformed root cultures (frequently called "hairy roots", in

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analogy to the natural disease of the same name) have received increasing attention recently since (*inter alia*) they have high genetic and biochemical stability (being theoretically initiated from a single cell), fast growth in hormone-free media, and high productivity (Abou Zid and Orihara, 2005; Georgiev *et al.*, 2007, 2008). Following intense research efforts, transformed root cultures of more than 450 different plant species have now been induced (Georgiev *et al.*, 2008) and shown to be potentially capable of producing several hundreds of compounds in commercially attractive quantities (Georgiev *et al.*, 2007). A key step in the development of commercial technologies based on hairy roots is scaling-up, since large-scale cultures are essential for cost-efficient production of targeted compounds, and the cultures must be maintained in a suitable physiological state. Hence, appropriate monitoring of the cultivation process is essential. For this purpose, flow cytometry could be useful, since it can be used to analyse large numbers of single cells in plant cultures, providing information (*inter alia*) on the distribution of the cells in different phases of the cell cycle (which can help to identify and quantify active and non-active cycling subpopulations). In addition, it can provide information on a wide range of relevant variables, including the cells' DNA, RNA, protein, and pigment contents, their size and shape, intracellular pH, membrane integrity and enzymatic activities. Further, cells with particular properties can be selected by cytometric cell sorting (Yanpaisan *et al.*, 1998). Thus flow cytometry was used for biomonitoring approaches during cultivation of cell suspensions of sunflower (Haas *et al.*, 2008).

We have previously reported the adaptation of transformed root cultures of *Harpagophytum procumbens* (Pedaliaceae) to submerged cultivation and studied the growth of the transformed roots in a 3-L bubble column bioreactor using the RITA® temporary immersion system (Georgiev *et al.*, 2006, 2008; Ludwig-Mueller *et al.*, 2008). We have also developed a purification scheme for isolating several high-value phenylethanoid glycosides from them: β-OH-verbascoside, verbascoside, leucosceptoside A, and martynoside (Gyurkovska *et al.*, 2010). The present study was undertaken to evaluate the potential of Devil's claw hairy roots to accumulate phenylethanoid glycosides in two other cultivation systems, and to assess the utility of flow cytometry for cell cycle analysis of hairy roots.

Material and Methods

Hairy root culture

The *Harpagophytum procumbens* hairy root cultures used to initiate the cultures examined in this study had been maintained for >5 years on solid Murashige and Skoog (MS) medium (Duchefa, Haarlem, The Netherlands, Cat. No. M0222), supplemented with 30 g/L sucrose and 5.5 g/L plant agar (Duchefa, Cat. No. P1003), at 26 °C, with a 21-d period of subcultivation.

Experimental scheme

Devil's claw hairy root cultures used in the experiments were grown (A) in liquid MS medium in 250-mL Erlenmeyer flasks with 20% net volume shaken at 110 rpm, in the dark, at 26 °C and (B) in a stirred tank reactor of 3 L capacity (BioFlo 110, New Brunswick, USA). The bioreactor, equipped with a propeller impeller (speed of 70 rpm), was operated in batch mode at 26 °C with an air flow rate of 1.0 L/min. The inoculum amounts were ~6.5 g dry weight/L.

Hairy root growth and kinetics

The growth and growth kinetics of the roots in the shake-flasks and bioreactor were assessed by harvesting cultures at the times after inoculation indicated in Figs. 1 and 5. On each occasion, the hairy roots were separated from the culture medium by filtration, and the pH value and conductivity of the medium were measured using an InnoLab pH720 pH meter (WTW, Weilheim, Germany) and Qcond 2400 Conductivity Meter Set (VWR International, Darmstadt, Germany), respectively. The final fresh and dried biomasses of the harvested roots were then gravimetrically determined (the latter after drying at 60 °C to constant weight), and their growth kinetics parameters were calculated as follows:

$$dX/dt = \mu X, \quad (1)$$

$$\mu = \ln (X/X_0)/\Delta t, \quad (2)$$

$$t_d = \ln 2/\mu, \quad (3)$$

where X_0 and X are the initial and final biomasses (g/L), respectively, Δt is the culture time interval (d), μ is the specific growth rate (1/d), and t_d is the doubling time (d).

Metabolite extraction and HPLC analysis

Frozen root tissue samples were extracted with methanol (solid/liquid ratio 1:50) in an ultrasonic bath (3 × 15 min), pooled, filtered (through 0.2-μm filters), and directly injected into an HPLC system, to quantify their phenylethanoid contents. The HPLC system consisted of an 1525 binary HPLC system controlled by Breeze software version 3.30 SPA, a 2487 dual λ absorbance detector (both from Waters Corp., Milford, MA, USA), and a Discovery® HS reverse phase (C18) column (250 mm × 4.6 mm i.d., 5 μm particle size; Supelco, Bellefonte, PA, USA). The metabolites were separated using a mobile phase consisting of methanol/water 10:90 at a flow rate of 1.0 mL/min for 5 min, followed by a linear gradient to 50:50 at a flow rate of 1.1 mL/min over 10 min. After each separation the column was washed using 100% methanol at a flow rate of 0.8 mL/min, then equilibrated to the starting composition (methanol/water 10:90), at a flow rate of 1.0 mL/min for 5 min. Eluting phenylethanoids were detected, and quantified, by monitoring the eluate at a wavelength of 330 nm. In addition, β-OH-verbascoside, verbascoside, leucosceptoside A, and martynoside were isolated from other portions of extracts of Devil's claw hairy roots, using a Polyamide 6 column, followed by Lobar columns RP8 and RP18, then identified by ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectroscopy using a Bruker AV 600 spectrometer, and comparing the resulting spectra with reported data (Miyase *et al.*, 1982; Sticher and Lahloub, 1982; Erzos *et al.*, 2002). Pure compounds were used to prepare calibration curves.

Flow cytometry (FCM)

The flow cytometric analyses were carried out using a CyFlow® SL Blue (20 mW solid-state laser at 488 nm) flow cytometer (Partec GmbH, Münster, Germany), operated with FloMax (Partec GmbH) software. Prior to analysis the flow cytometer was aligned using signals from fluorescent beads (Fluoresbrite 2.7 μm; Polysciences Inc., Warrington, PA, USA), as the red fluorescence was collected through a 630-nm high-pass filter. Cells from portions of the hairy roots obtained from the shake-flask cultures, at each harvesting occasion, were extracted and their nuclei were stained. Systematic investigations with numerous extraction and staining buffers, varying incubation times, the incorporation of centrifugation steps in the sample treatment, and the addition of several agents (polyvinylpyrrolidone, β-mercaptoethanol, ascorbic acid) to the buffers showed that the best results were obtained using general purpose buffer [GPB, 0.5 mM spermine · 4 HCl, 30 mM sodium citrate, 20 mM MOPS, 80 mM KCl, 20 mM NaCl, 0.5% (v/v) TritonX-100, pH 7.0], as described by Loureiro *et al.* (2007). For extraction and staining approx. 50 mg of hairy root tissue were chopped in GPB with a sharp razor blade, filtered trough a 30-μm mesh filter (Partec GmbH), incubated for 7 min, and analysed as described above.

To reduce noise from debris, signals from the nuclei were gated inside the scatter *vs*. red fluorescence dot plots, allowing nuclei to be clearly identified by their known fluorescence intensity/scattered light pattern. It should be noted that the coefficient of variation (CV) of the signal intensities was high (up to 20%), although the staining and extraction parameters were optimized, showing that hairy root tissue is not straightforward to be analyzed flow cytometrically.

Statistical analysis of the data

The data presented are averages obtained from two independent experiments for bioreactor cultures and three independent experiments with two replicates for shake-flask cultures. FCM and HPLC measurements were done in triplicate. The results are presented as means ± standard deviation (S.D.).

Results and Discussion

Shake-flask cultures

Growth parameters of the shake-flask cultivation of transformed Devil's claw root cultures in liquid MS medium are presented in Fig. 1. The cultures exhibited strong growth with an exponential phase between the 6th and 14th day of cultivation, and their biomass was highest (15.6 g/L) at the end of the exponential phase of growth. The highest specific growth rate of the transformed roots was 0.243 – 0.249 1/d (Fig. 1) with a doubling time of ~67 h. As previously observed, the roots grew mainly in size, without forming many lateral branches (Georgiev *et al.*, 2006; Ludwig-Mueller *et al.*, 2008), unlike most hairy root cultures. Strong yellow pigmentation of the roots was also observed.

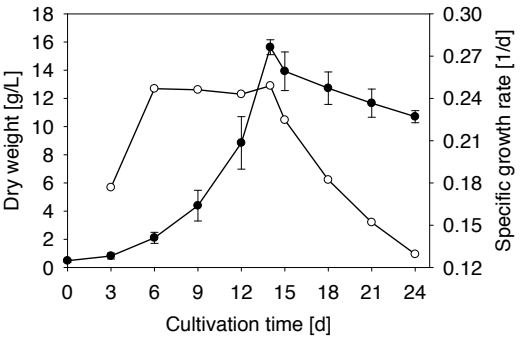

Fig. 1. Time courses of dry weight (●) and specific growth rate (○) of *Harpagophytum procumbens* hairy roots, cultivated in shake-flasks. Mean values ± S.D.

Flow cytometry was used to measure cell cycle parameters in the shake-flask cultures (Fig. 2). All examined samples showed a predominance of $G_0 + G_1$-phase cells, with a nuclear DNA content equivalent to the holoploid genome of Devil's claw cells. We did not detect any distinct S-phase or G_2 + M-phase cells (except on the 9th day of cultivation, when small portions of cells with DNA contents exceeding $G_0 + G_1$-phase cells were detected), probably because little or no cell division (and thus cell cycling) occurred in the hairy roots. This is not surprising since the *Harpagophytum procumbens* hairy root clone does not form many lateral branches of the root tips, which are the only parts of the roots that are likely to contain meristematic (actively diving) cells, thus only small fractions of the cells in the hairy root tissue are likely to be actively cycling. Indeed, the portions of meristematic cells in the samples examined by FCM were that small that no cycling cells were generally detected. This assumption is supported by the fact that Neumann *et al.* (1998) who synchronized hairy roots from *Pisum sativum* investigated the first millimeter of the root tips only.

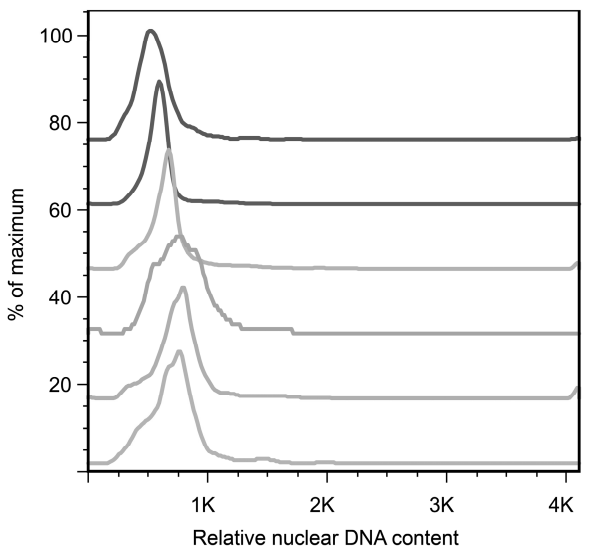

Fig. 2. DNA histograms of *Harpagophytum procumbens* hairy roots during submerged cultivation in shake-flasks (from bottom to top: day 3, 6, 9, 12, 15, and 18 of cultivation).

Our data clearly indicate that the observed increases in biomass during the hairy root cultivations arose from increases in the mass of the cells rather than cell division. We also observed slight shifts in the position of the peaks (Fig. 2), probably caused by variations in the concentrations of secondary metabolites in the extracts that influenced the chromatin structure and hence the dyeability of the nuclei. The presence of high concentrations of these cytosolic substances is furthermore suspected to be the reason for the comparatively high CVs of the DNA peaks. Further studies will focus on developing multiparametric cell cycle analysis techniques, which should provide additional information for distinguishing between cycling and quiescent cells (Yanpaisan *et al.*, 1999; Haas *et al.*, 2008).

The capacity of transformed *Harpagophytum procumbens* roots to produce secondary metabolites was investigated by determining their intracellular phenylethanoid contents. Verbascoside was found to be the major phenylethanoid glycoside (Fig. 3). The maximal verbascoside content in the transformed Devil's claw roots was observed in the early stationary phase of growth (1.12 mg/g dry weight), while martynoside, β-OH-verbascoside, and leucosceptoside A contents were maximal (at 0.87, 0.33, and 0.20 mg/g dry weight, respectively) in the late stationary phase (between the 18th and 21st days of cultivation; Fig. 4). While verbascoside, martynoside, and leucosceptoside A are reportedly accumulated in hairy root cultures of *Scutellaria baicalensis*, *Paulownia tomentosa*, and *Gmelina arborea* (Zhou *et al.*, 1997; Wysokinska and Rozga, 1998; Dhakulkar *et al.*, 2005), there are no previous reports of the accumulation of β-OH-verbascoside in any hairy root culture.

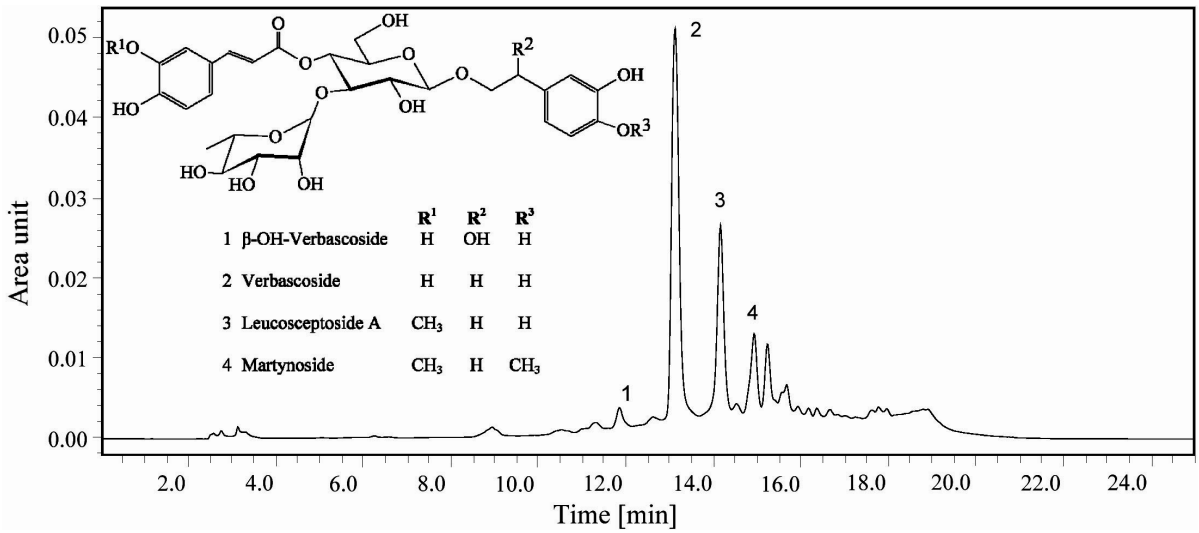

Fig. 3. Representative HPLC chromatogram and chemical structure of phenylethanoids isolated from *Harpagophytum procumbens* hairy roots.

Stirred tank reactor cultures

Bioreactor cultivation represents a critical step in the development of technologies for commercial production of metabolites by hairy root cultures (Georgiev *et al.*, 2007). Thus, in recent years several types of bioreactor have been developed for hairy root culture (Eibl and Eibl, 2008), which should ideally be compatible with the unique physiological characteristics of hairy roots, readily scalable and cost-effective. To meet these requirements we decided to use a standard stirred tank bioreactor, equipped with a propeller impeller, operating at lower agitation speed (70 rpm).

A significant problem associated with scaling up hairy root cultures is that it is impossible to take representative samples from the root masses. Thus, the *Harpagophytum procumbens* hairy roots were cultivated in the bioreactor for 14 d and 21 d. Slightly higher biomass amounts were accumulated after a 14-days cultivation (8.98 g/L), than after 21-days cultivation (8.35 g/L). The accumulated dry biomass was also lower than in shake-flask cultures, but ~30% higher compared to amounts obtained in previous cultivations of Devil's claw hairy roots in a 3-L bubble column bioreactor and RITA® temporary immersion systems (Georgiev *et al.*, 2008; Ludwig-Mueller *et al.*, 2008). This is presumably because the agitation in a stirred tank reactor improves the mass transfer of nutrients and oxygen. The high sensitivity of transformed root cultures to mechanical stress (Georgiev *et al.*, 2007) should also be considered, thus an appropriate impeller design – working at low agitation speed – should be chosen. It should also be mentioned that careful optimization of the conditions in the bioreactor (*e.g.* cultivation temperature, agitation, and air flow rate) is a power-

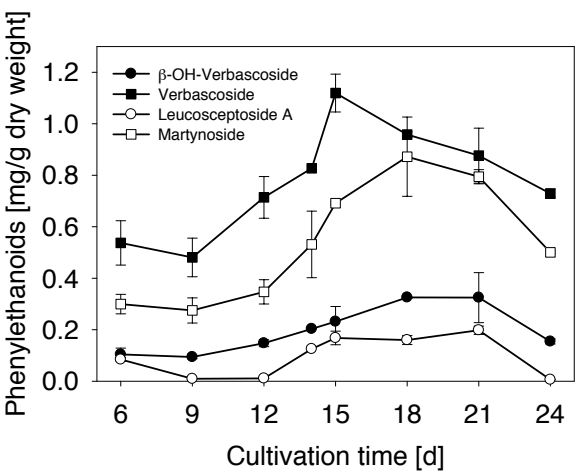

Fig. 4. Time courses of *β*-OH-verbascoside, verbascoside, leucosceptoside A, and martynoside accumulation in *Harpagophytum procumbens* hairy roots, cultivated in shake-flasks. Mean values ± S.D.

ful strategy for enhancing biomass accumulation (Georgiev *et al.*, 2009). The higher biomass accumulation in the stirred tank reactor – compared to other above-mentioned cultivation systems – together with its low capital costs and relative easy scalability might make this system highly suitable for hosting Devil's claw hairy roots.

Changes in dissolved oxygen (DO2) levels were also followed during the hairy roots' cultivation in the stirred tank reactor. The DO2 levels rapidly decreased until day 7 of cultivation (from 100% to 40%) and then remained constant until the end of the cultivation process (~38.5%). When the highest biomass of the roots in the bioreactor was recorded (at day 14), levels of DO2 were below 40% of air saturation.

In contrast to the roots' biomass, which was higher at day 14 of cultivation than at day 21, levels of secondary metabolites were higher at day 21 (Fig. 5). The maximum recorded content of verbascoside – the major phenylethanoid in Devil's claw hairy roots – was the same as in shake-flask cultures, while *β*-OH-verbascoside and martynoside contents were lower. It should also be mentioned that the transformed roots' leucosceptoside A content was 1.6-times higher in the 3-L stirred tank reactor cultures than in the shake-flask cultures. This is interesting since leucosceptoside A has been found to have strong antioxidant activity in oxygen radical absorbance capacity ($ORAC_{FL}$) assays (Georgiev *et al.*, 2010), hence its elevated levels in bioreactor cultures of Devil's claw hairy roots might improve their biological value. The levels of the other two phenylethanoid glycosides – *β*-OH-verbascoside and martynoside – were slightly lower than in shake-flask cultures.

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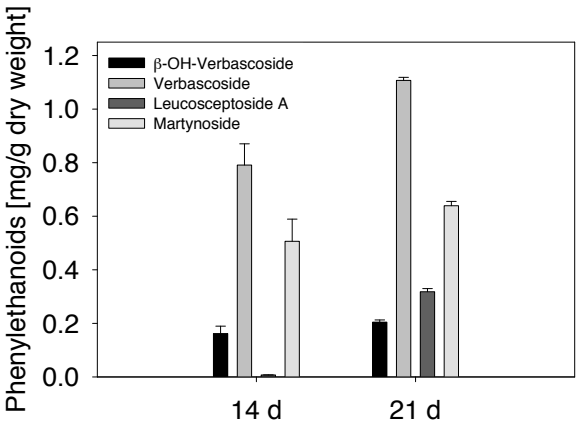

Fig. 5. Phenylethanoid contents in *Harpagophytum procumbens* hairy roots at days 14 and 21 of cultivation in the 3-L stirred tank reactor. Mean values ± S.D.

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