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CARDENOLIDES OF *DIGITALIS OBSCURA*: THE EFFECT OF PHOSPHATE AND MANGANESE ON GROWTH AND PRODUCTIVITY OF SHOOT-TIP CULTURES

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Abstract—Cardenolide composition of leaves from wild and micropropagated elite plants of *Digitalis obscura* (genotype T4) has been investigated and no qualitative differences were found among their major cardenolides (series A). All of the detected glycosides belong to the digitoxose-type cardenolides. Genins represented less than 2% of the overall content, while lanatoside A was the predominant cardenolide (*ca.* 65%) in all samples. The cardenolide yield of micropropagated *D. obscura* plants depended on the age and development of the cultures, but productivity of long-term cultures (2 years) was quite similar to that of the parent plant. Changes in the concentrations of phosphate or manganese ions in the nutrient media did not significantly affect the biosynthetic capacity of shoot-tip cultures, which was not correlated with increases or decreases observed in growth and development of the regenerated shoots or in their contents in photosynthetic pigments.

INTRODUCTION

Micropropagation is important to improve the production of medicinal plants because it offers a great potential for massive cloning of elite genotypes. In this respect, we have recently developed protocols for multiplying and maintaining high-yielding *Digitalis obscura* plants (genotype T4), and demonstrated the genetic stability of the clones grown in long-term shoot-tip cultures [1]. In the present work we have identified and quantified major cardenolides throughout the culture period (2 years), establishing a comparison with those produced by the parent plant.

Information concerning the factors regulating secondary metabolism is as important as the selection of high-producing lines in increasing the production of secondary metabolites [2]. For this reason, we previously determined the influence of different growth regulators on *D. obscura* shoot-tip cultures [1, 3] and now we have studied the possible effects of phosphate and manganese, which substantially affected growth and/or cardenolide production in different *Digitalis* species [4–7].

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RESULTS AND DISCUSSION

D. obscura *cardenolides*

Qualitative analysis performed by direct chromatographic comparison with standards under different HPLC conditions allowed the identification of the seven major cardenolides in the wild genotype T4 and *in vitro* regenerants. All of the detected glycosides belonged to the digitoxose-type cardenolides with the predominant ones being those of the series A (Table 1). Digitoxigenin, purpureaglycoside A, evatromonoside, digitoxigenin-bis-digitoxoside, lanatoside A, digitoxin and gitoxigenin were found in different concentrations in all samples examined. Gitoxin and digoxin were found in only trace amounts in the wild plant. By contrast, Brisa *et al.* [8] identified cardenolides of the series C in both wild plants and morphogenic cultures of *D. obscura*, which is also in contrast with the results obtained by Lichius [9] in *D. obscura* plants from Morocco where this author did not identify any metabolite corresponding to the series C.

In the three types of samples quantified (wild plant and *in vitro* regenerants from 9- and 24-month-old cultures) genins were the minor cardenolides representing less than 2% of the overall content, while lanatoside A was the predominant cardenolide (*ca.*

Table 1. Cardenolide content ($\mu g\ g^{-1}$ dry wt) in wild *D. obscura* (T4) and derived shoot cultures

	Wild plant	Culture (9 months)*	Culture (2 years)*
Digitoxigenin	2 ± 0	10 ± 2	3 ± 1
Purpureaglycoside A	223 ± 58	59 ± 11	74 ± 26
Evatromonoside	48 ± 8	119 ± 37	26 ± 2
Lanatoside A	1973 ± 34	537 ± 105	1554 ± 271
Digitoxigenin bis-digitoxoside	75 ± 18	85 ± 47	79 ± 16
Digitoxin	584 ± 85	40 ± 13	516 ± 18
Gitoxigenin	41 ± 4	13 ± 3	49 ± 1
Gitoxin	tr	—	—
Digoxin	tr	—	—
Total Content	2946 ± 206	863 ± 156	2301 ± 288

Values are combined means (±S.D.) from three extracts with two analyses each. tr = trace amount.

*Time elapsed since culture establishment.

65%) in all samples analyzed (Table 1). Lanatoside A and purpureaglycoside A, both tetraglycosides, were the only primary glycosides found in *D. obscura* extracts. In relation with secondary glycosides it is pointed out that the digitoxin content was higher (over 20%) in field plant and long-term cultures (2 years) than in 9-month old regenerants (5%). However, by contrast evatromonoside and digitoxigenin bis-digitoxoside, representing about 15 and 10%, respectively, of the overall content in leaves from 9-month-old cultures significantly decreased (1–3%) in field plants and long-term cultures (Table 1).

Lichius [9] carried out HPLC determinations of cardiac glycosides in several species of genus *Digitalis*, including *D. obscura* where he also found series A cardenolides to be predominant (*ca.* 65%). Within this series, he did not detect digitoxigenin bis-digitoxoside although digitoxigenin glucomethyloside was identified. The relative contents determined by Lichius [9] for individual cardenolides also differ from those found in our analysis (Table 1), since he detected three major glycosides, lanatoside A, purpureaglycoside A and evatromonoside, each representing about 18% of the overall content in *D. obscura* field plants.

Although cardenolide patterns of 9-month-old *in vitro* regenerated plants and the parent plant were almost identical, the former presented a much lower content of cardiac glycosides (Table 1). This fact could be initially related to a negative influence of successive subcultures, resulting in a gradual decrease in the production of cardenolides, as previously observed in different *Digitalis* cultures [10]. However, there was not a loss in the biosynthetic capacity of the regenerants since the cardenolide yield of *D. obscura* plants derived from long-term cultures (2 years) was quite similar to that of the wild T4 plant (Table 1). Thus, it could be suggested that this variation in cardenolide content is dependent on the age and development of the cultures. In this respect the characteristic of the leaves, main sites of cardenolide biosynthesis, changed throughout the culture from soft, thin and rounded to hard, thick and long resembling those of seedlings or adult plants, respectively.

Distinct results to these found in *D. obscura* were achieved in *D. lanata* shoot cultures established from axillary buds [11]. In this experiment, no substantial changes in the cardenolide content of the shoots were observed over the course of more than 1 year, but it also suggested that there was a direct relationship between leaf size and productivity in several culture lines. Results obtained by these authors [11], as well as those previously reported by Schöner and Reinhard [12], demonstrated that high-yielding *D. lanata* plants propagated by axillary-bud culture showed good homogeneity in terms of development and cardenolide content.

With a similar method, elite genotypes of *D. obscura* have been micropropagated and maintained for a long time, and the identity of the donor plant and the regenerants confirmed by RAPD analysis [1]. Therefore, these stabilized cultures provide a good tool for studying cardenolides and factors involved in the biosynthetic process.

Effect of changes in the concentration of phosphate

Raising phosphate concentration to two or three times that of the control in *D. obscura* shoot-tip cultures did not significantly affect the biomass production or photosynthetic pigment content (Table 2). Nevertheless, the higher phosphate concentration employed (BM × 3Pi) favoured culture development and production of a higher number of shoots, with increased fresh weight and size, in relation to BM (Table 2). Similar results have been reported with shoot cultures of *D. purpurea* [13] and *D. lanata* [14] since the best responses were obtained with modified MS media containing phosphate concentrations increased over that of the original formulation. On the other hand, growth of *D. purpurea* [4] and *D. thapsi* [5] cell cultures increased in parallel to the concentration of this ion employed in the nutrient

Table 2. Effect of phosphate (Pi) concentration on morphogenesis and photosynthetic pigment production from shoot-tip cultures of *D. obscura* (T4)

	No. shoots per explant*	fr. wt (g explant $^{-1}$)*	Shoot length (mm)†	Chlorophylls (mg g fr. wt^{-1})†	Carotenoids (mg g fr. wt^{-1})†
BM	19.8 ab	0.88 ab	15.4 ± 0.9	1.33 ± 0.08	0.37 ± 0.02
BM × 2Pi	16.9 a	0.83 a	15.8 ± 2.5	1.40 ± 0.29	0.39 ± 0.08
BM × 3Pi	25.5 b	1.26 b	17.4 ± 2.2	1.38 ± 0.35	0.37 ± 0.11

*For each column, values followed by the same letter are not significantly different according to Tukey's test ($P = 0.05$).
†Values are combined means (±S.D.) from three independent experiments.

medium. The widely reported promoting effect of phosphate has not been observed in some exceptional cases, such as hairy root cultures of *Datura stramonium* [15] or cell suspension cultures of *Penstemon serrulatus*, [16] where high concentrations of this ion did not modify or even reduce culture growth.

With regard to cardenolide content (Table 3), we observed that the biosynthetic capacity of *D. obscura* cultures, although increased, was not significantly affected by phosphate in the range of concentrations tested (170–510 mg l^{-1}). It has been shown that the effect of this macronutrient on *in vitro* cardenolide production is variable depending on the concentration tested and the *Digitalis* species studied. Thus, varying levels of phosphate did not increase cardenolide yield of *D. thapsi* cultures [5], whereas raised concentrations, up to three times the value in MS medium, significantly favoured metabolite accumulation in shoot-forming cultures of *D. purpurea* [4]. Moreover, both works described significant lower productions when phosphate was either reduced or removed. Contrary results have been reported with *in vitro* cultures of different medicinal plants such as *Catharanthus roseus* [17], *Datura stramonium* [15], *Nicotiana tabacum* [18, 19] and *Peganum harmala* [20], where a significantly higher biosynthetic capacity was observed when phosphate was omitted or drastically reduced in the media.

In summary, this macronutrient can substantially affect growth and secondary metabolite production. Nevertheless, both effects are not usually correlated, which supports the idea of an antagonistic regulation of primary and secondary metabolism [21–24].

Effect of changes in the concentration of manganese ions

In this experiment we studied the effect of varying manganese concentrations (0.1, 1.0 and 10 mM) on growth and productivity of *D. obscura* shoot-tip cultures. Proliferation rates, fresh weight and length of the regenerated shoots as well as their contents in photosynthetic pigments depended on manganese concentration. Media containing the highest concentration (BM × 100 Mn^{2+}) were deleterious for *D. obscura* cultures, and all of the evaluated parameters drastically diminished (Table 4) making it impossible to determine the cardenolide content. By contrast, shoots grown on BM × 10 Mn^{2+} showed both a significantly higher development in length and increased chlorophyll and carotenoid contents compared with those regenerants grown in control cultures containing 0.1 mM manganese (Table 4).

Similar results have been previously reported in *Triticum aestivum* by Wilkinson and Ohki [25] who established that growth and chloroplast pigment concentrations were dependent on manganese concentration in nutrient solution. These authors suggested that decreased plant growth and pigment biosynthesis are related to manganese deficiency or

Table 3. Effect of phosphate (Pi) and Mn^{2+} concentrations on cardenolide content (μg g dry wt $^{-1}$) in shoot-tip cultures of *D. obscura* (T4)

	BM	BM × 2Pi	BM × 3Pi	BM × 10Mn^{2+}
Digitoxigenin	10 ± 2	10 ± 1	8 ± 2	5 ± 1*
Purpureaglycoside A	59 ± 11	48 ± 2	73 ± 15	82 ± 20
Evatromonoside	119 ± 37	102 ± 29	58 ± 16*	146 ± 54
Lanatoside A	537 ± 105	850 ± 186*	716 ± 103	604 ± 118
Digitoxigenin bis-digitoxoside	85 ± 47	78 ± 15	53 ± 17	63 ± 23
Digitoxin	40 ± 13	13 ± 3*	62 ± 10	15 ± 5*
Gitoxigenin	13 ± 3	42 ± 11*	25 ± 9	12 ± 2
Total content	863 ± 156	1144 ± 224	993 ± 168	927 ± 161

Values are combined means (±S.D.) from three extracts with 2 analyses each.
*Significantly different from BM.

Table 4. Effect of Mn^{2+} concentration on morphogenesis and photosynthetic pigment production from shoot-tip cultures *D. obscura* (T4)

	No. shoots per explant*	fr. wt (g explant^{-1})*	Shoot length (mm)†	Chlorophylls (mg g fr. wt^{-1})†	Carotenoids (mg g fr. wt^{-1})†
BM	19.8 b	0.88 b	15.4 ± 0.9	1.33 ± 0.08	0.37 ± 0.02
BM × $10Mn^{2+}$	18.3 b	1.01 b	17.7 ± 0.5	1.59 ± 0.14	0.45 ± 0.04
BM × $100Mn^{2+}$	7.6 a	0.25 a	9.6 ± 1.0	0.87 ± 0.07	0.25 ± 0.02

*For each column, values followed by the same letter are not significantly different according to Tukey's test ($P = 0.05$).
†Values are combined means (±S.D.) from three independent experiments.

toxicity, since this element is required or is highly beneficial for enzymes involved with the synthesis of gibberellins (*ent*-kaurene synthetase) and/or chloroplast pigments. According to this view, our results with 1 mM manganese (BM × 10 Mn^{2+}) would reflect a higher capacity of these cultures to produce pigments (Table 4) and gibberellins. This hormonal increase provokes 'in turn' a higher shoot development (Table 4) which corroborates similar results previously reported by ourselves [3] on the effects of exogenous GA_3 added to T4 shoot cultures.

The fact that manganese can stimulate some steps in the isoprenoid biosynthetic system [25] could explain a possible effect of this element on cardenolide biosynthesis. Nevertheless, manganese concentration did not significantly affect the production of these metabolites in shoot-tip cultures of genotype T4 (Table 3). A comparison with other *Digitalis* species demonstrates that our results are similar to those reported by Hagimori *et al.* [26] on *D. purpurea*, but differ from those obtained with *D. lanata* [27], *D. thapsi* [6] and *D. grandiflora* [7] where high manganese concentrations usually favoured cardenolide accumulation. Nevertheless only in the last work, carried out with soil-grown *D. grandiflora* plants, were simultaneous improvements in growth, development and cardenolide production achieved by manganese.

EXPERIMENTAL

Plant material. Vegetative shoots were harvested from a selected *Digitalis obscura* L. (genotype T4) and axillary buds used for *in vitro* culture establishment. Leaves from both wild genotype and *in vitro* regenerants (shoots or plantlets) were employed for cardenolide extraction.

In vitro *culture.* Axillary-bud cultures from genotype T4 were established according to Gavidia *et al.* [1, 3]. Shoot-tips (1 cm) isolated from proliferating cultures and devoid of developed leaves were subcultured on basal medium (BM) containing MS nutrients [28], 3% (w/v) sucrose and 0.8% (w/v) Difco–Bacto agar (pH 5.7) supplemented with 1 μM BA. Long-term cultures (2 years) were maintained by alternative subcultures on BM and on BM with 1 μM BA for 30 days each. Leaves were collected after 9 and 24 months in culture for cardenolide determinations. Alternatively, a subset of shoot tips were cultured on modified BM media where phosphate (Pi) concentration was raised to twice (BM × 2Pi) or three times (BM × 3Pi) that of the control (KH_2PO_4 = 170 mg l^{-1}), or the original Mn^{2+} concentration ($MnSO_4 \cdot 4H_2O$ = 16.9 mg l^{-1}) increased by a factor of 10 (BM × $10Mn^{2+}$) or 100 (BM × $100Mn^{2+}$). Media were supplemented with 5 μM BA (30 days). Regenerated shoots were subcultured on their respective modified media according the above multiplication chain, with a total culture period of 4 months. At this time cardenolides were evaluated while proliferation rates, shoot growth (fr. wt) and photosynthetic pigment content were recorded after 2 months. A total of 24 explants in three separate experiments were cultured for each nutrient medium. In all cases, cultures were maintained in a growth chamber at 26 ± 2° and a 16 hr photoperiod with light supplied by Sylvania (GTE Gro-lux, F36W/GRO) fluorescent tubes (80 μmol m^{-2} per sec irradiance at culture level).

Cardenolide determinations. In each different experiment, leaves were dried at 50° for 48 hr and extracted according to the method described by Wichtl *et al.* [29]. Extracts were vacuum evaporated, residue dissolved in 3 ml MeOH and then used for qualitative or quantitative HPLC determinations. The HPLC system was constructed from Shimadzu components (LC-6A pumps, SPD-6A UV detector, SCL-6A controller, Chromatopac CR-4A integrator, HIC-6A oven) coupled to a 20 μl injector (Rheodyne 7125). Cardenolides were separated at 30° on a 25 × 0.4 cm Tracer column packed with Spherisorb ODS-2 (5 μm), detected at 230 nm and determined by co-chromatography with commercial standards. The HPLC was performed under the following conditions: (a) digoxin and digoxigenin determinations, MeCN–H_2O non-linear gradient from 20:80 to 60:40 in 45 min, flow rate 1 ml min^{-1}; (b) gitoxigenin, evatromonoside, digitoxigenin bis-digitoxoside, digitoxin and lanatoside A determinations, MeCN–H_2O non-linear gradient from 30:70 to 60:40 in 55 min, 1 ml min^{-1}; (c) gitoxin, purpureaglycoside A and digitoxigenin determinations, MeCN–H_2O (37:63) 0.8 ml min^{-1}.

Photosynthetic pigment determinations. Pigments were extracted from fresh tissues with cold 80%

Me_2CO under dim light. Chlorophyll and carotenoid contents were calculated according to Arnon [30] and Guerin-Dumartrait [31], respectively.

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