

Xanthone biosynthesis and accumulation in *calli* and suspended cells of *Hypericum androsaemum*

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Abstract

Calli and suspended cells of *Hypericum androsaemum* accumulated high levels of 1,3,5,6 and 1,3,6,7 oxygenated xanthenes. The major compounds include simple oxygenated xanthenes or derivatives with prenyl, pyran or methoxyl groups, four of them being new natural compounds. A hypothetical biosynthetic scheme is proposed based on the isolated compounds and statistical analysis. Xanthone accumulation was influenced greatly by medium factors, namely hormone supplementation. *Calli* grown with 4.5 μM α -naphthaleneacetic acid (NAA) + 2.3 μM kinetin (KIN) had the highest specific xanthone production (1.3% biomass dry weight (DW)), whereas suspended cells grown in similar medium accumulated a lower amount (0.87% DW). *Calli* displayed a negative linear relationship between total xanthone accumulation and NAA concentration, in the range of 4.5–22.5 μM . However, in this range the xanthone 1,3,6,7/1,3,5,6 ratio and the biomass production showed a positive linear relationship with NAA concentration. Substitution of 4.5 μM of NAA by the same molar amount of 2,4-dichlorophenoxyacetic acid (2,4-D), in the presence of 2.3 μM of KIN, caused a decrease in xanthone accumulation in *calli*. The use of *N*⁶-benzyladenine (BA) instead of KIN reduced xanthone production, independently of the auxin used. This effect was attenuated when both hormones were present. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Hypericum androsaemum*; *Calli*; Cells suspension; Xanthenes; Phytohormones

1. Introduction

Hypericum androsaemum L. has been used widely in Portugal as a medicinal plant. Its pharmacological properties have been attributed to phenolic compounds. Due to uncontrolled harvesting as well as seasonal, climatic, and geographic restrictions the availability of wild plants from this species in nature is strongly limited. In vitro cultures may be an alternative choice when the active compounds are produced in such sys-

tems. They provide a way to study the biosynthesis of secondary metabolites and the factors that influence it giving some possibilities of controlled production. Detailed studies on the optimization of the culture medium, identification of the full range of plant secondary metabolites produced, stability of production and definition of biosynthetic pathways by in vitro cultures have been reported ([1–4], and refs. therein).

In a previous work we reported that the major phenolics produced by in vitro cultures of *H. androsaemum* are xanthenes [5]. This type of compound is widely found in the *Hypericaceae* family and has interesting pharmacological properties. Xanthenes exhibit strong and selective inhibition of monoamine oxidase A, in vitro cytotoxicity and in vivo antitumor activity, as well as antibacterial and antifungal activities ([6,7], and refs. therein).

Abbreviations: DW, biomass dry weight; NAA, α -naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indol-3-acetic acid; BA, *N*⁶-benzyladenine; KIN, kinetin; Xanthone 1,3,6,7/1,3,5,6 ratio, ratio of xanthenes with the oxygenation pattern 1,3,6,7 and xanthenes with the oxygenation pattern 1,3,5,6.

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In spite of the interesting properties of the xanthonones and the potentialities of in vitro cultures for production of secondary metabolites, information about how xanthonones accumulate in in vitro systems is scanty [8–10].

Manipulating culture conditions, particularly changes in phytohormones is a valuable tool for increasing the level of bioactive metabolites [11–13]. This paper reports the isolation and identification of the major xanthonones produced by *calli* and suspended cells of *H. androsaemum*. An evaluation of the changes in xanthonones as a function of changes in growth conditions, namely phytohormones, is reported also.

2. Material and methods

2.1. In vitro culture and phytohormone supplementation

Solid MS medium [14], containing 0.8% agar (Iberagar, J.M. Vaz Pereira, Lisboa, Portugal) and 20 g/l sucrose, for *calli*, or liquid MS medium (without agar), for cell suspensions, was used as basal medium.

Calli cultures of *H. androsaemum* L. were established from stem segments of in vitro shoots maintained on solid medium, supplemented with 4.5 μM indol-3-acetic acid (IAA) and 2.3 μM kinetin (KIN), induced from stem segments of wild plants, collected in May 1996 in the National Park of Peneda-Gerês in the North of Portugal. *Calli* cultures were initiated and maintained on solid medium supplemented with 4.5 μM of auxin and 2.3 μM of cytokinin. Several combinations of auxin/cytokinin were selected to study xanthone production: α -naphthaleneacetic acid (NAA)/KIN, NAA/*N*⁶-benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D)/KIN, 2,4-D/BA and 2,4-D/BA + KIN (in this case, with BA and KIN supplemented at equal amounts to give the final concentration of 2.3 μM of cytokinin). Additionally, combinations of 2.3 μM KIN with NAA contents of 4.5, 11.25 and 22.5 μM were assayed for xanthone production. *Calli* were maintained at $25 \pm 1^\circ\text{C}$ under a 16-h light photoperiod, with a photon flux of 30 $\mu\text{mol}/\text{m}^2$ per s provided by Osram Fluora fluorescent bulbs. Cultures were subcultured, at the end of exponential growth phase, at 5-week intervals. After the 3rd subcul-

ture, *calli* were stabilized showing, however, areas of different pigmentation as well as development of root primordia. At the end of exponential phase, after the 6th subculture, they were collected for xanthone isolation and analysis.

Cell suspension cultures were established, in liquid MS medium supplemented with 4.5 μM NAA and 2.3 μM KIN, from *calli* grown on solid MS medium with the same hormone supplementation. Cultures shaken at 120 rpm, under the same environmental conditions referred for *calli*, were subcultured every 3 weeks, at the end of exponential growth phase, by transferring 25 ml of cell suspension containing 2–2.5 g, biomass fresh weight, to a 500 ml flask containing 150 ml of fresh medium. After the 4th subculture cell suspensions were stabilized. After the 8th subculture, at the end of exponential growth phase, suspended cells were collected for xanthone isolation and analysis.

2.2. HPLC xanthone analysis

Ground freeze dried biomass (0.1–0.5 g) was extracted with 10 ml of an aqueous methanolic solution (80%), with sonication, for 20 min, at ambient temperature and the homogenate was filtered. The process was repeated twice, first with 5 ml of methanol and then with 5 ml of acetone. After that, the solutions were combined and evaporated to dryness. The residue was redissolved in 5 ml methanol and submitted to HPLC-DAD analysis as described before [5]. Quantification of the xanthonones was performed by the external standard method, at 260 nm, as mangiferin (Extrasynthese, Geney, France) equivalents.

2.3. Isolation and identification of xanthonones

A mixture of ground freeze-dried *calli* and suspended cells of *H. androsaemum* (~70 g), collected as described above, was first defatted with *n*-hexane in a Soxhlet apparatus, for 12 h, and then extracted with hot methanol (4 \times 200 ml) followed of hot acetone (2 \times 200ml). The extracts were combined and evaporated under reduced pressure at 40°C. The residue was redissolved in 20 ml of MeOH and then fractionated over a Lobar C18 column using an aqueous methanolic solution (10–95%) as eluent. The fractions containing xanthonones were identified by HPLC-DAD and submitted to semipreparative HPLC in order

to isolate them. Elution was carried out as described before [15]. Identification of the isolated xanthenes was carried out by mass spectrometry (EI-MS probe 70 eV), and by $^1\text{H-NMR}$ (300 MHz, acetone- d_6 , ppm/TMS) and $^{13}\text{C-NMR}$ (70.5 MHz, acetone- d_6 , ppm/TMS) spectrometry, as well as by UV spectroscopy study with the classical reagents [7,16].

2.4. Statistical analysis

All data were analysed statistically with the Statistica/Mac software (Statsoft, 1986–1991). Mean differences were analysed statistically running the one way analysis of variance test (ANOVA). The homogeneity of variances was tested by a Cochran's test. Post hoc comparisons were performed with the HSD Tuckey test. The relationship between xanthenes was analysed case-wise with a Pearson correlation coefficient. Furthermore, we constructed matrices containing the significant ($P < 0.05$) positive and negative correla-

tions between individual xanthenes for each type of culture analysed, suspended cells or *calli*. One matrix was constructed for each hormone medium variant tested.

The linear regression analysis was performed with the data analysis GrapPad Prism 1.0 package (GraphPad, 1994).

3. Results and discussion

3.1. Identified xanthenes

Twelve xanthenes were isolated from the methanolic extract of the in vitro biomass of *H. androsaemum*, and 11 of them were identified fully. These compounds comprise the majority of the xanthenes detected by HPLC-DAD (64–80% of the xanthone fraction). They include simple oxygenated xanthenes or derivatives with prenyl, pyran or methoxyl groups (Fig. 1). Compounds 1 and 2 showed the UV spectral characteristics of

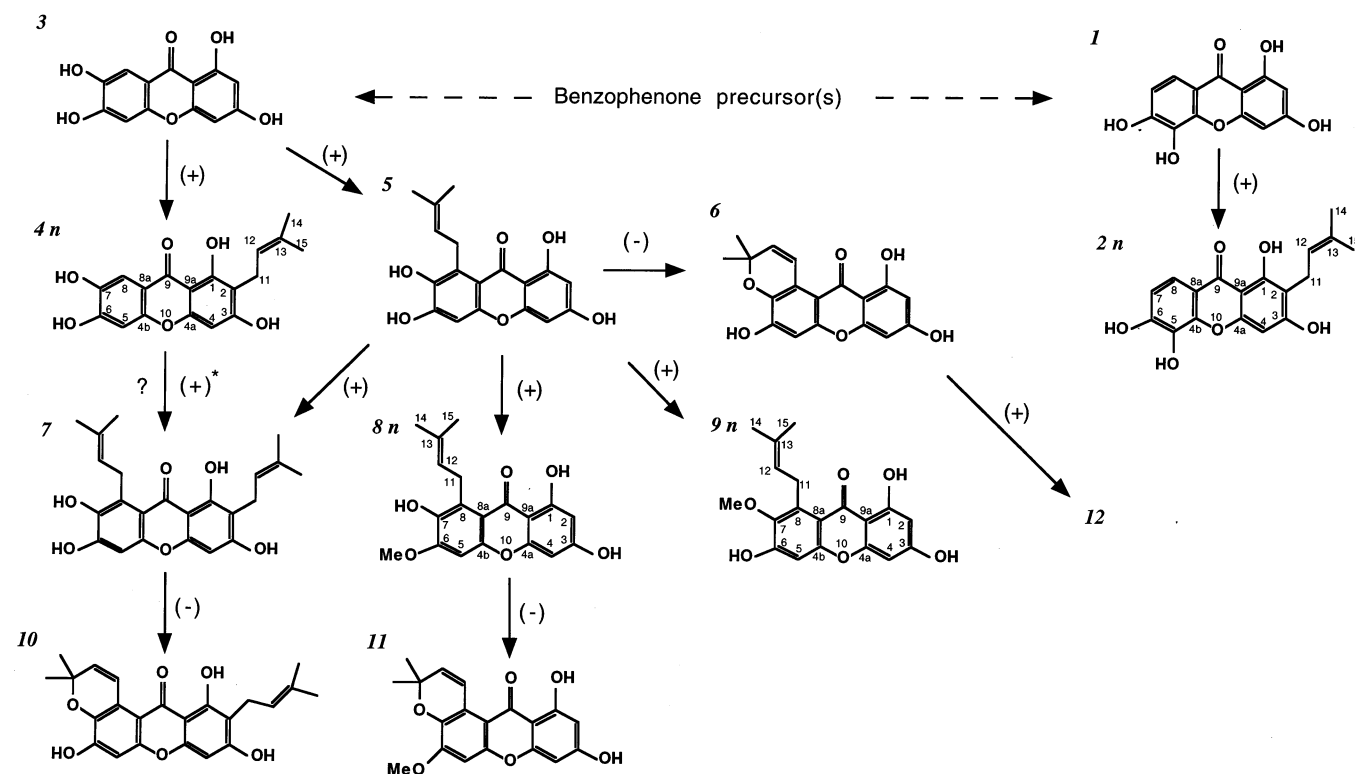


Fig. 1. Proposed pathway of xanthone biosynthesis in in vitro cultures of *H. androsaemum*. Significant ($P < 0.05$) positive (+) and negative (-) correlations between individual xanthenes are signalised ($n = 6$); (+) *, positive correlation for $0.13 < P < 0.22$; n , new compound. Compounds: (1) 1,3,5,6-tetrahydroxyxanthone; (2) 1,3,5,6-tetrahydroxy-2-prenylxanthone; (3) 1,3,6,7-tetrahydroxyxanthone; (4) 1,3,6,7-tetrahydroxy-2-prenylxanthone; (5) 1,3,6,7-tetrahydroxy-8-prenylxanthone; (6) toxylloxanthone B; (7) γ-mangostin; (8) 1,3,7-trihydroxy-6-methoxy-8-prenylxanthone; (9) 1,3,6-trihydroxy-7-methoxy-8-prenylxanthone; (10) garcinone B; (11) paxanthone; (12) derivative of compound 6 ($[M^+]$ peak at m/z 368).

Table 1
¹H-NMR and chromatographic data of compounds 2, 4, 8 and 9 (acetone-d₆, TMS as int. std.)

Position	2	4	8	9
2	–	–	6.21 (d, 1H, <i>J</i> = 1.9)	6.17 (d, 1H, <i>J</i> = 2.1)
4	6.51 (s, 1H)	6.34 (s, 1H)	6.33 (d, 1H, <i>J</i> = 1.8)	6.28 (d, 1H, <i>J</i> = 2.1)
5	–	6.86 (s, 1H)	6.86 (s, 1H)	6.91 (s, 1H)
7	6.95 (d, 1H, <i>J</i> = 8.4)	–	–	–
8	7.61 (d, 1H, <i>J</i> = 8.7)	7.62 (s, 1H)	–	–
11	3.33 (d, 2H, <i>J</i> = 7.2)	3.35 (d, 2H, <i>J</i> = 7.0)	4.12 (d, 2H, <i>J</i> = 6.3)	4.12 (d, 2H, <i>J</i> = 6.9)
12	5.26 (m, 1H)	5.29 (m, 1H)	5.29 (m, 1H)	5.25 (m, 1H)
Me-14	1.62 (s, 3H)	1.64 (s, 3H)	1.66 (s, 3H)	1.60 (s, 3H)
Me-15	1.76 (s, 3H)	1.73 (s, 3H)	1.84 (s, 3H)	1.80 (s, 3H)
–OCH ₃	–	–	3.86 (s, 3H, 6-OMe)	4.01 (s, 3H, 7-OMe)
R _t (HPLC)	34' 58"	33' 97"	36' 02"	36' 42"

the 1,3,5,6 oxygenated xanthenes, with band IV reduced to shoulder [17], while all the other identified xanthenes had an UV similar to mangiferin, typical of the 1,3,6,7 oxygenation pattern with a very well-defined band IV [18–20]. Data from ¹H- and/or ¹³C-NMR confirmed the oxygenation pattern and allowed the localization of the prenyl, pyran and methoxyl groups. Compounds 1, 3, 5 and 6 were isolated previously from *H. androsaemum* roots [18], while compounds 7 (γ -mangostin), 10 (garcinone B), and 11 (paxanthone) were isolated from *H. patulum* cell cultures [19,20]. The data obtained by us were in good agreement with those reported in the literature. As far as we are aware, compounds 2, 4, 8 and 9, although bearing the same skeletal features as all the other identified substances, are now reported for the first time and their ¹H-NMR data are presented in Table 1. Each of these compounds present MS and NMR signals consistent with the presence of a prenyl group. Compound 2 ($[M^+]$ at *m/z* 328) exhibited a pair of *ortho*-coupled at δ 6.95 and 7.61; these high values can only be due to protons located in the deshielded area of the carbonyl group [6] and this, together with the data from UV, confirmed the proposed oxygenation pattern. The prenyl group was located at C2 and not at C4, by comparison of the H4 δ value with those obtained for compound 7 and 10. Compound 4 ($[M^+]$ at *m/z* 328) showed signals for three uncoupled protons, which confirms the 1,3,6,7-oxygenation pattern and the prenyl group was located at C2 by the same reasoning as for compound 2. Compounds 8 and 9, in spite of having different HPLC retention times and UV spectra, had similar mass spectra ($[M^+]$ at *m/z* 342) and ¹H-NMR, with two *meta*-coupled protons on A ring (always lower δ shift

than those for B ring [19,20]), a singlet for one proton on B ring, a singlet for a methoxyl and signals for a prenyl group. The high δ value of protons in position C-11 (δ 4.12) indicates that the prenyl group is located at C8, in the neighborhood of the carbonyl group [18,20]. Besides, the whole set of values obtained for the prenyl group is in good agreement with those obtained for compound 5. The location of the methoxyl group was established to be at C-6, in compound 8, by NOE difference spectroscopy, which showed 14% enhancement of the H-5 (δ 6.86) on saturation of the methoxyl protons. Since compounds 8 and 9 are isomers and the UV study, with the classical reagents [7,16], showed that both have free hydroxyls at C-1 and C-3, the location of the methoxyl, in compound 9, must be at C-7. Compound 12 was not completely identified but the ¹H NMR spectra pointed to a derivative of compound 6 having another group linked to this structure, since the mass spectrum indicated a higher molecular weight ($[M^+]$ at *m/z* 368). On the HPLC-DAD analysis we have detected other minor xanthenes which have the same 1,3,6,7 and 1,3,5,6 oxygenation pattern as indicated by their UV profile.

Peters et al. [21] reported that cell suspension cultures of *H. androsaemum* accumulate several non-identified xanthenes, which, from preliminary spectroscopic data, are prenylated and/or C-glycosylated derivatives of 1,3,6,7-tetrahydroxanthone. According to the same authors, all the xanthenes detected in *H. androsaemum* lack a 5-oxo group. However, we identified also xanthenes with a 1,3,5,6 oxygenation pattern, indicating clearly that *H. androsaemum* cell cultures have the biosynthetic capability of producing 5-oxygenated xanthenes.

The reason for this discrepancy could be attributed to different culture conditions used. Although mangiferin is the major xanthone produced by the aerial part of wild *H. androsaemum* plants (results not shown), we did not detect this compound in in vitro biomass of this species. Other minor non-identified xanthones produced by the aerial part of wild *H. androsaemum* plants were also not found in in vitro biomass.

3.2. Hypothetical biosynthesis pathway of xanthones

Mangostin that was identified in our in vitro cultures (Fig. 1, compound 7) was identified also in *Garcinia mangostana* (Hypericaceae) [22]. Cinnamic acid, benzoic acid, *m*-hydroxybenzoic acid and malonic acid were found to be efficient precursors of this compound [22]. Beerhues and co-workers [21,23] have studied also the biosynthesis of xanthones in *H. androsaemum* cell suspensions. Their findings indicate that 3-hydroxybenzoic and benzoic acids are efficient precursors of an intermediate tetrahydroxybenzophenone that is subsequently converted to the xanthone backbone. Based on this information and in the compounds isolated by us we proposed the pathway shown in Fig. 1 for xanthone biosynthesis in in vitro cultures of *H. androsaemum*. The proposed scheme is supported also by statistical analysis (see Section 2). Significant correlations ($P < 0.05$) between individual xanthones connected by the arrows were observed in the matrices obtained (results not shown), independently of the type of culture and hormonal supplementation. The only exception is the putative conversion of xanthone 4 into xanthone 7 ($0.13 < P < 0.22$). This could indicate that xanthone 7 is originated preferentially from the conversion of 5 instead of 4. All correlations are positive except those that involve cyclization of the prenyl side chain (5, 7 and 8 into 6, 10 and 11, respectively) which have negative correlations (Fig. 1).

3.3. Xanthone accumulation in suspended cells versus calli

Although the biosynthetic pathway had remained unchanged, the specific accumulation of xanthones was influenced greatly by the hormonal supplementation and by the physical state of the

medium (Fig. 2). The highest specific production of xanthones ($\sim 1.3\%$ biomass dry weight (DW)) was observed in *H. androsaemum calli* grown with the supplementation of $4.5 \mu\text{M}$ NAA plus $2.3 \mu\text{M}$ KIN. Suspended cells grown with the same hormonal supplementation ($4.5 \mu\text{M}$ of NAA plus $2.3 \mu\text{M}$ of KIN) accumulated a lower xanthone amount ($\sim 0.87\%$ DW). Nevertheless, this value is higher than those reported for xanthone production by suspended cells of *Centaurium erythraea* and *C. littorale*, which were 0.7 and 0.3% DW, respectively [9]. A differential xanthone accumulation depending on the type of culture had already been observed in in vitro cultures of *C. erythraea* where the production of xanthones by cells suspension was higher than in calli [8].

Differences were also observed in individual accumulations of each compound (Table 2) and in the xanthone 1,3,6,7/1,3,5,6 ratio (Fig. 2). The higher total xanthone accumulation in calli grown with the supplementation of $4.5 \mu\text{M}$ NAA plus $2.3 \mu\text{M}$ KIN, relative to that observed for suspended cells, was due to the selective increase in accumulation of xanthones with the 1,3,6,7 pattern (Fig. 2 and Table 2). One exception was the compound 11 that accumulated in suspended cells in a yield

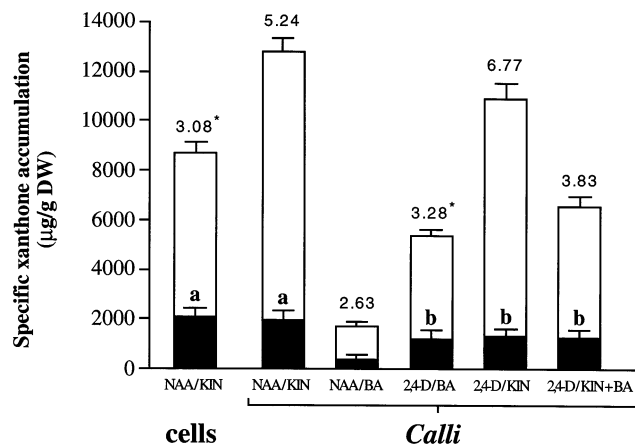


Fig. 2. Specific xanthone accumulation in calli and suspended cells of *H. androsaemum* (□, 1,3,6,7 and ■, 1,3,5,6 oxygenation patterns). Cultures were maintained with MS medium supplemented with $4.5 \mu\text{M}$ of auxin plus $2.3 \mu\text{M}$ of cytokinin, as described in Section 2. Results are means of six independent replicates. All the values are statistically different ($P < 0.05$) except those of xanthones 1,3,5,6, signalled with the same letter. On the top of the columns the mean values of the xanthone 1,3,6,7/1,3,5,6 ratio in calli and suspended cells of *H. androsaemum* are indicated. All the values are statistically different ($P < 0.05$) except those signalled with the asterisk (*).

Table 2
Specific xanthone accumulation in suspended cells and *calli* of *Hypericum androsaemum*^a

Xanthone	Cells NAA/KIN ($\mu\text{g/g DW}$); % tot	<i>Calli</i> NAA/KIN ($\mu\text{g/g DW}$); % tot	NAA 2.5/KIN ($\mu\text{g/g DW}$); % tot	NAA 5/KIN ($\mu\text{g/g DW}$); % tot	NAA/BA ($\mu\text{g/g DW}$); % tot	2,4-D/BA ($\mu\text{g/g DW}$); % tot	2,4-D/KIN/BA ($\mu\text{g/g DW}$); % tot	2,4-D/KIN ($\mu\text{g/g DW}$); % tot
1	45 \pm 6.6; 0.5	86 \pm 3.5; 0.7	37 \pm 6.7; 0.4	nd	nd	63 \pm 4.2; 1.2	22 \pm 1.8 ^a ; 0.3	22 \pm 2.4 ^a ; 0.2
2	193 \pm 34.0 ^a ; 2.2	533 \pm 28.4 ^b ; 4.2	487 \pm 21.2; 5.3	177 \pm 10.2 ^a ; 5.9	86 \pm 7.7; 5.1	251 \pm 17.8; 4.7	196 \pm 23.9 ^a ; 3.0	561 \pm 26.2 ^b ; 5.1
3	25 \pm 4.2 ^a ; 0.3	108 \pm 19.7; 0.8	75 \pm 10.7; 0.8	29 \pm 46 ^{a,b} ; 1.0	nd	26 \pm 3.5 ^a ; 0.5	37 \pm 4.2 ^{b,c} ; 0.6	40 \pm 4.1 ^c ; 0.4
4	102 \pm 10.4 ^a ; 1.2	158 \pm 16.8 ^b ; 1.2	138 \pm 11.3; 1.5	68 \pm 8.2 ^c ; 2.3	87 \pm 12.4 ^{a,c} ; 5.2	94 \pm 11.8 ^a ; 1.8	170 \pm 27.9 ^b ; 2.6	170 \pm 20.8 ^b ; 1.6
5	2006 \pm 192.1 ^a ; 23.0	2085 \pm 148.9 ^a ; 16.3	1400 \pm 66.1; 15.2	439 \pm 36.1 ^b ; 14.6	178 \pm 32.8; 10.6	508 \pm 25.9 ^b ; 9.5	589 \pm 18.7; 9.0	2125 \pm 164.5 ^a ; 19.4
6	1524 \pm 194.0 ^a ; 17.5	4256 \pm 337.0; 33.2	3332 ^b \pm 141.8; 36.2	1168 \pm 185.5; 38.4	416 \pm 82.5; 24.6	1593 \pm 139.1 ^a ; 29.7	1782 \pm 88.9; 27.2	3364 ^b \pm 287.7; 30.8
7	326 ^a \pm 31.1; 3.7	258 ^{a,b} \pm 41.2; 2.0	221 \pm 28.5b ^a ; 2.4	89 \pm 33.3 ^c ; 3.0	22 \pm 1.8; 1.3	77 \pm 18.2 ^{c,d} ; 1.4	57 \pm 13.6 ^d ; 0.9	476 \pm 17.6; 4.3
8	200 \pm 18.9 ^a ; 2.3	435 \pm 21.2; 3.4	202 \pm 21.7 ^a ; 2.2	54 \pm 15.7 ^b ; 1.8	25 \pm 13.3 ^b ; 1.5	38 \pm 6.9 ^b ; 0.7	66 \pm 6.8; 1.0	268 \pm 15.0; 2.4
9	152 \pm 13.4 ^a ; 1.7	279 \pm 12.7; 2.2	220 \pm 20.3; 2.4	82 \pm 7.0 ^b ; 2.7	93 \pm 7.6 ^b ; 5.5	158 \pm 18.7 ^a ; 2.9	307 \pm 19.2 ^c ; 4.7	306 \pm 14.9 ^c ; 2.8
10	177 \pm 20.3 ^a ; 2.0	158 \pm 15.4 ^a ; 1.2	129 \pm 10.1; 1.4	54 \pm 17.9 ^{b,c} ; 1.8	nd	66 \pm 6.6 ^b ; 1.2	29 \pm 13.8 ^c ; 0.4	234 \pm 10.6; 2.1
11	424 \pm 26.1; 4.9	253 \pm 20.0 ^a ; 2.0	230 \pm 11.7 ^a ; 2.5	157 \pm 12.6; 5.2	38 \pm 5.6; 2.2	67 \pm 12.4 ^b ; 1.2	74 \pm 12.9 ^b ; 1.1	555 \pm 36.4; 5.1
12	402 \pm 38.6; 4.6	699 \pm 74.2 ^a ; 5.4	515 \pm 42.4 ^b ; 5.6	176 \pm 14.2 ^c ; 5.8	161 \pm 34.3 ^c ; 9.5	560 \pm 30.5 ^b ; 10.4	1057 \pm 44.2; 16.1	625 \pm 90.8 ^{a,b} ; 5.7

^a Values are means of six independent replicates. In each column, the first and second value represent the mean \pm S.D. of xanthone content and the percentage of the compound relative to the total xanthenes, respectively. In each line, means followed by the same letters, were not statistically different ($P > 0.05$); nd, not detected. Hormonal concentrations were 4.5 μM for auxins and 2.3 μM for cytokinins. Exceptions for these supplementations were *calli* NAA 2.5/KIN and NAA 5/KIN whose NAA concentrations were 11.25 and 22.5 μM , respectively.

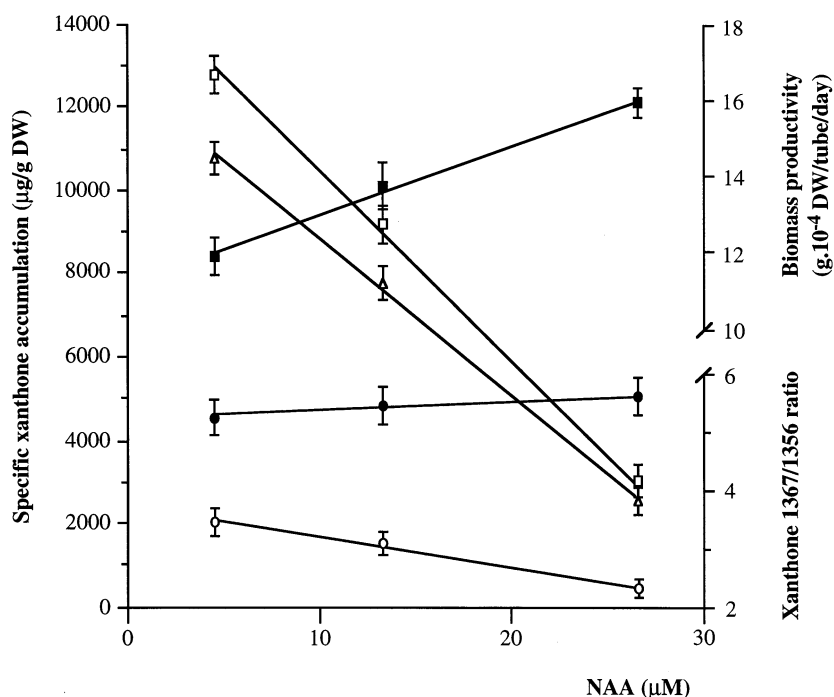


Fig. 3. Specific xanthone accumulation, biomass productivity and xanthone ratio in *calli* of *H. androsaemum* as a function of the NAA amount (4.5, 11.25 or 22.5 μM of NAA plus 2.3 μM of KIN, as described in Section 2). Results are means of six independent replicates and vertical bars indicate 95% confidence intervals to the average. The equations of the regression analysis and the respective r -squared are as follows: $y = -72.44x + 2385.73$; $r^2 = 0.987$ –○– 1,3,5,6 specific xanthone accumulation; $y = -373.48x + 12547.07$; $r^2 = 0.973$ –△– 1,3,6,7 specific xanthone accumulation; $y = -445.958x + 14939.55$; $r^2 = 0.968$ –□– total specific xanthone accumulation; $y = 0.016x + 5.19$; $r^2 = 0.981$ –●– 1,3,6,7/1,3,5,6 xanthone ratio; $y = 1.112x + 0.184$; $r^2 = 0.967$ –■– biomass productivity. In all equations the regression model is very significant ($P < 0.001$).

1.7-fold higher than that observed for *calli*. These results seem indicate that the cyclization process of the xanthone 11 was favored in cell suspensions and hampered in equivalent *calli*. On the contrary cyclization of 5 to 6 would be favored in *calli* and hampered in cell suspensions (Table 2). Cell cultures of *H. androsaemum* clearly accumulated lower amounts of xanthenes 1 and 2 (1,3,5,6 oxygenation pattern) than the correspondent *calli* (Table 2). Nevertheless, total 1,3,5,6 xanthone accumulation was not significantly different in both types of *H. androsaemum* cultures (Fig. 2). This was due to an increase in the production of other unidentified 1,3,5,6 xanthenes by suspended cells.

These results indicate that in spite of the fact that culture media and particularly phytohormones play an important role in regulation of secondary metabolism [11–13,24,25], a balance of several other factors also determines secondary metabolites accumulation. The cell suspensions of *H. androsaemum* are characterized mainly by isolated cells and small aggregates of two to ten cells, occasionally reaching 1 mm in diameter, in a

dedifferentiated state. However, in *calli* grown with the same hormonal supplementation a certain degree of differentiation occurred (see Section 2). This differentiation could have favoured xanthone production in *calli* of *H. androsaemum* whereas the opposite may have occurred in cell suspensions of this species. Lack of cellular differentiation is often considered as one of the major limitations in using dedifferentiated *calli* cells for secondary metabolite production [26]. Although plant cells are totipotent and hence should carry the genes for secondary metabolites as in the whole plant, these genes may not be expressed in a dedifferentiated state [27].

3.4. Effects of phytohormones on xanthone accumulation in *H. androsaemum calli*

The total and specific xanthone production, the xanthone 1,3,6,7/1,3,5,6 ratio as well as biomass *calli* production were influenced greatly by the phytohormones used, the type and ratios of auxin and cytokinin concentrations (Figs. 2–4; Table 2).

Some hormonal effects on the specific xanthone accumulation in *calli* seem be associated to biomass growth. The production of *calli* biomass was stimulated by the increase of NAA concentration, in the range studied (Fig. 3). A tight negative linear relationship occurred however, between total xanthone accumulation by *H. androsaemum calli* and NAA concentration. The replacement of 4.5 μM NAA by the same molar concentration of 2,4-D, in the presence of 2.3 μM KIN led to an increase in biomass production (Fig. 4) and to a decrease in the overall xanthone accumulation (Fig. 2). The lowest total xanthone accumulation and the highest *calli* biomass production occurred when BA substituted KIN in medium supplemented with 4.5 μM NAA (Figs. 2 and 4). The inverse relationship between the accumulation of secondary metabolites and the biomass growth such as occurred in these three assays is a common phenomenon referred already by other authors [13,26]. Apparently, 2,4-D, high levels of NAA, or BA in the presence of NAA, act in *H. androsaemum calli* directing carbon and energy flow preferentially to growth (primary metabolism) instead to xanthone production.

However, some other hormonal effects on specific xanthone accumulation, not associated to biomass growth, occurred in *H. androsaemum calli* suggesting that the phytohormones tested have different levels of action. Although xanthone production was negatively affected by the increase of NAA concentration, in the range studied, such

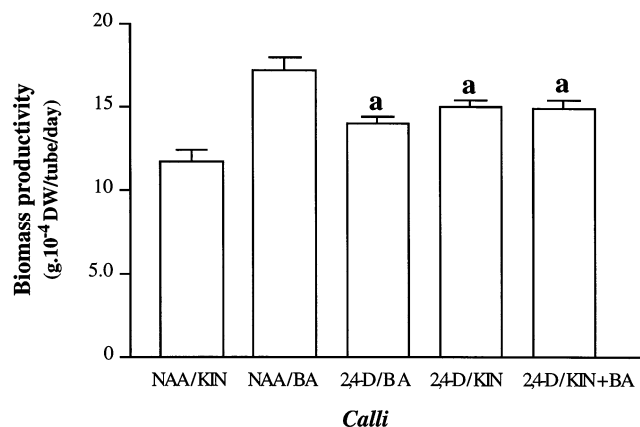


Fig. 4. Biomass productivity from *calli* of *H. androsaemum*. All *calli* were grown on MS medium supplemented with 4.5 μM of auxin plus 2.3 μM of cytokinin, as described in Section 2. Results are means of six independent replicates. All the values are statistically different ($P < 0.05$) except those signalled with the same letter.

inhibiting effect was lower in the relative accumulation of 1,3,6,7 xanthenes (Fig. 3). With exception for 6, 10 and 11, each identified xanthone followed the overall decreasing tendency with the increase of NAA concentration (Table 2). The relative percentage of the xanthenes 6, 10, and 11, which result from the cyclization of the prenyl side chain, increased with NAA concentration (Table 2). These results show that the cyclization of the isoprenyl side-chain was favored by increasing NAA concentration. The replacement of 4.5 μM NAA by the same molar concentration of 2,4-D, in the presence of 2.3 μM of KIN, showed a strong negative effect in the cyclization of compound 5 leading to a deep lowering in accumulation of 6 (Table 2), highly responsible for the overall decreasing in xanthone accumulation (Fig. 2). Such as it was above described xanthone accumulation decreased but a selective increase in the relative accumulation of 1,3,6,7 xanthenes occurred (Fig. 2), particularly those numbered as 7, 9, 10, and 11 (Table 2). The use of BA instead of KIN highly suppressed xanthone accumulation, independently of the auxin used (Fig. 2 and Table 2). The inhibition induced by BA was particularly strong in the accumulation of xanthenes with the 1,3,6,7 oxygenation pattern (Fig. 2). Consequently, this led to the lowest 1,3,6,7/1,3,5,6 xanthone ratios observed in *calli* maintained either with NAA/BA or with 2,4-D/BA. A negative effect resulting from the use of BA instead of KIN was also observed in the phenolic production by cell suspensions of *Cassia didymobotrya* [28]. The use of KIN in conjunction with BA slightly suppressed the inhibitory effect of BA in xanthone accumulation (Fig. 2). This was due exclusively to an increase in the production of 1,3,6,7 xanthenes. Changes on the expression and activity of several enzymes due to the action of auxins and cytokinins, including enzymes from the phenylpropanoid pathway, have been reported by other authors ([24,25], and refs. therein). It is probable that phytohormones act facilitating or hampering the expression and activity of specific xanthone enzymes influencing xanthone accumulation.

The present studies indicate that *in vitro* cultures of *H. androsaemum* have the potential to accumulate xanthenes and their production can be partially manipulated by changing the phytohormone supplementation. Some results indicate that phytohormones may act at the level of expression

and/or activity. Further studies are necessary however, to confirm it.

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