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EFFECTS OF PRECURSORS ON SERIALLY PROPAGATED *DIGITALIS LANATA* LEAF AND ROOT CULTURES

JOSEPH H. C. LUI and E. JOHN STABA

Department of Pharmacognosy, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455, U.S.A.

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Key Word Index—*Digitalis lanata;* Scrophulariaceae; organ culture: digoxin: plant growth regulators.

Abstract—Serially propagated *Digitalis lanata* leaf and root cultures established from germinated seeds were studied for digoxin production. Leaf cultures were grown and maintained in a medium containing benzyl adenine, and root cultures in the same medium with indoleacetic acid. A consistently high digoxin content, as determined by radioimmunoassay, is present in the leaf culture (9.0 mg % dry wt) and in root culture (1.9 mg % dry wt) as compared to unorganized cells (0.06 mg % dry wt). Leaf liquid cultures grew very rapidly as compared to the root cultures and the unorganized cell suspension cultures. The concentration of digoxin increased in both leaf and root cultures by adding to the medium either sodium glycocholate, cholesteryl acetate, or progesterone. Smilageninacetate increased the digoxin content of root cultures but not that of leaf cultures. Lanosterol and 5β-androstan-3,17-dione did not significantly increase the concentration of digoxin. Deoxycholic acid was toxic to the tissues studied.

INTRODUCTION

Cardenolides have been isolated from a number of species of digitalis [1]. Tissue cultures established from digitalis or other cardenolide-containing plants fail to produce cardenolides [2–4], contain trace amount of cardenolides [5–7], or lose their ability to produce cardenolides upon subculture [8]. Furuya *et al.* [9] reported that leaves regenerated from *Digitalis purpurea* callus cultures produced both digitoxin and purpurea glycoside A. This report is concerned with the establishment of root, leaf and unorganized cell suspension cultures of *D. lanata* Ehrh. and their ability to produce digoxin upon repeated subculture or in the presence of potential cardenolide precursors.

RESULTS

Leaf cultures

Cultures were initiated from cotyledon seedlings, i.e. those with cotyledons but without true leaves, on Murashige and Skoog's revised tobacco medium (RT medium) [10] containing agar and 10 ppm benzyladenine (BA). After 4 weeks, the cultures were transferred into RT liquid medium containing 5 ppm BA and grown under a 16 hr day-light cycle. In approximately 2 weeks, leaf cultures without roots were established and grew very rapidly, requiring subculturing every 4 weeks. The 4 week growth index (final tissue fresh wt/inoculum fr. wt) for the leaf cultures was 20 ± 2.2 (Table 1). Digoxin production was significant (9.0 ± 1.6 mg % dry wt.) and maintained over 10 passages (Fig. 1).

Root cultures

Cultures were initiated from cotyledon seedlings on RT medium containing agar and 3 ppm indoleacetic acid (IAA). After 4 weeks, the cultures were transferred into RT liquid medium containing 3 ppm IAA and grown in the dark. In approximately 2 weeks, root cultures without leaves were established. The 4 week growth index for this dark-grown root culture was 4.2 ± 0.4 (Table 1). Digoxin production of 1.9 ± 0.14 mg % dry wt was maintained over 10 passages (Fig. 1). The growth of this culture doubled when exposed to light (500 f.c.), but the

Table 1. Growth and digoxin content of *Digitalis lanata* cultures and plants

Material	Growth medium*	Growth index†	Digoxin (mg % dry wt)
Cultures (4 weeks old)			
Leaves from *in vitro* plantlet	RT-0	—	33 ± 2.0
Leaf culture	RT-BA5	20 ± 2.2	9.0 ± 1.6
Root culture (dark-grown)	RT-IAA3	4.2 ± 0.4	1.9 ± 0.14
Root culture (light-grown)	RT-IAA3	8.7 ± 0.5	1.5 ± 0.09
Unorganized cells	RT-0.1	5.9 ± 0.3	0.06 ± 0.005
Soil-grown plants (26 weeks old)			
Leaf	—	—	230 ± 36
Root	—	—	20 ± 2.4

* Murashige and Skoog's revised tobacco tissue culture medium with no growth regulator (RT-0), 5 ppm benzyladenine (RT-BA5), 3 ppm indoleacetic acid (RT-IAA3), and 0.1 ppm 2,4-dichlorophenoxy acetic acid (RT-0.1).

† Growth index = 4-week-old final fr. wt/initial inoculum fr. wt.

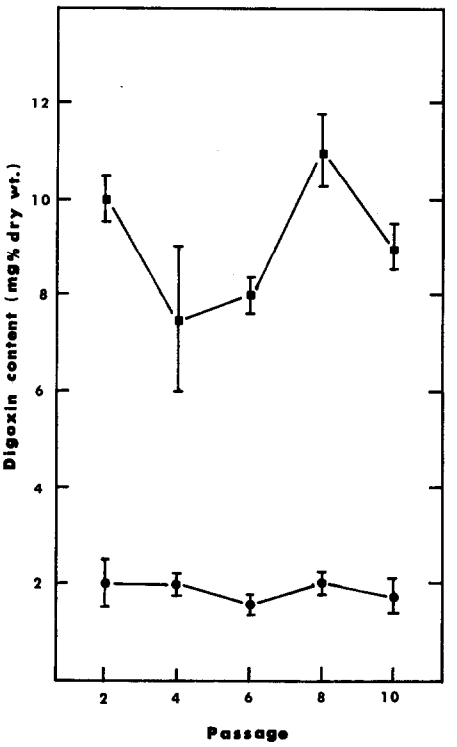

Fig. 1. Digoxin contents of *Digitalis lanata* Ehrh. organ subcultures. ■——■, Leaf culture; ●——●, root culture.

digoxin content did not change significantly (Table 1).

Unorganized cell suspension cultures

Unorganized (callus) cultures were initiated on RT medium containing 1 ppm 2,4-dichlorophenoxy acetic acid (2,4-D) from the cotyledon seedlings. After 4 weeks, the cultures were transferred into RT liquid medium containing 0.1 ppm 2,4-D. In about 2 weeks, suspension cultures were established that required subculturing every 4 weeks. The 4 week growth index for the suspension cultures was 5.9 ± 0.3. Digoxin was consistently present in a very low concentration of $6.0 \times 10^{-2} \pm 10^{-3}$ mg % dry wt (Table 1).

Precursor studies

Seven possible digoxin precursors (sodium glycocholate (I), deoxycholic acid (II), cholesteryl acetate (III), lanosterol (IV), progesterone (V), 5β-androstan-3,17-dione (VI) and smilagenin acetate (VII)) were added to leaf, root and cell culture medium. Digoxin production in leaf and root cultures increased approximately 3-fold by the addition of either 0.05% sodium glycocholate, or 0.01% cholesteryl acetate or 0.01% progesterone. Addition of smilagenin acetate (0.01%) to the medium increased digoxin production 4-fold in root cultures but not in leaf cultures. Digoxin production was not affected by precursor addition in unorganized cell suspension except for a 2-fold increase observed by the addition of progesterone (Table 2). Deoxycholic acid was toxic in the experimental condition used and resulted in the blackening of leaf or root tissues after 3 days of incubation.

In vitro *plantlets*

Cotyledon seedlings of *D. lanata* were grown on RT agar medium without growth regulators (RT-0 medium). These plantlets were grown under the same light and temperature conditions as leaf cultures. The concentration of digoxin varied from 18 to 44 mg % dry wt over a 12 week period (Fig. 2).

Soil-grown plants

Plants of *D. lanata* were grown in soil under the same light and temperature conditions as the leaf cultures. Leaves and roots were collected from 26-week-old plants and assayed for digoxin. The digoxin content of plant leaves was $2.3 \times 10^{2} \pm 36$ mg % dry wt and that of plant roots was 20 ± 2.4 mg % dry wt (Table 1).

Table 2. Effect of precursors on digoxin production in *Digitalis lanata* cultures*

	Digoxin content (mg % dry wt)		
	Leaf culture	Root culture	Unorganized cell
Controls			
RT medium	4.0 ± 0.2	2.0 ± 0.3	$4.0 \times 10^{-2} \pm 5.0 \times 10^{-3}$
RT medium with EtOH (4 ml/l.)	3.8 ± 0.3	1.9 ± 0.2	$3.9 \times 10^{-2} \pm 4.0 \times 10^{-3}$
RT medium with DMF (4 ml/l.)	3.7 ± 0.2	1.9 ± 0.1	$3.8 \times 10^{-2} \pm 4.0 \times 10^{-3}$
Precursors†			
0.05%			
I	11 ± 1.3	5.9 ± 0.5	$5.2 \times 10^{-2} \pm 7.0 \times 10^{-3}$
II	Toxic	Toxic	Toxic
0.01%			
III	12 ± 1.1	6.1 ± 0.1	$4.4 \times 10^{-2} \pm 2.0 \times 10^{-3}$
IV	4.0 ± 0.1	1.8 ± 0.1	$3.9 \times 10^{-2} \pm 2.0 \times 10^{-3}$
V	9.4 ± 0.9	8.8 ± 0.7	$7.6 \times 10^{-2} \pm 9.0 \times 10^{-3}$
VI	4.1 ± 0.2	2.6 ± 0.3	$3.1 \times 10^{-2} \pm 3.0 \times 10^{-3}$
VII	4.4 ± 0.3	8.5 ± 0.5	$4.2 \times 10^{-2} \pm 4.0 \times 10^{-3}$

* Leaf cultures were grown in RT-BA5 medium; root cultures were grown in RT-IAA3 medium; unorganized cell cultures were grown in RT-0.1 medium; the 4th-passage cultures were used in this experiment.

† Precursors added in ethanol or DMF (see Experimental): I, sodium glycocholate; II, deoxycholic acid; III, cholesteryl acetate; IV, lanosterol; V, progesterone; VI, 5β-androstan-3,17-dione; VII, smilagenin acetate.

Data represent means of 4 replicates followed by the s.e.

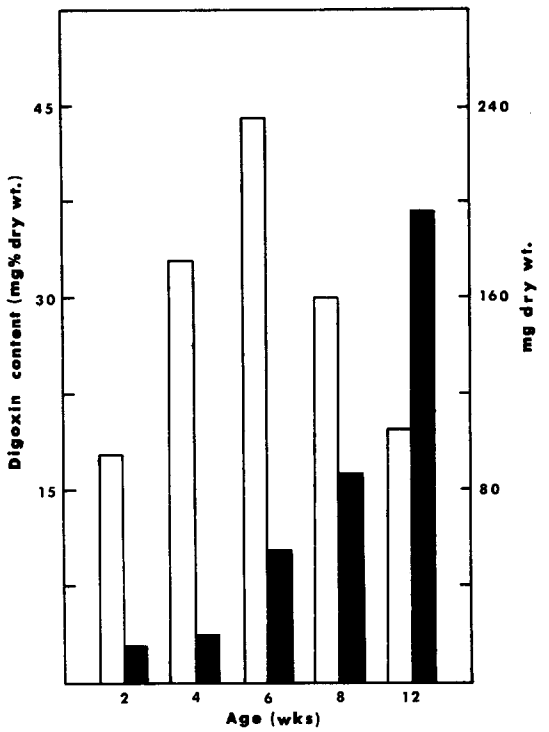

Fig. 2. Leaves' growth and digoxin content of *Digitalis lanata* Ehrh. *in vitro* plantlets at various times. □, Digoxin content (mg % dry wt); ■, dry wt of leaves from 4 *in vitro* plantlets.

DISCUSSION

Based on the experimental results (Table 1), the three established tissue lines have an ability to produce digoxin. Unorganized cell suspension cultures contain trace amounts of digoxin, and that of leaf cultures about 15 times less digoxin than the 26-week-old soil-grown plant. Evan *et al.* [11] reported low concentration of cardenolides present in young (1- to 4-month-old) *D. purpurea* plants, and the highest cardenolides concentration in approximately 24- to 28-week-old plants. Leaves from *D. lanata* plantlets grown on RT-0 agar medium over a 12 week period contained a significantly lower amount of digoxin (Fig. 2) than the 26-week-old soil-grown plants (Table 1). The low digoxin concentration present in the leaf culture, as compared to soil-grown plant leaves or roots, may be due in part to either its age or its stage of development.

Semi-purified chloroform and chloroform–methanol (1:1) extracts of leaf and root cultures were applied to TLC silica gel G plates. The cardenolide patterns of the extracts were similar to those obtained from the extracts of plant leaf and plant root. However, the primary cardiac glycosides, lanatoside A, B and C, were significantly lower in the organ cultures than the plant leaf. In contrast, cardiac genins such as digitoxigenin and gitoxigenin appeared relatively high in the leaf and root cultures.

EXPERIMENTAL

Digitalis lanata *cultures. D. lanata* leaf, root and callus cultures were established from aseptically germinated seeds (University of Washington, School of Pharmacy, Seattle, WA 98195). Cotyledon seedlings, i.e. those with cotyledons but without true leaves, were transferred onto RT medium containing agar and BA (10 ppm), IAA (3 ppm) or 2,4-D (1 ppm). After *ca* 4 weeks, the cultures were transferred into a 250 ml Erlenmyer flask containing 50 ml of liquid medium with BA (5 ppm), IAA (3 ppm) or 2,4-D (0.1 ppm), respectively, and placed on a gyrotory shaker (78 rpm) at 25°. Cultures that contained BA and 2,4-D were grown under a 16 hr day-light cycle (500 f.c., Fluorescent Plant-Grow Bulbs, Sears, 40 W, cool-light). Cultures containing IAA were grown in the dark.

Precursors study. Leaf, root and cell suspension cultures of *D. lanata* used for the precursor study were grown in 250 ml Erlenmyer flasks containing 50 ml RT liquid medium with either 5 ppm BA, 3 ppm IAA or 0.1 ppm 2,4-D, respectively. The inoculum for leaf and root cultures was *ca* 0.9 ± 0.1 and 0.18 ± 0.01 g fr. wt for the cell suspension cultures. After 10 days of growth, precursors dissolved in either EtOH or dimethyl formamide (DMF) were added directly to the medium to form a fine suspension in the liquid medium. Precursors: I—sodium glycocholate (5β-cholanic acid-3α,7α,12α-triol-*N*-(carboxymethyl)-amide sodium salt); II—deoxycholic acid (5β-cholanic acid-3α,12α-diol sodium salt); III—cholesteryl acetate (5-cholesten-3β-ol acetate); IV—lanosterol (8,24,(5α)-cholestadien-4,4,14α-trimethy-3β-ol); V—progesterone (4-pregnen-3,20-dione); VI—5β-androstan-3,17-dione; VII—smilagenin acetate (5β,20α,22α,25D-spirostan-3β-ol acetate). Sodium glycocholate and deoxycholic acid stock solns were 125 mg/ml of 70% EtOH, and progesterone and 5-androstan-3,17-dione stock solns were 25 mg/ml EtOH. Lanosterol, cholesteryl acetate and smilagenin acetate stock solutions were 25 mg/ml DMF. EtOH and DMF in the concn and conditions used did not significantly inhibit cellular growth (Table 2). The cultures were then incubated for 1 additional week under the condition previously described.

Analysis. Extraction. 1.0 g of either lyophilized digitalis cultures or plant tissues was suspended in 70% EtOH (20 ml) and shaken for 12 hr. The filtrate (extract I) was stored at 4° until assayed by RIA.

Semi-purified extracts (extract II) for TLC were prepared as follows: EtOH extracts (extract I) were defatted with petrol (30–60°) and then mixed with an equal vol. of H_2O. Saturated lead subacetate soln was added dropwise until no further precipitation occurred. The ppt. was removed by centrifugation and the excess Pb in the supernatant was precipitated by addition of 10% Na-Pi soln. The mixture was again centrifuged. The supernatant was adjusted to pH 5.2, extracted with $CHCl_3$ (3 ×), and $CHCl_3$–MeOH (1:1) (3 ×). The extracts were combined, dried (Na_2SO_4), and evapd to dryness under red. pres. The residue was dissolved in 1.0 ml of $CHCl_3$–MeOH (1:1).

TLC. Semi-purified extracts (extract II) prepared from the various experimental growth conditions and standards (lanatoside A, B and C (from ICN–K & K Laboratory Planview, N.Y.), digitoxin, digoxin, ditoxin, digitoxigenin, digoxigenin, gitoxigenin (from Sigma, Chemical Co., St. Louis, MO.), diginatin and diginatigenin (from Sandoz A.G., Basel, Switzerland) were chromatographed on Si gel G plates. The plates were developed at room temp. to a distance of 13 cm using CH_2Cl_2–MeOH–formamide (89.5:9.5:1) solvent system [12]. The plates were examined under UV light and sprayed with either anisaldehyde, Kedde, trichloroacetic acid, 3,5-diamino-benzoic acid or Xanthydrol reagent [13].

Radioimmunoassay (RIA). The RIA procedure used for extract I was as reported by Nickel *et al.* [14]. Tritiated digoxin (>8 Ci/mmol) and digoxin antiserum were from New England Nuclear (North Billerica, MA 01862). The precursor's zone (sodium glycocholate (I), R_f 0–0.05; deoxycholic acid (II), R_f 0.35–0.40; cholesteryl acetate (III), R_f 0.90–0.95; lanosterol (IV),

R_f 0.90–0.95; progesterone (V), R_f 0.80–0.85; 5β-androstan-3,17-dione (VI), R_f 0.75–0.80, smilagenin acetate (VII), R_f 0.85–0.90) was removed by scraping from the appropriate TLC plate. The remaining surface area was also removed, eluted with 20% EtOH, and assayed for digoxin.

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