Factors Affecting Callus Production and Glycosidal Content of Leaf Tissue Culture of *Digitalis lanata* Ehrh.

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Abstract

Grecian Foxglove, *Digitalis lanata* Ehrh. (Scrophulariaceae), is a perennial or biennial plant, which is a major source of digoxin and digitoxin. Its leaves are used for the preparation of these cardiac active glycosides for the treatment of cardio vascular disorders. This study was built on a suggestion that in *Digitalis* the explanted cells already contain all the genetic information that is required for the manufacture of digitoxin and digoxin. Accordingly, this genetic information will be present in callus from explant micro-culture. Activation by external factors such as light, the number of sub-cultures and type and age of the explants, should lead to stimulation, not only of callus size, but also the production of glycosides.

Segments of series explants taken from different Digitalis plant organs (shoot tip, leaf, hypocotyl and root), 2 and 4 weeks old, obtained from plants grown in vitro and of 8, 12 and 20 weeks from plants cultivated in vivo culture, were cultured on aseptic MS basal solid medium containing 5.0 mg/L 2.4-D and 0.5 mg/L BA. On the other hand, callus derived from different explants was recultured (in initial amounts of 49 – 63 mg) on the same new sterilised MS medium in order to increase the mass of callus. The cultures were then maintained in a growth chamber at $26^{o} \pm 2\ C^{o}$ and subjected to four different light photoperiods as follows: 10 hr. light /14 hr. dark cycle, 14 hr. light/10 hr. dark cycle, 16hr.light/ 8hr.dark cycle, and 18 hr. light/ 6 hr. dark cycle.

The obtained results show that the greatest callus production, as well as the highest amount of glycosides, occurred in callus derived from 2 or 20 week old leaf explants among all the examined types and ages of explants. Repeating the subculturing three times decreased dramatically the dry weight of callus, but it significantly favoured digoxin and digitoxin content. The optimal callus growth was obtained at 16 hr. light/day but the best glycosidal content was achieved when callus was exposed to 18hr. light/day.

INTRODUCTION

Digitalis lanata Ehrh (foxglove) is one of the most important medicinal plants and belongs to the family Scrophulariaceae. Its leaves are used for the preparation of the lanatoside (digoxin and digitoxin) glycosides, which are considered as the principal drugs available for treatment of cardio – vascular disorders (Trease and Evans, 1978). Secondary products associated with callus cultures consisting of undifferentiated masses of callus can be initiated from parenchymatous tissues of shoots, roots and other plant structures. In addition, the genetic information required for the manufacture of secondary metabolites is also present in the undifferentiated cells and when activated should lead to the production of these ingredients. Consequently, this aspect of callus culture with the aim of growing a particular plant tissue on a commercial scale under controlled conditions for the production of valuable metabolites will be a great achievement. Unfortunately, explant type and age are limiting factors in calli production. However, Lui and Staba (1979) detected cardenolides from root and leaf cultures of *Digitalis lanata*. Leaf cultures grow very rapidly and have a higher content of glycosides. This was

indicated also by Karting et al. (1979), Rucker (1983), Cacno et al. (1991), Brisa et al.(1991), Cellarova and Honcariv (1991) and Ladd et al. (1993) on *Digitalis.* On the other hand, the quantity of glycosiodal content in the callus decreased with the successive subculturing (Karting et al. 1976; Karting et al. 1983), yet the increased amount of the recultured callus might identify the decline occurred in the active ingredient due to the sequence subculturing .

Furthermore, little critical work has been carried out on the effects of light on callus formation of *Digitalis*, but a number of reports have shown that the light intensity and photoperiods have an important role on callus formation and secondary products synthesis in some other medicinal plants (Hesegawa et al. 1973; Reichling and Becker 1976; Murashige 1977; Knobloch et al. 1982; Bhatt et al. 1983; Nigra et al. 1989). In *Digitalis*, Hagimori et al. (1982) reported that the stimulatory effect of light on digitoxin formation was achieved by allowing the callus longer periods of light exposure. Scheibner et al. (1987) found that the optimum cardenolide amount was produced with white light and a photoperiod of 12 hours. Accordingly, this study focuses on the ability of some external factors such as light, explant type, age and number of subcultures to alter the callus production and its glycosidal content.

MATERIALS AND METHODS

This study was carried out in the Tissue Culture Laboratory of the Vegetable Department, Horticulture Research Institute, Dokki, and in the Tissue Culture Laboratory of the Horticulture Department, Faculty of Agriculture, Al-Azhar University during the seasons of 1994, 1995 and 1996.

D. lanata seeds used in this study were obtained from the Faculty of Pharmacy, University of Jordan, Amman, Jordan and were cultivated in two different ways, as follows:

Laboratory Cultivation (in vitro)

Seeds were washed several times with sterile distilled water and immersed in 20 % commercial clorox solution (1 % sodium hypochlorite) for 20 minutes and then washed three times with sterile distilled water in a laminar air flow hood to remove the residual sodium hypochlorite solution. Seeds were cultured in five sterile jars containing 25 ml of sterile MS solid medium and incubated at 25 ^{o}C for 7 - 10 days.

Field Cultivation (in vivo)

D. lanata seeds were sown in mid-November in shallow pots, covered with a thin layer of soil, carefully and regularly irrigated with water and kept in the lath house until emergence. Seedlings were transferred into 15 cm clay pots after they had formed about five leaves. One month later, the adaptive seedlings were transplanted into 30 cm pots and left until the flowering stage.

All the agricultural practices were done whenever necessary and the following experiments were carried out to study the effect of different external factors on callus formation, growth and glycosidal content.

Experiment I.: Effect of Explant Type on Callus Production and its Glycosidal Content

This experiment was designed to study the ability of different *D. lanata* organs (shoot tip, leaf, hypocotyl and root) to produce the highest value of callus with optimum glycosidal contents. To achieve these objectives, two week-old *D. lanata* seedlings grown in vitro were used to prepare uniform explants from the different organs (shoot tips, leaves, hypocotyls and roots) under sterilise conditions. The excised explants were cultured aseptically in 1.5 x 15 cm test tubes containing 10 ml of MS basal solid medium (solidified by adding 8 % agar).

Media were supplemented with 5.0 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D.), and benzyladenine (BA) at a level of 0.5 mg/L. The pH value was adjusted to 5.8 by

adding a suitable amount of 0.1 N HCl using a pH meter. The cultures were incubated in a growth chamber at $26^{o} \pm 2\ ^{o}C$ and 1500 lux white artificial light using cool white fluorescent lamps for 16 hr. light/day. Each treatment consisted of 20 test tubes and was replicated three times. After 45 days, callus dry weights were determined and the callus index was calculated according to the following formula, (nxG/N)X100 (Wakhlu and Barna, 1989).

Digoxin and digitoxin contents (µg/g dry weight) were determined as dried callus produced from explants grown on MS solid medium using RIA according to Farghaly (1992).

Experiment II.: Effect of Explant Age on Callus Production and Its Glycosidal Content

Segments of series explants differing in age (2 and 4 weeks-old) were taken from leaves of Digitalis seedlings grown in vitro. The excised explants were cultured aseptically on MS basal solid medium containing 5.0 mg/L 2,4-D. and 0.5 mg/L BA as utilised in the first experiment. The cultures were incubated at $26 \pm 2\ ^{o}C$ under 16 hr. light/day. Three replicates were used for each treatment with 20 test tubes for each replicate. After 45 days of incubation, samples of calli were taken for callus dry weight and determination of its glycoside content.

On the other hand, leaf explants from 8, 12 and 20 week-old plants cultivated in the field (*in vivo*) were excised and cultured aseptically on MS solid basal medium containing 5.0 mg/L of 2,4-D and 0.5 mg/L of BA and kept under the same conditions of temperature and light. Four replicates were used for each treatment with 20 test tubes for each replicate and the following data were recorded after 15, 30 and 45 days from callus manipulation: callus fresh weight, callus relative growth percentage calculated after 45 days according to Djoko and Thornburg (1992); callus growth rate (g/day) was calculated every 15 days during a period of 45 days according to Morsi and Abd El Gawad (1961), the callus increase value was recorded according to Szőke et al. (1970, 1979) and Krajewska et al (1982), as well as callus glycosidal content.

Experiment III.: Effect of Photoperiods on Callus Production and its Glycosidal Content

This experiment focused on examining the effect of the length of light exposure on callus production and its glycosidal content. For this reason, pieces of callus (48 - 50 mg) were cultured in jars containing the same MS solid basal medium used in experiment II.

Twenty-seven jars each containing 20 ml of MS medium were used for each treatment and were divided into 3 replicates of 9 jars each. The cultures were then maintained in a growth chamber at $26^{o} \pm 2\ ^{o}C$ and subjected to four different light photoperiods as follows:

10 hr. light /14 hr. dark cycle, (b) 14 hr. light / 10 hr. dark cycle, (c) 16 hr. light / 8 hr. dark cycle and (d) 18 hr. light / 6 hr. dark cycle. Callus fresh weight was determined every 15 days, and thecallus growth rate, and callus increase value were also measured. The glycosidal contents were estimated after 30 and 45 days from the start of incubation according to the RIA method.

The experimental design used in all the experiments was of a completely randomised design. Data were statistically analysed using ANOVA 2 or 3 ways of analysis with the help of a computer, while the L.S.D test was done as described by Snedecor and Cochran (1982) at 0.01 and 0.05 probability to verify differences between means of treatments.

Experiment IV.: Effect of Subculture Times on Callus Production and its Glycosidal Content

Callus derived from different explants of 4 week-old seedlings cultured in vitro, under the same conditions as experiment II, was recultured in an initial amount of 49 - 63

mg on the same new sterilised MS medium used in experiment II. This experiment was arranged in groups of jars divided into 3 replicates of 10 jars each and incubated under $26^{o} \pm 2°C$ and 16 hr. light/day. Culture was repeated in three subcultures at intervals of 30 days under the same conditions. The growth data for callus production as well as the glycosidal content were recorded before each subculture.

RESULTS AND DISCUSSION

Effect of Explant Type on Callus Production and Glycosidal Content of *D. lanata* Seedlings Cultivated in vitro

Data concerning callus dry weight and callus index as affected by the different explants used in this study are tabulated in Table 1. The results clearly showed that the highest callus index value was recorded for leaf explants followed by hypocotyl explants, while the lowest callus index was derived from root explants.

Rapid growth of leaf cultures was confirmed by the findings of Lui and Staba (1979), who mentioned that leaf cultures of *D. lanata* seedlings grow very rapidly as compared with root cultures. However, callus dry weight data showed a different direction than that of the callus index. The root explants produced significantly heavier callus dry weight compared with the other explants, despite the fact that it produced the lowest value for callus index. This may be due to the root explant not producing a greater number of callused cultures compared to other explants, while it yielded the biggest mass of callus.

Moreover, the highest content of digoxin and digitoxin (1.05 and 2.42 µg/g/d. wt. respectively), was extracted from callus produced from hypocotyl explants (Table, 1). In contrast, root explants tended to produce the lowest content of digoxin and digitoxin (0.067 and 0.055 µg/g/d. wt. respectively), although they gave the highest yield of callus dry weight (Table 1). Furthermore, shoot tip and leaf explants produced moderate values for digoxin and digitoxin contents, which were between the values for the hypocotyl and root. Moreover, the yield of digoxin and digitoxin per explant indicated that the digoxin and digitoxin content per explant had the same trend, as well as their formation, in dried callus whereas, the highest digoxin and digitoxin values/explant were obtained in callus derived from hypocotyl explants, but the root explants gave the lowest value, while shoot tip and leaf explants came in between.

In this connection, Kartnig et al. (1979) reported that calli of *D. purpurea* roots produced cardenolides, but in different patterns and in lower concentrations than callus cultures set up from over ground parts of the same plants.

Effect of Explant Age on Callus Production and its Glycosidal Content of *D. lanata* Seedlings Grown in vitro and in vivo

The variations in callus production as callus fresh and dry weight, relative growth percentage, callus growth rate and callus increase value as a response to the explant are illustrated in Table 2. Statistical analysis of variance for the obtained results clearly showed that the changes in callus production parameters were significant, affected due to the different explant ages and from which source the explants were taken (the in vitro and in vivo cultivation).

These results indicated that the highest callus dry weight was recorded for callus derived from 4 weeks-old explant (0.072 g), followed by callus derived from 20 week-old explants (0.152 g) for in vitro and in vivo cultivation, respectively. On the other hand, the lowest callus dry weight was achieved with callus derived from 2 weeks-old explants (0.055 g).

In addition, the dry weight of *D. lanata* callus derived from leaf explants cultivated in vivo indicated that the highest callus dry weight was produced from 20 week-old leaf explants (0.15 g), followed by callus from 12 and 8 week-old leaf explants (0.13 and 0.10 g respectively).

The variations in callus relative growth percentage were significantly affected

according to the physiological age of the explant, whereas the highest values (2602 %) were recorded for 2 week-old explants taken from in vitro grown seedlings and for 12 weeks-old explants taken from in vivo grown seedlings (3256 %).

The same trend was also observed regarding callus growth rate (g/day) and callus increase value; their values increased constantly to reach the maximum with explants taken from seedlings 2 weeks and 12 or 20 weeks old grown in vitro and in vivo cultivation, respectively.

Hughes (1981) stated that the physiological age of explants affected the type and extent of morphogenesis. The youngest and less differentiated meristematic tissues would give a successful culture, while tissues older than meristems would give a higher degree of morphogenic competence than meristamatic tissue .

Data presented in Table 3 illustrates the variations in digoxin and digitoxin contents in callus derived from the different explants taken from in vitro and in vivo grown seedlings.

It was clear that digoxin and digitoxin formation through *D. lanata* callus was significantly affected by explant physiological age for seedlings grown in vitro. Two week-old explants had less potential to form glycosides in their tissues compared to 4 week-old explants.

In this context, callus derived from 20 week-old explants produced the highest digoxin and digitoxin values concerning explants taken from seedlings grown in vivo cultivation. This means that the explant source distinguishably affected secondary products in *D. lanata*.

The highest digoxin level was recorded for callus produced from 20 week-old leaf explants (1.29 µg/g/d. wt.), followed by callus resulting from 12 and 8 week-old leaf explants (0.54 and 0.43 µg/g/d. wt., respectively). The highest content of digitoxin was achieved with callus derived from 20 week-old leaf explant (3.16 µg/g/d. wt.), followed by callus from 12 and 8 weeks-old leaf explant (0.14 and 0.057 µg/g/d. wt.), respectively, concerning the explants grown in vivo (Table 3).

In addition, the highest value of digitoxin was from callus derived from 2 week-old seedlings (0.023 µg / explant), while the highest amount of digoxin was in callus obtained from 4 week-old seedlings (0.028 µg / explant).

Two week-old explants tended to stimulate digoxin and digitoxin formation in the callused tissues more than 4 week-old explants (Table 3). This agrees with Lui and Staba (1979), who stated that the digoxin concentration presented in the leaf culture may be related to either its age or its stage of development.

In addition, the highest yield of digoxin and digitoxin per explant presented in callus derived from leaf explants cultivated in vivo when produced with callus derived from 20 week-old leaf explants (0.20 and 0.50 mg/explant respectively). The younger explant gave the lowest production. This was in agreement with the finding of Lui and Staba (1981).

It was evident from the results that the glycosidal content of *D. lanata* was affected by the age of the explant. In this connection, Lui and Staba (1981) reported that the concentration of digoxin in the leaf culture, as determined by radioimmuno-assay, varied from 25-44 % (dry weight) over 12 weeks as a result of the increase in age. The lower levels presented in younger leaf cultures may be related to their rapid growth.

Effect of Photoperiods on Callus Production and its Glycosidal Content

Data of Table 4 illustrates that the callus mass production reached its maximum value (1.09 g) when it was subjected to 16 hr. light/day, and it dropped to 0.63 g when the photoperiod was extended to 10 hr light/day.

On the other hand, the optimal condition for callus dry weight formation was achieved when callus was exposed to 16 hr light/day (0.133g). This explained that *Digitalis* callus growth is sensitive to photoperiod, whereas the photoperiod of 16 hr. light/day was better than the short period of 10 hr light/day as well as the longest period of 18 hr light/day.

In addition, callus which was exposed to 16 hr light/day produced the highest significant value of callus relative to growth percentage (3216 %) but, the lowest relative growth percentage (1806 %) was obtained from callus subjected to 18 hr light/day.

Moreover, callus growth rate increased constantly due to the different light photoperiods reaching its maximum value (0.035 g/day) under 16 hr. light/day; the growth rate then declined to 0.019 g/day when the photoperiod was raised to 18 hr. light/day (Table 4).

The variation of the light exposure period affected significantly the callus increase value. Increasing the photoperiod from 10 to 16 hr/day caused a visible increase in this parameter, reaching its maximum value (20.73), with a photoperiod of 16 hr/day. The increase of the photoperiod for more than 16 hr/day up to 18 hr/day significantly decreased callus increase value to 12.131.

In conclusion, it seemed that light has an important role in callus formation. These results confirmed the findings of Hesegwa et al. (1973) who reported that 16 hr light/day was the optimum daily light period for callus formation in *Asparagus*.

However, digoxin formation in callus negatively responded when increasing the length of the photoperiod up to 16 hr. light/day, while increasing the photoperiod up to 18 hr. light/day caused a slight increase in digoxin content compared with the above mentioned period. The highest content of digoxin (0.032 µg/g/d. wt.) was formed in callus which had been subjected to the shortest photoperiod of 10 hr. light/day.

The variation in digitoxin formation as affected by photoperiod treatments had the same response as digoxin in that increasing the photoperiod decreased the amount of digitoxin reaching its minimum value with 16 hr. light/day treatment, but its level rose quickly reaching its highest level of 0.38 µg/g/d. wt. with 18 hr light/day (Table 5).

In addition, it seemed that digitoxin accumulation differed markedly with photoperiod treatments, while the content of digoxin remained almost unchanged within the examined light photoperiods.

In this respect, Hagimori et al. (1982) reported that the stimulatory effect of light on digitoxin formation allows the callus cell to be closer to the status of the leaf cell and the instant increase in digitoxin content of the callus occurs in the longest period of light exposure. This could be interpreted as light inducing the formation of proplastids, which contain the cardenolide biosynthesis system. This means that the digitoxin increase was mainly due to the morphological change which occurred in callus growth rather than the direct effect of light. These suggestions for *Digitalis* were also confirmed by Ohlsson et al. (1983).

On the other hand, digoxin content calculated as µg/explant had a reverse response for photoperiod elongation than that of its content measured as µg/g/dry weight. The yield of digoxin per explant had a slight change with elongation of light exposure period. This may be due to the variation between callus dry weight giving a counter balance to the digoxin content among all the examined photoperiods. The highest amount of digoxin/explant was obtained when callus was exposed to 16 hr light/day (0.0031 µg / explant).

The highest value of digitoxin / explant was recorded with 18 hr. light/day (0.029 µg/explant).

Effect of Subculturing Times on Callus Production and its Glycosidal Content of *D. lanata* Seedlings Cultivated in vitro

Callus production in this study was expressed as dry weight in Table 6. It is observed from the data that the dry weights for all calli decreased with increasing subculture times. The highest amount of callus dry weight was obtained from callus derived from explants at the first subculture (0.15 g). Repeating subculturing twice and thrice decreased the callus dry weight by about 57 to 75 %, respectively from the first subculturing.

The lowest callus dry weight was observed with callus derived from root explants (0.073g). Leaf and shoot tip explants promoted callus formation significantly more

among all the subculturing times compared to the other hypocotyl or root explants. Shoot tip explants gave more callus dry weight(0.16 g) than leaf explants (0.15 g), especially in the first subculture. The differences were highly significant (Table 6).

Furthermore, the highest amount of digoxin was established by the subsequent culturing of the initial callus derived from leaf or/and hypocotyl explants, which produced the same average value for digoxin (0.044 µg/g/d. wt.). However, leaf explants had formed the highest value / explant (0.004 µg / explant).

The same trend holds true for digitoxin content. Its average was 0.31 µg/g/d. wt. or 0.019 µg / explant for the callus derived from leaf explants for the three subculturings. The lowest average for both digoxin and digitoxin / dry weight or / explant were obtained from callus derived from root subcultures.

Our results are confirmed by the findings of Elmar and Meinhart (1976) who reported that the main site of cardenolide storage and synthesis in *Digitalis* occurs in the leaves and are not prominent in roots.

The continuous re-culturing of callus induced its glycosidal content, which reached its maximum in the third subculture period. The highest accumulation of digoxin or digitoxin was observed when callus was subcultured from leaf explants and repeated 3 times, which gave 0.058 and 0.67 µg/g/d. wt. for digoxin and digitoxin, respectively (Table 6).

Table 6 also shows that the highest average of digoxin /explant was achieved with callus yielded in the first subculture (0.005 µg/explant). This might be due to the increase which occurred in callus dry weight of the first subculture.

Furthermore, the highest average for digitoxin /explant was achieved with the callus produced in the third subculture (0.011 µg/explant) as a result of the high digitoxin level of the third subculture itself and not to the callus dry weight. It was clear that leaf callus gave the best result for digoxin and digitoxin yield / explant (0.004 and 0.019 µg / explant respectively), as the total yield of glycosides was dependant upon both dry weight of callus, the amount of glycosides and consequently any factors affecting the callus dry weight or glycoside biosynthesis and its accumulation in plant tissues.

The results were in agreement with Seidal and Reinhard (1987) and Moldenhauer et al. (1990), but it did not agree with the finding of Kartnig et al. (1976) on *Digitalis*, who reported that the number and quantity of cardenolides decreased with successive subculturing. The variations in callus dry weight were significant at two levels of significance due to the incubation periods, explant types and their interactions. The first subculture was the best for producing the highest callus dry weight (0.15 g), which decreased by increasing the subsequent numbers of subcultures, regardless of explant type.

Statistical analysis of variance showed that the highest callus dry weight (0.16 g) was recorded for shoot tip explants which had been incubated for one month under 26 $\pm$2° C temperature and 16 hr. light/day conditions, while the lowest value (0.020 g) was obtained from root explants at the third subculture. Leaf explants formed a normal average amount of callus as compared to the hypocotyl and root explants, but it was still significantly less than that formed by shoot tip explants.

CONCLUSIONS

The objectives of this study were to investigate the effect of type and age of explant, time of light exposure (photoperiods) and subculturing times on *D. lanata* callus production and its glycosidal content grown either in vivo and / or in vitro cultivation.

D. lanata seeds were cultivated in both laboratory and lathhouse. Seedlings were used as a source for explants in the different serial of experiments.

Our results showed that leaf explants of *D. lanata* are a valuable starting point for callus production, especially if they are taken from 2 or 12 week-old seedlings grown in vitro or in vivo, respectively, and cultured on MS solid medium supplemented with 5.0 mg/L 2,4 D + 0.5 mg/l BA and incubated for 45 days at 26+2° C and 16 hr light / day.

Leaf cultures gave the biggest value for callus production, in most cases derived

from hypocotyl, while root cultures gave the lowest value.

The most suitable callus production in the form of fresh weight, callus growth rate and callus increase value was achieved in cultures of 2 week-old leaf explants grown in vitro or 12 weeks-old explants grown in vivo.

Moreover, callus formation depends remarkably on subculturing time and explant source. The minimum value for callus production was obtained from the third subculture and the highest yield (g/dry weight) was produced from the first subculture. Yet, the highest amount of callus dry weight was formed by shoot tip produced callus at the first subculturing, while root cultures gave the lowest value, especially at the third subculturing.

In addition, changes in the photoperiod other than 16 hours seemed to be unsuitable for callus formation. However, cultures exposed to 16 hours of photoperiod tended to produce the highest callus production, especially with 45 days of light incubation.

Another remarkable point is that the efficiency of digoxin and digitoxin production in *D. lanata* appeared to be strongly dependent on the examined limiting factors.

An important practical conclusion from our results in this case is that the highest production of digoxin and digitoxin detected on callus dry weight was achieved with hypocotyl explants grown on MS medium supplemented with 5.0 mg/L 2,4-D and 0.5 mg/L BA and from two week-old leaf explants taken from in vitro grown seedlings or from 20 weeks-old leaf explants prepared from in vivo grown plants.

Increasing the time of subculture led to an increase in digoxin and digtoxin production. In addition, the highest digoxin formation was achieved in the shortest photoperiod (10 hr. light /day).

A linear increase in digitoxin content was correlated with increasing the photoperiod, reaching a maximum with 18 hr. light /day.

Digoxin formation responded differently to photoperiod treatments depending on the base of determination. The shortest photoperiod (10 hr. light/day) was favoured for this process when determined as mg/g/d. wt., while 16 hr light/day photoperiod was more suitable when measured as mg/explant.

From this study the researchers suggest that using the tissue culture technique is a very important tool to produce effective material in vitro for medicinal plants. This technique has a high economical value, especially in the case of production of secondary metabolites free from pesticide contamination for local handling and export.

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Tables

Table 1. Effect of explant type on callus production, digoxin and digitoxin content derived from *Digitalis lanta* seedlings

Explant type		Callus index	Callus dry wt.	Digoxin		Digitoxin	
				µg/g/dry weight	µg/explant	µg/g/dry weight	µg/explant
Shoot tip		109.25	0.052	0.559	0.029	0.928	0.043
Leaf		126.6	0.061	0.190	0.011	0.247	0.013
Hypocotyl		118.6	0.058	1.047	0.055	2.416	0.126
Root		97.05	0.077	0.067	0.004	0.055	0.003
LSD	**1%**	2.46	0.0024	-	-	-	-
	5%	1.82	0.0018	-	-	-	-

Table 3. Effect of explant age on digoxin and digitoxin content in callus derived from *Digitalis lanata* seedlings grown in vitro and in vivo cultivation

Explant source	Age by weeks	Digoxin		Digitoxin	
		µg/g/dry weight	µg/explant	µg/g/dry weight	µg/explant
Laboratory cultivation (in vitro)	2	0.296	0.016	0.433	0.023
	4	0.400	0.028	0.083	0.006
Field cultivation (in vivo)	8	0.432	0.044	0.057	0.006
	12	0.543	0.072	0.137	0.018
	20	1.296	0.205	3.158	0.499

Table 2. Effect of explant age on callus production derived from *D. lanata* seedlings growing in vitro and in vivo

Explant source	Age (weeks)		Intial Weight (g)	Callus fresh wt. (g)	Callus dry wt. (g)	Callus relative growth	Callus growth rate	Callus increase value
Laboratory cultivation (in vitro)	2		0.050	0.830	0.053	2602.0	0.028	15.61
	4		0.055	0.672	0.072	1974.0	0.024	11.22
	LSD	5%	Ns	0.028	0.0012	93.1	0.0028	0.47
		1%	ns	0.038	0.0008	155.20	0.0030	0.54
Field cultivation (in vivo)	8		0.049	0.881	0.103	2638.7	0.028	16.98
	12		0.052	1.064	0.132	3256.0	0.037	19.46
	20		0.050	1.00	0.152	3418.0	0.039	19.01
	LSD	5%	ns	0.017	0.002	61.5	0.0011	0.34
		1%	ns	0.024	0.004	93.1	0.0014	0.47

Table 4. Effect of photoperiod (hrs) on callus product derived from *Digitalis lanata* seedlings

Photoperiod Light/day (hrs)		**Initial wt of callus (g)**	**callus fresh wt (g)**	**callus dry wt. (g)**	**callus relative growth %**	**callus rate (g/day)**	**callus increase value**
10		0.049	0.801	0.086	2110.2	0. 022	15.34
14		0.050	0.952	0.113	2582.0	0.028	18.03
16		0.050	1.086	0.133	3216.0	0.035	20.73
18		0.048	0.630	0.074	1806.2	0.019	12.13
L S D	**5%**	ns	0.0209	0.0025	ns	0.0010	0.426
	1%	ns	0.0283	0.0034	ns	0.0013	0.578

Table 5. Effect of photoperiod (hrs) on digoxin and digitoxin content in callus derived from *Digitalis lanata* seedlings

Photoperiod	**Digoxin content**		**Digitoxin content**	
Light/day (hrs)	**µg/g/dry weight**	**µg/explant**	**µg/g/dry weight**	**µg/explant**
10	0.032	0.0026	0.255	0.0221
14	0.026	0.0029	0.177	0.0204
16	0.023	0.0031	0.122	0.0164
18	0.028	0.0021	0.378	0.0291

Table 6. Effect of subculture time on callus production (as dry weight), digoxin and digitoxin contents derived from different explants of *Digitalis lanata* seedlings cultured in vitro

Data	Callus dry weight (g)				Digoxin content								Digitoxin content							
					µg/g/dry weight				µg/explant				µg/g/dry weight				µg/explant			
Subculture time	1	2	3	main	1	2	3	main	1	2	3	main	1	2	3	main	1	2	3	main
Shoot tip	0.157	0.065	0.050	0.090	0.025	0.023	0.048	0.032	0.004	0.001	0.002	0.002	0.022	0.025	0.160	0.069	0.003	0.002	0.008	0.004
Leaf	0.150	0.062	0.047	0.086	0.040	0.035	0058	0.044	0.006	0.002	0.003	0.004	0.105	0.167	0.667	0.313	0.016	0.010	0.031	0.019
Hypocotyl	0.139	0.068	0.033	0.080	0.037	0.041	0.055	0.044	0.005	0.003	0.002	0.003	0.053	0.071	0.107	0.077	0.007	0.005	0.004	0.005
Root	0.144	0.057	0.020	0.073	0.031	0.042	0.044	0.039	0.004	0.002	0.001	0.002	0.038	0.062	0.084	0.051	0.005	0.003	0.002	0.003
Average	0.147	0.063	0.037	0.082	0.013	0.015	0.031	0.019	0.005	0.002	0.002	0.002	0.054	0.081	0.254	0.127	0.007	0.005	0.011	0.008

L.S.D	1%	5%
explant	0.0033	0.0025
subculture	0.0029	0.0021
explant X subculture	0.0058	0.0043