Phytochemistry, 1977 Vol 16, pp 610–611 Pergamon Press. Printed in England

RESTORATION OF CARDENOLIDE-SYNTHESIS IN REDIFFERENTIATED SHOOTS FROM CALLUS CULTURES OF *DIGITALIS PURPUREA*

MASAO HIROTANI and TSUTOMU FURUYA
School of Pharmaceutical Sciences, Kitasato University, Minato-Ku, Tokyo 108, Japan

(*Received* 20 *August* 1976)

Key Word Index—*Digitalis purpurea*; Scrophulariaceae; tissue cultures; redifferentiation; digitoxin; Purpurea glycoside A.

Abstract—No cardenolide was detected in cultured cells and roots of root forming callus of *Digitalis purpurea* under such conditions as addition of auxins, varied sugar concentration of the medium and illumination. Cardenolides such as digitoxin and purpurea glycoside A were found in leaves from the regenerated shoot from callus cultures. The manifestation of cardenolide-synthesizing potentiality seems to be closely related to some process of leaf development.

Tissue cultures from cardenolide-producing plants have been established but no information is available on their cardenolide content [1–3]. Earlier work [4–6] has shown that steroids are readily metabolized into various products in the callus tissues of *D. purpurea*. Nevertheless, the *Digitalis* callus failed to give detectable amounts of cardenolides in its suspension cultures which contained sterols believed to be precursors for cardenolide biosynthesis.

There are some studies of the auxin [7] and light effects [8] on the production of secondary metabolites. In some cases, redifferentiation in callus tissues is a prerequisite for the production of secondary metabolites [9, 10]. In the present work a survey was carried out on the influence of cultural conditions and on organ differentiation in the production of cardenolides and phytosterols.

The ability of *Digitalis purpurea* callus tissues to synthesize cardenolides and phytosterols was examined. Cardenolides were not detectable in any extract of these cultures but campesterol, stigmasterol and sitosterol were detected in the *n*-hexane and $CHCl_3$ fractions of the extracts from all callus cultures (Table 1). In general sitosterol was the major component. The origin of the callus and the conditions of cultivation such as the substitution of an auxin in the medium (2,4-D, NAA and IAA) and/or illumination (about 25 000 lx for 16 hr/day and about 6000 lx, continuous light) and/or the different concentrations of sucrose (3%–0.25%) had no effect on the cardenolide-synthesizing ability and phytosterol constitution. Thus, the potency of *Digitalis* callus for biosynthesis of cardenolides and phytosterol was essentially not influenced by such changes in the experimental conditions.

Table 1. Detection of cardenolides and phytosterols in various callus cultures

Callus	Auxin in medium (mg/l)	Sucrose in medium (%)	Illumination*	Fr. wt of materials (g)	Cardenolides	Relative amounts of phytosterols		
						Campesterol	Stigmasterol	Sitosterol
Dig 1	2,4-D(1)	3	–	119.5	–	+	++	+++
	IAA (1)	3	–	141.4	–	+	++	+++
	NAA (1)	3	–	108.9	–	+	++	+++
	—	3	–	110.0	–	+	+	+++
	2,4-D (1)	3	+	109.9	–	+	++	++
	IAA (1)	3	+	107.0	–	+	++	+++
	NAA (1)	3	+	114.6	–	+	++	+++
	—	3	+	146.4	–	+	++	+++
DL 1	2,4-D (1)	3	–	102.5	–	+	++	+++
	2,4-D (1)	3	+	101.1	–	+	++	+++
DS 1	2,4-D (1)	3	–	97.8	–	+	++	+++
B 1	–	3	+	106.0	–	+	++	+++
	–	0.5	+	125.7	–	+	++	++
	–	0.25	+	152.9	–	+	++	++

* Illumination for callus B about 6000 lx continuous light; otherwise about 25 000 lx for 16 hr per day.

Table 2. Four original callus strains of *Digitalis purpurea*

Strain	Date of isolation	Growth regulators (conc mg/l.)	Origin	Description (Culture in dark)
Dig. 1	Feb. 1968	2,4-D (1)–kinetin (0.1)	Seedling	Wet and soft; pale yellow
DL 1	June 1973	2,4-D (1)–kinetin (0.1)	Leaf	Compact; pale greenish–yellow
DS 1	Aug. 1974	2,4-D (1)–kinetin (0.1)	Seedling	Compact; pale greenish–yellow
IS	Aug. 1974	IAA (1)–kinetin (0.1)	Seedling	Root-forming

Root redifferentiation was easily induced in most strains by transfer to the 1 ppm IAA–0.1 ppm kinetin medium in darkness. IS (original root-forming callus; see Experimental) was used as the source of redifferentiated roots. Bud formation was sometimes observed in cultures containing the 0.1 ppm IAA–1 ppm kinetin medium and on illumination. After allowing primordial leaf-like structures to develop, the shoots were transferred to basal medium containing 3% sucrose. Further development of the leaves (20–40 mm long) was achieved by culture for about 3 weeks under the 16 hr period of illumination (about 6000 lx) at 25°, but no root formation could be induced. The aerial parts of the cultures were harvested. Materials thus obtained, that is root-forming calluses (IS) regenerated leaves (S) and leaves (L) and roots (R) from the original plants were used for examination.

Cardenolides could not be detected in the fractions from IS and R. TLC of the $CHCl_3$ fractions of extracts from S and L showed the presence of several compounds, two with similar mobilities to authentic samples of digitoxin (R_f 0.31 and 0.52) and purpurea glycoside A (R_f 0.04 and 0.11), with TLC solvent systems (2) and (3), respectively. Digitoxigenin (R_f 0.41 and 0.56) was also detected in the extract of L but not in the extract from S.

The main phytosterol from IS and S was stigmasterol. In the extract from L only campestrol was detectable. Lamba and Staba [11] described the formation of root and leaf structures in callus of *D. lanata*, but they did not examine the chemical components of these redifferentiated tissues. The present study demonstrated that the potency for cardenolide production was restored in the shoot but not in the root redifferentiated from *Digitalis* callus tissues.

Hart *et al.* [12] reported that volatile sesquiterpenes typical of the original *Pogostemon cablin* plant were not detectable in regenerated plantlets from a callus which did not produce them and that the morphology of these plantlets was unlike that of the parent plant, although glandular tricheomes were present as in the parent plant.

These facts suggest that non-production of cardenolide in the *Digitalis* callus under various culture conditions is not due to disappearance of the genetic information for cardenolide biosynthesis but is due to suppression of its expression. Cardenolide production seems strictly to correlate with the formation of leaf-like structures.

EXPERIMENTAL

Plant materials. Four original strains of callus tissues (Dig 1, DL 1, DS1 and IS) were derived from seedlings and leaves of *Digitalis purpurea* (Table 2). They were grown on the modified Murashige and Skoog's tobacco medium containing 3% sucrose and subcultured every 3 weeks. Callus B, an auxin-cytokinin-habituated callus was obtained from callus DL 1 as described by Syōno and Furuya [13]. In addition, callus S, with differentiated leaves, was obtained from the IS strain. In IS strain, bud formation was sometimes observed in cultures with the 0.1 ppm IAA–1 ppm kinetin medium in the light. Plants of *D. purpurea* were raised from seeds. Leaves and roots from about 10-month-old plants grown in a field were used for examination.

Extraction and detection of cardenolide and phytosterol. Harvested materials were homogenized in a blender with 300 ml MeOH. Each homogenate was filtered and the residue was re-extracted 2 × in the same way. Filtrates were combined and MeOH was evaporated *in vacuo* and extracted 3 × each with *n*-hexane (300 ml in total), $CHCl_3$ (300 ml in total) and $CHCl_3$:MeOH (2:1). These extracts were separately washed with H_2O, dried, evapd to dryness, and examined (TLC and GLC) for phytosterols (*n*-hexane and $CHCl_3$ fractions) and cardenolide ($CHCl_3$ and $CHCl_3$–MeOH fractions). TLC was on Si gel and the solvent systems used for detection of (1) phytosterols, (2) digitoxigenin and digitoxin, and (3) purpurea glycoside A were (1) C_6H_6–EtOAc, 4:1, (2) $CHCl_3$–MeOH, 9:1, (3) $CHCl_3$–MeOH, 6:1, respectively. GLC was on a 2 m × 3 mm glass column packed with 1% SE-30 on Gas-Chrom Q at 220° and 55 ml/min of N_2.

Acknowledgements—The authors thank Professor H. Suzuki, Faculty of Science, Tokyo Kyoiku University, and Associate Professor K. Syōno, University of Tokyo, for their helpful discussions. We are grateful to Dr. J. Okano, Daiichi Seiyaku Co. Ltd. for the authentic sample of purpurea glycoside A, to Mr. S. Kobayashi, Experiment Station for Medicinal Plant Studies, University of Tokyo, for his supply of *Digitalis purpurea*.

REFERENCES

1. Staba, E. J. (1962) *J. Pharm. Sci.* **51**, 249.
2. Furuya, T., Hirotani, M. and Shinohara, T. (1970) *Chem. Pharm. Bull.* **18**, 1080.
3. Pilgrim, H. (1972) *Pharmazie* **27**, 121.
4. Furuya, T., Hirotani, M. and Kawaguchi, K. (1971) *Phytochemistry* **10**, 1013.
5. Furuya, T., Kawaguchi, K. and Hirotani, M. (1973) *Phytochemistry* **12**, 1621.
6. Hirotani, M. and Furuya, T. (1975) *Phytochemistry* **14**, 2601.
7. Furuya, T., Kojima, H. and Syōno, K. (1971) *Phytochemistry* **10**, 1529.
8. Corduan, G. and Reinhard, E. (1972) *Phytochemistry* **11**, 917.
9. Ikuta, A., Syōno, K. and Furuya, T. (1974) *Phytochemistry* **13**, 2175.
10. Tabata, M., Yamamoto, H., Hiraoka, N. and Konoshima, M. (1972) *Phytochemistry* **11**, 949.
11. Lamba, S. S. and Staba, E. J. (1963) *Phyton* **20**, 175.
12. Hart, J. W., Woodcock, G. J. and Wilson, L. (1970) *Ann. Bot. London* **34**, 789.
13. Syōno, K. and Furuya, T. (1974) *Plant Cell Physiol.* **15**, 7.