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Morphogenesis in leaf, hypocotyl and root explants of *Digitalis thapsi* L. cultured in vitro

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Abstract

The effects of the auxins 2,4-D, NAA and IAA either alone or in combination with kinetin or BA were investigated to assess the morphogenetic potential of leaf, root and hypocotyl explants of *Digitalis thapsi*. Calluses were obtained from the three explants in basal medium without the addition of growth regulators and in leaves, the calluses formed roots. Application of 2,4-D, NAA or BA increased callus formation. The presence of NAA induced root formation and that of BA induced shoot formation via callus interphase. Indole-3-acetic acid alone only induced the generation of roots in the hypocotyl callus. Kinetin was ineffective in all the explants tested. Combinations of NAA with kinetin or BA were more effective in inducing organogenesis in leaf explants. Optimum responses were obtained in hypocotyl and root explants by using IAA in combination with BA, the highest rate of shoot regeneration being observed in hypocotyl explants.

Rooting of the differentiated shoots was readily achieved in media without growth regulators. Regenerated plantlets were transferred to soil and grew with a survival rate of 70%.

Abbreviations: BA – benzyladenine, 2,4-D – 2,4-dichlorophenoxyacetic acid, IAA – indoleacetic acid, Kin – kinetin, NAA – naphthaleneacetic acid

Introduction

Owing to the commercial interest in cardenolides produced by species of the genus *Digitalis*, much work has been done on in vitro culture of several of the species of this genus (Luckner & Diettrich 1988). The aim has been to achieve an economically viable production of the metabolites by cultured cells.

The best results have been obtained with the species *D. lanata* (Alfermann et al. 1985; Luckner & Diettrich 1988). However, to date, no production system of sufficient enconomic interest for commercial applications has been developed.

One of the alternatives for improving the yield of such substances from cultured cells is the study of alternative species. In the present work we report on conditions for establishing callus cultures and achieving de novo organogenesis from different explants of *D. thapsi*, a species that may be of interest in the production of cardenolides (San Feliciano et al. 1988).

Materials and methods

Seeds from *Digitalis thapsi* L. were surface disinfested with 3% sodium hypochlorite and 0.1% Tween 20 for 20 min, rinsed exhaustively with

distilled water and germinated aseptically on 1% agar-water (Difco Bacto). The seedlings were transferred to glass tubes with sterile basal medium (BM) containing Murashige & Skoog nutrients (1962), and 3% sucrose. The pH of medium was adjusted to 5.7 and 1% (w/v) agar was added prior to autoclaving at 120°C for 20 min. The cultures were kept in growth chambers at 26°C ± 2 with a 16-h photoperiod (20 μmol m^{-2} s^{-1}).

When the seedlings were 30 days old, hypocotyl, root and leaf segments were cultured in Petri plates containing 30 ml of BM supplemented with varying concentrations (0, 0.1, 0.5, 1, or 2 mg l^{-1}) of the auxins 2,4-D, IAA or NAA, various concentrations (0, 0.1, 0.5, 1, 2, 3, 4 or 5 mg l^{-1}) of the cytokinins BA or kinetin, or different combinations of both types of both regulators. These compounds were added to the medium prior to autoclaving. The plates were incubated under the conditions indicated previously and observed weekly. Final data were recorded after 40 days. All data are the averages of the experiments with 30 explants per treatment.

Regenerated shoots longer than 2 cm were separated from the explants and transferred to tubes containing BM. After rooting, and when the shoots were longer than 7 cm, plantlets were transplanted to pots filled with a soil mixture consisting of equal parts of vermiculite and peat moss. The plantlets were adapted to growth chamber conditions as above with gradual exposure to reduced relative humidity by progressively removing a glass cover over a period of 2–3 weeks.

Results and discussion

Morphogenetic responses in leaf explants

Foliar explants grown on basal medium formed callus and roots in a low percentage (Table 1). Supplementation with IAA failed to elicit any morphogenetic responses. Incorporation of 2,4-D (2 mg l^{-1}) or NAA (0.5, 1 and 2 mg l^{-1}) resulted in callus formation after 3–4 weeks of culture. It should be noted that 0.5 mg l^{-1} of NAA also promoted root formation in the callus.

Kinetin alone did not cause any morphogenetic response. The presence of high concentrations (3,4 and 5 mg l^{-1}) of BA promoted callus formation and shoot organogenesis from the foliar explants (Table 1).

In some cases exposure of leaf explants to combinations of cytokinins and auxins improved

Table 1. Influence of various growth regulators on morphogenesis in leaf explants of *Digitalis thapsi*.

Growth regulator (mg l^{-1})		Explants forming callus (%)	Callus forming roots (%)	Callus forming shoots (%)	No. shoots per callus[a]
None		13	31	–	–
2,4-D	0.1	–	–	–	–
	0.5	–	–	–	–
	1	–	–	–	–
	2	90	–	–	–
NAA	0.1	–	–	–	–
	0.5	87	100	–	–
	1	81	77	–	–
	2	86	–	–	–
BA	0.1	–	–	–	–
	0.5	–	–	–	–
	1	10	30	–	–
	2	12	–	–	–
	3	20	–	11	+
	4	8	–	35	+
	5	5	–	50	+

[a] +: 1–5 shoots per callus; ++: 6–10 shoots per callus; +++: more than 10 shoots per callus.

the responses. Again IAA, in combination with Kin or BA, was ineffective. When 2,4-D was used in combination with any one of the cytokinins only improved callus formation. Substitution of IAA, or 2,4-D by NAA in media containing BA resulted in root and shoot regeneration when BA concentrations were high (3 or 5 mg l^{-1}) (Table 2). Shoots were also observed on a medium containing 1 mg l^{-1} NAA and 0.5 or 1 mg l^{-1} kinetin.

These data show that the morphogenetic capacity of leaf explants is higher with a synthetic auxin (NAA) than with the natural one (IAA). This result is in agreement with results reported for *Digitalis obscura* (Pérez-Bermúdez et al. 1984) and *D. purpurea* (Rücker et al. 1981).

Morphogenetic responses in root and hypocotyl explants

As in the case of foliar explants the absence of growth regulators in the basal medium promoted callus formation in hypocotyls and root explants. In no case was root formation observed.

The addition of IAA induced swelling and

Table 2. Effect of different NAA/cytokinin combinations on morphogenesis in leaf explants of *Digitalis thapsi*.

Auxins (mg l^{-1})		Cytokinins (mg l^{-1})		Explants forming callus (%)	Callus forming roots (%)	Callus forming shoots (%)	No. shoots per callus[a]
NAA	0.1	BA	0.1	–	–	–	–
			0.5	–	–	–	–
			1	–	–	–	–
			3	78	13	9	+
			5	55	31	6	+
	0.5		0.1	4	–	–	–
			0.5	8	–	–	–
			1	–	–	–	–
			3	58	22	6	+
			5	64	20	10	+
	1		0.1	6	–	–	–
			0.5	7	–	–	–
			1	26	14	–	–
			3	24	29	14	+
			5	80	90	40	++
	2		0.1	60	17	–	–
			0.5	50	8	–	–
			1	46	8	–	–
			3	97	26	10	+
			5	75	75	5	+
	1	Kin[b]	0.1	46	100	–	–
			0.5	25	75	31	+
			1	41	100	20	+
			3	45	100	–	–
			5	75	100	–	–
	2		0.1	10	–	–	–
			0.5	6	–	–	–
			1	–	–	–	–
			3	–	–	–	–
			5	–	–	–	–

[a] +: 1–5 shoots per callus; ++: 6–10 shoots per callus; +++: more than 10 shoots per callus.
[b] NAA (0.1 and 0.5 mg l^{-1}) together with either concentration of Kin did not promote any response.

later necrosis of root explants. However, hypocotyl explants formed callus and roots. Calluses from these two explants were formed in the presence of 2,4-D, NAA and BA alone (Table 3). Furthermore, NAA or high concentrations of BA favoured the induction of roots in the resulting callus. For both explants, as was seen in leaves, 3, 4 and 5 mg l^{-1} of BA alone also promoted the formation of shoots.

The presence of BA together with IAA or NAA in the media resulted in slight (NAA/BA) to moderate (IAA/BA) shoot regeneration. The best responses were obtained with 0.1–0.5 mg l^{-1} IAA (Table 4) or 0.1 mg l^{-1} NAA (Table 5) in combination with BA concentrations higher than 0.5 mg l^{-1}. It should be noted that in these explants the IAA/BA combination was more effective in the induction of organogenesis than in leaf explants.

Compared with BA, kinetin was less effective for organogenesis since shoot formation was sometimes observed (6%) with 0.1 mg l^{-1} NAA-0.5 mg l^{-1} Kin. Similar results have been reported for root explants of *D. obscura* (Pérez-Bermúdez et al. 1983).

From these results, one can conclude that shoot and root regeneration was supported better in hypocotyl explants than in leaves or roots.

Regenerated shoots from the three explants were isolated and transferred to basal medium. Under these conditions all shoots rooted. The establishment of in vitro grown plants in soil was easily achieved. Utilizing the technique described in materials and methods, about 70% of the rooted plantlets survived.

Arrillaga et al. (1986) have shown that IAA is a good agent for promoting somatic embryogenesis in hypocotyl segments from *D. obscura* seedlings. Similar results were obtained with root explants of the same species (Pérez-

Table 3. Influence of various growth regulators on morphogenesis in hypocotyl and root explants of *Digitalis thapsi*.

Growth regulator (mg l^{-1})		Explants forming callus (%)		Callus forming roots (%)		Callus forming shoots (%)		No. shoots per callus[a]	
		H[b]	R[b]	H	R	H	R	H	R
None		6	26	–	–	–	–	–	–
2,4-D	0.1	22	27	–	–	–	–	–	–
	0.5	16	42	–	–	–	–	–	–
	1	28	56	–	–	–	–	–	–
	2	100	100	–	–	–	–	–	–
IAA	0.1	32	–	66	–	–	–	–	–
	0.5	22	–	46	–	–	–	–	–
	1	50	–	83	–	–	–	–	–
	2	50	–	43	–	–	–	–	–
NAA	0.1	49	90	100	100	–	–	–	–
	0.5	85	100	100	100	–	–	–	–
	1	70	100	75	100	–	–	–	–
	2	60	100	11	47	–	–	–	–
BA	0.1	–	–	–	–	–	–	–	–
	0.5	–	–	–	–	–	–	–	–
	1	–	–	–	–	–	–	–	–
	2	62	71	100	100	–	–	–	–
	3	94	93	50	47	12	13	++	+
	4	100	94	59	100	29	12	++	+
	5	76	84	9	–	45	9	+	++

[a] +:1–5 shoots per callus; ++: 6–10 shoots per callus; +++: more than 10 shoots per callus.
[b] H: hypocotyl; R: root.

Table 4. Morphogenetic responses induced by different combinations of growth regulators in hypocotyl and root explants of *Digitalis thapsi*.

Growth regulator ($mg\,l^{-1}$)				Explants forming callus (%)		Callus forming roots (%)		Callus forming shoots (%)		No. shoots per callus[a]	
				H[b]	R[b]	H	R	H	R	H	R
2,4-D	0.1	BA	0.1	41	56	–	–	–	–	–	–
			0.5	64	70	48	100	–	–	–	–
			1	90	100	50	100	6	–	+	–
			3	56	56	23	45	–	–	–	–
			5	47	81	37	30	–	15	–	+
	0.5		0.1	97	100	–	–	–	–	–	–
			0.5	98	98	–	–	–	–	–	–
			1	86	100	–	–	–	–	–	–
			3	100	80	–	–	–	–	–	–
			5	58	81	–	–	–	15	–	+
	1		0.1	80	97	–	–	–	–	–	–
			0.5	80	98	–	–	–	–	–	–
			1	71	100	–	–	–	–	–	–
			3	100	100	–	–	–	–	–	–
			5	100	100	–	–	–	–	–	–
	2		0.1	56	100	–	–	–	–	–	–
			0.5	48	62	–	–	–	–	–	–
			1	81	96	–	–	–	–	–	–
			3	88	100	–	–	–	–	–	–
			5	63	63	–	–	–	–	–	–
IAA	0.1	BA	0.1	40	–	–	–	–	–	–	–
			0.5	31	40	–	–	60	17	+++	+++
			1	50	25	–	40	–	–	–	–
			2	87	86	23	100	23	–	++	–
	0.5		0.1	25	13	–	–	–	–	–	–
			0.5	50	25	16	25	37	–	++	–
			1	36	35	33	100	83	33	++	+
			2	29	56	–	11	60	11	++	++
	1		0.1	39	–	57	–	14	–	+	–
			0.5	53	–	33	–	33	–	+++	–
			1	50	63	75	–	25	–	+	–
			2	40	4	16	–	–	–	–	–
	2		0.1	12	18	100	100	–	–	–	–
			0.5	33	43	–	100	–	–	–	–
			1	26	5	75	–	–	–	–	–
			2	29	19	–	–	–	–	–	–

[a] +: 1–5 shoots per callus; ++: 6–10 shoots per callus; +++: more than 10 shoots per callus.
[b] H: hypocotyl; R: root.

Bermúdez et al. 1987). Also, Kuberski et al. (1984) triggered adventive embryogenesis and development of embryos in tissue cultures from *D. lanata* using media supplemented with 2,4-D or NAA. However, in the three explants of *D. thapsi* studied, despite the 96 different hormonal treatments employed, we never observed the formation of embryoid structures.

Finally, since the callus origin may be an important factor affecting cardenolide produc-

Table 5. Morphogenetic responses induced by different combinations of NAA and BA in hypocotyl and root explants of *Digitalis thapsi*.

Growth regulator ($mg\ l^{-1}$)				Explants forming callus (%)		Callus forming roots (%)		Callus forming shoots (%)		No. shoots per callus[a]	
				H[b]	R[b]	H	R	H	R	H	R
NAA	0.1	BA	0.1	95	91	90	98	5	–	+	–
			0.5	100	100	100	100	11	6	+	+
			1	100	100	100	98	5	11	+	+
			3	100	100	62	100	23	6	+++	+
			5	100	100	73	100	6	6	+++	+
	0.5		0.1	93	100	100	100	–	–	–	–
			0.5	80	73	45	100	–	–	–	–
			1	95	95	72	100	–	–	–	–
			3	63	70	50	66	20	8	+	+
			5	94	94	47	100	–	–	–	–
	1		0.1	95	72	100	100	–	–	–	–
			0.5	53	100	15	100	–	–	–	–
			1	88	100	34	100	–	–	–	–
			3	73	87	36	54	–	–	–	–
			5	68	90	9	31	9	–	+	–
	2		0.1	100	100	13	18	–	–	–	
			0.5	82	100	–	44	–	–	–	–
			1	94	100	31	70	–	–	–	–
			3	85	75	–	58	–	–	–	–
			5	55	76	27	56	–	–	–	–

[a] +: 1–5 shoots per callus; ++: 6–10 shoots per callus; +++: more than 10 shoots per callus
[b] H: hypocotyl; R: root.

tion in *Digitalis* spp (Kartnig et al. 1976), we have established several callus lines from these explants. The results of experimentation with these will be published in a forthcoming paper.

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