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Identification and quantification of phenolic compounds in *Hypericum perforatum* L. transgenic shoots

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Abstract Regeneration of transgenic shoots was achieved from *Hypericum perforatum* L. hairy roots on hormone-free MS/B$_5$ medium for a period of 4 weeks under a photoperiod of 16-h light. A control experiment was set up with root segments obtained from in vitro grown seedlings. Investigations have been made to study the production of phenolic compounds in non transgenic and transgenic shoot cultures. Six groups of phenolic compounds such as phenolic acids, flavonols, flavan-3-ols, naphtodianthrones, phloroglucinols, and xanthones were recorded in the transgenic shoots. Chlorogenic acid was found as the most representative phenolic acid in shoot extracts. With regard to the class of quercetin derivatives in transformed shoots, quercetin 6-*C*-glucoside usually dominated among the glycosides followed by quercitrin and hyperoside. The analysis of flavan-3-ols in transgenic shoots resulted in the identification of epicatechin and proanthocyanidin dimers. One of the main achievements in this study was considerably enhanced hypericin and pseudohypericin production in transgenic shoots. The concentration of identified naphtodianthrones was about 12-fold higher in transformed shoots compared to control. Chromatographic analysis of phloroglucinols in transgenic shoots resulted in the identification of hyperforin, while its homolog adhyperforin was detected in traces. A twofold higher content of hyperforin was observed in transgenic shoots compared to control. Although mangiferin was found as the main representative xanthone in shoot extracts, several other xanthones identified as γ-mangostin isomers, trihydroxy-1-methoxy-*C*-prenyl xanthone, garcinone E, and banaxathone E were de novo synthesized in transformed shoots. Therefore, *H. perforatum* transgenic shoots could be considered as a source for rapid and increased production of naphtodianthrones and other specific phenolic compounds.

Keywords Hairy roots · *Hypericum perforatum* L. · Naphtodianthrones · Phenolic compounds · Transgenic shoots

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Introduction

St. John's wort (*Hypericum perforatum* L.) is a well-known traditional medicinal plant naturally occurred in relatively dry temperate zones of Europe, West Asia, and North Africa. This plant has received considerable interest in recent years due to the increased market demand for *Hyperici herba* crude material as a source of natural bioactive pharmaceuticals. *Hypericum* extracts contain a complex mixture of bioactive metabolites, mainly naphtodianthrones (hypericin and pseudohypericin), phloroglucinols (hyperforin and adhyperforin), and flavonoids with a broad spectrum of biological effects (Kirakosyan et al.

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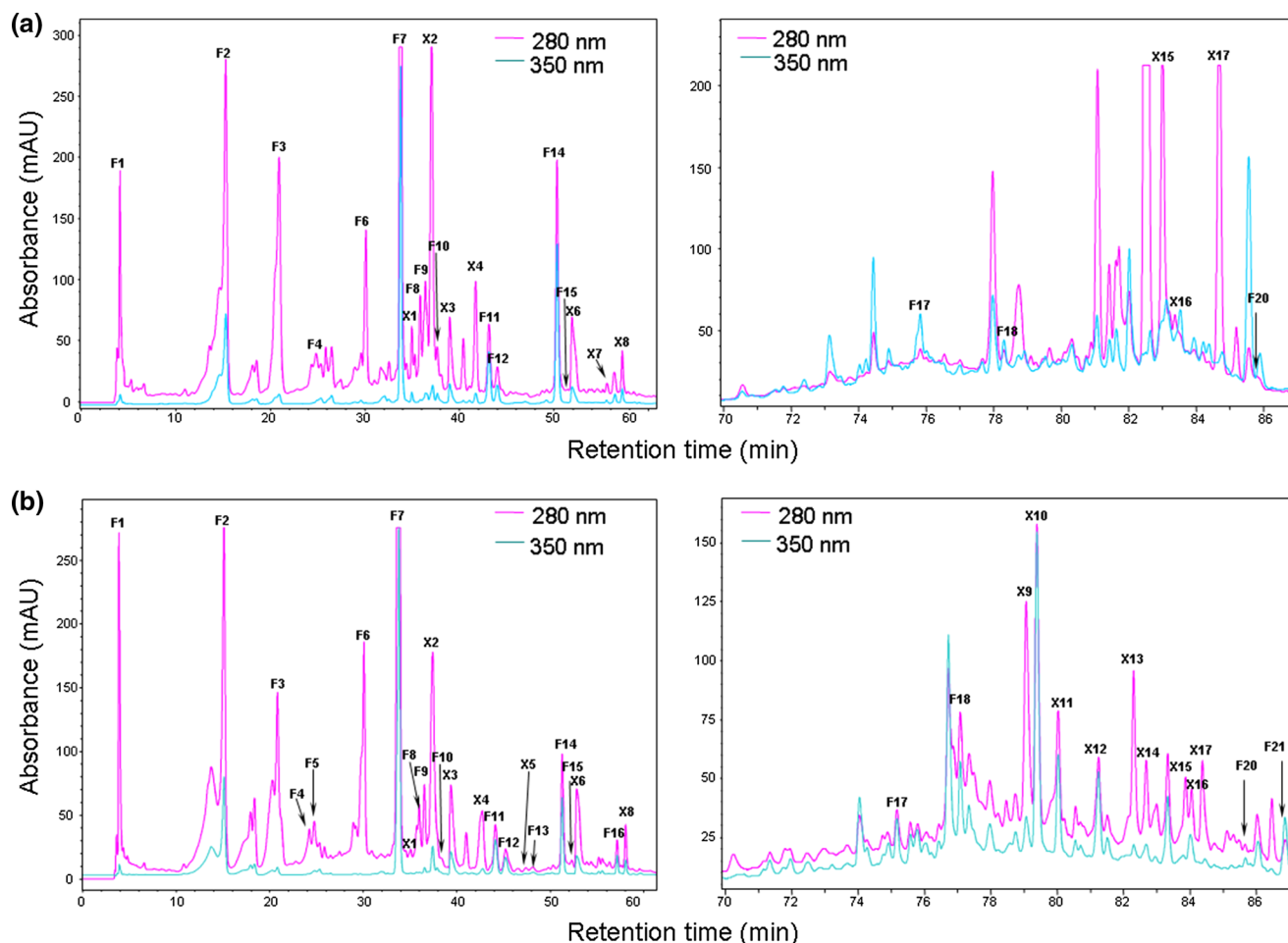
Fig. 1 Chromatograms of *Hypericum perforatum* control (**a**) and transgenic shoots (**b**) monitored at 280 and 350 nm for detection of phenolic compounds. *Compound symbols* correspond to those indicated in Table 3

2004). Several pharmacological studies have been published concerning anti-inflammatory, hepatoprotective, antiviral, antimicrobial, antioxidant, antitumoral, and wound-healing activity of *Hypericum* extracts (Bombardelli and Morazzoni 1995; Barnes et al. 2001; Medina et al. 2006). The most significant use of *Hypericum* pharmaceutical preparations comprise symptomatic treatment of mild-to-moderate depression and recently good perspectives emerged also in the field of major depression (Sarris et al. 2012; Solomon et al. 2013). It has been shown that antidepressant action of this plant is due to the synergistic effects of hyperforins, hypericins, and flavonoids (Bilia et al. 2002; Butterweck 2003).

The pharmaceutical industry is currently supplied with plant material produced through field cultivation to maximize both yield and quality of phytochemicals responsible for therapeutic properties (Büter et al. 1998). However, chemical variability of *H. perforatum* field-grown plants has been strongly influenced by genetic, physiological, metabolic, ecological, and environmental

factors (Cellárová 2003; Košuth et al. 2003; Kirakosyan et al. 2004). Moreover, field-grown plant material is usually susceptible to contamination by pollutants and infestation by fungi, bacteria, viruses, and insects that can compromise the quality of bioactive products. For these reasons, it is difficult to produce chemically consistent *H. perforatum* plants and to obtain high-quality standardized extracts containing stable quantities of hypericins, hyperforins, and flavonoids (Murch and Saxena 2006). As a consequence of the great commercial potential of this species, attempts have been focused on improvement of secondary metabolite production by application of in vitro culture methods (Zobayed et al. 2003). The deliberate stimulation of specific bioactive metabolites within carefully regulated in vitro cultures provides an excellent form for extensive investigation of metabolic pathways under highly controlled micro-environmental regimes (Karuppusamy 2009).

Plant genetic transformation offers opportunity to introduce new qualities into medicinal and aromatic plants.

Fig. 2 Chromatograms of *Hypericum perforatum* control (**a**) and transgenic shoots (**b**) monitored at 590 nm for detection of naphtodianthrones. *Compound symbols* correspond to those indicated in Table 3
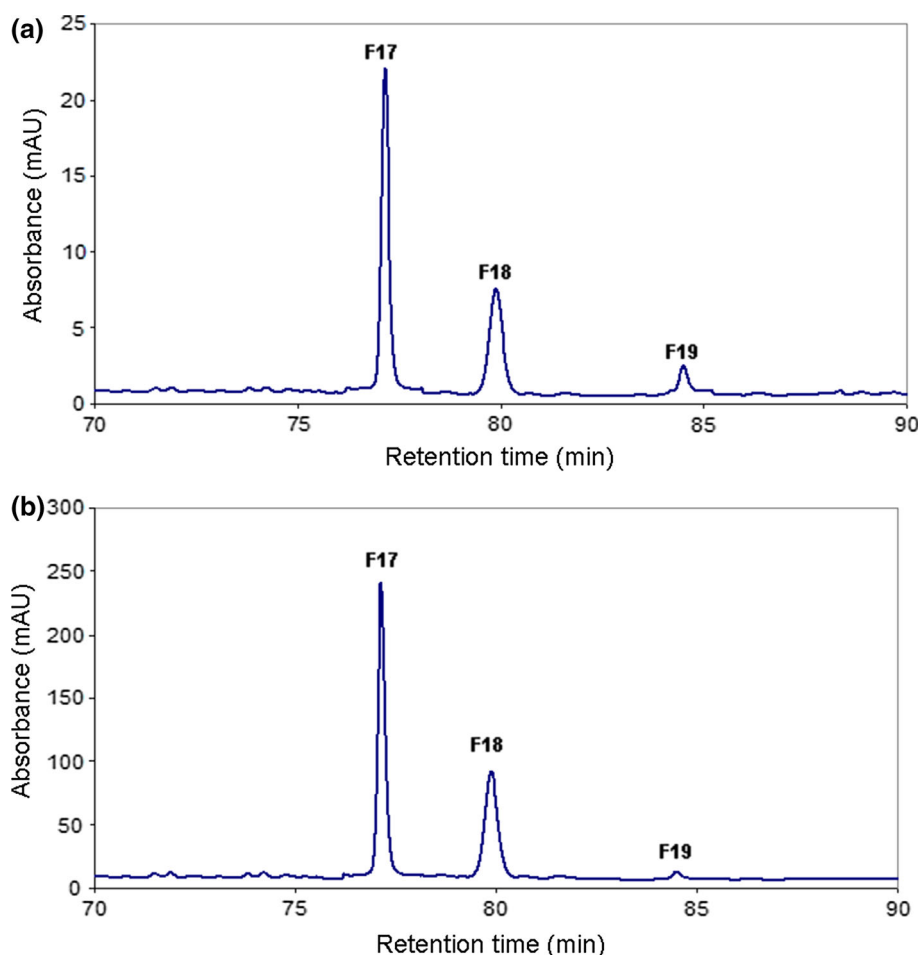
Agrobacterium rhizogenes, a gram-negative phytopathogenic soil bacterium, is well known for its ability to transfer its T-DNA from the root-inducing (Ri) plasmid to the host genome, thereby causing formation of hairy roots (HR). HR cultures are considered as a promising source of physiologically consistent plant tissues for standardized production of root-specific metabolites due to their biochemical and genetic stability (Guillon et al. 2006). Until now, only *A. rhizogenes* (Di Guardo et al. 2003; Vinterhalter et al. 2006; Komarovská et al. 2009) and biolistic-mediated (Franklin et al. 2007) transformation procedures for few *Hypericum* species have been applied. Wild agropine strain *A. rhizogenes* ATCC 15834 was used in the first successful transformation of *H. perforatum* (Di Guardo et al. 2003). Also, an efficient transformation protocol of this species was reported with *A. rhizogenes* A4M70GUS (Vinterhalter et al. 2006). Two other *Hypericum* species (*H. tomentosum* and *H. tetrapterum*) were successfully transformed with *A. rhizogenes* ATCC 15834 and A4 (Komarovská et al. 2009). Recently, we have developed an efficient *A. rhizogenes* A4-mediated transformation system for *H. perforatum* which lead to the formation of HR

cultures (Tusevski et al. 2013). *H. perforatum* HR exhibited high potential for spontaneous regeneration into whole transgenic plants (Di Guardo et al. 2003; Vinterhalter et al. 2006). Studies on successful regeneration of HR have stimulated interest in developing procedures for analyses of phytochemical composition in transgenic plants. However, only few studies have been focused on secondary metabolite production in *H. perforatum* HR (Tusevski et al. 2013) and HR-regenerated plants (Di Guardo et al. 2003; Bertoli et al. 2008; Koperdáková et al. 2009a). We have previously studied the phenolic profile of *H. perforatum* HR with respect to phenolic acids, flavonol glycosides, flavan-3-ols, flavonoid aglycones and xanthones (Tusevski et al. 2013). In this view, we have reported that *H. perforatum* HR cultures represent a promising experimental system for enhanced xanthone production. Even though *Hypericum* HR are system of choice for commercial scale of xanthones, these cultures are not suitable for production of the most important bioactive compounds (hypericins and hyperforins) usually distributed in the aerial parts of the plant. The presence of some specific secondary metabolites (hypericin, hyperforin, chlorogenic acid, rutin, hyperoside,

quercitrin, and quercetin) was already reported in the literature for *H. perforatum* transgenic shoots (Di Guardo et al. 2003; Bertoli et al. 2008). Even if work had been carried out on these topics, our study provided complementary information on the identification and quantification of different classes of phenolic compounds in *H. perforatum* transgenic shoots. The present study described culture conditions for establishment of *H. perforatum* transgenic shoots through *A. rhizogenes* mediated HR cultures. Transgenic shoots were evaluated according to their morphology and production of phenolic compounds (phenolic acids, flavonols, flavan-3-ols, naphtodianthrones, phloroglucinols, and xanthones).

Materials and methods

Plant material

Seeds from *H. perforatum* were collected from wild plants growing in a natural population in the National Park Pelister at about 1,394 m asl. Voucher specimen (Number 060231) of *H. perforatum* is deposited in the Herbarium at the Faculty of Natural Sciences and Mathematics, University "Ss. Cyril and Methodius"-Skopje, Republic of Macedonia (MKNH). As previously reported (Gadzovska et al. 2005), seeds were surface sterilized and cultured on MS macro and oligoelements (Murashige and Skoog 1962), B_5 vitamin solution (Gamborg et al. 1968), supplemented with 3 % sucrose, and solidified with 0.7 % agar. In vitro cultures were maintained in a growth chamber at 25 ± 1 °C under a photoperiod of 16-h light, irradiance at 50 µmol m^2 s^{-1}, and 50–60 % relative humidity.

Establishment of transgenic shoots

Establishment of *H. perforatum* HR cultures was described in our previous study (Tusevski et al. 2013). Briefly, HR cultures were induced from root segments of in vitro-grown seedlings, after co-cultivation with *Agrobacterium rhizogenes* strain A4. The transgenic nature of HR cultures was confirmed by PCR analysis of the presence of *rol*B sequences from T_L-DNA of *A. rhizogenes* Ri plasmid (Tusevski et al. 2013). Transformed roots were maintained by subculturing at 28-day intervals on MS/B_5 hormone-free medium in the dark. One HR line exhibiting the highest growth potential was previously selected for HPLC/DAD/ESI–MS^n analysis (Tusevski et al. 2013). In this study, we used the same HR line for establishment of transgenic shoot cultures. For this purpose, HR segments (about 1.5 cm length without root tip) were excised and cultivated on solid MS/B_5 medium without phytohormones. The

cultures were maintained in a growth chamber at 25 ± 1 °C under a photoperiod of 16-h light, irradiance at 50 µmol m^2 s^{-1}, and 50–60 % relative humidity. A control experiment was set up with root segments apart the root tip obtained from in vitro grown seedlings. After 4 weeks, HR and control explants exhibited high potential for spontaneous shoot regeneration. Regeneration frequency of explants, number of adventitious shoots per explant, and length of adventitious shoots in control and HR explants were determined. Emergent control and HR-regenerated shoots of size 1.5–2.0 cm were excised from the root tissues and transferred on solid MS/B_5 medium supplemented with 0.2 mg L^{-1} N^6-benzyladenine (BA) for incessant shoot growth. Shoot cultures were monthly subcultured on the same medium and maintained under a 16-/8-h light/dark photoperiod. After the third subculture, control and transgenic shoots were used for evaluation of morphometric parameters and phytochemical composition. The daily growth index (DGI) (final fresh weight-initial fresh weight/days of culture) was measured. The productivity coefficient (PC) was calculated by multiplying the daily growth index per dry weight yield (DGI × DWY). Furthermore, the morphological characterization was evaluated by length of shoots, number of leaf couples, and number of dark glands per leaf. These morphological data were used to calculate the index of compactness [IC = number of leaf couples per shoot/shoot length (cm)] (Bertoli et al. 2008). Leaves at similar developmental stage (five uppermost fully developed leaf couples) from each shoots were used for measurement of number of dark glands per leaf (Koperdáková et al. 2009a). Thereafter, control and transgenic shoots were harvested, frozen in liquid nitrogen, and then stored at −80 °C, until phytochemical analysis.

HPLC/DAD/ESI–MS^n analysis

Phenolic profile was investigated in 30-day-old control and transgenic shoot cultures. Phenolic compounds extraction from freeze–dried lyophilized and powdered plant material was performed as previously reported by Tusevski et al. (2013). Briefly, phenolic compounds were extracted from powdered plant material (0.2 g) with 80 % (v/v) methanol in ultrasonic bath for 30 min at 4 °C. Methanolic extracts were centrifuged (15 min at 12,000 rpm) and the supernatant was filtered through Sep-pack C_{18} cartridges before HPLC analysis. The HPLC system was equipped with an Agilent 1100 series diode array and mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser, and G1315B photo-diode array detector, controlled by ChemStation software (Agilent, v.08.03). Chromatographic separations were carried out on

150 × 4.6 mm, 5 μm XDB-C18 Eclipse column (Agilent, USA). The mobile phase consisted of two solvents: water–formic acid (A; 99:1 v/v) and methanol (B) in the following gradient program: 90 % A and 10 % B (0–20 min), 80 % A and 20 % B (20–30 min), 65 % A and 35 % B (30–50 min), 50 % A and 50 % B (50–70 min), 20 % A and 80 % B (70–80 min), and continued with 100 % B for a further 10 min. Each run was followed by an equilibration period of 10 min. The flow rate was 0.4 mL min^{-1} and the injection volume 10 μL. All separations were performed at 38 °C. Formic acid (HCOOH) and methanol (CH$_3$OH) were HPLC grade solvents (Sigma-Aldrich, Germany). The HPLC water was purified by a Purelab Option-Q system (Elga LabWater, UK). The commercial standards chlorogenic acid, rutin, quercetin, kaempferol, catechin, epicatechin, phloroglucinol, hypericin, pseudohypericin, and xanthone (Sigma-Aldrich, Germany) were used as reference compounds. The reference compounds were dissolved in 80 % methanol. The concentration of the stock standard solutions was 1 mg mL^{-1} and they were stored at −20 °C. Spectral data from all peaks were accumulated in range 190–600 nm, and chromatograms were recorded at 260 nm for xanthones and hyperforins, at 280 nm for flavan-3-ols, at 330 nm for phenolic acids, at 350 nm for flavonols, and at 590 nm for hypericins. Peak areas were used for quantification at wavelengths where each group of phenolic compounds exhibited an absorption maximum. The HPLC system was connected to the Agilent G2445A ion-trap mass spectrometer equipped with electrospray ionization (ESI) system and controlled by LCMSD software (Agilent, v.6.1.). Nitrogen was used as nebulizing gas at a pressure level of 65 psi and the flow was adjusted to 12 L min^{-1}. Both the heated capillary and the voltage were maintained at 350 °C and 4 kV, respectively. MS data were acquired in the negative ionization mode. The full scan mass covered the mass range from m/z 100 to 1,200. Collision-induced fragmentation experiments were performed in the ion trap using helium as a collision gas, with voltage ramping cycle from 0.3 up to 2 V. Maximum accumulation time of the ion trap and the number of MS repetitions to obtain the MS average spectra were set at 300 ms and 3, respectively. Identification of the component peaks was performed by the UV/Vis, MS and MS^2 spectra, and retention times of the abovementioned available standards.

Statistical analysis

The experiments were independently repeated twice under the same conditions and all analyses were performed in triplicate. The data were expressed as mean values with a standard deviation (±SD). In statistical evaluation, the Student's t test was used for the comparison between two independent groups using SPSS statistical software program (SPSS version 11.0.1 PC, IL, USA). Differences were considered significant at $p < 0.05$.

Results

Establishment of transgenic shoots

H. perforatum transgenic shoots were regenerated from HR explants on MS/B$_5$ medium without phytohormones under a 16-/8-h light/dark photoperiod. Control root explants obtained from in vitro grown seedlings were also cultured under the same conditions. HR and control explants did not exhibit a significant difference in the regeneration frequency (about 65 %) (Table 1). At the beginning of culture, green shoot primordia developed thrust out of the root surface without visible callus formation. HR did not show a significant difference in the number of shoots per explant compared to corresponding control roots (Table 1). Transgenic shoots evaluated in our study showed normal size and morphology like those regenerated from control roots. Moreover, no significant difference in shoot length was observed between HR and control regenerated shoots (Table 1).

In order to stimulate further growth of transgenic and control shoots, they were cultured on MS/B$_5$ medium supplemented with 0.2 mg L^{-1} BA medium. After 20–30 days of culture on this medium, multiple shoots were obtained directly from apical or axillary buds. The productivity coefficients (PC) calculated by using DGI and DWY were very similar between multiplied transgenic and control shoots (Table 2). Transgenic and control shoots did not show significant differences for IC values. These morphological data were confirmed by calculating the number of leaf couples per shoot and the length of shoots. Unlike these morphometric parameters, the number of dark glands on the margins of leaves (Table 2) was significantly higher in transgenic shoots (17.3 ± 1.7) compared to control ones (11.7 ± 1.9). Overall, transgenic shoots did not show any differences in grown pattern and morphology except the number of dark glands per leaf compared to control shoots.

Table 1 Morphometric parameters of control and transgenic *Hypericum perforatum* shoots on solid hormone-free MS/B$_5$ medium

Root cultures	Regeneration frequency (%)	Number of shoots per explant	Shoot length (cm)
Control roots	62.2 ± 8.5	3.1 ± 1.8	3.7 ± 1.1
Hairy roots	67.4 ± 3.8	4.5 ± 2.1	3.5 ± 0.8

Table 2 Morphometric parameters of control and transgenic *Hypericum perforatum* shoots on solid MS/B_5 medium supplemented with 0.2 mg L^{-1} BA

Shoot cultures	DGI	DWY (%)	PC	Number of leaf couples	Shoot length (cm)	IC	Number of dark glands
Control shoots	0.12 ± 0.04	14.9 ± 2.3	0.018 ± 0.002	8.4 ± 0.3	3.7 ± 0.5	2.3 ± 0.2	11.7 ± 1.9
Transgenic shoots	0.16 ± 0.03	15.5 ± 1.9	0.024 ± 0.003	9.8 ± 1.3	3.8 ± 0.2	2.6 ± 0.19	17.3 ± 1.7*

DGI daily growth index, *DWY* dry weight yield, *PC* productivity coefficient, *IC* index of compactness

* Denoted values indicating significant differences between data ($p < 0.05$)

HPLC/DAD/ESI–MS^n analysis

The HPLC/DAD/ESI–MS^n technique was used to analyze the secondary metabolite profile in *H. perforatum* control and transgenic shoots. Six groups of phenolic compounds such as phenolic acids, flavonols, flavan-3-ols, naphtodianthrones, phloroglucinols, and xanthones were recorded in shoot extracts (Table 3). Their identification was based on the typical UV/Vis spectral data and LC/MS in the negative ionization mode [M–H]$^-$ with the subsequent MS^2, MS^3 and MS^4 analysis for further identification with reference to similar data previously reported (Zhang et al. 2007; Pappeti et al. 2008; Ferreres et al. 2008; Silva et al. 2005; Conceição et al. 2006; Miketova et al. 1998; Cuyckens et al. 2001; Piperopoulos et al. 1997; Piovan et al. 2004; Tolonen et al. 2002; Dias et al. 2000; Franklin et al. 2009). The HPLC analysis of secondary metabolites revealed marked differences between control and transgenic shoots.

Phenolic acids

HPLC chromatograms confirmed the presence of four phenolic acids (**F1**, **F2**, **F3,** and **F5**) in shoot extracts (Table 3; Fig. 1). Compound **F1** was identified as quinic acid, taking into account its MS^n fragmentation pattern and literature data (Zhang et al. 2007). The molecular ion fragmentation of 3-caffeoylquinic acid (**F2**) yielded fragment ions corresponding to quinic acid (*m/z* 191) and caffeic acid (*m/z* 179) moieties (Pappeti et al. 2008). 3-*p*-coumaroylquinic acid (**F3**) and 3-feruloylquinic acid (**F5**) were readily distinguished by their cinnamic acid-derived MS^2 base peaks. Quinic acid, 3-caffeoylquinic acid and 3-*p*-coumaroylquinic were detected in both control and transgenic shoots, while 3-feruloylquinic acid was presented only in transgenic cultures. 3-caffeoylquinic acid (chlorogenic acid) was found as the most representative phenolic acid in *H. perforatum* transgenic shoots. A 1.3-fold increase of 3-caffeoylquinic acid was observed in transgenic shoots compared to control. In addition, transgenic shoots showed a capability for de novo production of 3-feruloylquinic acid. The total amount of identified

phenolic acids in transgenic shoots was significantly higher compared to control shoots.

Flavonols

In *H. perforatum* control and transgenic shoots, flavonols were represented with quercetin and kaempferol derivatives (Table 3; Fig. 1). Compound **F7** can be identified as *C*-glycoside of quercetin due to detected deprotonated molecular ion and its MS^2 fragment ions characteristic of mono-*C*-hexosyl flavones (Ferreres et al. 2008). The UV-spectrum and MS data of compound **F9** were consistent with those of kaempferol 3-rhamnoside. According to its UV and mass spectra, compound **F10** was identified as kaempferol 6-*C*-glucoside. Compound **F11** had MS data consistent with those of quercetin derivative with a hexose C3 (Silva et al. 2005). Therefore, this compound was tentatively identified as hyperoside (quercetin 3-*O*-galactoside). Taking into account the mass spectra, compounds **F12** and **F15** were distinguished as quercetin and kaempferol derivatives with rutinose at C3, respectively (Conceição et al. 2006). The absence of intermediate fragmentation between the deprotonated molecular ion and the aglycone ion is indicative of an interglycosidic linkage 1 → 6 (Cuyckens et al. 2001); therefore these compounds were putatively identified as quercetin 3-*O*-rutinoside (rutin) and kaempferol 3-*O*-rutinoside (Silva et al. 2005). According to its UV and mass spectra, compound **F13** was tentatively identified as quercetin 3-*O*-pentoside. Compound **F14** had UV-spectrum and MS data (Silva et al. 2005) consistent with those of quercitrin (quercetin 3-rhamnoside). The identification of compound **F16** was confirmed by spiking with a commercial standard of quercetin and confirmation of its MS data. In transgenic shoots, flavonols were observed to be qualitatively and quantitatively distinct from those of the corresponding control shoots. It is important to point out that only quercetin 6-*C*-glucoside was quantified in significantly higher amount in transgenic shoots compared to control cultures. Moreover, quercetin 3-*O*-pentoside and flavonoid aglycone quercetin were de novo synthesized in transgenic shoots. On the other side, kaempferol 6-*C*-glucoside, hyperoside

Table 3 Retention times, UV and mass spectral data of phenolic compounds in *Hypericum perforatum* control and transgenic shoots

Peak	Compounds	t_r (min)	UV (nm)	[M–H]⁻ (m/z)	–MS² [M–H]⁻ (m/z)	Control shoots mg 100 g⁻¹ DW ± SD	Transgenic shoots mg 100 g⁻¹ DW ± SD
Phenolic acids							
F1	Quinic acid	3.9	262, 310	191	173, **127**	30.26 ± 1.22	27.65 ± 2.54
F2	3-Caffeoylquinic acid[a]	13.7	240, 294sh, 326	353	**191**, 179, 135	315.63 ± 18.29	406.09 ± 22.92
F3	3-p-coumaroylquinic acid	19.9	314	337	191, **163**	111.38 ± 9.71	128.28 ± 13.09
F5	3-Feruloylquinic acid	25.3	314	367	**193**	ND	10.74 ± 0.67
Total						457.27 ± 29.22	572.76 ± 39.22*
Flavonols							
F7	Quercetin 6-C-glucoside	33.9	256, 356	421	331, **301**	152.97 ± 10.26	197.32 ± 15.68*
F9	Kaempferol 3-O-rhamnoside	37.3	256, 264 352	431	**285**	16.89 ± 1.33	16.39 ± 1.17
F10	Kaempferol 6-C-glucoside	37.7	256, 266, 350	405	315, **285**	8.52 ± 0.42	3.78 ± 0.33*
F11	Hyperoside (quercetin 3-O-galactoside)	43.8	264, 296sh, 354	463	**301**	38.75 ± 5.39	29.19 ± 1.64*
F12	Rutin (quercetin-3-O-rutinoside)[a]	44.9	263, 298sh, 356	609	**301**	19.04 ± 2.01	20.46 ± 1.61
F13	Quercetin 3-O-pentoside	48.2	256, 294sh, 356	433	**301**	ND	2.11 ± 0.09
F14	Quercitrin (quercetin 3-O-rhamnoside)[a]	51.3	254, 354	447	**301**	99.82 ± 12.44	40.92 ± 2.61*
F15	Kaempferol 3-O-rutinoside	52.2	256, 266, 350	593	**285**	t_r	t_r
F16	Quercetin[a]	57.1	256, 372	301	273, 229, **179**, 151	ND	7.77 ± 0.56
Total						335.99 ± 31.85	317.94 ± 23.69
Flavan-3-ols							
F6	(epi)catechin[a]	29.9	280	289	**245**, 205	61.75 ± 2.56	121.96 ± 9.97*
F4	Proanthocyanidin dimer	24.5	280	577	559, 451, **425**, 407, 289	0.76 ± 0.03	20.04 ± 1.04*
F8	Proanthocyanidin dimer	36.8	280	577	559, 451, **425**, 407, 289	158.87 ± 23.19	72.95 ± 3.81*
Total						221.38 ± 25.78	214.95 ± 14.82
Naphtodianthrones							
F17	Pseudohypericin[a]	76.7	288, 325, 465, 580	519	487, 421	7.84 ± 0.82	85.76 ± 4.23*
F18	Hypericin[a]	78.2	288, 325, 465, 580	503	**405**	2.14 ± 0.02	25.83 ± 1.89*
F19	Protopseudohypericin	85.3	285, 375, 550	521	**423**	0.69 ± 0.04	2.15 ± 0.01*
Total						10.67 ± 0.88	113.74 ± 6.13*
Phloroglucinols							
F20	Hyperforin[a]	85.5	285	535	**383**	2.44 ± 0.02	5.15 ± 0.03*
F21	Adhyperforin	86.9	285	549	**397**	ND	t_r
Xanthones							
X1	Mangiferin C-prenyl isomer	36.1	238, 260, 312, 372	489	**327**, 399	537.38 ± 23.76	190.03 ± 21.66*
X2	Mangiferin	37.3	238, 256, 312, 362	421	331, 301, **258**	3,818.37 ± 167.54	3,169.80 ± 277.72*
X3	Xanthone derivative 1	39.5	258, 308, 368	323	243	446.39 ± 59.09	764.61 ± 44.12*
X4	Xanthone derivative 2	42.8	/	449	275	1,025.20 ± 92.91	283.05 ± 30.08*

Table 3 continued

Peak	Compounds	t_r (min)	UV (nm)	$[M–H]^-$ (m/z)	$–MS^2$ $[M–H]^-$ (m/z)	Control shoots mg 100 g^{-1} DW $\pm$ SD	Transgenic shoots mg 100 g^{-1} DW $\pm$ SD
X5	Xanthone derivative 3	46.2	242, 306	367	**287**	ND	35.24 $\pm$ 2.96
X6	Xanthone derivative 4	52.9	/	337	322, **257**	858.86 $\pm$ 99.76	747.94 $\pm$ 56.61
X7	1,3,6,7-Tetrahydroxyxanthone dimer	53.9	238, 254, 312, 364	517	517, 469, 447, 379, **257**	74.69 $\pm$ 3.82	ND
X8	Xanthone derivative 5	58.1	/	525	403, 385, 367, 343, **301**	285.15 $\pm$ 43.12	343.24 $\pm$ 40.26
X9	γ-mangostin isomer	78.9	260, 316, 370	395	351, **339**, 326, 283	ND	401.81 $\pm$ 18.93
X10	Trihydroxy-1-methoxy-C-prenyl xanthone	79.4	260, 286, 314	341	**326**	ND	434.69 $\pm$ 25.88
X11	γ-mangostin isomer	80.0	246, 262, 320	395	351, 339, 326, **283**	ND	239.84 $\pm$ 14.05
X12	Garcinone E	81.2	256, 286, 332	463	394, 351, **339**, 297, 285	ND	155.56 $\pm$ 18.64
X13	Xanthone derivative 6	82.2	262, 288, 322	431	/	ND	225.00 $\pm$ 20.15
X14	Banaxanthone E	82.6	252, 302, 330	477	419, 393, **339**, 297	ND	108.77 $\pm$ 8.86
X15	Xanthone derivative 7	83.6	270, 330, 400	467	398, 383, 327, 271, 234	1,846.8 $\pm$ 184.28	137.06 $\pm$ 10.12*
X16	Garcinone C	83.9	286, 340	413	369, 344, **301**, 233	1,281.64 $\pm$ 78.38	79.00 $\pm$ 6.88*
X17	Xanthone derivative 8	84.4	254, 284, 326	481	**412**, 397, 327, 271, 234	1,726.47 $\pm$ 142.01	166.65 $\pm$ 9.81*
Total						**11,900.95 $\pm$ 894.67**	**7,482.29 $\pm$ 606.73***

MS^2 ions in bold indicate the base peak. For information on peak numbers see Figs. 1 and 2

ND not detected, *DW* dry weight, *sh* shoulder, t_r retention time

* Denoted values indicating significant differences between data ($p < 0.05$)

[a] Compounds identified with authentic standards

and quercitrin were in significantly lower amounts in transgenic shoots compared to control ones. However, the total contents of identified flavonols were almost equal in transgenic and control shoots.

Flavan-3-ols

The HPLC analysis confirmed the presence of three flavan-3-ols (**F4**, **F6**, and **F8**) in control and transgenic shoots (Table 3; Fig. 1). The mass spectrum in full scan mode showed a deprotonated molecule, MS^2 ions and UV spectrum characteristic for epicatechin (**F6**). Compounds **F4** and **F8** showed characteristic fragmentation pathway by retro Diels–Alder reaction (Miketova et al. 1998) and were recognized as proanthocyanidin dimers. A twofold increase of epicatechin and 26-fold increase of proanthocyanidin dimer (**F4**) was observed in transgenic shoots compared to control. In contrast, a twofold decrease of proanthocyanidin dimer (**F8**) was found in transgenic cultures. However, the total amount of flavan-3-ols remained unchanged in both transgenic and control shoots.

Naphtodianthrones

Among the class of naphtodianthrones, hypericin, pseudohypericin, and protopseudohypericin were identified in control and transgenic shoots (Table 3; Fig. 2). The HPLC–MS analysis of naphtodianthrone compounds (**F17**, **F18**, and **F19**) have given collision induced fragment ion spectra identical to those reported by Piperopoulos et al. (1997) and Piovan et al. (2004), allowing a clear identification of these compounds in the samples. The UV/DAD spectra of compounds **F17** and **F18** showed identical absorption maxima typical for pseudohypericin and hypericin, respectively (Tolonen et al. 2002). In addition, the identity of these compounds was verified by comparison of their ESI mass spectrum and the HPLC retention time with an authentic standard of hypericin. The UV and mass spectra of compound **F19** were consistent with those of protopseudohypericin (Tolonen et al. 2002). In transgenic shoots, from 11- to 12-fold increase of hypericin and pseudohypericin were observed compared with control. In addition, a threefold increase of protopseudohypericin was also found in transgenic shoots. Transgenic shoot cultures accumulated significantly higher levels of total naphtodianthrones than control shoots.

Phloroglucinols

Chromatographic analysis of phloroglucinols resulted in the identification of hyperforin and adhyperforin (Table 3; Fig. 1). The UV/DAD spectra and MS^2 fragmentation patterns of compounds **F20** and **F21** were consistent with the structures of hyperforin and adhyperforin, respectively

(Tolonen et al. 2002; Piovan et al. 2004). A twofold increase of hyperforin was observed in transgenic shoots compared to control. In contrast, adhyperforin existed only in trace amounts in transgenic shoots, while its presence was not confirmed in the samples of control shoots.

Xanthones

Seventeen xanthones were detected in the methanolic extracts of *H. perforatum* shoot cultures and nine of them were fully identified by ESI–MS (Table 3; Fig. 1). These included simple oxygenated xanthones or derivatives with prenyl, pyran, or methoxy groups. According to MS fragmentation patterns and literature data (Cuyckens et al. 2001; Ferreres et al. 2008), compounds **X1** and **X2** were putatively identified as mangiferin-C-prenyl isomer and mangiferin, respectively. The mass spectrum of compound **X7** indicated that this compound is a dimer of 1,3,6,7-tetrahydroxyxanthone. Compounds **X9** and **X11** were putatively identified as isomers of γ-mangostin (1,3,6,7-tetrahydroxyxanthone-C-bis-prenyl), since they have an identical molecular ion but different UV spectra and retention times. Taking into account the spectral data and comparison to previously published data (Dias et al. 2000; Conceição et al. 2006; Franklin et al. 2009), we can tentatively assign compound **X10** as trihydroxy-1-methoxy-C-prenyl xanthone. The mass spectra of compounds **X12**, **X14**, and **X16** showed loss of a prenyl residue C_4H_8 and two prenyl residues, therefore, these xanthones were identified as garcinone E, banaxanthone E, and garcinone C, respectively. Several other peaks (**X3–X6**, **X8**, **X13**, **X15**, and **X17**) were categorized as xanthone derivatives, but were not fully identified.

Results from this study demonstrated marked qualitative and quantitative variations in the production of xanthones between control and transgenic shoots. In this view, xanthones in transgenic shoots could be distinguished in four groups: (1) compounds whose quantity increased (**X3**, **X8**), (2) compounds whose quantity decreased (**X1**, **X2**, **X4**, **X6**, **X15–X17**), (3) compounds that were not detectable (**X7**), and (4) compounds that were de novo synthesized (**X5**, **X9–X14**). It is worth nothing that mangiferin was the major xanthone in transgenic shoots, which accounted for 42 % of the total xanthones. However, transgenic shoots showed significantly lower amounts of total detected xanthones compared to control shoots.

Discussion

Establishment of transgenic shoots

HR induction and shoot regeneration are important prerequisites for successful production of transgenic plants

using *A. rhizogenes.* In our laboratory, *H. perforatum* HR cultures were induced by co-cultivation of root explants with *A. rhizogenes* strain A4 to study the production of phenolic compounds (Tusevski et al. 2013). In the present work, HR segments were cultivated on MS/B$_5$ medium without phytohormones under a photoperiod of 16-h light and spontaneous shoot regeneration was observed after 4 weeks. However, HR did not show any significant difference in the frequency of adventitious shoot formation compared to control roots. Further phenotypic assessment was done by measuring the number of shoots per root explant and the shoot length (Table 1). A significant difference in the morphogenetic response between control and HR explants was not observed. In addition, control and HR-regenerated shoots demonstrated similar growth patterns. These findings indicate that the source tissue did not affect the level of competence for adventitious shoot formation. Moreover, depletion of nutrient media during continuous growth and hormonal disbalance in transformed tissues can be considered as responsible factors for spontaneous regeneration of shoots (Koperdáková et al. 2009b; Mehrotra et al. 2013). Various potential for regeneration of transgenic shoots from *H. perforatum* HR cultures was already reported in the literature (Di Guardo et al. 2003; Vinterhalter et al. 2006; Bertoli et al. 2008). In this view, the regenerative potential of *H. perforatum* HR cultures differs with respect to the light requirement; consequently, shoot regeneration can be promoted by light (Di Guardo et al. 2003) or is light independent (Vinterhalter et al. 2006). With regard to the shoot morphology, *H. perforatum* HR-regenerated shoots have shown either a normal (Vinterhalter et al. 2006) or altered phenotype (Di Guardo et al. 2003). As presently established, transgenic shoots had normal morphology like those regenerated from *H. perforatum* HR induced by infection with *A. rhizogenes* A4M70GUS (Vinterhalter et al. 2006). On the other side, transgenic shoots regenerated from *H. perforatum* HR transformed with *A. rhizogenes* ATCC 15834 exhibited typical "HR phenotype" including dwarfism, shorter internodes, increased branching, reduced apical dominance, and small and wrinkled leaves (Di Guardo et al. 2003). The observed differences in morphological features of transgenic shoots could be attributed to unique transformation events comprising different insertion sites and activation of transgene expression or/and different origin of transformed cells (Koperdáková et al. 2009a).

In an attempt to improve the growth of *H. perforatum* transformed shoots, cytokinin BA was used as a effective promoter of shoot multiplication. With respect to the growth parameters, control and transgenic multiplied shoots showed similar PC values (Table 2). The PC values reported in our study were relatively lower than those observed by Bertoli et al. (2008) in different *H. perforatum* HR-regenerated plant lines. These authors suggested that PC value is correlated with tissue hydration and the growth of transgenic shoots in the liquid culture immersion. Likewise, number of leaf couples and shoot length defining IC value did not show a significant difference between control and transgenic multiplied shoots (Table 2). It is worth noting that transgenic multiplied shoots showed significantly increased number of dark glands per leaf compared to control (Table 2). The characteristic multicellular dark glands were early developed on the leaf margins of both control and transgenic multiplied shoots. We have already reported that multiplication of *H. perforatum* in vitro shoots has been related to leaf morphogenesis and apparition of dark oil glands on the margins of leaves described as a multicellular reservoir of hypericins (Gadzovska et al. 2005, 2013). Such an apparition of multicellular dark glands has also been observed on the leaves of *H. perforatum* transgenic shoots (Di Guardo et al. 2003; Bertoli et al. 2008; Koperdáková et al. 2009a). The direct mode of shoot regeneration, as observed in *H. perforatum*, is a desirable trait when the aim is to study the production and accumulation of bioactive compounds in transgenic plants.

Production of phenolic compounds in transgenic shoots

Transformed shoots have recently been a matter of great interest as a source of bioactive metabolites synthesized in the aerial part of many medicinal plants (Floryanowicz-Czekalska and Wysokinska 2000). This study was focused on detailed phenolic profile of *H. perforatum* transgenic shoots using HPLC/DAD/ESI–MS^n method. The complete survey of shoot extracts revealed the presence of six different classes of phenolic compounds such as phenolic acids, flavonols, flavan-3-ols, naphtodianthrones, phloroglucinols, and xanthones. The HPLC profiles obtained in the course of this work clearly evidenced a distinct phenolic production between control and transgenic shoots.

Phenolic acids

Concerning production of phenolic compounds, *H. perforatum* transgenic shoots exhibited a superior potential for accumulation of phenolic acids. Among the four identified phenolic acids, 3-caffeoylquinic acid (chlorogenic acid) was found to be the major phenolic acid in the samples of transgenic shoots. In this view, Bertoli et al. (2008) selected transgenic plant lines which have been the most productive in chlorogenic acid. As an important antioxidative compound, chlorogenic acid has been previously identified in different *H. perforatum* in vitro cultures such as root cultures (Tusevski et al. 2013), adventitious roots cultivated in bioreactor (Cui et al. 2010) and shoot cultures (Dias et al. 1999). Furthermore, chlorogenic acid has also

been detected in other *Hypericum* in vitro cultures such as *H. undulatum* shoot cultures (Rainha et al. 2013) and *H. ternum* micropropagated plants (Pinhatti et al. 2010).

Flavonols

Hypericum species are widely known for its richness in flavonoids, especially by the flavonols derived from quercetin and kaempferol glycosilation. In our study, quercetin derivatives represented a major class of flavonols in *H. perforatum* transgenic shoots. With regard to the class of quercetin derivatives in transgenic shoots, quercetin 6-*C*-glucoside usually dominates among the glycosides followed by quercitrin and hyperoside. Several differences can be pointed out when comparing the flavonol production in transgenic shoots evaluated here with those reported by Bertoli et al. (2008). In accordance, these authors found that *H. perforatum* transgenic plants produced rutin, hyperoside, quercetrin, and quercetin as presently established in our experiment. On the other hand, transgenic shoots evaluated in our study showed a capability to produce kaempferol derivatives such as kaempferol 3-*O*-rhamnoside, kaempferol 6-*C*-glucoside, and kaempferol 3-*O*-rutinoside. Taking in account the results from our previous study (Tusevski et al. 2013) for flavonol composition in *H. perforatum* transformed roots, we found that HR produced only quercetin derivatives, while kaempferol glycosides have not been detected. Considering the quercetin derivatives, *H. perforatum* transgenic shoots produced the same type of those usually found in shoot cultures such as rutin, hyperoside, isoquercitrin, quercitrin and quercetrin (Dias et al. 1999; Pasqua et al. 2003; Klejdus et al. 2013; Gadzovska et al. 2013). Recently, flavonol glycosides have also been identified in *H. undulatum* shoot cultures (Rainha et al. 2013) and *H. ternum* regenerated plantlets (Pinhatti et al. 2010). Regarding the flavonoid aglycones in transgenic shoots, our data showed the presence of quercetin but the aglycone kaempferol was not detected. Identification of aglycone quercetin in transgenic shoots represents a potentially interesting finding, since it is well known that quercetin is a biologically active flavonoid that interacts synergistically with other bioactive substances (Mertens-Talcott and Percival 2005).

Flavan-3-ols

The HPLC–MS analysis of flavan-3-ols in transgenic shoots resulted in the identification of epicatechin and proanthocyanidin dimers but the flavanol catechin was not detected. We have previously shown that *H. perforatum* transgenic roots accumulated significant amounts of monomeric flavan-3-ols, such as catechin and epicatechin (Tusevski et al. 2013). In this view, *H. perforatum* HR

cultures can be suggested as better producers of flavan-3-ols than transgenic shoots. However, the presence of both monomeric flavanols and proanthocyanidin dimers had been previously confirmed in shoots and calli of *H. erectum* (Yazaki and Okuda 1990) and *H. undulatum* shoot cultures (Rainha et al. 2013). Furthermore, catechin and epicatechin play important role as antioxidants and can exert marked medicinal effects (Nurulain 2006).

Naphtodianthrones

As far as the authors are aware, hypericin production has been intensively studied in cell, tissue, and organ cultures of *H. perforatum*. A detailed study of Pasqua et al. (2003) on the accumulation of bioactive metabolites in *H. perforatum* undifferentiated cell cultures, compare with shoot cultures, clearly demonstrated that organ differentiation is necessary to obtain hypericins. In addition, a relationship between the biosynthesis of naphtodianthrones and the morphogenesis, formation and number of dark glands on the leaves has been already reported for *H. perforatum* shoot cultures (Gadzovska et al. 2005; Liu et al. 2007a). On the other side, there are many data indicating the presence of hypericins in undifferentiated cells and callus cultures (Kirakosyan et al. 2000; Walker et al. 2002; Conceição et al. 2006; Kartnig et al. 1996). Our previous results on *H. perforatum* suspended cells (Gadzovska et al. 2007, 2013) have shown that the presence of dark glands was not absolutely necessary for hypericin and pseudohypericin production and could occur on photoperiod conditions (16 h). In this context, Bais et al. (2002) suggested that the hypericin pool is localized within a vacuolar compartment in cultured *H. perforatum* cells. Therefore, it can be assumed that dark glands are not limiting factor for production of hypericins in *H. perforatum* in vitro cultures.

A number of in vitro culture approaches have been adopted to improve the production of hypericins in *Hypericum* species. *Hypericum* cell suspensions, callus cultures, shoots, and plantlets have been established to study the overproduction of naphtodianthrones using phytohormones (Gadzovska et al. 2005; Liu et al. 2007a), elicitors (Kirakosyan et al. 2000; Sirvent and Gibson 2002; Walker et al. 2002; Conceição et al. 2006; Liu et al. 2007b; Gadzovska et al. 2007, 2013; Gadzovska-Simic et al. 2012), precursor feeding (Liu et al. 2007b) and physical stimuli (Odabas et al. 2009; Sooriamuthu et al. 2013; Varghese and Sooriamuthu 2013). Although *H. perforatum* in vitro cultures are known to produce naphtodianthrones, there has been little work to investigate whether these compounds are inducible by genetic transformation.

In this study, HPLC analyses revealed that pseudohypericin, hypericin, and protopseudohypericin were the only naphtodianthrones present in shoot extracts. Regarding the

quantitative aspect, the concentration of identified naphtodianthrone pigments was about 12-fold higher in transgenic shoots compared to control. Pseudohypericin was found as the main naphthodianthrone in transgenic shoots, usually present in 3.5-fold higher amounts than hypericin. Our findings indicated that the amounts of hypericins positively correlate with the number of dark glands per leaf when comparing the control and transgenic shoots. Accordingly, a large number of studies have also demonstrated the correlation between dark glands and hypericin levels (Briskin and Gawienowski 2001; Piovan et al. 2004; Zobayed et al. 2006). The dark glands are considered as the limiting factors for production of the active compounds (Pasqua et al. 2003) probably due to necessity of dark glands for deposition of the synthesized hypericin. Consequently, the number of dark glands per leaf could be used as a rapid screening marker for selection of the experimental conditions for optimization of hypericin yields in *H. perforatum* transgenic shoot cultures (Bertoli et al. 2008). Therefore, we can assume that *A. rhizogenes*-mediated transformation markedly affect the formation of dark glands in HR-regenerated transgenic shoots. In this view, only few studies have been focused on hypericin assay in *H. perforatum* HR-regenerated shoots (Di Guardo et al. 2003; Bertoli et al. 2008; Koperdáková et al. 2009a). Hypericin content in *H. perforatum* transgenic shoots evaluated in this study was similar to that reported by Bertoli et al. (2008). On the other hand, Koperdáková et al. (2009a) showed much lower total hypericin content in ex vitro cultivated *H. perforatum* transgenic plants due to reduced number and size of dark glands on leaves and petals. Outgoing results showed that transgenic shoots produced hypericin and pseudohypericin content up to fivefold higher than the best reported from our previous studies for in vitro multiplied shoots (Gadzovska et al. 2005, 2013). This is the most important advantage of transgenic shoots as an in vitro model for studying the biosynthetic pathways of naphtodianthrones within a short cultivation time. Altogether, these results indicated that *H. perforatum* transgenic shoots represent a promising experimental system for enhanced production of naphtodianthrones.

Phloroglucinols

Phloroglucinol derivatives are widely distributed in the genus *Hypericum*. Two closely related compounds hyperforin and adhyperforin have been found as the main phloroglucinols in *H. perforatum* (Karppinen et al. 2007). There were strong indications that hyperforin is synthesized in the translucent glands and their delimiting cells (Soelberg et al. 2007). Although regulation of hyperforins synthesis is not yet fully understood, there is substantial

progress in the elucidation of biotechnological aspects, especially for their enhanced production using in vitro cultures. Many studies have been performed to improve the production of hyperforins in *Hypericum* shoot, plantlet, meristem, and seedling cultures by using elicitation (Sirvent and Gibson 2002; Zobayed et al. 2003; Pavlík et al. 2007; Charchoglyan et al. 2007; Liu et al. 2007a), optimization of culture conditions (Kirakosyan et al. 2004; Sooriamuthu et al. 2013; Odabas et al. 2009) and precursor feeding (Karppinen et al. 2007).

In this study, HPLC–MS analysis of phloroglucinols in transgenic shoots resulted in the identification of hyperforin, while its homolog adhyperforin was detected in traces. A twofold higher content of hyperforin was observed in transgenic shoots compared to control ones. In this view, hyperforin had been previously identified in transgenic shoots of *H. perforatum* (Bertoli et al. 2008) in amounts higher than those reported here. To the best of our knowledge, the accumulation of bioactive molecules in shoot cultures has been shown to be dependent on tissue differentiation and a further degree of leaf development is necessary to obtain hypericins and hyperforins (Pasqua et al. 2003). In the current study, the overproduction of naphtodianthrones and phloroglucinols in transgenic shoots could be related to an *A. rhizogenes*-mediated transformation rather than to the differentiation step. As reported previously (Bertoli et al. 2008) for *H. perforatum* transgenic shoots, it is possible to select high-producing plants of secondary metabolites by screening a large population of independent lines after *A. rhizogenes* transformation.

Xanthones

One of the main achievements in this study was the identification of xanthones as the major phenolic fraction in shoot extracts. Among the class of xanthones, mangiferin was the main representative xanthone produced in transgenic shoots. Although mangiferin was found along with several other xanthones in both control and transgenic shoots, new xanthones appeared in the transgenic shoots. In this view, two γ-mangostin isomers, trihydroxy-1-methoxy-*C*-prenyl xanthone, garcinone E, banaxathone E, and two xanthone derivatives were de novo synthesized in transformed shoots. Such an accumulation of xanthones in *H. perforatum* transgenic shoots is curious, since it is well known that these metabolites are usually found in the roots, but trace amounts can also be found in the aerial parts of the plants (Erdelmeier et al. 2000). Recent studies showed that *Hypericum* in vitro cultures have the potential to accumulate xanthones and their production can be manipulated by the hormonal supplementation (Dias et al. 2000) or/and by the culture type (Pasqua et al. 2003). A differential xanthone accumulation depending on the type of

culture had already been observed in *H. perforatum* undifferentiated calli (Pasqua et al. 2003; Mulinacci et al. 2008) and suspended cells (Dias et al. 1999) but not in regenerated shoots. Curiously, mangiferin was only detected in *H. undulatum* shoot cultures (Rainha et al. 2013) revealing the possibility for the existence of several other xanthones. The reason for this finding could be attributed to different culture conditions used and phytohormone supplementation. Recently, the potential of *H. perforatum* adventitious root cultures for improving xanthone accumulation has been investigated (Tocci et al. 2012). We have also shown that *H. perforatum* transgenic roots are a promising biotechnological system for mass production of xanthones (Tusevski et al. 2013). In spite of the bioactive properties of xanthones and the potentialities of in vitro cultures for production of secondary metabolites (Beerhues and Berger 1995), information about how xanthones accumulate in in vitro systems is scanty.

Conclusion

In conclusion, we have developed an efficient system for regeneration of *H. perforatum* HR cultures which leads to the formation of transgenic shoots producing various groups of phenolic compounds. Distinct phenolic profile between control and transgenic shoots was shown as detailed for the first time. Transgenic shoots showed biosynthetic potential for the production of specific secondary metabolites such as quinic acid derivatives, quercetin and kaempferol derivatives, epicatechin and proanthocyanidin dimmers, naphtodianthrones, phloroglucinols, and xanthones. More importantly, transgenic shoots synthesized and stored significant quantities of hypericin and pseudohypericin. Therefore, *H. perforatum* transgenic shoots represent a promising experimental system for obtaining qualitatively and quantitatively standardized extracts. Further studies are necessary to exploit the biosynthetic potential of transgenic shoots, focusing on the production of other specific bioactive metabolites.

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