In Vitro Cell. Dev. Biol. 27P:143–146, July 1991

1054-5476/91 $01.50+0.00

MICROPROPAGATION OF JUVENILE AND ADULT *DIGITALIS OBSCURA* AND CARDENOLIDE CONTENT OF CLONALLY PROPAGATED PLANTS

SONSOLES VELA, ISABEL GAVIDIA, PEDRO PÉREZ-BERMÚDEZ, AND JUAN SEGURA[1]

Department of Plant Biology, Faculty of Pharmacy, University of Valencia, Av. Blasco Ibañez 13, 46010 Valencia, Spain

(Received 12 March 1991; accepted 6 June 1991; editor E. J. Staba)

SUMMARY

Cultures of *Digitalis obscura* L. were established from axillary buds of mature plants or leaves of seedlings obtained under aseptic conditions. Explants were cultured on Murashige and Skoog medium containing benzyladenine and/or naphthaleneacetic acid. Shoot proliferation from axillary buds was not affected by seasonal fluctuations in the stock plants and increased relative to the cytokinin concentration, but auxin reduced the multiplication rate. Differentiation of somatic embryos and adventitious buds from cultured leaves required naphthaleneacetic acid alone or combined with benzyladenine, respectively. Cardenolide pattern and content of the regenerated plants were determined by high performance liquid chromatography and radioimmunoassay, respectively. Several cardenolides of series A and C were identified in the regenerants; no significant differences were found in the cardenolide patterns. Digoxigenin derivatives were found in all clonally propagated plants, but the amount of these glycosides was much higher in those obtained from axillary buds. This is the first report on micropropagation of *D. obscura* from mature plants.

Key words: Digitalis obscura; in vitro culture; shoot proliferation; cardenolides.

INTRODUCTION

Most of the commercially important secondary products, including *Digitalis* glycosides are either not formed in sufficiently large quantities or not at all by plant cell cultures. The reasons for these disappointing results have been widely discussed and some ways to overcome the limitations of production have been suggested (Robins and Rhodes, 1988). As in other *Digitalis* species (Luckner and Diettrich, 1988; Rücker, 1988), de novo synthesis of cardenolides in *Digitalis obscura* L. cultures seems to be closely related to morphologic differentiation (Brisa et al., 1991). In the present work we use organized cultures to generate or improve the yield of cardenolides by in vitro cultures of *D. obscura*. Tissues employed were leaves of axenic seedlings and axillary buds of field-grown plants. A protocol for micropropagation of sexually mature plants of *D. obscura* is also described.

MATERIALS AND METHODS

Tissue Culture

Culture conditions. Unless otherwise stated, the basal medium (BM) employed for culture contained MS constituents (Murashige and Skoog, 1962), 2% sucrose, and 0.8% Difco-Bacto agar, and pH of 5.8. The media were autoclaved at 121° C (1 kg · cm^{-2}) for 20 min and then distributed in 15 × 2.5-cm glass tubes (25 ml/tube). Cultures were kept in growth chambers at 26 ± 2° C and 16 h photoperiod with light supplied by Sylvania Gro-lux fluorescent lamps (20 W · m^{-2}). Tissues were subcultured to the corresponding fresh media each 30 days, and final data evaluated after 4 mo. in culture.

Leaf culture. *Digitalis obscura* L. (Scrophulariaceae) seedlings were grown from seeds germinated under sterile conditions as described by Pérez-Bermúdez et al. (1983). The first pair of leaves from 30-day-old plantlets were excised and placed with adaxial surface to the medium. Growth regulators employed were 2.7 or 5.4 μM naphthaleneacetic acid (NAA) alone or in combination with 8.8 μM benzyladenine (BA). After an induction period of 60 days, the tissues were transferred to BM for shoot and plant development. A total of 48 explants in three separate experiments were cultured for each treatment.

Axillary bud culture. Vegetative shoots were harvested in autumn and spring seasons from sexually mature *D. obscura* field-grown plants. Leaves were removed and the buds, attached to the stem, rinsed in 0.5% NaOCl (30 min) and maintained under running tap water for 12 h. They were then submerged for 15 min in an aqueous solution of 1% Benomyl and kept at 4° to 6° C for 2 to 7 days. Isolated buds were sterilized by two successive treatments, 70% ethanol (45 s) and 3% NaOCl with 0.1% Tween 20 (15 min), and finally rinsed with sterile distilled water. Meristems surrounded by two to three leaf primordia (0.2 to 0.5 mm) were dissected out and cultured on BM with 3% sucrose and 0.7% agar. Explants were first incubated for 45 days on media with BA (0, 0.5, 2, 5, and 20 μM) and NAA (0 or 0.5 μM); subsequently, developed buds were transferred to BM. A total of 12 explants were cultured for each treatment and experiments repeated twice in 2 consecutive years.

[1] To whom correspondence should be addressed.

TABLE 1

MORPHOGENIC RESPONSES FROM CULTURED LEAVES OF *DIGITALIS OBSCURA* SEEDLINGS[a]

Hormones, μM		Caulogenesis		Rhizogenesis		Embryogenesis	
NAA	BA	%CE	Shoots/ Explant	%RE	Roots/ Explant	%EE	Embryos/ Explant
0.0	0.0	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0 ± 0.0
2.7	8.8	56	9.7 ± 0.8	—	—	—	—
5.4	8.8	46	7.1 ± 0.4	—	—	—	—
2.7	0.0	—	—	80	11.9 ± 2.8	56	5.1 ± 1.4
5.4	0.0	—	—	83	17.0 ± 1.1	31	2.0 ± 0.5

[a] Values (±SD) are the average of three experiments with 16 replicates each; %CE, %RE, %EE = percentages of caulogenic, rhizogenic, and embryogenic explants.

Cardenolide Extraction and Determination

Extraction. A minimum of six samples (0.5 to 1 g) from regenerated plants in each different culture were dried at 40° C for 48 h before extraction according to the method described by Wichtl et al. (1982). Extractions were performed from samples collected at the end of the in vitro culture period.

High performance liquid chromatography (HPLC) analysis. For description of the equipment and conditions employed *see* Brisa et al. (1991).

Radioimmunoassay (RIA) analysis. Digoxigenin-derivative content was determined as described by Weiler and Zenk (1976). Standard solutions were prepared from analytical grade digoxin (Sigma, St. Louis, MO). Antidigoxin antiserum and ^{3}H-labeled antigen (digoxin) were purchased from Sigma and DuPont, (Wilmington, DE) respectively. Bound and free antigen were separated by dextran-coated charcoal precipitation. Radioactivity was measured in a LKB Wallac 1217 Rackbeta counter. The amounts were expressed in terms of micrograms of digoxin-equivalents per gram dry weight.

Results and Discussion

Micropropagation

Leaf cultures. Three kinds of morphogenic responses were induced from cultured leaves of *D. obscura*: callus formation, organogenesis (root and bud differentiation), and somatic embryogenesis. Callus formation was noticed within 2 wk in culture and indirect organogenesis or embryogenesis after 3 and 7 wk, respectively. Adventitious bud differentiation required the simultaneous presence of both NAA and BA in the induction media. In contrast, rhizogenesis and somatic embryogenesis were promoted by NAA alone. Further subculture to hormone-free medium enhanced either shoot elongation or embryo development to plants (Table 1).

These results constitute the first report on the capacity of cultured *D. obscura* leaves to regenerate plants via somatic embryogenesis and also substantiate some aspects already published by us on the regulation of in vitro morphogenesis from several juvenile explants of this species (Pérez Bermúdez et al., 1983, 1984; Brisa and Segura, 1989).

Axillary bud culture. Shoot multiplication from material of adult origin apparently occurs as a result of the release of axillary buds from apical dominance. Within 2 to 3 wk of culture the first visible sign was swelling of the explants and gradual proliferation of preexisting buds in the absence of callus formation. Bud proliferation took place when medium was supplemented with BA or combinations of this cytokinin with NAA. This auxin alone failed to elicit shooting. After transfer to hormone-free medium, buds developed into shoots.

As is shown in Table 2, the proliferative capacity of cultured axillary buds increased relative to the BA concentration and optimum shoot formation (ca. 30 shoots/explant) was obtained with 5 to 20 μM of this cytokinin. The analysis of variance also showed that seasonal fluctuations in the stock plant did not affect shoot yield, although the presence of NAA in the induction medium significantly reduced the response promoted by BA (Table 2). It has been suggested that auxins strengthen apical dominance at the expense of shoot proliferation (Hildebrandt and Harney, 1983). This effect may explain the lower multiplication rates of *D. obscura* cultures growing on NAA-supplemented media.

The system of in vitro propagation of *D. obscura* via axillary bud culture described here is comparable in its efficiency of shoot proliferation to others performed on *D. lanata* (Erdei et al., 1981; Schöner and Reinhard, 1986; Diettrich et al., 1990) and *D. thapsi* (Herrera et al., 1990). Concerning growth regulators effects, our results agree with those obtained by Diettrich et al. (1990) because

TABLE 2

EFFECTS OF BA, NAA, AND HARVEST SEASON ON THE PROLIFERATIVE CAPACITY (NO. SHOOTS/EXPLANT) OF AXILLARY BUD CULTURES OF *DIGITALIS OBSCURA*

BA, μM	Harvest Season	NAA, μM 0.0	NAA, μM 0.5	Mean[a]
0.0	autumn	0.0	0.0	
	spring	0.0	0.0	0.0*
0.5	autumn	6.7	2.8	
	spring	3.2	2.1	3.7*
2.0	autumn	16.7	11.6	
	spring	14.2	9.5	13.0**
5.0	autumn	36.1	20.7	
	spring	27.1	22.9	26.7†
20.0	autumn	32.3	25.8	
	spring	29.9	25.7	28.4†
	Mean[a]	16.6**	12.1*	

[a] Values followed by the same symbol are not significantly different according to Tukey's test ($P = 0.05$).

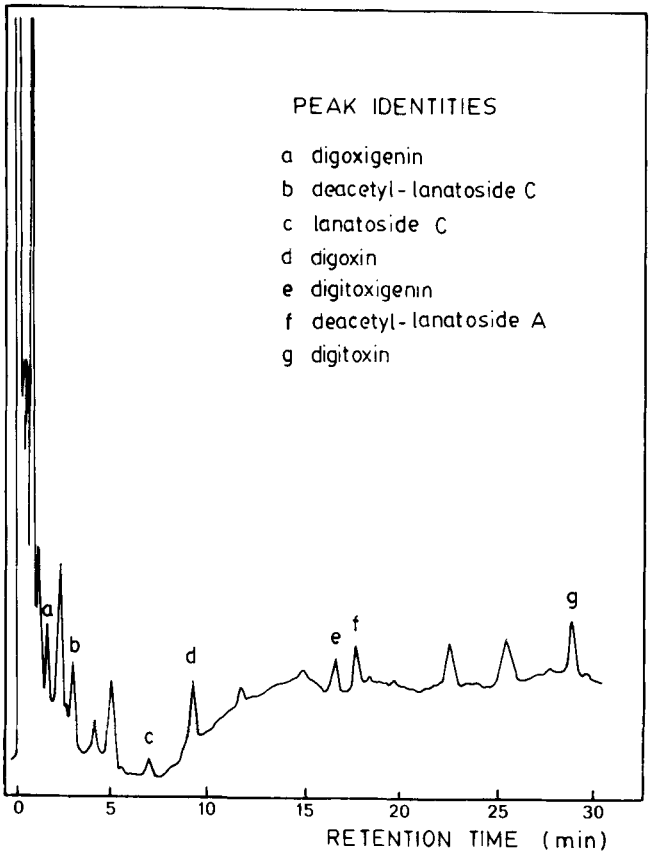

FIG. 1. HPLC cardenolide separation of an extract from *D. obscura* plants regenerated through axillary bud culture.

the presence of a cytokinin, but not an auxin, was necessary for shoot proliferation. Nevertheless, previous works on *D. lanata* (Erdei et al., 1981; Schöner and Reinhard, 1986) reported the addition of an auxin to be favorable for the growth of the cultures. Contrary effects to these found in *D. lanata* and *D. obscura* were achieved in *D. thapsi* shoot tip cultures (Herrera et al., 1990). In this species, shoot multiplication was dependent on the presence of auxins and slightly favored by the addition of BA or kinetin, although the authors did not study the effect of cytokinins alone.

Cardenolide Content of Clonally Propagated Plants

Cardenolide pattern of plants grown from explants of adult or juvenile origin were similar as determined by HPLC (Fig. 1). Most extracts from regenerated plants contained digoxigenin, deacetyl-lanatoside C, lanatoside C, and digoxin (series C) and digitoxigenin, deacetyl-lanatoside A, and digitoxin (series A). This pattern basically resembled that of the leaves of the mother plant (Brisa et al., 1991). In this respect, Schöner and Reinhard (1986) also observed that micropropagated *D. lanata* plants showed good homogeneity and were identical with their parent plant in terms of digoxin-glycoside content.

Radioimmunoassay analysis substantiated the presence of digoxigenin derivatives in the in vitro obtained plants (Tables 3 and 4), but the amount of these cardenolides was lower than that reported

TABLE 3

CARDENOLIDE CONTENT IN PLANTS OF *DIGITALIS OBSCURA* REGENERATED THROUGH AXILLARY BUD CULTURES ESTABLISHED IN AUTUMN[a]

BA, μM	NAA, μM	μg Digoxin Equivalents/ g Dry Weight
0.5	0.0	26.0 ± 9.9
0.5	0.5	not analyzed
2.0	0.0	25.4 ± 8.9
2.0	0.5	24.5 ± 12.2
5.0	0.0	22.7 ± 6.5
5.0	0.5	16.4 ± 7.7
20.0	0.0	23.3 ± 7.3
20.0	0.5	15.5 ± 8.0

[a] Values (±SD) are the average of at least six determinations.

by Brisa et al. (1991) in field-grown plants (ca. 129 μg digoxin equivalents/g dry weight). The digoxin content of the regenerants mainly depended on the origin of the explants used for culture establishment. The higher digoxin accumulation was found in plants obtained from axillary buds, and no differences were observed between cultures initiated in autumn or spring. Neither BA concentrations nor the presence of NAA provoked appreciable changes in the cardenolide content of these cultures (Table 3). In leaf cultures, plants differentiated through somatic embryogenesis produced more digoxigenin derivatives than those developed from adventitious buds (Table 4). This differential behavior could be first related to the higher development of the embryo-derived plants at the time of harvest. On the other hand, adventitious shoots showed a certain degree of vitrification, which also could negatively affect their biosynthetic capacity. In this respect, we have recently found that vitrified plants regenerated from hypocotyl cultures of *D. obscura* show drastic reductions in both chlorophyll and digoxin-derivative contents (unpublished results).

To date, only explants of juvenile origin have been used for in vitro propagation of *D. obscura* (Pérez-Bermúdez et al., 1983, 1987; Arrillaga et al., 1986; Brisa and Segura, 1987, 1989). However, these explants are not available from elite plants that have been identified in the field. This is the first successful report on micropropagation of sexually mature plants of *D. obscura*. The elevated multiplication rates obtained, and the capability of the regenerants to accumulate appreciable amounts of series C cardenolides, would

TABLE 4

CARDENOLIDE CONTENT IN PLANTS OF *DIGITALIS OBSCURA* REGENERATED FROM CAULOGENIC (CC) OR EMBRYOGENIC (EC) LEAF CULTURES[a]

Growth Regulators, μM		μg Digoxin Equivalents/g Dry Weight	
NAA	BA	CC	EC
2.7	8.8	2.2 ± 0.2	—
5.4	8.8	1.9 ± 0.1	—
2.7	0.0	—	5.5 ± 1.1
5.4	0.0	—	5.3 ± 1.5

[a] Values (±SD) are the average of at least six determinations.

be of great interest not only for rapid cloning of valuable genotypes but also to obtain biomass for cardenolide production. Embryogenic or caulogenic cultures from juvenile explants of *D. obscura* also represent a suitable material for selection experiments and somaclonal variation studies aimed to improve the yield of cardenolides.

References

Arrillaga, I.; Brisa, M. C.; Segura, J. Somatic embryogenesis and plant regeneration from hypocotyl cultures of *Digitalis obscura* L. J. Plant Physiol. 124:425–430; 1986.

Brisa, M. C.; Segura, J. Isolation, culture and plant regeneration from mesophyll protoplasts of *Digitalis obscura* L. Physiol. Plant. 69:680–686; 1987.

Brisa, M. C.; Segura, J. Morphogenic potential of mechanically isolated single cells from *Digitalis obscura* L. callus. Plant Cell Tissue Organ Cult. 19:129–139; 1989.

Brisa, M. C.; Pérez-Bermúdez, P.; Falcó, J. M., et al. Morphogenesis and cardenolide formation in *Digitalis obscura* cultures. J. Plant Physiol. 137:635–637; 1991.

Diettrich, B.; Mertinat, H.; Luckner, M. Formation of *Digitalis lanata* clone lines by shoot tip culture. Planta Med. 56:53–58; 1990.

Erdei, I.; Kiss, Z.; Maliga, P. Rapid clonal multiplication of *Digitalis lanata* in tissue culture. Plant Cell Rep. 1:34–35; 1981.

Herrera, M. T.; Cacho, M.; Corchete, M. P., et al. One step shoot tip multiplication and rooting of *Digitalis thapsi* L. Plant Cell Tissue Organ Cult. 22:179–182; 1990.

Hildebrandt, V.; Harney, P. M. In vitro propagation of *Syringa vulgaris* "Vesper". HortScience 18:432–434; 1983.

Luckner, M.; Diettrich, B. Cardenolides. In: Constabel, F.; Vasil, I. K., eds. Cell culture and somatic cell genetics of plants, vol. 5. San Diego: Academic Press; 1988:193–212.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497; 1962.

Pérez-Bermúdez, P.; Cornejo, M. J.; Segura, J. In vitro propagation of *Digitalis obscura* L. Plant Sci. Lett. 30:77–82; 1983.

Pérez-Bermúdez, P.; Brisa, M. C.; Cornejo, M. J., et al. In vitro morphogenesis from excised leaf explants of *Digitalis obscura* L. Plant Cell Rep. 3:8–9; 1984.

Pérez-Bermúdez, P.; Falcó, J. M.; Segura, J. Morphogenesis in root-tip meristem cultures of *Digitalis obscura*. J. Plant Physiol. 130:87–91; 1987.

Robins, R. J.; Rhodes, M. J. C., editors. Manipulating secondary metabolism in culture. England: Cambridge University Press; 1988.

Rücker, W. *Digitalis* spp.: in vitro culture, regeneration, and the production of cardenolides and other secondary products. In: Bajaj, Y. P. S., ed. Biotechnology in agriculture and forestry, vol. 4. Berlin: Springer-Verlag; 1988:388–418.

Schöner, S.; Reinhard, E. Long-term cultivation of *Digitalis lanata* clones propagated in vitro: cardenolide content of the regenerated plants. Planta Med. 52:478–481; 1986.

Weiler, E. W.; Zenk, M. H. Radioimmunoassay for the determination of digoxin and related compounds in *Digitalis lanata*. Phytochemistry 15:1537–1545; 1976.

Wichtl, M.; Wichtl-Bleier, W.; Mangkudidjojo, M. Hochleistungs-flüssigkeits chromatografhische analyse von *Digitalis*-blattextrakten. J. Chromatogr. 247:359–365; 1982.

The financial support of CICYT, Madrid, Spain (project no. PB89-0419) is gratefully acknowledged.