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EMBRYOGENESIS AND CARDENOLIDE FORMATION IN TISSUE CULTURES OF *DIGITALIS LANATA*

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Abstract—*Digitalis lanata* strain VII from filament callus grew in small cell colonies in nutrient media with high auxin activity Upon increasing the cytokinin-auxin ratio in the medium it formed large numbers of adventive embryos from which plantlets were regenerated in hormone-free media At the globular state the embryos turned green if irradiated and started to produce cardiac glycosides The extent of embryo development and cardiac glycoside production depended on a suitable hormone regime as well as on the C and N sources in the medium The cardenolide content of the irradiated embryos increased within three weeks from about zero to about 0 1 μg/mg dry weight (about 1 mg/l culture) It reached about 0 15 μg/mg dry weight (4 mg/l culture) during plantlet regeneration

INTRODUCTION

Several groups have shown that *in vitro* cultivated cells of *Digitalis lanata* L and other *Digitalis* species grown in an undifferentiated state either produce insignificant amounts of cardenolides or lose their ability for cardenolide formation during repeated subcultivation [1–9] Regeneration of embryos [10] or shoots [7, 11–13], however, triggers cardenolide biosynthesis under suitable conditions Regeneration of adventive embryos was shown to proceed with high frequency in a strain of *D lanata* (strain V) obtained in a rather complicated way in a screening programme for haploid *D lanata* cell cultures [10] In this paper we report on a new strain (*D lanata* strain VII) developed in a planned manner which possesses a capability for cardenolide formation and accumulation similar to that of strain V We describe also conditions for optimum embryogenesis and cardenolide accumulation

RESULTS

Origin and propagation of D lanata *strain VII*

Strain VII was derived from a flower bud filament of *D lanata* cultivar 'Dresdener' on a nutrient medium containing 5 mg/l 2,4-D [14] The primary callus formed on this medium was removed and grown in suspension culture in a modified Murashige-Skoog medium with reduced 2,4-D concentration (1 mg/ml, NM I-M) It formed relatively dense cell colonies which in some cases contained cores of small, polyhedric, plasma-rich meristematic cells with large nuclei, surrounded by enlarged, round parenchymatic cells (Fig 1A) The doubling time of the cells in nutrient medium NM I-M was about 48 hr

Embryogenesis and cardenolide formation

Like strain V [10], strain VII formed large amounts of adventive embryos and plantlets under suitable hormone conditions On changing the medium used for propagation, which had a high auxin-cytokinin ratio (NM I-M), to media with reduced or no auxin activity (NM II-M and NM V-M) adventive embryos developed from the meristematic areas The embryonic structures turned green if irradiated at a certain stage of development and formed plantlets if cultivated in a hormone-free medium (NM VI-M) In irradiated cultures the following stages were distinguished during plantlet regeneration meristematic areas → white globular structures → green globular structures → heart-shaped and torpedo-shaped embryoid structures with root and shoot poles → fully developed adventive embryos → plantlets (Fig 1)

The fully developed white globular structures, the green globular structures and the later stages of embryo and plantlet regeneration were able to synthesize and accumulate compounds giving a positive reaction with digitoxin-specific antibodies These compounds were shown to be mainly digitoxin derivatives by means of TLC and HPLC [F Hering, unpublished results] In the following they are designated collectively as cardenolides The cardenolides present in the investigated tissue cultures of *D lanata* were found exclusively in the cell material This was in agreement with the fact that most cardenolides added to the cultures are accumulated in the cells [15]

The best results in terms of embryogenesis and cardenolide accumulation were obtained if the ratio of the

Abbreviations BA, benzyladenine, CBA, 4-chlorophenoxy butyric acid, 2,4-D, 2,4-dichlorophenoxy acetic acid, DBA, 4-(2,4-dichloro)-phenoxy butyric acid, DE, digitoxin equivalents, DPA, 3-(2,4-dichloro)-phenoxy propionic acid, IAA, indoleacetic acid, IBA, indolebutyric acid, Ki, kinetin, NAA, naphthaleneacetic acid, NM, nutrient medium

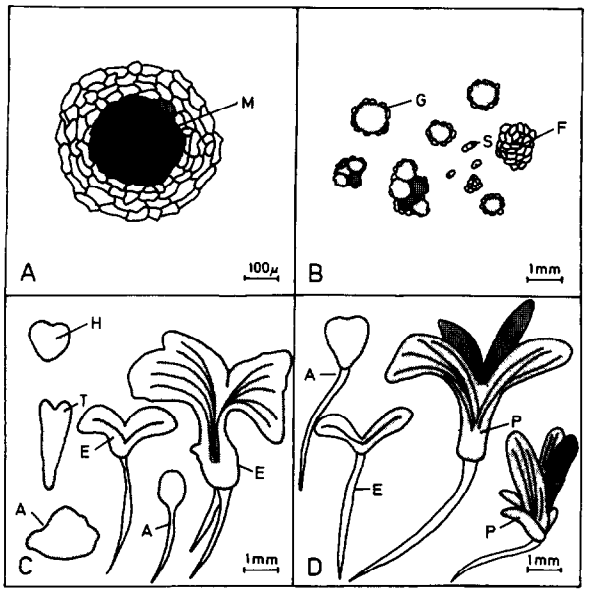

Fig 1 Morphological structures in cultures of *D lanata* strain VII cultivated in NM I-M, NM II-M, NM V-M and NM VI-M as described in the Experimental (A) Cell colony with meristematic core (M) grown in NM I-M (B) Population of cell colonies after cultivation in NM II-M for a period of 4 weeks G, globular structures built up from meristematic cells, F, friable cell colonies, S, single cells (C) Morphological structures after a growth period of 4 weeks in NM V-M H, heart-shaped structures, T, torpedo-shaped structures, E, fully developed embryos, A, anomalous structures (D) Morphological structures after a growth period of 4 weeks in NM VI-M P, plantlets, E, fully developed embryos, A, anomalous structures

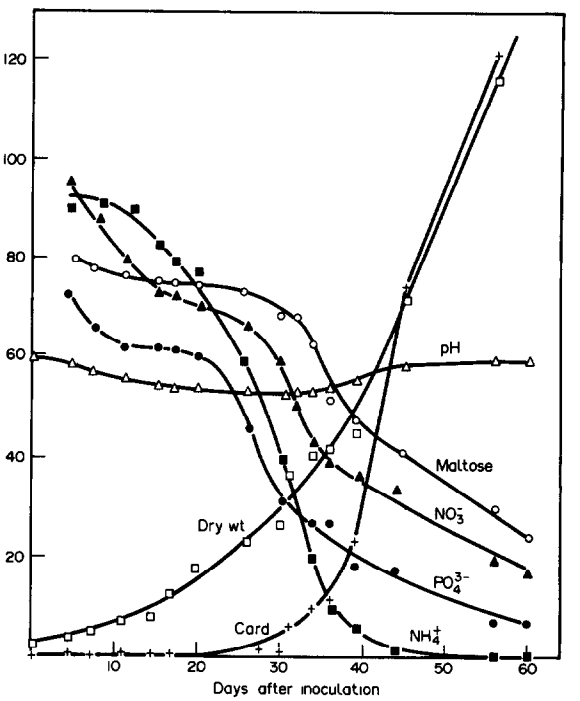

Fig 2 Growth and cardenolide accumulation of *D lanata* strain VII in nutrient medium NM V-M as described in the Experimental All values are given in g/l □——□, Dry wt (100 = 10), ■——■, NH_4^+ (100 = 0 128), ○——○, maltose (100 = 30), ●——●, P_i (100 = 0 170), △——△ pH (100 = 10), ▲——▲, NO_3^- (100 = 3 28), +——+, cardenolides (100 = 0 001)

cytokinin/auxin activity of the nutrient media was increased in two steps and a medium (NM II-M) containing a week auxin (NAA) was used to trigger embryo formation The direct transfer of cells from NM I-M (containing 1 mg/l 2,4-D) to NM V-M (containing no auxins) resulted in a severe inhibition of growth, embryogenesis and cardenolide accumulation

Large numbers of small globular structures ($\phi < 1$ mm) made up of meristematic cells (Fig 1B) were obtained if the cells were grown for four weeks in NM II-M with a regular exchange of the medium The numbers of these structures in the inoculum determined the rate of cardenolide accumulation in the following developmental phases The larger friable cell colonies present in the suspension were removed, as they were unable to develop into cardenolide synthesizing and accumulating structures This was achieved by passing the cultures through a sieve (0 4 × 0 8 mm mesh) to give a filtrate containing large numbers of the globular structures and only small numbers of single cells and of the friable cell colonies

Also critical for the further development of the cultures was the number of globular structures used for inoculation of NM V-M The optimal number was equivalent to about 0 2 mg dry wt/ml culture, which on the one hand allowed conditioning of the medium and on the other hand avoided inhibition of further development which occured at high densities of the globular structures

In nutrient medium V-M the small globular structures increased considerably in size, became heart- and torpedo-shaped and turned green if irradiated with white light After about four weeks fully developed torpedo-shaped embryos had developed which contained roots and thick rigid leaf-like structures (Fig 1C) Cardenolide accumulation started after about 30 days and continued for more than 20 days (Fig 2) At the end of this period a cardenolide content of about 0 1 μg/mg dry wt (≙ 1 0 μg/ml culture) was reached

The influence of the medium on embryogenesis and cardenolide accumulation

Of significance for embryo development and cardenolide accumulation in NM V-M were the hormones and the C and N sources With maltose, embryogenesis and cardenolide accumulation were higher than with any of the other sugars used With sucrose both processes were considerably repressed In media containing sucrose, fructose and glucose appeared during cultivation [10, 15] In contrast in the cultures containing maltose no free glucose or fructose was detected In further experiments it will be tested whether addition of fructose or glucose to maltose-grown cultures inhibits embryogenesis and cardenolide accumulation, i e morphological and chemical cell specialization as in many microbial cultures [16]

With respect to the N source, optimum growth, embryo formation and cardenolide accumulation were obtained

with NH_4^+ and NO_3^-, whereas, for instance, with L-glutamine all three were strongly reduced Of importance was the ratio of NH_4^+/NO_3^- The best results were obtained with an excess of NO_3^- (NH_4^+ NO_3^- 1 10, Table 1) Obviously a small amount of NH_4^+ was necessary as an initial source of readily available nitrogen NH_4^+ was taken up faster from the medium than NO_3^- (cf Fig 2) It was replenished during further cultivation by reduction of NO_3^-, which supplied the cultures for a relatively long period with a steady stream of NH_4^-

With respect to the hormones, about 1 mg/ml of BA or Ki in nutrient medium V-M were most suitable (Table 2) Addition of small amounts of auxins to nutrient solutions containing cytokinins did not influence embryo formation and cardenolide accumulation, but in higher concentrations auxins inhibited both processes The administra-

Table 1 Influence of the NO_3^-/NH_4^+-ratio on embryo development and cardenolide accumulation in *D lanata* tissue cultures

Ratio NO_3^-/NH_4	Dry wt (mg/ml culture)	Embryogenesis	Cardenolides (μg DE/ml culture)
0*	0 7	−	0 01
2	7 2	+ +	0 60
5	7 5	+ + +	0 84
10†	6 8	+ + +	0 99
50	4 5	+	0 21
100	3 5	+	0 19
∞	2 2	+	0 20

D lanata strain VII was cultivated in NM I-M and NM II-M as given in the Experimental It was then grown in NM V-M in which the NH_4^+/NO_3^--ratio was altered by use of different amounts of NH_4NO_3 and KNO_3 (total nitrogen 60 mM)

*Instead of NH_4NO_3/KNO_3, NH_4Cl was used as nitrogen source

†NM V-M as standard

Table 2 Influence of different hormones on embryo development and cardenolide accumulation in *D lanata* tissue cultures

Hormones (mg/l)				Dry wt (mg/ml culture)	Embryogenesis	Cardenolides (μg DE/ml culture)
BA	0 1			8.7	+	0 55
BA	0 5			7 9	+ +	0 64
BA*	1 0			9 1	+ + +	1 12
BA	2 0			5 0	+ +	0 11
BA	5 0			0 9	+	0 002
Ki	0 1			0 9	−	0 002
Ki	0 5			4 8	+ +	0 38
Ki	1 0			8 6	+ + +	1 02
BA	1 0	NAA	0 01	8 4	+ + +	1 00
BA	1 0	NAA	0 1	6 0	+ +	0 60
BA	1 0	IAA	0 01	4 8	+	0 29
BA	1 0	IAA	0 1	5 4	+	0 32
Ki	1 0	NAA	0 01	5 0	+ + +	1 06
Ki	1 0	NAA	0 1	5 6	+ +	0 70
NAA	1 0					
IAA	1 0					
IBA	1 0	Severe inhibition of growth, no embryogenesis,				
DBA	1 0	no formation of cardenolides				
CBA	1 0					
DPA	2 0					

D lanata strain VII was cultivated in NM I-M and NM II-M as given in the Experimental It was then grown for 30 days in NM V-M to which instead of 1 mg/l BA the given hormones were added

*NM V-M as standard

tion of different auxins without cytokinins or use of the nutrient solution without hormones inhibited growth, embryogenesis and cardenolide accumulation

It was found that if the media containing BA or Ki was removed after about three weeks of cultivation, i e at the developmental stage at which the embryogenic structures turned green under illumination, and replaced by a hormone-free medium (NM VI-M) then small plantlets were formed in the following weeks This demonstrated that the green embryogenic structures at a certain stage of development were able to produce all the hormones necessary for further development in contrast to earlier stages which needed an exogenous hormone supply A direct transfer of cultures from NM II-M to NM VI-M, for instance, resulted in cessation of growth and development

If the medium was replaced every week after 30 days of cultivation the cardenolide content in the cultures reached 0 15 μg/mg dry wt In the leaf fraction the maximum cardenolide accumulation was 0 45 μg/mg dry wt It was of interest that during cultivation in NM VI-M the dry wt increased considerably yielding a cardenolide content of 4 μg/ml culture

The plantlets grown submerged differed from normal seedlings by possessing thick, rigid leaves, and frequently by the existence of more than one root and shoot pole, and by deformations etc (Fig 1D) However, normal plantlets were regenerated if they were further cultivated on NM VI-M solidified with agar These plantlets could be adapted to non-sterile conditions and growth in earth demonstrating that the abnormal structures were caused at least in part by unphysiological conditions during embryogenesis, and as far as they were caused by genetic deviations may be normalized during further plantlet development

The dependence of cardenolide biosynthesis and accumulation on irradiation

If globular structures pregrown in NM II-M were further cultivated in NM V-M their cardenolide content depended strongly on irradiation with white light (Table 3) Though the morphological structures in non-irradiated and irradiated cultures were similar (embryogenesis is a process independent of light), the non-irradiated structures were devoid of chlorophyll and chloroplasts and never contained more than very small amounts of cardenolides Already a daily six-hour-period of irradiation with about 2000 lx increased the cardenolide content about 10-fold Continuous irradiation gave the highest cardenolide accumulation (increase about 100-fold) This light dependence of cardenolides accumulated in embryogenic structures was in agreement with previous results obtained with *D lanata* strain V [10] and with results found with *D purpurea* shoot cultures [13]

With respect to the effect of irradiation on cardenolide accumulation in *Digitalis* cell cultures, it should be emphasized that morphological specialization (formation of globular embryogenic structures at a certain stage of development, see above) or the regeneration of leaves [13] was necessary for a considerable cardenolide accumulation Illuminated, green unorganized cell cultures of *D purpurea*, for instance, did not accumulate larger amounts of cardenolides [13] On the other hand irradiation was not a prerequisite for cardenolide biosynthesis, though the cardenolide content of non-irradiated cultures usually was much smaller than that of irradiated cultures (Table 3, [13]) It will be the aim of further experiments to elucidate the role light plays in the increase of cardenolide accumulation in more detail

Table 3 Influence of illumination on cardenolide accumulation in *D lanata* tissue cultures

Light (hr/day)	Dry wt (mg/ml culture)	Cardenolides (μg DE/ml culture)
0	7 2	0 008
6	4 4	0 08
12	5 6	0 18
18	7 6	0 54
24	7 2	0 94

D lanata strain VII was cultivated in NM I-M and NM II-M as given in the Experimental During cultivation in NM V-M the cultures were illuminated for different periods

DISCUSSION

D lanata strain VII accumulates, under the experimental conditions described in this paper, about the same amount of cardenolides as is found in the other most productive *Digitalis in-vitro*-cultures (Table 4) In accordance with refs [17] and [18], it was derived from a plant selected for its high cardenolide content It should be mentioned, however, that this plant produced a high portion of digoxin derivatives at the end of the first vegetation period, the time point of analysis, whereas the adventive embryos and plantlets formed *in vitro* contain mainly digitoxin derivatives This is in agreement with results on the cardenolide fraction of *D lanata* seedlings and young plants, which were shown to contain either no digoxin derivatives or only small amounts [19–24]

The results described in this paper show that adventive embryos may accumulate *in vitro* nearly the same amounts of cardenolides as plantlets (or shoots) Since microscopical examination indicates that the globular structures which start cardenolide accumulation are rather simple in construction, they might be more suitable for the investigation of the mechanisms triggering cardenolide accumulation than shoots or plantlets which comprise several types of cells showing differences in cardenolide accumulation [25, 28] and probably biosynthesis Furthermore the adventive embryos are easier to handle biotechnologically than the more complex structures Embryogenic strains may therefore be of special value in the further investigation of cardenolide formation in *Digitalis in-vitro*-cultures

EXPERIMENTAL

Biological material The embryogenic strain VII of *D lanata* was used in the experiments [14] It was propagated on NM I [10] for about 2 years prior to the start of the work described in this paper

Nutrient media Basic nutrient medium (mmolar) maltose 83 3, NH_4NO_3 20 6, KNO_3 18 8, $CaCl_2$ 3 0, KH_2PO_4 1 25, Na_2EDTA $2H_2O$ 0 10, H_3BO_3 0 10, $MnSO_4$ 0 10, $ZnSO_4$ 0 03, $MgSO_4$ $7H_2O$ 1 5, $FeSO_4$ $7H_2O$ 0 18, (rest μmolar) KJ 5 0,

Table 4 Maximum cardenolide contents of *Digitalis in vitro* cultures

Plant/strain	Material cultivated	Cardenolide content (μg/mg dry wt)	Cardenolide content (μg/ml culture)	References
D lanata				
strain VII	Plantlets	0 15	4 0	This paper
strain VII	Adventive embryos	0 1	1 0	This paper
strain V	Adventive embryos	0 1	1 5	[10]
D lanata	Shoots	0 1		[12]
D purpurea	Shoots	0 05	0 8	[26]
D grandiflora	Adventive embryos	0 05		[27]

Na_2MoO_4 $2H_2O$ 1 0, thiamine 1 5, pyridoxine 2 4, nicotinamide 8 2, myo-inositol 555, L-ala 333, L-arg 17 8, L-asn 25 8, L-asp 12 8, L-gln 2 0, L-glu 107, gly 62 6, L-his 0 32, L-hydroxypro 9 5, L-leu 37 7, L-lys HCl 10 7, L-met 0 3, L-phe 0 3, L-pro 16 9, L-ser 121, D,L-thr 34 4, L-tyr 0 3, L-val 19 6

Modified nutrient media NM I-M The amount of KH_2PO_4 in the basic medium was increased to 680mg/l and 1 0mg/l 2,4-D and 0 02 mg/l Ki were added NM II-M 1 0mg NAA and 0 02 mg Ki were added to the basic medium NM V-M The amount of KNO_3 in the basic medium was adjusted to 4930 mg/l, that of NH_4NO_3 to 450mg/l and 1 0mg/l BA was added NM VI-M The basic nutrient medium was diluted with H_2O 1 1

Propagation of the cells Cells were cultivated for periods of 1 week in 500ml flasks containing 125ml NM I-M in rotary shakers (125rpm) at 25° in dim light 15–20ml cell suspension (≙ about 60mg dry wt) was used as inoculum

Embryogenesis 15–20ml of a cell suspension grown for 7 days in NM I-M (≙ about 60mg dry wt) were transferred into 125ml NM II-M and cultivated for 4 weeks in a rotary shaker (125rpm) at 25° in dim light Every week the nutrient medium was replaced by another 125ml NM II-M 15ml NM V-M in 100ml Erlenmeyer flasks were inoculated with cells precultivated for 4 weeks in NM II-M Structures passing through a sieve of 0 4 × 0 8 mm mesh width (≙ 2–4 mg dry wt) were used as inoculum The cultures were kept for 4 weeks on reciprocal shakers (80 strokes/min) at 25° and about 2000 lx (fluorescent tubes 'daylight white' and 'lumoflor 80' 1 1, Kombinat VEB Narva, Brand-Erbisdorf)

Plantlet regeneration The nutrient medium of cultures grown in NM V-M was replaced by 20ml NM VI-M This medium was replaced weekly with new NM VI-M during a period of 8 weeks The cultures were kept on a reciprocal shaker (80 strokes/min) at 25° and about 2000 lx (fluorescent tubes 'day-light white' and 'lumoflor 80' 1 1)

Determination of cardenolides To an aliquot of the homogenized culture 1 part of aq 96% EtOH was added The mixture was heated to 80° for 2 min, cooled and centrifuged The supernatant was used for the determination of digitoxin equivalents (and occasionally digoxin equivalents) by means of specific radioimmunoassays [10, 28] The extracted cell material was used for dry wt determination

Determination of nutrients Maltose 1ml diluted culture medium was heated with 0 1 ml 37% HCl to 100° for 1 hr Then the mixture was neutralized with NaOH and diluted with H_2O to 5ml The glucose formed was determined according to ref [29] Phosphate and ammonium were determined according to refs [30] and [31], respectively

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