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Callus induction, biomass growth, and plant regeneration in *Digitalis lanata* Ehrh.: influence of plant growth regulators and carbohydrates

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Abstract: The effect of plant growth regulators (PGRs) and carbohydrate sources on callus induction, callus growth, and plant regeneration in foxglove was examined. Explants were transferred onto MS medium with various levels of PGRs and carbohydrates to determine the optimum explant and effective combinations of PGR treatment. For callus induction 6.0 mg L^{-1} of α-naphthalene acetic acid (NAA) and 3.0 mg L^{-1} of benzyl-aminopurine (BA) were very responsive. Addition of cytokinins (BA and kinetin) at 0.5-3.0 mg L^{-1} to media containing NAA enhanced callus growth. Shoot regeneration was best achieved in MS + 6.0 mg L^{-1} of BA. Adenine sulphate (Ade) and casein hydrolysate (Ch) were added to the medium as a nitrogen source to improve plant growth and maximum growth was obtained on medium supplemented with 1.5 mg L^{-1} of kinetin + 0.5 mg L^{-1} of IAA + 500 mg L^{-1} of Ch. Carbohydrates also influenced callus production and shoot regeneration potentiality. Among all the tested carbohydrates (sucrose, maltose, fructose, and glucose) and concentrations (3.0-6.0 g L^{-1}), the optimum carbohydrate concentration was 3.0 g L^{-1} and was applied to all carbohydrate cases.

Key words: Callus, regeneration, carbohydrates, nitrogen source, rooting

Introduction

Digitalis L., also known as foxglove, belongs to the family Scrophulariaceae. The species of *Digitalis* are biennial or perennial herbs and contain important cardioactive compounds (glycosides) that are used to treat heart problems. The extract of *Digitalis* is used to strengthen cardiac contractility; it also controls the heart rate, particularly in cases of irregular atrial fibrillation. The plant exhibits a vagal effect on the parasympathetic nervous system, and as such is used in re-entrant cardiac arrhythmias and to slow the ventricular rate during atrial fibrillation (Luderitz, 2005). Glycosides are present in several species of *Digitalis*, including *D. lanata* Ehrh. and *D. purpurea* L. Among all the cardenolides (the term cardenolide refers to the glycosides that are used to treat cardiac disorders), digoxin is the most important and is used frequently.

Calli were induced from diverse organs in several *Digitalis* species seedlings and adult plants of different

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ages grown in vitro and ex vitro (Fujii et al., 1994). Several internal and external factors appear to influence callus induction and growth; of the various chemical factors, mineral nutrients and exogenous plant growth regulators (PGRs) are the most important that affect differentiation and growth of the plant (Hagimori et al., 1980; Tewes et al., 1982). In plant cell culture, growth and morphogenesis are also similarly controlled by the types and concentrations of PGRs, and the interactions between PGRs (Perez Bermudez et al., 1983; Arriliaga, 1986; Asemota et al., 2007). These groups of compounds often interact with other media components, such as sugar and nitrogen, in influencing biochemical and morphogenetic response (Jeannin et al., 1995; Ahn et al., 1996). In the present study we sought (1) to investigate the effect of PGRs on callus induction, callus growth, and shoot regeneration, (2) to evaluate the influence of different carbohydrates on morphogenesis, (3) to assess the effect of different nitrogen sources on regeneration, and (4) to monitor various biochemical changes during morphogenetic development.

Materials and methods

Plant material and culture conditions

Seeds of *Digitalis lanata* were collected during October-November 2003 from the province of Kashmir, India. The seeds were surface sterilised in 0.1% $HgCl_2$ for 5 min, and then rinsed 3 times with sterilised distilled water. Then 20-25 surface-disinfected seeds were placed in Magenta-7 vessels containing 50 mL of MS medium (Murashige & Skoog, 1962) without PGRs. Germinated seedlings were grown until they were 2-4 cm long. Various parts, such as stems, leaves, roots, and hypocotyls, were used as explants. All cultures were incubated under a 16-h photoperiod, illuminated by a cool white fluorescent lamp (F 40 T 12/CW/EG) with a photon flux density of 100 μmol m^{-2} s^{-1} at 25 ± 2 °C. The relative humidity (RH) was maintained at 60%-70%.

Experimental design

Culture medium for callusing

For callus induction explants were placed on MS medium supplemented with various auxins, such as 2,4-diclorophenoxy acetic acid (2,4-D), α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and chlorophenoxy acetic acid (CPA), alone or in combination with different concentrations of cytokinins (N^6-benzyladenine or kinetin). Tissues were routinely sub-cultured (every 3 weeks) in fresh medium with the respective PGRs and concentrations.

Shoot regeneration and growth

Indirect shoot regeneration was carried out on medium supplemented with various concentrations (3.0-6.0 mg L^{-1}) of BA or kinetin. For maximum shoot proliferation kinetin was combined with auxin (IAA, NAA); some media also contained casein hydrolysate (CH) and adenine sulphate (Ade) as a nitrogen source (500 mg L^{-1}).

Rooting

Rooting was induced by using excised shoots. Both liquid and solid MS media were tested. All the media were supplemented with various concentrations (0.0-6.0 mg L^{-1}) of IBA, IAA, and NAA in combination with cytokinins (0.0-3.0 mg L^{-1}). Data were scored in terms of roots per shoot and number of lateral roots.

Carbohydrates in medium

In addition to sucrose, 3 other carbohydrates (glucose, maltose, and fructose) were used at 2.0, 3.0, 4.0, and 6.0 g L^{-1} in MS medium to test the effects of these carbon sources during different developmental stages of morphogenesis.

Biochemical analysis

Estimation of chlorophyll and carotenoids

The chlorophyll and carotenoid in fresh leaves were estimated using dimethyl sulphoxide (DMSO), according to Hiscox and Israelstam (1979). Vials containing 100 mg of finely chopped fresh leaves in 7 mL of DMSO were covered with black paper and kept in oven at 65 °C for 1 h. The reaction mixture was transferred to a graduated tube and the final volume was made up to 10 mL by adding DMSO. This chlorophyll and carotenoids extract (3 mL) was placed in a cuvette and absorbance was read at 480, 510, 645, and 663 nm with a Perkin Elmer Lambda Bio 20 UV/Vis spectrometer (Switzerland). The chlorophyll concentration in mg L^{-1} of fresh weight was calculated following MacLachan and Zalik (1963).

Estimation of protein

Following the method of Bradford (1976), 0.5 g of fresh leaves were ground in a mortar and pestle with 1.5 mL of 0.2 M phosphate buffer (pH 7.0), and then centrifuged at 5000 rpm for 10 min at 4 °C. Then 0.5 mL of supernatant was added to 0.5 mL of TCA and centrifuged at 3300 rpm for 30 min. The supernatant was discarded; the pellet was washed twice with double distilled water (DDW) and dissolved in 1 mL of 0.1 N NaOH. Then 0.1 mL of aliquot was added to 5 mL of Bradford reagent and kept for 30 min. Absorbance was read at 595 nm. Protein concentrations were determined using bovine serum albumin as the standard.

Statistical analysis

Data on the effects of the PGRs on various parameters were analysed by one-way analysis of variance (ANOVA). Values are the means of 3 replicates from 2 separate experiments, and mean separation was determined using LSD at $P \leq 0.05$.

Results

Callus induction

Auxins are reported to induce calli when used alone or in combination with cytokinins. We therefore added several auxins to the media to induce calli. Profuse callus induction was observed with NAA alone, but with cytokinins callus induction was further improved. In *D. lanata,* the leaf explant was very responsive and induced profuse calli, compared to other explants, such as stems, hypocotyls, and roots (Table 1).

Callus growth

Maximum callus growth was noted in MS supplemented with 6.0 mg L^{-1} of NAA and 3.0 mg L^{-1} of BA (Figure 1A). Calli from leaves grew faster than calli from other sources. Callus fresh and dry weight was 3.58 and 0.29 g, respectively, when 12 weeks of cultures were measured (Table 2). Morphologically, calli were first light yellow and later turned green.

Table 1. Frequency of callus induction in *D. lanata* at different concentrations of auxins with 3 mg L^{-1} of BA.

PGRs (mg L^{-1})		Hypocotyl	Leaf	Stem	Root
2,4-D	4.0	40.78 ± 0.19^{c}	53.20 ± 0.41^{d}	32.65 ± 0.20^{c}	25.64 ± 0.20^{d}
	6.0	42.64 ± 0.09^{b}	56.33 ± 0.31^{c}	35.20 ± 0.35^{b}	27.42 ± 1.26^{c}
CPA	4.0	33.05 ± 0.38^{e}	39.22 ± 0.41^{g}	21.06 ± 0.19^{g}	17.61 ± 0.15^{g}
	6.0	35.60 ± 0.12^{d}	40.49 ± 0.23^{f}	22.54 ± 0.15^{ef}	19.04 ± 0.19^{f}
IAA	4.0	25.56 ± 0.19^{g}	40.66 ± 0.19^{f}	23.06 ± 0.35^{e}	22.47 ± 0.20^{e}
	6.0	29.98 ± 0.47^{f}	41.63 ± 0.32^{e}	25.56 ± 0.19^{d}	24.82 ± 0.14^{d}
NAA	4.0	43.25 ± 0.19^{b}	68.46 ± 0.16^{b}	40.66 ± 0.17^{a}	34.79 ± 0.36^{b}
	6.0	44.50 ± 0.15^{a}	88.06 ± 0.35^{a}	40.74 ± 0.08^{a}	37.03 ± 0.08^{a}
IBA	4.0	23.38 ± 0.19^{h}	40.10 ± 0.37^{fg}	22.28 ± 0.43^{f}	17.33 ± 0.16^{g}
	6.0	25.31 ± 0.18^{g}	41.86 ± 0.05^{e}	25.59 ± 0.08^{d}	18.55 ± 0.20^{fg}

Values represent the mean ± SE of 3 replicates and data were scored after 3 weeks. Within each column, values followed by the same superscript letter are not significantly different at P = 0.05, according to the LSD test.

Table 2. Callus biomass of *D. lanata* at the optimised concentration of auxins (6.0 mg L^{-1}) and BA (3.0 mg L^{-1})

Treatments	F.W. (4 weeks)	D.W. (4 weeks)	F.W. (8 weeks)	D.W. (8 weeks)	F.W. (12 weeks)	D.W. (12 weeks)
2,4-D	1.05 ± 0.05^{b}	0.12 ± 0.0^{a}	1.5 ± 0.07^{b}	0.22 ± 0.01^{c}	2.09 ± 0.03^{b}	0.27 ± 0.07^{a}
CPA	1.0 ± 0.02^{b}	0.09 ± 0.01^{b}	1.18 ± 0.08^{b}	0.14 ± 0.02^{b}	1.43 ± 0.15^{c}	0.19 ± 0.03^{c}
IAA	1.02 ± 0.05^{b}	0.1 ± 0.01^{b}	1.07 ± 0.06^{c}	0.14 ± 0.03^{b}	1.76 ± 0.07^{c}	0.22 ± 0.01^{b}
NAA	1.97 ± 0.09^{a}	0.14 ±0.02^{a}	2.28 ± 0.05^{a}	0.19 ± 0.01^{a}	3.58 ± 0.11^{a}	0.29 ± 0.02^{a}
IBA	0.47 ± 0.03^{c}	0.02 ±0.0^{c}	0.98 ± 0.04^{c}	0.05 ± 0.01^{d}	1.25 ± 0.02^{d}	0.1 ± 0.02^{d}

Values represent the mean ± SE of 3 replicates and data were scored after 3 weeks. Within each column, values followed by the same superscript letter are not significantly different at P = 0.05, according to the LSD test.

After 3 weeks of incubation calli became dark green and produced shoots.

Suspension culture and callus growth

A suspension culture was established from calli produced from leaves and hypocotyls. At low concentrations of 2,4-D (0.25-0.5 mg L^{-1}) we observed new callus induction and hypocotyl calli only induced 'embryo-like' structures (Figure 1B). Upon being plated on agar-solidified medium the suspended cells/aggregates proliferated to produce fresh calli, particularly when supplemented with 1.0 mg L^{-1} of 2,4-D (Table 3); the 'embryo-like' structures, however, did not progress to maturity**.**

Effect of various carbohydrates on callus growth

For callus induction and subsequent growth, MS with 6.0 mg L^{-1} of NAA and 3.0 mg L^{-1} of BA were used. Calli were cultivated with different concentrations of sugars and differential callus growth was observed. Maximum callus biomass (3.88 g fresh weight and 0.28 g dry weight) was obtained with 3% maltose after 12 weeks (Table 4). Minimum fresh and dry weight was observed in medium supplemented with 2% fructose (1.60 and 0.05 g, respectively). Thus, maltose induced maximum callus biomass, as compared to other tested sugars.

Regeneration from callus

Calli cultured in MS with NAA and BA turned green, and shoot regeneration was achieved in media containing different BA concentrations (Figure 1C). The 6.0 mg L^{-1} concentration was more effective for the emergence of shoots (Table 5). The regeneration efficiency was higher in leaf calli than in calli from other sources. To maintain continuous regeneration, regenerative calli were maintained with low-level BA (3.0 mg L^{-1}), as higher levels proved to be less productive for an extended period of culture. Maltose (at all concentrations) improved callus biomass (Figure 1D), but had no influence on regeneration. The number of shoots increased with time and within 12 weeks of culture a maximum of 133 shoots was obtained; however, in the same medium, shoots were produced in clusters and had reduced growth. To restore normal growth a variety of combinations of other PGRs and nitrogen sources were tested. Kinetin was better, as it improved both stem and leaf growth. We also used 2 additives and noted that Ch was more responsive than Ade. MS medium supplemented with 1.5 mg L^{-1} of kinetin + 0.5 mg L^{-1} of IAA + 500 mg L^{-1} of casein hydrolysate (Figure 1E) was an effective treatment for shoot growth (Table 6).

Effect of carbohydrates on regeneration

Various carbohydrate sources were tested and added to the media optimised with BA (6.0 mg L^{-1}) for regeneration (BA was the most potent cytokinin). Sugars at the 2% level had little or no effect on regeneration; calli turned brown and showed early necrosis. Maltose improved callus biomass only; it had no influence on regeneration. Fructose had a better impact on regenerating shoots than maltose did, especially at 3%, but 4% and 6% were less productive. Glucose and sucrose (3% and 6%) also showed their influence in developing micro shoots (Figure 1F). The highest number of shoots (133.00 ± 1.93) was observed in MS + 3% sucrose + 6.0 mg L^{-1} of BA (Table 7). Glucose also showed good regeneration ability and produced 72.40 micro shoots within 12 weeks of culture.

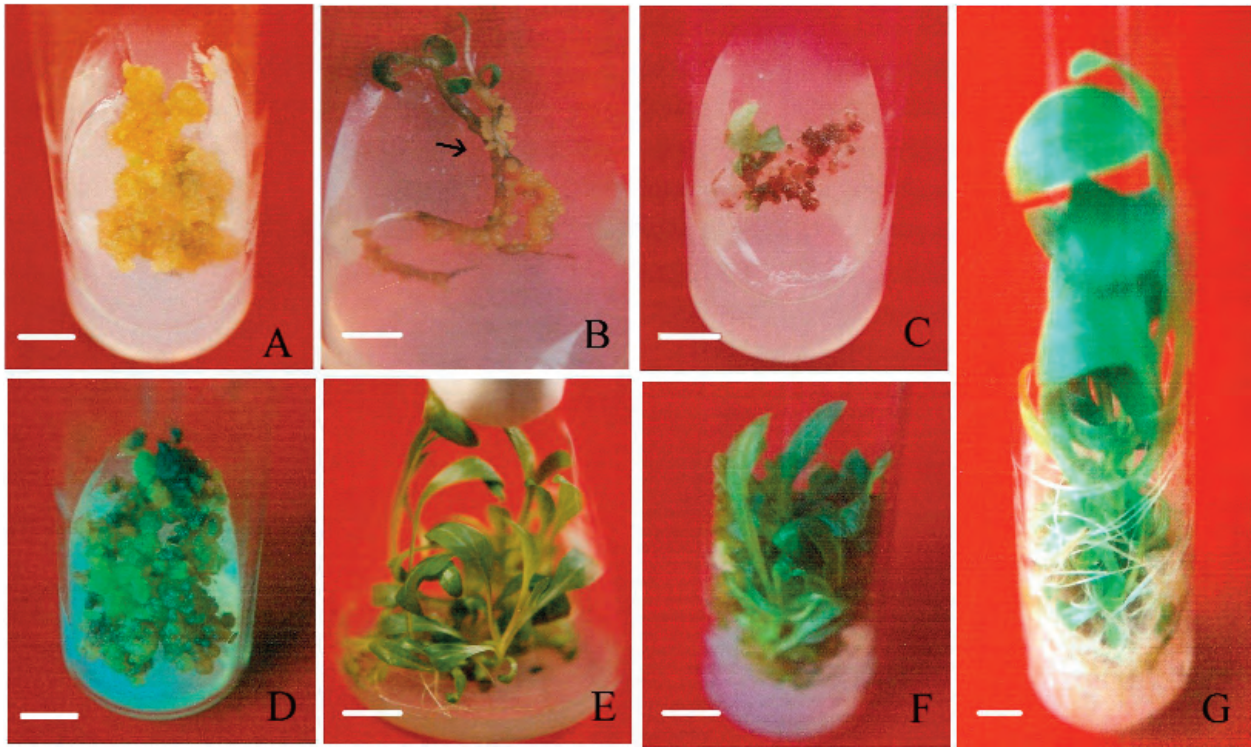

Figure 1. Callus induction and in vitro plant regeneration in *Digitalis lanata*.
A. Callus growth on MS with 6.0 mg L^{-1} of NAA + 3.0 mg L^{-1} of BA.
B. 'Embryo-like' (arrow head) structure developed on a hypocotyl.
C. Callus showing regeneration; MS contained 6.0 mg L^{-1} of BA.
D. Callus cultivated on maltose shows no regeneration; callus turned green.
E. Regenerated shoots in MS + 1.5 mg L^{-1} of KIN + 0.5 mg L^{-1} of IAA + 500 mg L^{-1} of Ch.
F. Regeneration in glucose (3%) amended medium (MS + 1.5 mg L^{-1} of KIN + 0.5 mg L^{-1} of IAA + 500 mg L^{-1} of Ch).
G. Rooting in agar-solidified MS with 6.0 mg L^{-1} of NAA + 3.0 mg L^{-1} of BA.
Bars: (A-F: 1 mm; G: 1 cm).

Different sugars and shoot growth

For shoot growth, MS containing 1.5 mg L^{-1} of kinetin + 0.5 mg L^{-1} of IAA + 500 mg L^{-1} of Ch was used. With 3% sucrose, both shoot length and the number of leaves were maximal (i.e. 7.82 cm and 10.80, respectively). In cultures supplemented with glucose and fructose (3%), fructose was less important and produced the minimum number of leaves (Table 8).

Root induction

The frequency of rooting was maximal in MS + 6.0 mg L^{-1} of NAA + 3.0 mg L^{-1} of BA (Figure 1G), which also showed good vigorous growth. In agar-solidified medium, the average number of roots was 11.20 versus 10.09 in liquid medium. Poor rooting response was noted in 6.0 mg L^{-1} of IBA + 3.0 mg L^{-1} of BA, which produced 3.80 and 3.00 roots/plant, with an average root length of 2.81 and 3.89 cm in liquid and solid medium, respectively. Of the 2 types of media (solid and liquid) tested for rooting (Tables 9, 10), the number of roots/shoot was higher in solid medium, whereas liquid medium promoted root growth.

Effect of carbohydrates on rooting

Among all the sugars tested, sucrose at 3% promoted the rooting process to the highest degree, followed by 4%, as both the number of roots and their growth improved (Table 11).

Table 3. Callus growth in *D. lanata* on MS medium with 2,4-D added. The hypocotyl callus from the suspension was cultured in petri dishes after 4, 8 and 12 weeks of incubation.

2,4-D concentrations (mg L^{-1})	F.W.	D.W.	F.W.	D.W.	F.W.	D.W.
0.5	0.53 ± 0.02^{d}	0^{c}	0.58 ± 0.09^{d}	0.02 ± 0.01^{d}	0.87 ± 0.12^{d}	0.04 ± 0.01^{c}
0.75	0.71 ± 0.11^{c}	0.03 ± 0.01^{b}	1.04 ± 0.12^{b}	0.06 ± 0.02^{c}	1.63 ± 0.11^{b}	0.10 ± 0.04^{b}
1.0	0.97 ± 0.05^{a}	0.06 ± 0.02^{a}	1.66 ± 0.10^{a}	0.10 ± 0.03^{a}	1.86 ± 0.07^{a}	0.16 ± 0.1^{a}
2.0	0.84 ± 0.11^{b}	0.05 ± 0.01^{b}	0.99 ± 0.12^{c}	0.09 ± 0.01^{a}	1.76 ± 0.09^{b}	0.17 ± 0.02^{a}
2.5	0.71 ± 0.14^{c}	0.04 ± 0.03^{b}	1.2 ± 0.05^{b}	0.08 ± 0.01^{b}	1.23 ± 0.11^{c}	0.11 ± 0.07^{b}

Values represent the mean ± SE of 3 replicates and data were scored after 3 weeks. Within each column, values followed by the same superscript letter are not significantly different at P = 0.05, according to the LSD test.

Table 4. Effect of different sugars on callus biomass growth in *D. lanata*. MS was supplemented with 6.0 mg L^{-1} of NAA and 3.0 mg L^{-1} of BA.

Treatments	F.W. (4 weeks)	F.W. (8 weeks)	F.W. (12 weeks)
S1 (2% Sucrose)	1.42 ± 0.13^{c}	1.79 ± 0.11^{d}	2.31 ± 0.23^{b}
S2 (3% Sucrose)	1.97 ± 0.05^{a}	2.98 ± 0.22^{a}	3.58 ± 0.19^{a}
S3 (4% Sucrose)	1.6 ± 0.05^{b}	2.78 ± 0.15^{a}	3.37 ± 0.16^{a}
S4 (6% Sucrose)	1.53 ± 0.05^{c}	2.03 ± 0.15^{c}	2.62 ± 0.16^{b}
G1 (2% Glucose)	1.22 ± 0.06^{d}	1.38 ± 0.08^{a}	1.97 ± 0.21^{a}
G2 (3% Glucose)	1.72 ± 0.09^{b}	2.46 ± 0.09^{b}	2.2 ± 0.24^{c}
G3 (4% Glucose)	1.47 ± 0.07^{c}	2.03 ± 0.14^{c}	3.3 ± 0.19^{a}
G4 (6% Glucose)	1.33 ± 0.10^{d}	1.68 ± 0.09^{d}	3 ± 0.21^{a}
M1 (2% Maltose)	1.28 ± 0.05^{a}	1.5 ± 0.10^{a}	1.78 ± 0.15^{d}
M2 (3% Maltose)	1.72 ± 0.12^{b}	2.46 ± 0.21^{b}	3.8 ± 0.21^{a}
M3 (4% Maltose)	1.47 ± 0.11^{c}	2.22 ± 0.17^{c}	3.2 ± 0.22^{a}
M4 (6% Maltose)	1.3 ± 0.16^{d}	1.68 ± 0.15^{d}	2.2 ± 0.19^{c}
F1 (2% Fructose)	0.57 ± 0.06^{f}	1.2 ± 0.12^{e}	1.6 ± 0.13^{d}
F2 (3% Fructose)	0.86 ± 0.09^{e}	1.82 ± 0.22^{d}	2.37 ± 0.11^{a}
F3 (4% Fructose)	0.65 ± 0.04^{e}	1.35 ± 0.14^{e}	2 ± 0.12^{c}
F4 (6% Fructose)	0.6 ± 0.05^{e}	1.0 ± 0.09^{e}	1.9 ± 0.18^{c}

Values represent the mean ± SE of 3 replicates and data were scored after 3 weeks. Within each column, values followed by the same superscript letter are not significantly different at P = 0.05, according to the LSD test.

Table 5. Shoot/callus mass (1.0 g) in *D. lanata* that developed at various BA concentrations.

BA (mg L^{-1})	4 weeks	8 weeks	12 weeks
(3.0)	63.80 ± 0.42^{b}	75.80 ± 0.42^{b}	88.40 ± 0.58^{b}
(4.0)	51.20 ± 0.67^{c}	55.80 ± 0.28^{c}	60.80 ± 0.42^{c}
(6.0)	77.80 ± 0.67^{a}	100.40 ± 1.06^{a}	133.00 ± 1.11^{a}

Values represent the mean ± SE, and data were scored for up to 12 weeks of culture. Within each column, values followed by the same superscript letter are not significantly different at P = 0.05, according to the LSD test.

Table 6. The growth of regenerated shoots (cm) in *D. lanata*. MS was amended with optimised PGRs and Ch/Ade (500 mg L^{-1}).

Optimised concentration of PGRs (mg L^{-1})	Shoot length (cm)		
	4 weeks	8 weeks	12 weeks
KIN (1.5) + IAA (0.5) + Ch	3.21 ± 0.40^{ab}	4.87 ± 0.11^{a}	7.66 ± 0.23^{a}
KIN (1.5) + IAA (0.5) + Ade	3.00 ± 0.12^{ab}	3.87 ± 0.06^{bc}	5.66 ± 0.18^{bc}
KIN (1.5) + NAA (0.5) + Ch	3.52 ± 0.19^{a}	4.04 ± 0.37^{b}	5.82 ± 0.38^{b}
KIN (1.5) + NAA (0.5) + Ade	2.98 ± 0.30^{ab}	3.44 ± 0.21^{bc}	5.06 ± 0.24^{bcd}
BA (1.5) + IAA (0.5) + Ch	3.18 ± 0.28^{ab}	4.01 ± 0.09^{b}	5.38 ± 0.82^{bcd}
BA (1.5) + IAA (0.5) + Ade	2.54 ± 0.29^{bc}	3.34 ± 0.08^{c}	4.56 ± 0.24^{cd}
BA (1.5) + NAA (0.5) + Ch	1.84 ± 0.41^{cd}	3.26 ± 0.32^{c}	4.92 ± 0.41^{bcd}
BA (1.5) + NAA (0.5) + Ade	1.23 ± 0.06^{d}	2.42 ± 0.19^{d}	3.98 ± 0.09^{d}

Values are the mean ± SE and data were scored for up to 12 weeks of culture. Within each column, values followed by the same superscript letter are not significantly different at P = 0.05, according to the LSD test.

Table 7. Effect of different sugars on inducing shoots in *D. lanata*. MS was supplemented with optimised 6.0 mg L^{-1} of BA.

Sugars	%	4 weeks	8 weeks	12 weeks
Sucrose	3	77.80 ± 0.67^{a}	100.40 ± 1.06^{a}	133.00 ± 1.11^{a}
	4	55.80 ± 0.23^{c}	60.80 ± 0.23^{c}	64.40 ± 0.43^{c}
	6	50.60 ± 0.46^{d}	55.60 ± 0.33^{e}	58.40 ± 0.69^{e}
Glucose	3	65.00 ± 0.36^{b}	67.20 ± 0.23^{b}	72.40 ± 0.69^{b}
	4	54.60 ± 0.77^{c}	58.00 ± 0.51^{d}	62.00 ± 0.00^{d}
	6	47.00 ± 0.36^{e}	53.60 ± 0.27^{f}	57.60 ± 0.27^{e}
Fructose	3	49.20 ± 0.67^{d}	50.00 ± 0.00^{g}	50.60 ± 0.46^{f}
	4	20.23 ± 0.64^{f}	21.23 ± 0.01h^{h}	24.43 ± 0.12^{g}
	6	15.34 ± 0.012^{g}	18.01 ± 0.011^{i}	20.56 ± 0.023^{h}

Values represent the mean ± SE and data were scored for up to 12 weeks of culture. Within each column, values followed by the same superscript letter are not significantly different at P = 0.05, according to the LSD test.

Biochemical analysis at various morphogenetic stages

Various biochemical parameters (chlorophyll, chlorophyll a, chlorophyll b, carotenoids, and soluble proteins) were examined in order to monitor the association between such compounds with different morphogenetic stages, such as callus, regenerative callus, and complete plantlet.

Chlorophyll in calli, in regenerative calli, and in microshoots

It is known that chlorophyll is a major pigment in green plants and is in cultures of *Digitalis* species. Early stages of development were associated with lower levels of chlorophyll. With the progression of development chlorophyll content increased considerably. We noted that chlorophyll and

Table 8. Effect of different sugars on shoot growth and the number of leaves in *D. lanata*. MS medium contained 1.5 mg L^{-1} of KIN + 0.5 mg L^{-1} of IAA + 500 mg L^{-1} of Ch.

Treatments	4 weeks		8 weeks		12 weeks	
	SL	LN	SL	LN	SL	LN
S2 (3% Sucrose)	3.52 ± 0.13[a]	4.8 ± 0.17[a]	4.84 ± 0.18[a]	9.6 ± 0.14[a]	7.82 ± 0.13[a]	10.8 ± 0.1[a]
S3(4% Sucrose)	3.03 ± 0.06[b]	3.54 ± 0.06[b]	3.54 ± 0.04[b]	3.75 ± 0.08[b]	3.88 ± 0.33[c]	4.15 ± 0.08[b]
S4(6% Sucrose)	2.45 ± 0.12[c]	2.54 ± 0.14[c]	2.99 ± 0.13[c]	2.84 ± 0.17[c]	3.42 ± 0.24[c]	3.24 ± 0.13[c]
G2 (3% Glucose)	2.16 ± 0.13[d]	1.6 ± 0.14[d]	3.52 ± 0.13[b]	2.4 ± 0.18[d]	4.8 ± 0.17[b]	3.0 ± 0.06[c]
G3(4% Glucose)	1.74 ± 0.12[e]	0.8 ± 0.17	2.38 ± 0.10	2.2 ± 0.14[d]	2.92 ± 0.05[d]	2.4 ± 0.14[d]
G4(6% Glucose)	2.15 ± 0.19[d]	1.8 ± 0.09[d]	2.67 ± 0.11[c]	2.8 ± 0.12[d]	3.15 ± 0.18[d]	3.4 ± 0.21[c]
F2(3% Fructose)	1.36 ± 0.26[d]	2.0 ± 0.09[cd]	2.18 ± 0.12[d]	2.6 ± 0.14[d]	2.85 ± 0.12[d]	3.2 ± 0.11[c]
F3(4% Fructose)	1.02 ± 0.10[e]	1.23 ± 0.11[e]	1.33 ± 0.21[e]	1.3 ± 0.09[e]	1.41 ± 0.13[e]	1.37 ± 0.08[e]
F4(6% Fructose)	0.99 ± 0.08[e]	1.04 ± 0.10[e]	1.12 ± 0.09[e]	1.14 ± 0.07[e]	1.24 ± 0.06[e]	1.18 ± 0.12[e]

Values represent the mean ± SE of 3 replicates and data were scored after 3 weeks. Within each column, values followed by the same superscript letter are not significantly different at P = 0.05, according to the LSD test.

Table 9. The number and length of *D. lanata* roots when regenerated shoots (callus derived) were cultured on agar medium supplemented with various PGRs.

Treatments (mg $L^{-1)}$	4 weeks		8 weeks		12 week	
	RN	RL	RN	RL	RN	RL
N_1BA_1 (1.5 NAA + 0.5 BA)	2.6 ± 0.14[d]	1.97 ± 0.15[d]	3.8 ± 0.14[d]	3.16 ± 0.15[c]	7.0 ± 0.16[b]	3.93 ± 0.14[d]
N_2BA_2 (2.0 NAA + 1.5 BA)	4.8 ± 0.17[b]	2.57 ± 0.13[b]	5.6 ± 0.12[b]	4.09 ± 0.11[b]	7.2 ± 0.17[b]	6.29 ± 0.13[b]
N_3BA_3 (3.0 NAA + 1.5 BA)	6.2 ± 0.14[a]	6.52 ± 0.15[a]	8.4 ± 0.18[a]	8.31 ± 0.13[a]	11.2 ± 0.17[a]	10.24± 0.13[a]
IA_2BA_2 (3.0 IAA + 1.5 BA)	3.0 ± 0.09[c]	2.43 ± 0.19[b]	4.8 ± 0.10[c]	4.17 ± 0.25[b]	6.2 ± 0.26[c]	4.69 ± 0.29[c]
IA_3BA_3 (6.0 IAA + 3.0 BA)	2.2 ± 0.11[d]	2.17 ± 0.12[c]	4.0 ± 0.18[d]	2.97 ± 0.11[d]	5.4 ± 0.15[d]	4.01 ± 0.19[d]
IB_3BA_3 (6.0 IBA + 3.0 BA)	1.8 ± 0.20[e]	1.89 ± 0.15[d]	2.6 ± 0.19[e]	2.98 ± 0.16[d]	3.8 ± 0.13[d]	2.81 ± 0.21[e]

Values represent the mean ± SE of 3 replicates and data were scored after 3 weeks. Within each column, values followed by the same superscript letter are not significantly different at P = 0.05, according to the LSD test.

Table 10. Effect of liquid medium on rooting and root growth. MS was amended with various PGRs.

Treatments (mg L^{-1})	4 weeks		8 weeks		12 week	
	RN	RL	RN	RL	RN	RL
N_1BA_1 (1.5 NAA + 0.5 BA)	1.6 ± 0.10^{e}	2.5 ± 0.04^{c}	2.2 ± 0.09^{d}	3.18 ± 0.13^{c}	3.8 ± 0.04^{c}	4.1 ± 0.12^{a}
N_2BA_2 (2.0 NAA + 1.5 BA)	4.2 ± 0.11^{b}	3.28 ± 0.11^{b}	5 ± 0.08^{b}	4.68 ± 0.12^{b}	5.4 ± 0.17^{b}	5.7 ± 0.11^{a}
N_3BA_3 (3.0 NAA + 1.5 BA)	6 ± 0.14^{a}	5.51 ± 0.12^{a}	7.2 ± 0.14^{a}	7.74 ± 0.11^{a}	10.09 ± 0.10^{a}	10.98 ± 0.14^{a}
IA_2BA_2 (3.0 IAA + 1.5 BA)	3.2 ± 0.12^{c}	2.35 ± 0.16^{c}	3.4 ± 0.16^{c}	4.28 ± 0.10^{b}	4.0 ± 0.13^{c}	4.36 ± 0.16^{a}
IA_3BA_3 (6.0 IAA + 3.0 BA)	2.4 ± 0.11^{d}	1.62 ± 0.11^{d}	3.4 ± 0.12^{c}	3.39 ± 0.18^{c}	5.0 ± 0.12^{b}	4.59 ± 0.14^{a}
IB_3BA_3 (6.0 IBA + 3.0 BA)	1.35 ± 0.13^{e}	1.4 ± 0.12^{d}	2.4 ± 0.17^{d}	1.77 ± 0.1^{d}	3.0 ± 0.10^{d}	3.89 ± 0.09^{a}

Values represent the mean ± SE of 3 replicates and data were scored after 3 weeks. Within each column, values followed by the same superscript letter are not significantly different at P = 0.05, according to the LSD test.

Table 11. Effect of different sugars on rooting and root growth in *D. lanata*. MS was supplemented with 6.0 mg L^{-1} of NAA and 3.0 mg L^{-1} of BA.

Sugars	%	4 weeks		8 weeks		12 week	
		RN	RL	RN	RL	RN	RL
Sucrose	3	6.00 ± 0.00^{a}	5.51 ± 0.24^{a}	7.20 ± 0.21^{a}	7.74 ± 0.37^{a}	10.09 ± 0.10^{a}	9.98 ± 0.50^{a}
	4	4.00 ± 0.23^{b}	4.50 ± 0.04^{bc}	5.40 ± 0.09^{b}	5.00 ± 0.17^{c}	7.00 ± 0.23^{b}	6.53 ± 0.12^{b}
	6	3.80 ± 0.36^{b}	4.35 ± 0.24^{c}	5.00 ± 0.22^{bc}	5.04 ± 0.39^{c}	6.20 ± 0.00^{c}	5.37 ± 0.17^{c}
Glucose	3	4.00 ± 0.00^{b}	4.83 ± 0.09^{b}	5.08 ± 0.08^{bc}	5.92 ± 0.14^{b}	6.00 ± 0.18^{c}	6.82 ± 0.06^{b}
	4	2.98 ± 0.27^{c}	2.82 ± 0.11^{de}	4.22 ± 0.11^{c}	3.77 ± 0.20^{e}	4.40 ± 0.46^{d}	4.80 ± 0.45^{d}
	6	2.40 ± 0.27^{cd}	2.54 ± 0.09^{ef}	3.32 ± 0.11^{d}	3.12 ± 0.19^{f}	4.02 ± 0.23^{d}	4.10 ± 0.16^{d}
Fructose	3	2.60 ± 0.28^{cd}	4.16 ± 0.21^{c}	3.60 ± 0.15^{d}	4.46 ± 0.26^{d}	4.60 ± 0.27^{d}	4.67 ± 0.27^{cd}
	4	2.32 ± 0.05^{d}	3.23 ± 0.12^{d}	2.89 ± 0.01^{e}	3.76 ± 0.03^{e}	3.00 ± 0.06^{e}	3.99 ± 0.01^{d}
	6	2.00 ± 0.00^{d}	2.32 ± 0.12^{f}	2.47 ± 0.04^{e}	2.65 ± 0.07^{g}	2.58 ± 0.01^{e}	2.88 ± 0.02^{e}

Values are the mean ± SE, and data were scored for up to 12 weeks of culture. Within each column, values followed by the same superscript letter are not significantly different at P = 0.05, according to the LSD test.

Table 12. Protein content in different *D. lanata* tissues, as influenced by different carbon sources.

Treatment	%	Tissues			
		C	RC	RS	RO
Sucrose	3	1.88 ± 0.04^{b}	5.29 ± 0.11^{b}	8.50 ± 0.26^{a}	3.00 ± 0.18^{ab}
	4	1.67 ± 0.023^{c}	3.99 ± 0.20^{d}	6.32 ± 0.01^{c}	2.76 ± 0.01^{bc}
	6	1.58 ± 0.035^{d}	4.76 ± 0.09^{c}	5.35 ± 0.15^{d}	2.53 ± 0.24^{c}
Glucose	3	1.95 ± 0.064^{a}	5.60 ± 0.23^{a}	8.77 ± 0.25^{a}	3.25 ± 0.11^{a}
	4	1.88 ± 0.012^{b}	5.05 ± 0.01^{b}	6.74 ± 0.01^{b}	2.64 ± 0.01^{c}
	6	1.83 ± 0.020^{b}	4.10 ± 0.08^{d}	4.95 ± 0.06^{e}	1.15 ± 0.20^{f}
Maltose	3	1.60 ± 0.012^{d}	-	-	-
	4	1.56 ± 0.006^{d}	-	-	-
	6	1.42 ± 0.01^{e}	-	-	-
Fructose	3	1.23 ± 0.01^{f}	3.02 ± 0.012^{e}	3.33 ± 0.59^{f}	1.85 ± 0.81^{d}
	4	1.05 ± 0.012^{g}	2.95 ± 0.035^{e}	3.04 ± 0.029^{f}	1.62 ± 0.046^{de}
	6	1.00 ± 0.017^{g}	2.89 ± 0.006^{e}	3.00 ± 0.012^{f}	1.46 ± 0.012^{ef}

C: Callus; RC: regenerating callus; RS: regenerated shoots; RO: complete plant; -: nil.
Values are the mean ± SE. Data were scored after 2 weeks of culture. Within each column, values followed by the same superscript letter are not significantly different at P = 0.05, according to the LSD test.

carotenoids were completely absent in calli. Regenerative calli had more chlorophyll and carotenoids (chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids: 1.14, 1.01, 2.15, and 0.92 mg g^{-1} of fr. wt., respectively) when 8-week-old cultures were analysed. We also observed that the content of different pigments was maximal in regenerated microshoots (2.17, 1.80, 3.97, and 0.98 mg g^{-1} of fr. wt., respectively).

Proteins

Regenerated (micro) shoots exhibited maximum protein, i.e. 8.50 mg g^{-1} of fr. wt., when 12-week-old cultures were analysed. Regenerating calli also had sufficient amounts of soluble protein.

Protein levels as influenced by carbohydrates

Glucose (3%) improved the level of protein at different stages of development, i.e. in calli, in regenerative calli, and in regenerated microshoots, as compared to the control (Table 12). The protein level was very low in cultures supplemented with 6.0% fructose.

Discussion

This study aimed to evaluate the effect of PGRs, various carbohydrates, and different nitrogen sources on morphogenesis in *Digitalis lanata* cultured in vitro. Callus initiation and growth were markedly stimulated by a high NAA concentration (6.0 mg L^{-1}), either alone or in combination with cytokinin (BAP or kinetin). This relatively high NAA concentration was shown to be equally successful for leaf explants in various other plants (Odewale et al., 1996; Asemoto et al., 2007). In an earlier report dense and green calli were observed in media containing NAA/BA, versus yellow white, friable calli induced on 2,4-D (Chow et al., 1990). In *Digitalis thapsi* L., induced calli were well maintained on medium supplemented with 0.5 mg L^{-1} of 2,4-D and 0.5 mg L^{-1} of BA for 2 years or more, with subcultures at every 4 weeks (Herrera et al., 1990). A wide variety of explants were used for the regeneration of shoots and regeneration frequencies of up to 98% were reported for foxgloves when leaf explant was cultured on medium supplemented with 3.0 mg L^{-1} of BA (Christey & Earle, 1991). A number

of explants have been tested for their regeneration ability, and our study revealed that leaves were the ideal source of explant for in vitro culture in *D. lanata*.

For cultured tissues, the requirement of exogenous PGRs depends on the endogenous level, which varies with organs, plant genotypes, and the phase of plant growth (Chand & Singh, 2004). Of the various cytokinins used, BA was more productive than kinetin in *Digitalis*; the superiority of BA over kinetin for multiple shoot formation has been demonstrated in many plants, including important medicinals (Sujatha & Dhingra, 1993; Siril & Dhar, 1997; Chand & Singh, 1999). A positive influence of cytokinins on shoot formation was observed: the number of shoots increased as the concentration of kinetin or BA increased. A similar relationship, i.e. higher levels of kinetin or BA resulting in more shoots, was observed in other plant genera (Singh & Chandra, 1983; Wong & Loh, 1987).

Casein hydrolysate (Ch) is a complex mixture of amino acids and ammonium salts. In *Digitalis* cultures Ch was added in order to improve in vitro response; in the present study we observed that the addition of 0.5 g L^{-1} of Ch increased cultural growth (fresh weight). Ch is known to stimulate the synthesis of cell wall material and promote cell expansion (Gray & Conger, 1985). A possible explanation for Ch regulating plant growth is its influence in controlling IAA oxidation (Trigiano & Conger, 1987). A high shooting percentage was observed due to the combined effect of IAA and Ch, as compared to IAA or Ch alone, suggesting that these 2 compounds work synergistically. In our experiments with *Digitalis*, NAA with BA were very effective for the induction of roots. Hu and Wang (1983) previously reported the maximum number of roots with profuse growth at a high concentration of NAA and BA. Nevertheless, D'Souza and Sharon (2001) noted that average root length was not greatly affected by increased concentrations of NAA (0.05-4.0 mg L^{-1}) in the rooting medium.

In recent years several reports demonstrated that various carbon sources influence in vitro morphogenesis in different plant species (Biahoua & Bonneau, 1999; Petersen et al., 1999; Fuentes et al., 2000). In the present study we also noted that the in vitro response, including regeneration, was influenced by the added carbon sources.

In general, sucrose and glucose accelerated the frequency of organogenesis. This improved regenerative behaviour was not always linked to carbohydrate nutritional status itself, but to the osmotic condition induced by carbohydrates (Lou & Kako, 1995; Nakagawa et al., 2001; Gaj, 2004). Carbohydrates control morphogenesis by acting as energy sources and also by altering the osmotic potential of the culture medium (Pritchard et al., 1991). Sucrose is considered the best sugar for plant tissue culture (Petersen et al., 1999; Fuentes et al., 2000; Neto et al., 2003). This may be due to its efficient uptake across the plasma membrane (Borkowska & Szezebra, 1991). In fructose-added media, cultures showed early necrosis in *D. lanata*; the toxic compounds present in the media might also cause callus browning. Buter et al. (1993) reported a toxic compound-5-(hydroxymethyl)-2-furaldehyde (HMF), a derivative of fructose-that was generated by high temperature during media sterilisation.

Biochemical studies at various morphogenetic stages show that biochemical reserves were higher in regenerated microshoots, which accumulated further at later stages of growth. In the present study we noted that sucrose concentrations above and below 3% decreased chlorophyll content significantly in *D. lanata* cultures. It has been thought that the addition of sucrose reduces 5-aminolaevulinic acid (ALA) synthesis, a precursor of the porphyrin molecule that forms part of chlorophyll, due to inhibition of the activity of the enzyme ALA synthase (Pamplin & Chapman, 1975). It has been observed that cells grown on supra-optimal concentrations of sucrose may lose the ability to synthesise chlorophyll forever, because at such concentrations plastids are converted into colourless amyloplasts (Hemphill & Venketeswaran, 1978). Joseph et al. (2004) recently reported alteration in the total chlorophyll content and/or chlorophyll a/b ratio in cultures. Upon a reduction in photosynthetic pigments, the photosynthesis and source-sink relationship would be disturbed, affecting starch biosynthesis and storage as well (Wang et al., 1997). The overall efficiency of regeneration was thus significantly reduced in media containing high concentrations of carbohydrates. In this investigation we demonstrated the influence of carbon sources on morphogenesis in *D. lanata*; the role of PGRs and nitrogen sources has also been summarised.

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