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Calcium restriction induces cardenolide accumulation in cell suspension cultures of *Digitalis thapsi* L.

Margarita Cacho, Margarita Morán, Jorge Fernández Tárrago, and Purificación Corchete

Departamento de Biología Vegetal, Laboratorio de Fisiología Vegetal, Facultad de Farmacia, Universidad de Salamanca, E-37007-Salamanca, Spain

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Summary. The removal of calcium ions from Murashige and Skoog culture medium induced a marked increase in the accumulation of cardenolides in cell suspension cultures of *Digitalis thapsi*. Cell viability was not affected although growth was slightly reduced. Strontium ions could substitute for calcium in inhibiting cardenolide production, this effect of calcium being reversed by the addition of $LaCl_3$ or ethyleneglycol-bis-(β-aminoethyl ether)-N,N′-tetraacetic acid. The results suggest that calcium, apart from its general effects on growth, may play a role in the regulation of cardenolide metabolism in a concentration dependent manner.

Key words: *Digitalis thapsi* - Calcium - Digitoxin - Digoxin - Suspension cultures

Abbreviations: BA, 6-benzylaminopurine; 2,4-D, 2,4-dichloro phenoxyacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N′-tetraacetic acid; FW, fresh weight; MS, Murashige and Skoog (1962).

Introduction

Morphologically undifferentiated cultures of several species of the *Digitalis* genus produce insignificant amounts of cardenolides, the expression of the biosynthetic capacity for these compounds being dependent upon morphological differentiation (Luckner and Diettrich 1985; Brisa et al. 1991).

As pointed out by Luckner and Diettrich (1991) the expression of secondary metabolism is integrated within the programs of gene expression and development leading to specialized cells or organs. However, direct triggering of secondary product biosynthesis could be achieved apart from the formation of complex morphological structures.

Cell suspension cultures of *Digitalis thapsi* accumulate low amounts of cardenolides, although they do not lose their biosynthetic capacity after repeated subcultures (Corchete et al. 1990). Manipulation of the components of the medium of a cell line derived from long term undifferentiated calli of this plant, yielded no substantial increase in cardenolide production except when calcium was eliminated from the medium or when lithium or high amounts of manganese ions were added (Corchete et al. 1991).

Calcium acts on *D. thapsi* cultures by inhibiting cardenolide accumulation and only complete removal of the ion is apparently effective in abolishing this inhibition. A similar effect has been reported in the accumulation of diverse secondary products in different cell cultures (Sierra et al. 1991; Indrayanto et al. 1993).

Because of the importance of calcium in the regulation of a number of physiological processes in plants (for review see Hepler and Wayne 1985), we were interested in investigating whether calcium could control cardenolide production in different strains of *D. thapsi*.

Materials and Methods

Callus cultures of *Digitalis thapsi* L. were established from leaves and hypocotyls of aseptically germinated seeds using Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 3% sucrose and 2,4-dichlorophenoxyacetic acid and N^6-benzyladenine as growth regulators (2 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BA for leaf-derived callus and 0.5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA for hypocotyl-derived callus). The pH of the media was adjusted to 5.7 and 1% agar was added prior autoclaving at 120° C for 20 min. The cultures were kept in growth chambers at 26° C± 2 with a 16 h photoperiod (20 μE m^{-2} s^{-1}). Calluses were subcultured every 4 weeks.

Suspension cultures were established from 4 month-old leaf and hypocotyl-derived calluses by transferring them to 250 ml Erlenmeyer flasks containing 50 ml of the same medium as for callus. Cultures were shaken on a rotary shaker 100 rpm under 16 h photoperiod and subcultured every 2 weeks, by transferring approximately 0.45 g FW. Experimental work, unless otherwise indicated, was done with 2 month-old suspension cultures.

Viability was measured according to the method described by Widholm (1972).

Correspondence to: P. Corchete

Fresh weight was determined after removing culture medium by filtration through a pre-weighed Whatman No.1 filter paper (Whatman Paper Ltd.) under suction. The dry weight was estimated after the same sample had been dried at 70° C for 48 h.

Determination of cardenolides by radioimmunoassay and calculation of culture growth index were carried out as described previously (Corchete et al. 1991).

Results

Calcium and growth

Figure 1 shows the changes, occurring over a 21 day period, in fresh and dry weight of the cell lines F2 and H2 derived from hypocotyl and leaf callus, respectively. Removal of calcium from the medium reduced the growth of both lines. However, cell viability was similar to that of cells growing in complete medium (see also Fig. 1); furthermore, the cultures could be maintained in calcium-depleted medium for four additional subcultures.

Calcium and cardenolide content

Although the cardenolide content, expressed in absolute amounts, after 3 days of culture was higher in the F2 line (Fig. 2), the production profile over the period of growth studied was similar in both lines: a reduction in the cardenolide content was observed on day 7 followed by an increase in the later periods studied (Fig. 2). On comparing these results with the growth curves shown in Figure 1 it can be concluded that minimum accumulation occurred when the cells were growing actively.

Transfer of the F2 and H2 lines to a medium depleted of calcium induced a noticeable increase in the formation of cardenolides. As shown in Figure 2 this increase was more pronounced when the suspensions grew less actively, i.e. at the beginning and at the end of the experimental period.

Since the cell lines could be maintained over two months in medium without calcium, we were prompted to study whether callus induction might be possible from hypocotyl and leaf explants in MS medium without calcium.

Under these conditions, both explants formed calluses after four weeks of culture, the morphology and colour (friable and pale-green) of these calluses being similar to those obtained in complete medium. Cell suspension cultures could be established from these calcium-starved calluses also in the absence of the cation, and these new established cell lines (named F2a and H2a respectively) could be subcultured at least 3 times.

As can be seen in Figure 3, growth of the F2a and H2a lines was less pronounced than that of F2 and H2, but the cardenolide content was significantly higher, the production profile being similar to that of the F2 and H2 lines.

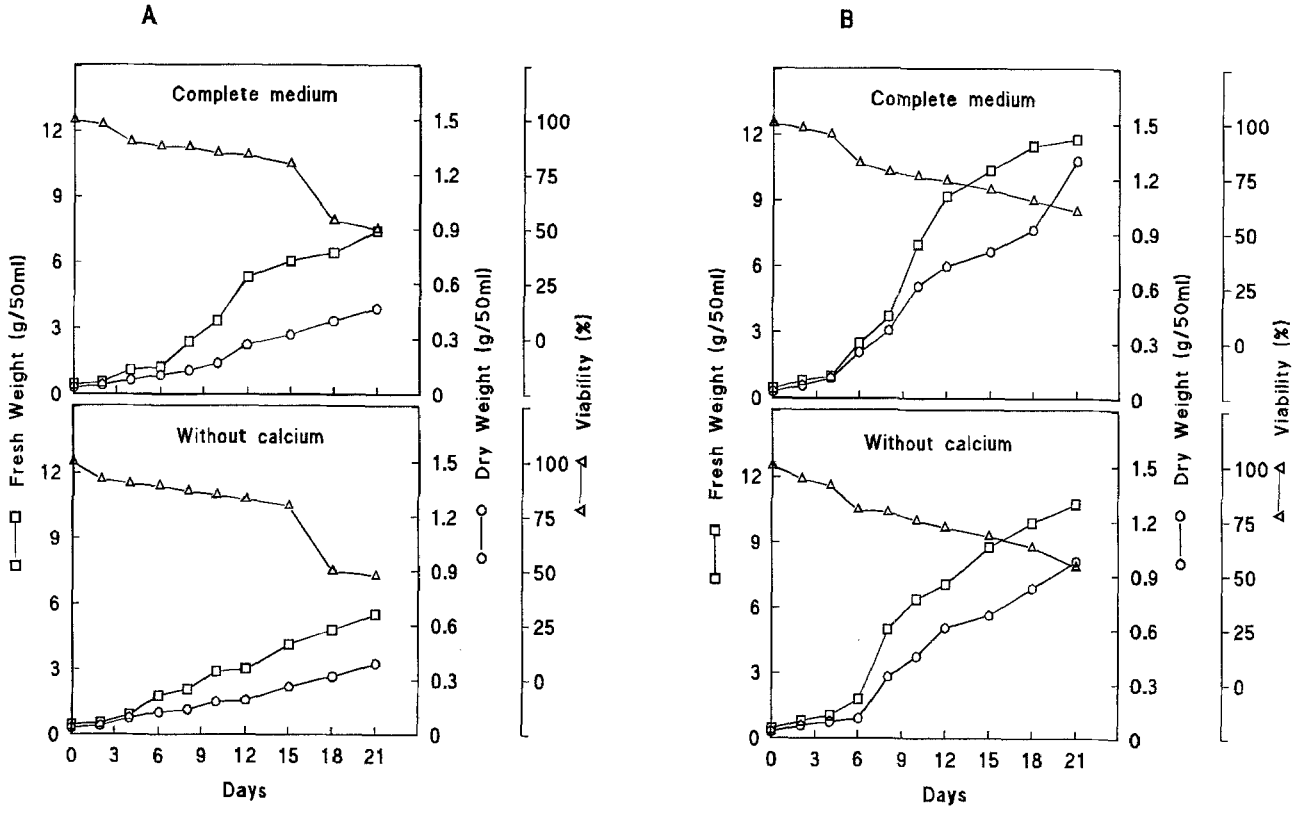

Fig.1. Growth of *Digitalis thapsi* cell cultures in complete and calcium-depleted medium. A: Leaf derived cell line (H2); B: Hypocotyl derived cell line (F2)

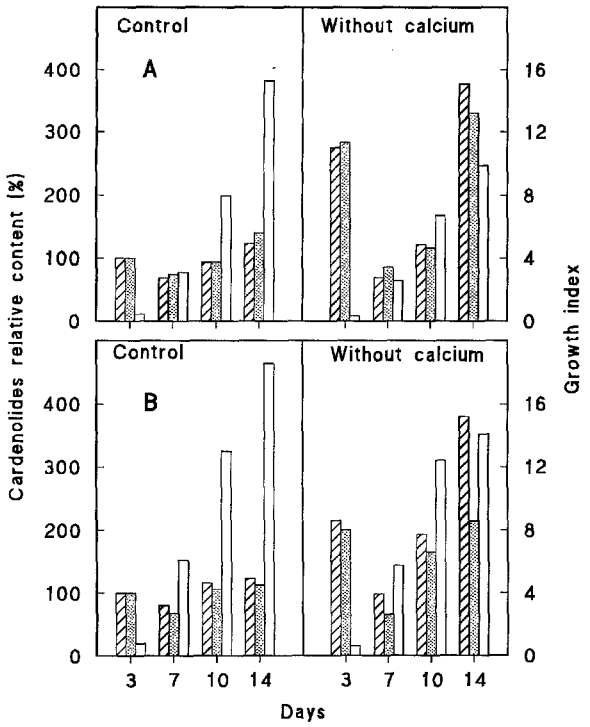

Fig.2. Cardenolide relative content (▨ digitoxin, ▩ digoxin) and growth index (□) of *Digitalis thapsi* cell cultures grown in complete (control) and calcium-depleted medium. A: cell line H2, absolute values for 100% (day 3 of control) = 39.8±3.29 ng g^{-1} FW digitoxin and 30.8± 2.86 ng g^{-1} FW digoxin; B: cell line F2, absolute values for 100% (day 3 of control) = 64.14±8.5 ng g^{-1} FW digitoxin and 56.28±9.6 ng g^{-1} FW digoxin (± standard deviation).

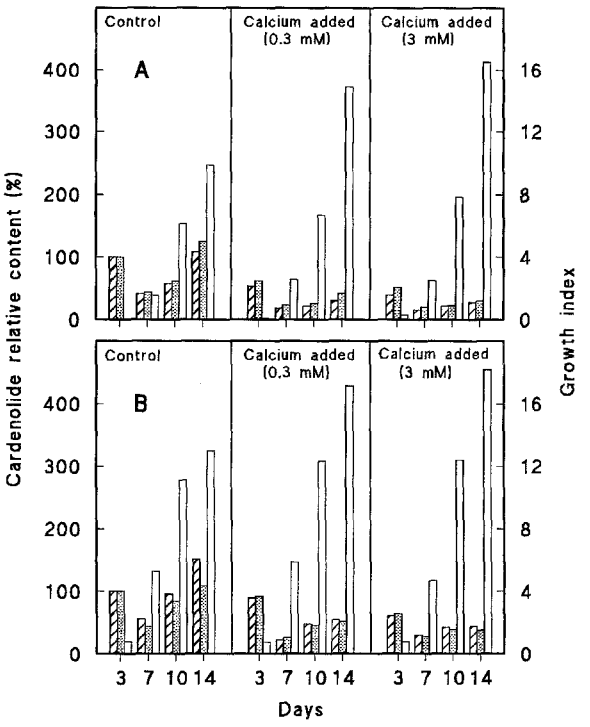

Fig.3. Cardenolide relative content (▨ digitoxin, ▩ digoxin) and growth index (□) of *Digitalis thapsi* cell cultures established in calcium depleted medium and grown in calcium-depleted (control) and calcium-supplemented medium. A: Leaf cell line (H2a), absolute values for 100% (day 3 of control) = 154.1±2.02 ng g^{-1} FW digitoxin and 96.5± 2.68 ng g^{-1} FW digoxin; B: Hypocotyl cell line (F2a), absolute values for 100% (day 3 of control) = 180.76± 7.51 ng g^{-1} FW digitoxin and 147.33± 4.05 ng g^{-1} FW digoxin (± standard deviation).

By transferring the F2a and H2a lines to media supplemented with different amounts of calcium an enhancement in growth was observed. However, cardenolide production decreased markedly and fell in parallel with the increase in concentration. (Fig. 3).

Effect of Strontium, EGTA and Lanthanum

The effect of these substances on the growth and cardenolide content in cell cultures of *D. thapsi* was tested on the F2 line, which, as shown previously, grew better and produced higher levels of cardenolides. All the data collected are shown in Table 1.

The F2 line was subcultured on Ca^{++} depleted medium to which 3 mM $SrCl_2$ had been added. Strontium can substitute for calcium in several physiological processes (Zook et al. 1987). In our cultures its effect on cardenolide accumulation was similar to calcium. However, the growth of the cells was significantly reduced. Also, in the presence of this ion the decrease in production on day 7 was not observed.

To investigate the possible involvement of calcium in inhibiting cardenolide accumulation, suspension cultures grown in complete medium were treated with different concentrations of EGTA, a Ca^{++}-specific ligand able to reduce the availability of extracellular calcium (Gilroy et al. 1986), and with La^{3+}, an ion thought to block the entrance of calcium into cells (Tester 1990).

In the presence of EGTA, the accumulation of cardenolides increased in a concentration-dependent manner. However, at the lowest concentration tested, a noticeable decrease in digitoxin and digoxin contents was observed after 10 days of treatment.

Lanthanum also exerted a promoting effect on cardenolide accumulation in a concentration and time-dependent manner, but in this case the lowest concentration employed was inhibitory only at the beginning of the experiment (3 days).

Discussion

The results reported here suggest that the accumulation of cardenolides in cultures of *Digitalis thapsi* is inhibited by calcium. The increased amounts of metabolites produced when Ca^{++} was removed from the culture medium do not seem to be exclusively related to an indirect effect by reducing growth or inducing aging processes, because cultures could be initiated and maintained for a reasonable period of time without a

Table 1. Effect of the initial $CaCl_2$, $SrCl_2$, $LaCl_3$ and EGTA concentration on cardenolide content and growth on the F2 cell line of *Digitalis thapsi*

				Digitoxin[a] (%)			Digoxin[b] (%)			Growth index		
Ca^{++}	Sr^{++}	La^{3+}	EGTA	Culture day			Culture day			Culture day		
(mM)	(mM)	(μM)	(μM)	3	7	10	3	7	10	3	7	10
3	–	–	–	100	76.6	109.4	100	67.7	106.8	0.74	6.63	14.07
–	–	–	–	203.3	99.7	170.7	197.3	77.5	170.4	0.63	5.52	14.55
–	3	–	–	97.2	95.5	98.9	105.1	106.4	96.52	0.63	2.35	8.61
3	–	0.14	–	52.6	101.1	112.9	61.3	106.6	123.9	0.67	3.58	13.8
3	–	0.28	–	170.6	237.7	285.2	163.4	110.7	230.2	0.67	3.4	18.5
3	–	0.56	–	125.6	182.1	231.7	153.8	227.7	378.2	0.75	4.1	11.97
3	–	–	1.25	127.1	104.5	71.8	169.7	116.9	27.1	0.79	5.32	15.17
3	–	–	5	307.6	176.9	207.6	140.7	126.3	163.5	0.76	3.36	14.56
3	–	–	10	458.2	364.1	368.4	365.6	322.1	370.8	0.72	4.1	13.47

[a] 100 % absolute value = 64.14±8.5 ng g^{-1} FW

[b] 100 % absolute value = 56.28±9.6 ng g^{-1} FW

significant loss in viability. Furthermore, Sr^{++}, which substantially reduced the growth of cultures, also inhibited cardenolide formation.

The results obtained with the cell lines established in medium without $CaCl_2$ (F2a and H2a) and the effect of EGTA on the F2 line grown in complete MS medium indicate that the inhibitory action of calcium seems to depend on its extracellular concentration and not on its total removal as reported previously (Corchete et al. 1991). A number of physiological processes in plants are controlled by this cation (for review see Hepler and Wayne 1985). However, the changes of Ca^{++} concentrations outside the cell, do not necessarily indicate that its action is associated with changes in its intracellular concentrations (Kauss 1987). In our cultures, the effect of La^{3+} suggests that the internal concentration of the ion may participate in the control of the accumulation of cardenolides in *D. thapsi*, without excluding the possibility of the coexistence of a general effect of calcium on cell surfaces.

Calcium ions seem to play a role in the control of the production of secondary metabolites, but in a different manner depending on the species. Calcium stimulates the accumulation of rishitine in potato tuber tissues (Zook et al. 1987) and of raucafricine in cell cultures of *Rauwolfia serpentina* (Schübel et al. 1989). However, as in *D. thapsi*, this cation negatively affects the production of alkaloids in cell suspension cultures of *Tabernaemontana divaricata* (Sierra et al. 1991), of hecogenin in callus cultures of *Agave amaniensis* (Indrayanto et al. 1993) and of digitoxine in cultures of *Digitalis purpurea* (Hagimori et al. 1983). In this latter case, lower $CaCl_2$ concentrations promoted shoot differentiation. However, no morphogenetic responses were observed in *D. thapsi* cells growing in calcium-depleted medium.

The data presented here open questions for future work on whether the concentration and/or fluctuations in internal calcium levels really exert some control on cardenolide production in *D. thapsi* and whether their negative action is due to a direct inhibition or an indirect one through the activation of a system which may compete for the synthesis of these secondary metabolites.

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