

Xanthenes from roots, hairy roots and cell suspension cultures of selected *Hypericum* species and their antifungal activity against *Candida albicans*

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Abstract

Key message Highest xanthone contents were found in *Hypericum pulchrum* and *H. annulatum* untransformed roots. The best anti-*Candida* activity was obtained for hairy roots extracts of *H. tetrapterum* clone 2 ATCC 15834.

Abstract Extracts of root cultures, hairy roots and cell suspensions of selected *Hypericum* spp. were screened for the presence of xanthenes and tested for their antifungal activity against *Candida albicans* strain ATCC 10231. At least one of the following xanthenes, 5-methoxy-2-deprenylrheediaxanthone; 1,3,6,7-tetrahydroxyxanthone; 1,3,5,6-tetrahydroxyxanthone; paxanthone; kielcorin or mangiferin was identified in methanolic extracts of the untransformed root cultures. The highest total xanthone content, with five xanthenes, was found in untransformed *H. pulchrum* and *H. annulatum* root cultures. Hairy roots and the controls of *H. tetrapterum* contained 1,7-dihydroxyxanthone, while hairy root cultures and the corresponding controls of *H.*

tomentosum contained toxyloxanthone B, 1,3,6,7- and 1,3,5,6-tetrahydroxyxanthone. Two xanthenes, cadensin G and paxanthone, were identified in cell suspension cultures of *H. perforatum*. Their content increased about two-fold following elicitation with salicylic acid. The anti-*Candida* activity of the obtained extracts ranged from MIC 64 to >256 $\mu\text{g ml}^{-1}$. Among the extracts of *Hypericum* untransformed roots, the best antifungal activity was obtained for extracts of *H. annulatum* grown under CD conditions. Extracts of hairy roots clones A4 and 7 ATCC15834 of *H. tomentosum* and clone 2 ATCC15834 of *H. tetrapterum* displayed inhibition of 90 % of *Candida* growth with 256 $\mu\text{g ml}^{-1}$. Extracts from chitosan-elicited cells did not show antifungal activity.

Keywords *Hypericum* · Xanthone · Root cultures · Hairy roots · Cell suspensions · Antifungal activity

Introduction

Members of the genus *Hypericum* L. are distributed worldwide, but are found predominantly in temperate zones of the Earth. Up to the present, almost 500 species of the genus have been documented (Nürk et al. 2013), but only ca. 40 % of them have been phytochemically characterized (Crockett and Robson 2011). Extracts of the species of the genus *Hypericum*, natural sources of bioactive compounds, display numerous activities in multiple bioassays (recently reviewed by Wolffe et al. 2014). Among the many secondary metabolites produced by *Hypericum* spp., xanthenes are becoming the focus of interest in current research because of their powerful antioxidant, antibacterial, antitumoral and antifungal

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activities (reviewed by Fotie and Bohle 2006; El-Seedi et al. 2010; Tala et al. 2013).

In *Hypericum*, xanthenes are mainly accumulated in the roots. In fact, the first xanthone identified in *Hypericum* spp. was maculatoxanthone from the roots of *H. maculatum* Crantz (Arends 1969). The occurrence of hydroxy- and methoxy- substituted xanthenes in roots of *H. androsaemum* L. is mentioned in the study of Nielsen and Arends (1979). Thereafter, four new xanthenes were isolated from the roots of *H. roeperanum* Schimp. ex A. Rich. (Rath et al. 1996). Crockett et al. (2011) isolated three xanthenes from root extracts of *H. perforatum*: 1,6-dihydroxy-5-methoxy-40,50-dihydro-40,40,50-trimethylfurano-(20,30:3,4)-xanthone; 4,6-dihydroxy-2,3-dimethoxyxanthone and cis-kielcorin, and a new inhibition of the plant pathogenic fungi *Phomopsis obscurans* and *Plasmopora viticola* was observed (Crockett et al. 2011). Moreover, the antifungal activity of *H. perforatum* root extracts has been evaluated against a broad panel of human fungal pathogens, including *Candida* spp. (Tocci et al. 2011). *Candida* is a yeast that causes the greatest number of fungal diseases. Infections caused by *Candida* spp. affect 70–75 % of women at least once during their life (Sobel 2007).

In vitro root cultures represent suitable biotechnological tools for the production of standardised extracts and for the isolation of bioactive substances (Pasqua et al. 2005; Tocci et al. 2012). The biotechnological potential for xanthone production in elicited root cultures of *H. perforatum* has been widely studied (Tocci et al. 2011, 2013b). Tusevski et al. (2013a, b) also determined significant quantities of xanthenes in hairy roots of *H. perforatum* following transformation by *Agrobacterium rhizogenes*. Xanthenes have also been identified as major metabolites in cell suspension cultures of *H. perforatum* (Pasqua et al. 2003). To improve xanthone biosynthesis in cell suspension cultures, several biotic elicitors such as *Colletotrichum gloeosporioides*, *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes* and chitosan have been applied (Conceição et al. 2006; Franklin et al. 2009; Tocci et al. 2010; Tusevski et al. 2014). Other studies have cited the use of elicitors, such as salicylic acid, to stimulate hypericins and hyperforins in cell suspension cultures (Gadzovska et al. 2013).

The aims of this study were (1) to determine the xanthone profile in root culture extracts of several previously unstudied *Hypericum* species; (2) to evaluate the capacity for xanthone production of *H. tetrapterum* Fries. and *H. tomentosum* L. hairy roots; (3) to ascertain the effect of elicitation of a cell suspension culture of *H. perforatum* with chitosan and salicylic acid on xanthone content and; finally, (4) to assess the antifungal activity of these extracts against *Candida albicans*.

Materials and methods

Plant material and culture conditions

Root and hairy roots cultures

Roots of eight species of the genus *Hypericum* (*H. humifusum* L., *H. annulatum* Moris., *H. tetrapterum* Fries., *H. tomentosum* L., *H. maculatum* Crantz., *H. rumeliacum* Boiss., *H. pulchrum* L. and *H. kouytchense* H. Lév.) were selected for this experiment. The roots were isolated from in vitro-grown plants derived from stock cultures cultivated on hormone-free medium containing Murashige-Skoog's (MS) salt mixture (Murashige and Skoog 1962), Gamborg's B5 vitamins (Gamborg et al. 1968), 30 g l⁻¹ sucrose and 2 mg l⁻¹ glycine. The culture medium was solidified with 7 g l⁻¹ agar, and the pH was adjusted to 5.6 before autoclaving. Isolated roots were transferred into liquid medium of the same composition. Root cultures were grown in Erlenmeyer flasks at 23 ± 1 °C, 40 % relative humidity under either continuous darkness (CD) or exposed to a 16/8 (light/dark) photoperiod with artificial irradiance of 26 μmol m⁻² s⁻¹ on a rotary shaker (120 rpm). The root cultures with the growth index (GI) <2 at the end of culture period were excluded from further experiments. Consequently, only *H. annulatum* was cultivated under conditions of both continuous darkness and 16/8 photoperiod, whereas the remaining root cultures were grown as follows: (CD) *H. humifusum*, *H. kouytchense*, *H. pulchrum*; (16/8) *H. tetrapterum*, *H. maculatum*, *H. tomentosum*, *H. rumeliacum*. The standard and hairy root cultures were subcultured every 18–23 days. The growth dynamics of the listed *Hypericum* species were determined gravimetrically by measuring fresh weight increases on the 2nd, 5th, 7th, 9th, 12th, 14th, 16th, 19th and 22nd days. The initial weight of all samples was adjusted to 0.5 g FW of roots, which were immersed into 10 ml of hormone-free liquid MS media. The GI was calculated as follows:

$$GI = \frac{\text{Initial fresh weight} - \text{Final fresh weight}}{\text{Initial fresh weight}}$$

Roots were harvested from the liquid media at the end of the culture period, dried in an oven at 40 °C until reaching a constant weight (48–72 h). This dry biomass (DW) was used for preparation of extracts for xanthone analysis and antifungal testing against *C. albicans* strain ATCC 10231. Hairy root cultures of *H. tomentosum* and *H. tetrapterum* had been previously established via transformation with wild-type agropine strains of *Agrobacterium rhizogenes*, A4 and ATCC 15834 (American Type Cultures Collection), and their transgenic character was proved (Komarovská et al. 2009) (Table 1). Before using the hairy

Table 1 Hairy root clones of *H. tomentosum* and *H. tetrapterum*

Sample	Species	Clone name	Rhizogenes strain	Rol genes
1	<i>H. tomentosum</i>	Control	–	–
2	<i>H. tomentosum</i>	Clone 1	ATCC 15834	<i>rolA,C</i>
3	<i>H. tomentosum</i>	Clone 2	ATCC 15834	<i>rolC</i>
4	<i>H. tomentosum</i>	Clone 4	ATCC 15834	<i>rolA,B,C</i>
5	<i>H. tomentosum</i>	Clone 7	ATCC 15834	<i>rolC</i>
6	<i>H. tomentosum</i>	Clone e	A4	<i>rolB,C</i>
7	<i>H. tomentosum</i>	A4	A4	<i>rolA,C</i>
8	<i>H. tetrapterum</i>	Control	–	–
9	<i>H. tetrapterum</i>	Clone 1	A4	<i>rolA,B,C</i>
10	<i>H. tetrapterum</i>	Clone 2 ATCC	ATCC 15834	<i>rolB,C</i>
11	<i>H. tetrapterum</i>	Clone 2 A4	A4	<i>rolA,B,C</i>
12	<i>H. tetrapterum</i>	Clone 4	ATCC 15834	<i>rolA,B,C</i>

root cultures in this experiment, the presence of the *rol* genes (*rolA* + *rolB*) was repeatedly determined by PCR amplification using specific primers as described by Zdravkovic-Korac et al. (2004); *rolC*, as described by Di Guardo et al. (2003), and *rolD*, by Komarovská et al. (2009). Roots grown under photoperiod conditions showed the lowest biomass increases among all species tested so the hairy roots were grown only in continuous darkness.

The culture conditions in the dark and the procedure for determination of growth kinetics were the same as for the root cultures described above.

Initiation, characterization and elicitation of cell suspensions

Cell suspension cultures, initiated from callus cultures of leaf origin, were derived from diploid *H. perforatum* plants. Two grams of whitish soft callus was resuspended in 100 ml of liquid medium containing MS macro and microelements (Murashige and Skoog 1962), Gamborg's B5 vitamins (Gamborg et al. 1968), 30 g l⁻¹ sucrose and 2 mg l⁻¹ glycine, supplemented with 4.5 μM 2,4-dichlorophenoxyacetic acid, 0.46 μM kinetin and 0.44 μM 6-benzylaminopurine (Pretto and Santarém 2000), adjusted to pH 5.6 prior to autoclaving. Cell suspensions were placed on an orbital shaker at 120 rpm and cultured at 23 ± 1 °C, 40 % relative humidity under continuous darkness, which is beneficial for biomass production. The cultures were subcultured every 10–13 days. The growth dynamics were determined by the dry weight increase in cell biomass harvested on the 2nd, 4th, 6th, 8th, 10th and 12th days of culture after drying for 3 days at 40 °C, according to Tocci et al. (2012). The growth index was calculated as described in Sect. "Root and hairy roots cultures". On the 4th day of subculture, the level of cell ploidy was assessed. One millilitre of general purpose buffer (Loureiro et al. 2007) was added to the cell

suspension in a ratio of 1:1, and the suspension was filtered through nylon sieve mesh with a pore size of 76 μm. 50 μl RNase was then added, along with the same amount of propidium iodide. Cells in test-tubes were chilled on ice for 20 min and then quantitatively analysed using a flow cytometer (PartecCyFlow ML, Germany). The cell suspension cultures were elicited on the 4th day of subculture with 10 mg l⁻¹ chitosan dissolved in 0.1 % acetic acid (CH) (Fluka, Iceland), 50 μM SA salicylic acid (SA) (Duchefa, Netherlands) and combinations thereof.

HPLC analysis of xanthenes

One hundred milligrams of dried untransformed roots, hairy roots and cell suspensions were ground with pestle and mortar and extracted three times with methanol (HPLC grade, Carlo Erba Milan, Italy) at a solvent: biomass ratio of 10 ml: 100 mg DW, then transferred into an ultrasonic water bath for 20 min. Subsequently, the extracts were placed in a dark location and macerated overnight at room temperature. Extracts were filtered and dried with a rotavapor. The dry extracts were re-dissolved in 2 or 4 ml of methanol and then analysed by gradient high-performance liquid chromatography (HPLC). Xanthenes in cell suspension cultures were analysed 1, 3 and 5 days after the addition of an elicitor. Cells without treatment were referred to as controls. The HPLC analysis of the extracts was performed using an instrument consisting of a pump (Waters 1525 Binary HPLC Pump) equipped with a UV detector (Waters 2487 Dual λ Detector) and a reverse-phase C18 column (4.6 × 150 mm; 5 μm; waters). The extract capacity was determined by linear gradient elution, using a modified version of a method described by Dias et al. (1999). The mobile phase consisted of 100:0.1 (v/v) water:formic acid (component A) and methanol (component B) (Carlo Erba, Milan, Italy) (gradient elution: time 0, 90 % A and 10 % B; time 2 min, 70 % A, 30 % B; time

8 min, 60 % A, 40 % B; time 13 min, 50 % A, 50 % B; a plateau of 2 min; time 23 min, 30 % A, 70 % B; a plateau of 5 min; time 38 min, 20 % A, 80 % B; a plateau of 12 min; time 55 min, 5 % A, 95 % B; a plateau of 10 min; time 70 min, 90 % A, 10 % B), with a flow rate of 1.0 ml min^{-1} and an injection volume of $20 \mu\text{l}$. The absorbance of the compounds at 260 and 320 nm was measured using the external standard method. As a references, a mixture of 1,3,6,7-tetrahydroxyxanthone and 1,3,5,6-tetrahydroxyxanthone, kielcorin, toxyloxanthone B, paxanthone, cadensin G, all of which had been previously purified and spectroscopically characterized in the laboratory of the University Sapienza in Rome, Italy, was used. 1,7-dihydroxyxanthone and 5-methoxy-2-deprenylrheedi-xanthone B were extracted from regenerated roots of *H. perforatum* subsp. *angustifolium*. Biyouxanthone D was added to the described standard xanthone mixture for HPLC analysis of hairy root culture extracts. Xanthones were quantified as equivalents of paxanthone (Tocci et al. 2011, 2012) at 260 nm and all measurements were made in triplicate.

Antifungal susceptibility tests

For the antifungal evaluation, *C. albicans* strain ATCC 10231 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and used in tests. The strain was stored and grown in accordance with the procedures published by the Clinical and Laboratory Standards Institute (CLSI, 2008). The broth microdilution method was performed to evaluate the antifungal activity according to a standardised method for yeast (CLSI, 2008). Fluconazole (Sigma Aldrich, St. Louis, Missouri, USA) was used as a positive control. Fluconazole and dry root or cell extracts were diluted to a final concentration as follows: the final amounts ranged from 0.125 to $64 \mu\text{g ml}^{-1}$ for fluconazole and, for the total dry sample extracts, from 0.5 to $256 \mu\text{g ml}^{-1}$. The extracts were initially dissolved in dimethyl sulfoxide at concentrations 100 times higher than the highest desired test concentration and then diluted in the test medium to the appropriate concentration. The *C. albicans* ATCC 10231 was cultivated on Sabouraud dextrose agar and incubated at $35 \text{ }^\circ\text{C}$. An inoculum was taken after 24 h of microorganism cultivation. Colonies were subsequently resuspended in 5 ml of sterile saline solution and vortexed for 15 s. The density of the cells was adjusted spectrophotometrically measuring, at 560 nm, to obtain a *C. albicans* stock suspension of $1\text{--}5 \times 10^6 \text{ cells ml}^{-1}$. Microdilution trays containing $100 \mu\text{l}$ of serial two-fold dilutions of fluconazole or cell/root extracts in RPMI 1640 medium (Sigma Aldrich, St. Louis, Missouri, USA) were inoculated with the *C. albicans* suspension adjusted to a final inoculum concentration of $5.0 \times 10^2\text{--}2.5 \times 10^3$

cells ml^{-1} . The trays were incubated at $35 \text{ }^\circ\text{C}$ and *C. albicans* growth was observed after 48 h. The minimal inhibitory concentration 50 (MIC50) was the lowest concentration that showed $\geq 50 \%$ growth inhibition compared with the drug-free control. The MIC90 was the lowest drug concentration that caused $\geq 90 \%$ growth inhibition compared to the control, and the MIC100 was equal to 100% growth inhibition.

Results

Growth characteristics of the cultures

The growth of the untransformed roots (Fig. 1a, b) and hairy roots (Fig. 1c, d) was determined by assessing the fresh weight (FW) increase and the growth index (GI). As expected, the growth of hairy roots was faster than that of the corresponding untransformed roots. While the biomass of untransformed roots of *H. tetrapterum* or *H. tomentosum* increased towards the end of the subculture 2–3 times, depending on the light conditions, the hairy root biomass was enhanced 2- to 10-fold, depending on the clone. The highest root biomass (GI = 8.44) was detected in hairy roots of *H. tomentosum* clone e, transformed by *A. rhizogenes* A4 (Fig. 1c). However, no correlation between the biomass production and the type of integrated *rol* gene(s) was evident. Among the untransformed root cultures of *Hypericum* species, the highest biomass production was detected in dark-grown *H. annulatum* (GI = 3.6) (Fig. 1a). Cell suspensions of *H. perforatum* were characterized by their cell morphology, growth kinetics and ploidy level. They consisted mainly of cell aggregates of variable size and single spherical and oval-shaped cells with diameters ranging from 25 to $120 \mu\text{m}$ and large vacuoles. The subculture interval lasted 12 days, with exponential growth occurring between the days 4 and 8 (Fig. 1C). The GI at the end of subculture reached the value of 1.99. The cell suspension cultures were also variable as to the ploidy level. Flow cytometry screening revealed several ploidy levels (2C, 4C, 8C, 16C) with a prevalence of tetraploid cell populations.

Xanthone profile of the untransformed roots of *Hypericum* spp. grown under CD conditions

Phytochemical analysis of in vitro-grown untransformed roots cultured under CD conditions revealed great inter-specific variation in both the total content of xanthones and the presence of individual xanthone types (Table 2). The root cultures of *H. pulchrum* and *H. annulatum*, cultured under CD conditions, were characterized by having the highest total xanthone content and five xanthones were

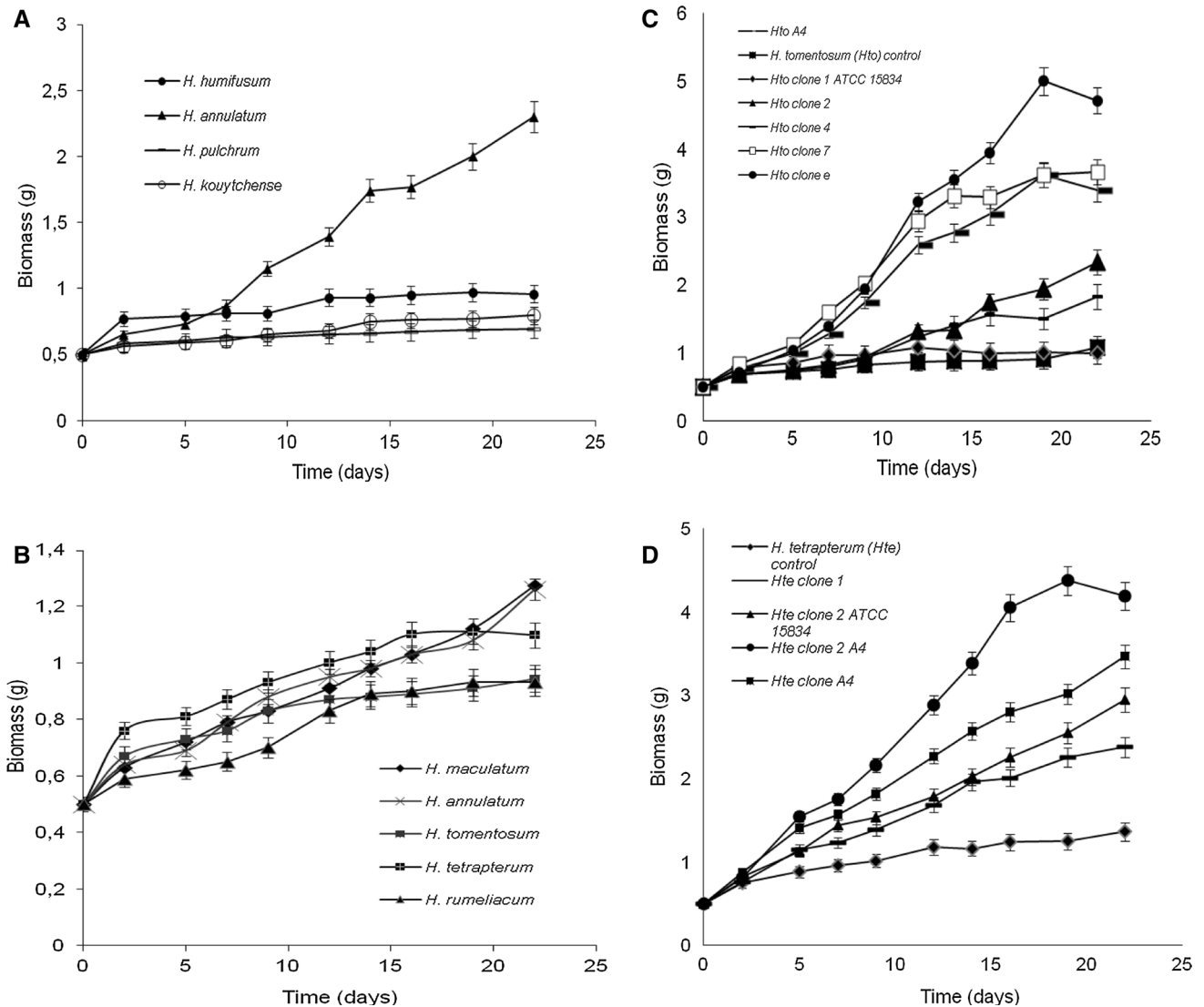


Fig. 1 Growth kinetics of untransformed roots cultured in the dark (a) and 16/8 h photoperiod (b), hairy roots of *H. tomentosum* (c), hairy roots of *H. tetrapterum* (d) control cell suspension cultures (e).

Error bars represent standard deviation (SD) based on triplicate measurements of biomass fresh weight. 16/8 photoperiod; CD continuous dark; HTo *H. tomentosum*, HTe *H. tetrapterum*

identified: 3 xanthenes occurred in both species, paxanthone was present in *H. pulchrum* and methoxy-2-deprenylrheediaxanthone B occurred in *H. annulatum*. It is noteworthy that the root cultures of *H. pulchrum* had the highest content of xanthenes, but the slowest growth of all investigated cultures. Only one xanthone was identified in the extracts of *H. humifusum* and the lowest total xanthone content was determined in the root cultures of *H. kouytchense*.

Xanthone profile of the untransformed roots of *Hypericum* spp. grown under 16/8 h photoperiod

In the root cultures of five *Hypericum* spp. cultured under 16/8-h photoperiod, the total xanthone content varied

between 0.5 and 1.4 mg/g DW with the highest content identified in *H. tomentosum*, which was characterized by slow growth. While the presence of mangiferin was detected in all investigated species, methoxy-2-deprenylrheediaxanthone B was found only in *H. tetrapterum*. 1,3,6,7 and 1,3,5,6-tetrahydroxanthones were determined in all species except for *H. rumeliacum*. The content of these xanthenes in *H. tomentosum* was about 2 to 3 times higher than in *H. tetrapterum*, *H. maculatum* and *H. annulatum* (Table 3).

Xanthone profile of hairy roots

In all hairy roots clones of *H. tetrapterum*, 1,7-dihydroxyxanthone was identified at a concentration about 30 times

Table 2 Xanthenes identified in methanolic root extracts of selected *Hypericum* species cultured under continuous darkness conditions

Species	Total xanthone content \pm SE (mg g ⁻¹ DW)	Xanthenes	Content of single xanthenes \pm SEM (mg g ⁻¹ DW)
<i>H. pulchrum</i>	4.877 \pm 0.282	Kielcorin	2.219 \pm 0.211
		Paxanthone	2.068 \pm 0.203
		Mangiferin	0.501 \pm 0.035
		1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone	0.089 \pm 0.008
<i>H. annulatum</i>	2.737 \pm 0.157	Methoxy-2-deprenylrheediaxanthone B	1.345 \pm 0.077
		Kielcorin	0.513 \pm 0.036
		Mangiferin	0.449 \pm 0.032
		1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone	0.430 \pm 0.031
<i>H. humifusum</i>	0.744 \pm 0.047	Paxanthone	0.744 \pm 0.047
<i>H. kouytchense</i>	0.057 \pm 0.009	1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone	0.057 \pm 0.009

Data are the mean \pm standard error (SE) of three replicates

Table 3 Xanthenes identified in methanolic root extracts of selected *Hypericum* species cultured under 16/8 h photoperiod conditions

Species	Total xanthone content \pm SE (mg g ⁻¹ DW)	Xanthenes	Content of single xanthenes \pm SE (mg g ⁻¹ DW)
<i>H. tomentosum</i>	1.370 \pm 0.079	1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone	0.906 \pm 0.055
		Mangiferin	0.464 \pm 0.033
<i>H. tetrapterum</i>	0.739 \pm 0.047	1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone	0.451 \pm 0.033
		Methoxy-2-deprenylrheediaxanthone B	0.150 \pm 0.027
		Mangiferin	0.138 \pm 0.012
<i>H. annulatum</i>	0.537 \pm 0.037	1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone	0.273 \pm 0.064
		Mangiferin	0.264 \pm 0.016
<i>H. rumeliacum</i>	0.514 \pm 0.036	Mangiferin	0.514 \pm 0.036
<i>H. maculatum</i>	0.468 \pm 0.033	1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone	0.366 \pm 0.028
		Mangiferin	0.102 \pm 0.009

Data are the mean \pm standard error (SE) of three replicates

higher than in the corresponding control (Table 4). The extracts of *H. tomentosum* hairy roots contained toxyloxanthone B, 1,3,6,7- and 1,3,5,6-tetrahydroxyxanthone (Table 4). In contrast to *H. tetrapterum*, the total xanthone content in extracts of the transgenic clones of *H. tomentosum* did not exceed the content in the control by more than two-fold. It should be noted that the control roots grown under photoperiod (Table 3) or under dark conditions (Table 4), both for *H. tetrapterum* and *H. tomentosum*, contained different xanthenes.

Production of xanthenes in elicited cell suspension cultures of *H. perforatum* L

Xanthone production was determined in cell suspensions on the 5th, 7th and 9th days of culture, corresponding to the 1st, 3rd and 5th days after elicitation. Control cell suspension cultures produced on average 0.238 ± 0.012 mg g⁻¹ DW

of total xanthenes. The highest total xanthone content was detected 24 h after elicitation with SA, and gradually decreased until the beginning of the stationary phase (Fig. 2). In contrast, elicitation with CH or CH with SA resulted in only slight increase of the total xanthone content as compared to the control. Two xanthenes, paxanthone and cadensin G, were identified in all samples. At the beginning of the elicitation treatment, the content of the latter was several times higher, while the content of both xanthenes was comparable at the onset of the stationary phase.

Antifungal activity of *Hypericum* extracts

The antifungal activity of the extracts from *Hypericum* untransformed root cultures, hairy roots and cell cultures against *C. albicans* ATCC 10231 was evaluated (Table 5). Among the *Hypericum* untransformed roots, the best antifungal activity was obtained for *H. annulatum* grown under

Table 4 Xanthenes identified in methanolic extracts of *H. tetrapterum* and *H. tomentosum* hairy roots cultured under continuous darkness conditions

	Clone	Total xanthone content \pm SE (mg g ⁻¹ dw)	Xanthone	Xanthone content \pm SE (mg g ⁻¹ dw)
<i>H. tetrapterum</i>	Control	0.005 \pm 0.005	1,7-dihydroxyxanthone	0.005 \pm 0.005
	Clone 1	0.123 \pm 0.014		0.123 \pm 0.014
	Clone 2 ATCC	0.157 \pm 0.022		0.157 \pm 0.022
	Clone 2 A4	0.143 \pm 0.011		0.143 \pm 0.011
	Clone 4	0.112 \pm 0.019		0.112 \pm 0.019
<i>H. tomentosum</i>	Control	0.97 \pm 0.014	Toxyloxanthone B	0.78 \pm 0.009
			1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone	0.19 \pm 0.019
			Biyouxanthone D	0.56 \pm 0.022
			Toxyloxanthone B	0.66 \pm 0.024
			1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone	0.29 \pm 0.023
	Clone 1	0.95 \pm 0.023	Biyouxanthone D	0.82 \pm 0.013
			Toxyloxanthone B	0.51 \pm 0.018
			1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone	0.16 \pm 0.013
	Clone 2	0.67 \pm 0.015	Toxyloxanthone B	0.81 \pm 0.029
			1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone	0.26 \pm 0.020
			Biyouxanthone D	0.85 \pm 0.022
	Clone 4	1.07 \pm 0.024	Toxyloxanthone B	1.21 \pm 0.033
			1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone	0.32 \pm 0.021
			Toxyloxanthone B	0.64 \pm 0.019
	Clone 7	1.53 \pm 0.026	1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone	0.17 \pm 0.018
Toxyloxanthone B			1.67 \pm 0.043	
1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone			0.35 \pm 0.024	
Clone E	0.81 \pm 0.019	Toxyloxanthone B		
		1,3,6,7 and 1,3,5,6-tetrahydroxyxanthone		
A4	2.02 \pm 0.036			

Data are the mean \pm standard error (SE) of three replicates

CD conditions, with MIC 50 value of 128 $\mu\text{g ml}^{-1}$. Anti-*Candida* activity was also observed for hairy roots extracts of *H. tomentosum* (clone A4 and clone 7 ATCC 15834) and *H. tetrapterum* (clone 2 ATCC15834 and clone 2 A4), with MIC50 s ranging from 128–256 $\mu\text{g ml}^{-1}$ and from 64 to 128 $\mu\text{g ml}^{-1}$, respectively. No antifungal activity against *C. albicans* was found for extracts of elicited and non-elicited *H. perforatum* cells (Table 5).

Discussion

Xanthenes are among the most common compounds isolated from the plants of the genus *Hypericum*, which is considered as a rich source of xanthenes and derivatives that have a wide range of pharmacological effects (reviewed by Fotie and Bohle 2006; El-Seedi et al. 2010; Tala et al. 2013). Xanthenes are present predominantly in the roots and, with some exceptions, can be found in the aerial parts (Kitanov and Nedialkov 1998). Since the first isolation of maculatoxanthone from *H. maculatum* (Arends 1969), several new xanthenes have been isolated and identified from *H. perforatum* (Crockett et al. 2011; Li

et al. 2013), *H. riparium* (Tala et al. 2013) and *H. oblongifolium* (Ali et al. 2014). Underground plant organs of *H. perforatum*, grown under controlled in vitro conditions, accumulate 27 times more xanthenes than the roots of wild plants (Tocci et al. 2013a). In this study, we analysed xanthone accumulation in the root cultures of nine *Hypericum* species that had not previously been investigated. Four of them were cultured under CD and five under 16/8 h photoperiod conditions. Only one species, *H. annulatum*, was cultured under both CD and 16/8-h photoperiod conditions. The species grown under photoperiod conditions always showed lower total xanthone content. The highest total xanthone content, with five xanthenes identified, was recorded in the root cultures of *H. pulchrum* and *H. annulatum*, respectively, both cultured under continuous darkness. Root cultures of *H. annulatum* were characterized by the highest growth index of the nine studied species, both under CD and 16/8-h photoperiod conditions, but under conditions of darkness, xanthone content was much higher than under photoperiod conditions (2.7 and 0.5 mg/g DW, respectively). The extracts obtained from *H. annulatum* roots, cultured under CD conditions, displayed good antifungal activity against *C.*

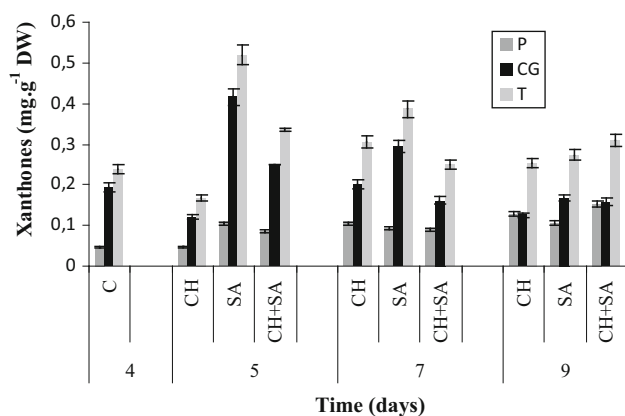


Fig. 2 The content of xanthenes (mg g^{-1} DW) in cell suspension cultures. Figure shows production of xanthenes in control samples (the 4th day) and samples after elicitation on the 5th, 7th and 9th day of culture. Error bars represent SE (standard errors) based on three independent measurements. C control, CH elicitation with chitosan, SA elicitation with salicylic acid and combination of them SA+CH; P paxanthone, CG cadensin G, T total xanthone content; DW dry weight

albicans. Root cultures of *H. tomentosum*, *H. tetrapterum*, *H. annulatum*, *H. rumeliacum* and *H. maculatum*, cultured under 16/8-h photoperiod conditions, did not vary markedly as to the biomass increase and composition and total content of xanthenes with the exception of *H. tomentosum* with the highest total xanthone content.

The biosynthesis of xanthenes in the in vitro-cultured roots seems to be sensitive to exogenous stimuli such as nutrients or hormones (Tocci et al. 2012) or elicitors (Tocci et al. 2011, 2013b), the application of which may result in increased (sometimes transient) production of xanthenes. Comparison of the total xanthone content and the number of identified individual xanthenes in the roots of *H. annulatum* cultured under both CD and 16/8-h photoperiod conditions revealed about five times higher total xanthone content and higher diversity of xanthenes in CD condition-cultured roots.

A high diversity of xanthenes was previously detected in the transgenic hairy roots of *H. perforatum* (Tusevskiet al. 2013a), which makes the system promising for enhanced xanthone production. The authors also reported on altered biosynthetic potential of hairy root cultures grown in the dark and under photoperiod conditions (Tusevski et al. 2013b). We have analysed the content of xanthenes in several clones of hairy root cultures of *H. tomentosum* and *H. tetrapterum* developed in our laboratory. The total content of xanthenes in hairy roots of both species showed a positive correlation with the detected antifungal activity, although the spectrum of individual xanthenes identified in the extract differed. In all hairy root clones of *H. tetrapterum*, we identified 1,7-dihydroxyxanthone while in the transgenic clones of *H. tomentosum*, we found, along with

1,3,6,7- and 1,3,5,6-tetrahydroxyxanthone, biyoxanthone D and toxylloxanthone B in higher quantities than reported from root cultures of *H. perforatum* (Tocci et al. 2013b).

Xanthenes are compounds that are synthesized even by undifferentiated cells. They are the main metabolic products in suspension cultures and undifferentiated calli (Pasqua et al. 2003).

In cell suspension cultures of *H. perforatum*, we identified two xanthenes, paxanthone and cadensin. Paxanthone was first isolated from cell cultures of *H. patulum* by Ishiguro et al. (1993). Later, Ishiguro et al. (1995) isolated prenylated xanthenes and their cyclization products from the cell cultures of the same species. The accumulation of high levels of tetrahydroxyxanthenes in calli and suspended cell cultures of *H. androsaemum*, dependent on hormone supplementation, and *H. perforatum* was reported by Dias et al. (2000, 2001). El-Mawla (2005) identified 1,7-dihydroxyxanthone, 1,3,7-trihydroxyxanthone and three tetrahydroxyxanthenes in cell cultures of *H. gnidioides* grown in Gamborg's B5 medium in the dark. A high content of xanthenes in undifferentiated calli of *H. perforatum* was reported by Mulinacci et al. (2008). In order to increase xanthone biosynthesis in cultured cells, we applied two elicitors, SA and CH, either alone or in combination. Although we recorded more than a two-fold increase in xanthone content 24 h after the application of SA, this increase did not persist during the following days of culture. The weak response to elicitor treatment may be due to the genetically and morphologically heterogeneous composition of the culture. The use of fungal or bacterial elicitors yielded better results. Conceição et al. (2006) treated cell cultures of *H. perforatum*, primed with SA or MeJA, with the fungal elicitor *Colletotrichum gloeosporioides*, and recorded an increased xanthone production of 12-fold as compared to the control. A similar increase was achieved by Franklin et al. (2009), who used the elicitor *Agrobacterium tumefaciens*. In addition, these authors were able to identify four *de novo* synthesized xanthenes, indicating that xanthenes play a dual function: acting in plant defence to protect against biotic stress and oxidative damage and as phytoalexins to impair the pathogen growth. Our accumulated results indicate that increased xanthone synthesis is a part of systemic defence response in *Hypericum* species.

In summary, this work presents new results of xanthone production by root cultures of several previously unstudied *Hypericum* species. In addition, xanthone production was studied in transgenic clones of two *Hypericum* species, *H. tomentosum* and *H. tetrapterum*, which along with previously reported *H. perforatum* transgenic hairy root cultures, are among the most promising experimental systems for the study of the regulation of xanthone biosynthesis and prospective biotechnological production. Extracts from

Table 5 Antifungal activity of untransformed roots, hairy roots and cell suspension cultures against *Candida albicans* ATCC 10231

System	Species	Culture conditions	<i>Candida albicans</i> ATCC 10231		
			MIC ₅₀	MIC ₉₀	MIC ₁₀₀
UR	<i>H. maculatum</i>	16/8	>256	>256	>256
UR	<i>H. humifusum</i>	CD	256	>256	>256
UR	<i>H. annulatum</i>	CD	128	256	256
UR	<i>H. annulatum</i>	16/8	256	256	>256
UR	<i>H. tomentosum</i>	16/8	256	256 to >256	>256
UR	<i>H. tetrapterum</i>	CD	>256	>256	>256
UR	<i>H. tetrapterum</i>	16/8	>256	>256	>256
UR	<i>H. pulchrum</i>	CD	>256	>256	>256
UR	<i>H. kouytchense</i>	CD	256	256	256
UR	<i>H. rumeliacum</i>	16/8	256 to >256	256	>256
HR	<i>H. tomentosum</i> (HTo) control	CD	>256	>256	>256
HR	HTo clone 1 ATCC 15834	CD	256	>256	>256
HR	HTo clone 2	CD	>256	>256	>256
HR	HTo clone 4	CD	256	>256	>256
HR	HTo clone 7 ATCC 15834	CD	128–256	>256	256 to >256
HR	HTo clone e	CD	256 to >256	>256	>256
HR	HTo A4	CD	128	256	>256
HR	<i>H. tetrapterum</i> (HTe) control	CD	>256	>256	>256
HR	HTe clone 1	CD	>256	>256	>256
HR	HTe clone 2 ATCC 15834	CD	64–128	128–256	256 to >256
HR	HTe clone 2 A4	CD	128 to >256	256 to >256	>256
HR	HTe clone 4	CD	>256	>256	>256
CS	<i>H. perforatum</i> control	CD	>256	>256	>256
CS	<i>H. perforatum</i> SA	CD	>256	>256	>256
CS	<i>H. perforatum</i> CH	CD	>256	>256	>256
CS	<i>H. perforatum</i> SA + CH	CD	>256	>256	>256

UR untransformed roots, HR hairy roots, CS cell suspension cultures, HTo *H. tomentosum*, HTe *H. tetrapterum*, SA salicylic acid, CH chitosan, SA+CH salicylic acid+chitosan treatment, 16/8-photoperiod, CD continuous dark, nt not tested, MIC₅₀, MIC₉₀ and MIC₁₀₀ minimal inhibitory concentration needed to inhibit the growth of 50, 90 and 100 % of *Candida albicans* ($\mu\text{g ml}^{-1}$)

samples with the highest contents of xanthenes exhibited antifungal activity against *C. albicans*.

Author contribution statement DZ, AM, JH, performed all the in vitro experiments, acquired the data and prepared the figures and tables; AM performed the cytofluorimetric analysis on cell suspensions, AM, DZ and GDA carried out the chemical extraction and HPLC analysis of the in vitro -grown material; AV acquired and interpreted chemical data; FDD and GS performed and interpreted the antifungal activity tests; EC and GP coordinated all stages of the experimental work and drafted the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

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

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