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INCREASING PRODUCTIVITY OF DIGOXIN CONTENT IN DIGITALIS LANATA EHRH "*IN VITRO*"

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ABSTRACT

Axillary buds Cultures of *Digitalis lanata* Ehrh were established from seedling, obtained under aseptic conditions. Shoot tip and single node explants were cultured on MS basal solid medium with various of BA concentrations. Shoots proliferation and growth were significantly influenced by BA levels and explant type. The highest amount of shoots was obtained on medium supplemented with 5.0 µM of BA, while digoxin and digitoxin contents affected by high level of BA (17.5 µM). A further subculture to medium containing high levels of BA promoted shoot growth and suppressed glycoside contents. Production of digoxin and digitoxin improved upon transferring shoots onto half strength on MS medium, vitrified shoots were appeared as well. Interestingly, addition progesterone as precursor, led to enhancement of digoxin and digitoxin contents, especially on rooted shoots.
Keywords: *Digitalis lanata* Ehrh, Digoxin, Cardiac glycosides, Growth regulators, Progesterone, Precursors. Medium strength.

INTRODUCTION

Many higher plants are major source of natural products used as pharmaceutical compounds, agrochemical, flavours and fragrances ingredient and pesticides. In fact, the most of prescription drugs used in the world is extracted from plants or is based on chemical structures of compounds produced by plants. The supply of these compounds is limited by the availability of the plant. *Digitalis lanata* plant belonging to family schrophulreacea is valuable for cardiac glycosides production. Cardio-active glycosides appear in several plant families which mostly are not related to each other; the majority of the research into cardiac glycosides revolves around the usage of *Digitalis* and its manipulation for enhanced secondary metabolites formation. *Digitalis* cardenolides, especially digoxin and digitoxin are important in medicine and cardenolide production by cultured cell of *Digitalis.* Owing to the commercial interest in cardenolides produced by species of the genus *Digitalis*, much work has been done on in vitro culture of several species of this genus, the aim has been to achieve an economically viable production of the metabolites by cultured cells (Luchner & Diettrich, 1988). However, no updated production system of sufficient economic interest for commercial application has been developed. One of the alternatives for improving the yield of such substances from cultured cells is the study of alternative species.(Cacho *et al*., 1991). This research seeks to produce these valuable compounds through an alternative means, using tissue cultures of *Digitalis lanata* Ehrh. Cell and tissue culture would provide a means of producing these valuable drug molecules more consistently, independent of weather or season and at a higher concentration than that

found in the plant. Shoot tip culture has been widely used for rapid propagation of many species due to its advantage over traditional methods. Moreover, the multiple shoots produced in the culture can be used as an alternative source of metabolites specially, in plants where the production is dependent on morphogenesis (Herrera, *et al.,* 1990). Plant tissue culture technique has become a powerful tool in plant biotechnology. The potential of plant tissue culture for plant propagation and production of secondary metabolites has itself provided substantial for researcher (Dicosmo *et al*., 1995). In previous study, it was examined the effect of certain growth regulators, carbon sources, some major and minor-elements of MS basal medium used, and precursor of the cardenolide biosynthesis (Bosila, 2001). The overall vision of this research is to meet the needs and demands of important plant-derived pharmaceuticals by using plant tissue culture. However, the objectives of this research are to overcome the current challenges in the production of valuable plant-derived drugs (digoxin and digitoxin) by enhancing production at the cellular level and tissue.

MATERIALS AND METHODS

This study was carried out in the plant tissue culture laboratory, Horticulture dept., Al Azhar University, Cairo, Egypt, through the period from 2005 to 2006.

Establishment of culture

Shoots initiation

Seeds of *Digitalis lanata* Ehrh were surface disinfected with 1% sodium hypochlorite and three drops of tween 20 for 15 minutes, and then rinsed exhaustively with sterile distilled water. The seeds were germinated aseptically on MS basal medium solidified by 0.7% of agar (Difco bacto) and 3 % sucrose. The pH value of medium was adjusted to 5.8 prior to autoclaving at 121 ^{0}C for 20 minutes. The cultures were kept in growth chamber at 26± 2 ^{0}C with a 16 h. photoperiod by cool florescent lamp.

When the seedling were 15 days-old, shoot tip and single node explants were cultured in jar (ca. 370 ml) containing 50 ml of sterile MS basal solid medium supplemented with various concentrations (2.5, 5.0, 7.5, 15.0, 17.5 and 25.0 µM) of BA (6- benzyl adenine). All jars were incubated under the conditions indicated previously and observed weekly. Final data were recorded after four weeks. All data were the average of 27 explants per treatment. Shoots fresh and dry weight (g/explant) was recorded, glycoside contents as digoxin and digitoxin were determined by HPLC method (mg/g.dry.weight) as well. Superior multiple shoots were derived from shoot tip explant, subcultured every 4 weeks for three months on MS basal solid medium with 17.5 µM of BA to produce shoots enough for the following experiments

Effect of BA concentrations on shoot growth and Digoxin content.

The best result deals with glycoside contents were selected for this experiment. Multiple shoots which were derived from shoot tip explants, cultured on MS solid medium supplemented with 0.5, 2.5, 5.0, 7.5 and 10.0

µM. All cultures were incubated for 8 weeks. Data were recorded as shoot kinetic growth, fresh and dry weight (g),length, number of shoot, leaf number, number of nodes, root number and length, callus weight, digoxin and digitoxin content as well (mg/g.dry.weight)

Effect of MS salt strength medium on shoot growth and digoxin content.

Multiple shoots were cultured on different strength (¼ , ½ , ¾ and 1) of MS basal solid medium growth regulators-free. All cultures were incubated for 4 weeks and data were recorded as shoot fresh and dry weight (g/explant) were recorded, glycoside contents as digoxin and digitoxin.

Effect of precursor (progesterone) on glycoside content as digoxin and digitoxin.

This experiment was carried out to study the impact of residual of BA an progesterone on digoxin and digitoxin content and the importance of rooting in absorption process *in vitro*. Obtained shoots (in third subculture) were divided into two groups. The first was grown on MS solid medium plus NAA in rate of 4 mg/l to induce the roots on shoots (pilot experiment was carried out before). The second group was cultured on the same medium with 25µM BA to devoid rooting. All cultures were grown on MS medium containing different concentrations of BA (0.0, 2.5 and 5.0 µM) for two weeks, then both groups were inoculated with 5ml progesterone in rate of 100 mg/l and incubated for another two weeks. Data was taken as glycoside content as digoxin and digitoxin in all treatments.

For all experiments, the medium was adjusted to pH 5.8 and solidified by 0.7% agar and dispensed into jars (50 ml). The cultures were then autoclaved at 1.3 kg/cm^2 pressure for 20 minutes. Following inoculation, all the cultures were maintained in a 16 h photoperiod, having light intensity of 2000 lux from florescent lamp at temperature 26±2 ºC. Subculture was carried out at regular intervals to induce number of shoots. Data were recorded as mentioned above,

Measurement of Kinetic growth

1. Fresh weight of shoots determined by weighing shoots after washing with distilled water (to remove the adhering).
2. Dry weight of shoots was determined by weighing samples after drying an oven at 60ºC until the weight became constant, according to Balbaa, *et al.* (1974).
3- Length & number of shoot, leaf number, number of nods, root number and length, callus weight, as well.

Chemical analysis

Quantitative analysis of all samples was carried out in the plant tissue culture laboratory, Horticulture dept., Al Azhar University, Cairo, Egypt.

Preparation of samples

Glycoside contents as digoxin and digitoxin were performed on dry shoot samples derived from all treatments tested by HPLC as mg/g.dry.weight (mg/g.D.W.)

Shoot samples were completely dried, accurately weighed and each was transferred to a 50 ml beaker. A 10 ml aliquot of 100% methanol was added to each sample, and then the solution was agitated on shaker overnight. The resulting extracts were filtered. Each extract was completely

dried and subsequently re-suspended in 1 ml methanol in preparation for injection into high performance liquid chromatography (Sep-PakC_{18} cartridge, MeCN-MeOH-H_2O (20:1:50) and uv detection (220 nm).All chemicals that was used for analytical method and authentic compounds purchased form Sigma company, Egypt.

Statistical analysis:

All experiments were conducted under controlled conditions and followed complete randomized design (CRD) with three replications and 9 jar for each replicate. Duncan's multiple Range Test (Sndecor and Cochran, 1982) was used with the help of MSTATE software**.**

RESULTS AND DISCUSSION

Establishment of shoots

Different concentration of 6- benzyladenine (BA) were singly used on MS basal solid medium for optimizing multiple shoots regulation from shoot tip and single node explants (Table,1). The response was fluctuated in both type of explants. The optimum shoots proliferation was achieved after 30 days of culture, longer culture periods caused necrosis and vitrified shoots. Shoots proliferation and growth were observed in all levels of BA tested. Irrespective of their origin, shoots formation began after 7-10 days from incubation period. Among the various hormonal supplemented, the best response towards multiple shoots proliferation was observed from shoot tip and single node on MS medium fortified with 5.0 µM and 7.5 µM respectively, the average of shoot fresh weight from shoot tip was (0.389 g /explant) against 0.247 g from single node explant. Also, the results clearly observed that, increasing the amount of multiple shoots, especially with shoot tip explants.

On the other hand, both explants failed to form any new shoots on medium benzyladenine-free. Moreover, the poor response was observed in medium, cultured with single node with 25 µM of BA (0.053 g/explant). In general, from statistical analysis, it could be concluded that, fresh weight of shoots was gradually decreased by enhancing the level of BA, It's also evident that, addition of BA in rate of 2.5 or 5.0 µM was strongly affected fresh weight of shoots. On contrast, single node was relatively affected by moderate levels of BA, especially in rate of 7.5 and 15.0 µM. However, in these rates, no significant effect was observed. It was also recorded that, addition of BA higher than 15.0 µM was strongly decline fresh weight of shoots. However, although shoot tip explants appeared evident success, it was used in limited number in comparison with single node explants.

Concerning shoot dry weight, both explants took the same trends with BA concentrations and explant types. However, the different observation may be due to form callus around the base of shoots (Fig, 1) or and different humidity percentage of shoots. However, the highest amount (0.180 g/ explant) for both explants was achieved with 5.0 and 7.5 µM of BA for shoot tip and single node respectively.

From statistical analysis, it could be concluded that, although, the fresh and dry shoots took different values, they were significantly affected by low levels of BA. despite, BA plays a positive role for shoots proliferation, it also play a critical role for fluctuation of glycosides production. So, it must be used more suitable medium for both purpose. This result agrees with the finding Hu and Wang, (1983), they found that, the addition of cytokinins to the medium is necessary to achieve shoot tip multiplication. These results closely consist with Vela *et al*.(1991) on *Digitalis* plant, they found that, digoxigenen derivative were determined in all clonolly-propagated plant, but the amount of these glycosides was much higher in those obtained from axillary buds. Also, they found the de-novo synthesis of cardenolides in *Digitalis obscura* L cultures seems to be closely related to morphologic differentiation. One of the main problems facing the practical of plant cell culture for the production of phytochemicals is that, undifferentiated cell cultures don't often form sertain compounds. However, after morphogenesis for cultures, the regenerants are able to accumulate a lot of these compounds. In this concern, many invetigators have obtained Hypercin and hyperforin from shootlet meristem cultures in vitro from *Hypericum* plant (Kirakosyan *et al*. 2000 & 2004 and Murch *et al*. 2000)

Table (1): Effect of explant type on shoot growth and glycosides content of *Digitalis lanata* Ehrh grown on MS solid medium and supplemented with different levels of BA after 4weeks.

Explants	BA µM	Fresh weight g/explant	Dry weight g/explant	Digoxin mg/g.D.wt.
Shoot tip	**2.5**	0.245 b	0.170 a	0.327
	5.0	0.389 a	0.180 a	0.525
	7.5	0.170 c	0.070 b	0.158
	15.0	0.150 c	0.053 b	0.222
	17.5	0.110 c	0.040 b	1.959
	25.0	0.130 c	0.032 b	0.694
Single node	**2.5**	0.194 b	0.110 b	0.728
	5.0	0.085 cd	0.040 c	0.469
	7.5	0.247 a	0.180 a	0.227
	15.0	0.229 a	0.130 ab	0.398
	17.5	0.129 bc	0.040 c	0.640
	25.0	0.053 d	0.030 c	0.635

Different letters within columns indicate significant difference (P ≤ 0.05) according to Duncan's multiple range test.

Effect of BA concentrations on shoot growth and Digoxin content

Data in Figure (2, A-f) show the kinetic growth of shoots resulted in shoot tip explant and cultured on Ms basal medium with different levels of BA. From the results, all parameters tested affected by BA concentrations. Fig. (2-D) show that, BA in level of 5.0 µl achieved the longest shoot (6.5 cm.) when cultured on MS medium followed by 0.5 µl of BA (5.7 cm.), also the shoot number (Fig, 2-B) and leaf length, (Fig, 2-E) were affected by 0.5 µl of BA, whereas recorded the highest number (5.6 shoot/explant) in comparison with all treatments. While, increasing BA level till 7.5 µl, increase the leaves

number, (47.3/explant), (Fig.2-C), as well as shoot weight (Fig, 2-F) and of node numbers (Fig, 2-A) ((3.76 g/explant and 9.3 node/explant) respectively. Moreover, the root numbers (Fig. 2-G) and root length (Fig. 2-H) were affected by high level of BA and moderate, whereas recorded the highest value with 5.0 and 10.0 µl of BA. Callus weight (Fig. 2-I) was differently affected by levels of BA. Whereas, callus reach to maximum value on medium contained BA in rate of 7.5 µl (0.560 g/explant). Also, from noticeable results, it's clear that, the response for all parameters were differently affected by BA, but most effect took place with 5.0 and 7.5 µl of BA except in callus weight, BA in rate 10.0 µl formed high amount.

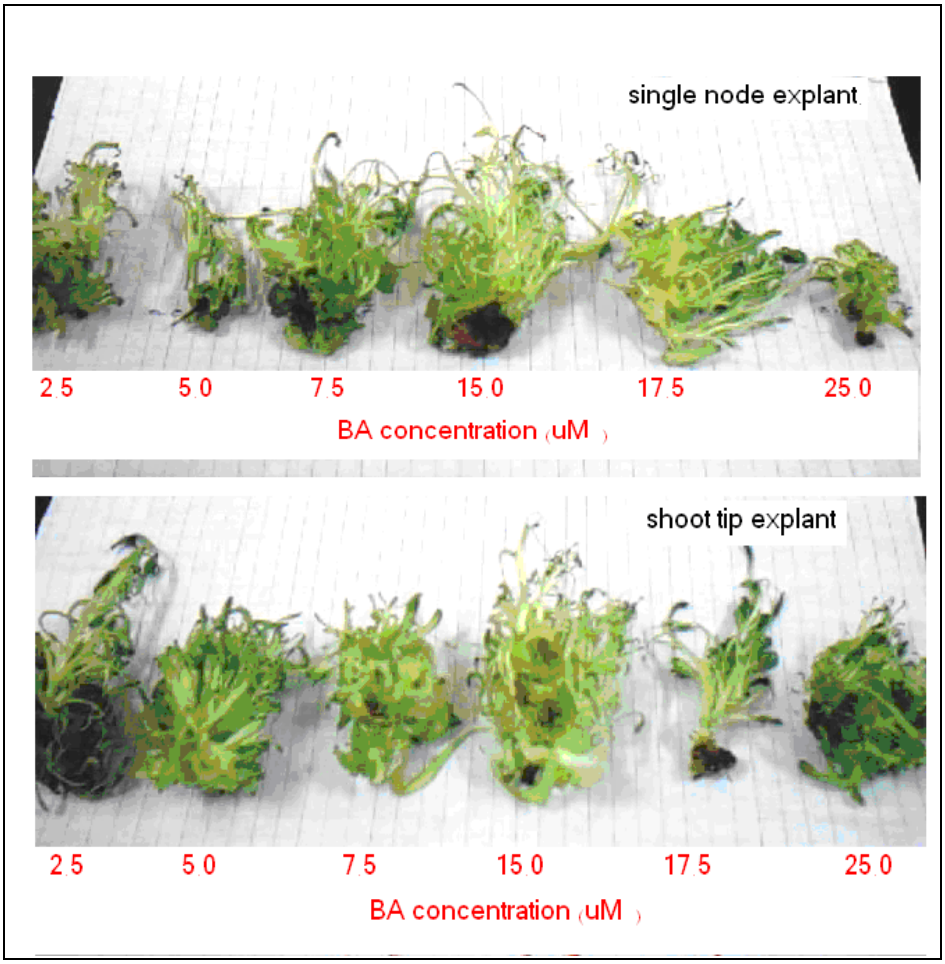

Fig.(1): Effect of explant type on shoot growth and glycosides content of *Digitalis lanata* Ehrh grown on MS solid medium and supplemented with different levels of BA after 4weeks (Photo).

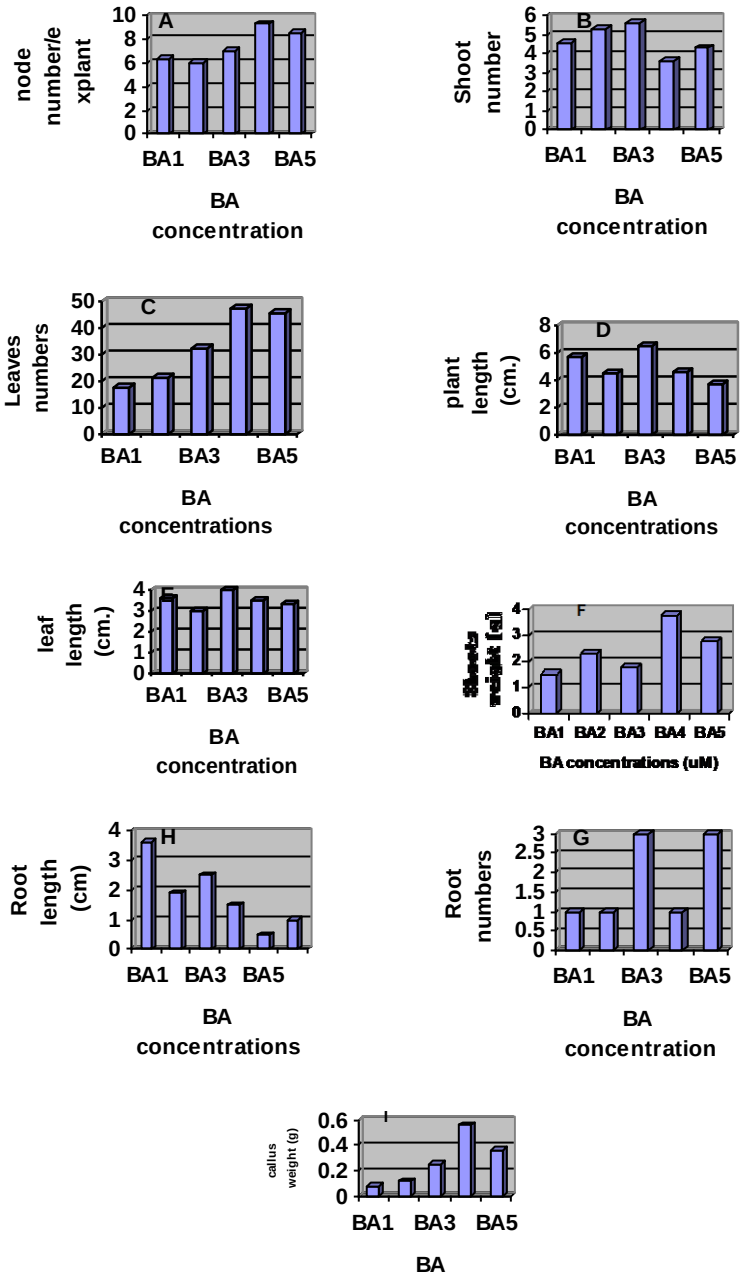

Figure (2): Effect of BA concentrations on shoot proliferation and growth, derived from shoot tip explants of *Digitalis lanata* Ehrh, grown on MS basal solid medium.

Where: BA1 is 0.5 µM, BA2 is 2.5 µM, BA3 is 5.0 µM, BA4 is 7.5 µM, and BA5 is 10.0 µM.

Concerning digoxin and digitoxin contents, Data in Table (2) indicate that, both low and high rate of BA achieved the highest amount (3.387 and 3.154 mg/g.D.W) with 0.5 and 10.5 µl of BA, respectively. These results reveal to the importance of BA levels and undifferentiated callus on digoxin and digitoxin contents. Whereas, in pervious study on *Digitalis lanata* (Bosila 2001), undifferentiated callus was recorded the low amount of digoxin and digitoxin contents (‹14 ng/g.D.W) in this concern, the result closely consist with Vela *et al*., (1991) on *Digitalis* plant, they found that, digoxigenen derivative were found in all clonally-prpagated plants, but the amount of these glycosides was much higher in those obtained from axillary buds. ……. So, the obtained results is considered worthwhile (three-fold of the control) for improve the digoxin and digitoxin production through tissue culture techniques.

Table (2): Effect of BA concentrations on shoot growth and glycosides content derived from *Digitalis lanata* Ehrh shoot tip explant.

BA µM	Shoot D.W. g/explant	Digoxin and digitoxin content	
		mg/g D.W.	% of control
0.5	0.370 b	3.387	131.43
2.5	0.270 c	0.314	12.18
5.0	0.460 a	0.188	7.30
7.5	0.350 bc	0.118	4.58
10.0	0.410 ab	3.154	122.39
ontrol	0.176 d	2.577	100.0

Different letters within columns indicate significant difference (P ≤ 0.05) according to Duncan's multiple range test.

Effect of MS salt strength medium on shoot growth and digoxin content.

Data in Table (3) show that, fresh weight of shoots was significantly affected by MS medium strength. Shoots growth as fresh weight was responded with increasing MS strength. Low rate of MS strength (¼ MS) was more effective for stimulating fresh amount of shoots in comparison with other MS strength tested due to callus formation on shoot base. So, the highest amount of shoots was recorded with ¼ MS medium (5.55 g/explant). Also, it's evident that, no significant between full strength and ½ MS because of callus formation on shoots base, too. In this respect, it could be concluded that, callus formation on shoot base was the critical factor for result variation. Also, type of callus affected fresh and dry weight, whereas, compact callus is heavier than friable as well as high humidity percentage in callus.

Concerning shoot dry weight, there is low significant between all treatments.With regard to glycoside contents as digoxin and digitoxin, it's evident that, shoots growth under ½ MS medium improved biosynthesis process and increase the glycosides contents, whereas shoots were grown on ½ MS medium achieved the highest amount of digoxin and digitoxin (3.39 mg/g.D.W), followed by ¼ MS medium (3.12 mg/g.D.W).This results parallel observations made on other plant systems; *Catharanthus roseus* (Madagascar periwinkle) and *Rhodiola sachalinensis* (cartic root) in which compact globular structures constitute a very good system for secondary metabolite synthesis (Verpoorte, 1996). Generally, ¾ MS and ½ MS medium increased digoxin and digitoxin contents more than three-fold of the control.

From the results, it could be concluded that, MS composition affect shoot growth and glycoside contents. In this concern, quite how these elements affect the digitoxin formathion is open to discussion, the effect of iodine on plant is obscure; Murashige and Skoog, (1962) decided KI level in their medium arbitrarily, the concentration of Cu^{++} in the MS medium is insufficient, in some cases. Ikeda *et al*., (1982) have reported that, a tenfold increase in Cu^{++} concentration promoted growth and cytohrome aa_3 content, affected digitoxin content. As well as the indirect effect, Hagimori *et al*., (1983) studied the effect of pH value on digitoxin content and found that, the most suitable value was 6 prior to autoclaving., since, MS medium is less than this value, Consequently glycoside may be contents was affected.

In previous study on different salts concentrations of MS medium were tested and the results reveal to digoxin and digitoxin positively affected by increasing this salts in comparison with MS basal medium (Bekhit, 1996). In this concern, further study should be carried out to cover this gap for increasing glycoside contents after addition and controlling in all parameters that are affected digoxin and digitoxin contents

Table (3): Effect of MS salt strength on shoot growth and glycosides content derived from *Digitalis lanata* Ehrh shoot tip exp.

MS salt strength	Shoot Growth		Digoxin &digitoxin Content (8wks)		Callus
	fresh weight /explant	dry weight g/explant	mg/g D.W	% of control	
¼ MS	5.55a	0.25a	3.12	300	+
½ MS	2.92b	0.23a	3.39	326	+
¾ MS	1.84c	0.15b	2.05	197	-
1 MS	3.35b	0.24a	1.04	100	-

Different letters within columns indicate significant difference (P ≤ 0.05) according to Duncan's multiple range test.
+ callus on shoot base
- no callus on shoot base

Effect of precursor (progesterone) on glycoside content as digoxin and digitoxin.

Data in Table (4) show that, generally, rooted-shoots appeared clearly success in digoxin and digitoxin production in comparison with shoot without roots. Also, rooted –shoots that were grown on MS basal medium hormone-free with 100mg/l of progesterone achieved the best results (0.808 mg/g.D.W). On contrast, the poor response was recorded on medium contained 2.5 mg/l of BA or progesterone-free (0.066 and 0.098 mg/g.D.W) respectively. It's evident also that, residual effect of BA, led to decrease digoxin and digitoxin contents, as well as presence of root may be improve the absorption efficiency, led to increasing the desired compounds.

From the results, it could be concluded that, This results closely agree with Fauconnier et al., (1996). They found that rooted plantlet of *Anthemis nobilis* contained essential oil four times more than in the shoot cultures without roots. Also many biotechnological strategies have been hypothesized and experimented for enhanced production of secondary

metabolites from plant. Some of these include media modification and precursor feeding (Namdeo *et al*. 2002, Rao&Ravishankar 2002 and Vanishree *et al*. 2004). In general, attempts to induce or increase the production of secondary metabolites by supplying precursors or intermediate compounds, have been effective in many cases (Silvestrini *et al*., 2002). Lindmann and Luckner (1997) reported that, *Digitalis lanata* and *D. purpurea* callus cultured rapidly transformed progesterone to pregnane. Moreover, Shoot-forming callus tissues of *Digitalis purpurea* accumulated an increase level of digoxin and/or digitoxin when progesterone was added to the medium. Addition of phenylalanine to *taxus* cultures, stimulation of taxol production (Fett-Neto *et al*.(1993). Also, Feeding ferulic acid to cultures of *Vanilla planifolia* resulted in increase in vanillin accumulation (Ramagonoli and Knorr, 1988). in *Hypericum perforatum,* Karppinen *et al*., (2007) improved the production of hyperforin and adhyperforin when the cultures feeding with 1-valine and 1-isoleucine. Hamza and Bekhit,(2008) found that, addition L-phenylalanine, L-tryptophane and L-tyrosine to *Sylibum marianum* culture, led to increase sylimarine content.

In general, precursor feeding has been a successful approach for enhanced production of secondary metabolites from plant cell grown *in vitro*. The concept is based on the idea that, any compound which an intermediate in or at the beginning of secondary metabolite biosynthetic route, stands a good chance or increase the yield of final product (Evans, 2001 and Nemado *et al*.,2007)

Table (4): Effect of Auxins, BA and Progesterone on Digoxin (mg/g.d.wt.) in *Digitalis lanata* Ehrh organ tissues

BA (µM)	Progestron (mg/l)	Digoxin & digitoxin contents (mg/g.D.W)	
		Shoots with root	Shoots without root
0.0	0.0	0.114	0.098
2.5	100	0.171	0.066
5.0	100	0.247	0.127
0.0	100	0.808	0.273

Conclusion

From all results, it could be concluded that, BA is considered critical factors for induce the most amounts of cardiac glycosides in singrism or high level. It's noticeable also, that, shoot tip culture has been widely used for rapid propagation of many species due to advantage over traditional methods. Moreover, the multiple shoot-produced in culture can be used as an alternative source of metabolites specially in plants where the production is dependent on morphogenesis. Also, the exogeneous supply of bio synthetic precursors to culture medium, increase the yield of the desired product. This approach is useful when the precursors are inexpensive. Although, generally the yield of cardiac glycosides in Digitalis tissues was very low, and moreover during the successive transfers of the cultured cells, the amount of cardenolides often decreased and disappeared. Nevertheless, shoot cultures as clearly shown for *Digitalis lanata* do in fact offer a more efficient production

system for secondary metabolites of interest than cultivating these plants in the field or collecting them from wild population or anywhere else.
Therefore, secondary metabolites formation can take place within a short cultivation time that allows one to lower overall production costs, while at the same time, produce a high- quality consistent products.

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INCREASING PRODUCTIVITY OF DIGOXIN CONTENT IN DIGITALIS LANATA EHRH "*IN VITRO*"

زيادة انتاجية الديجوكسين والديجيتوكسين فى نبات الديجيتالس معمليا

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اجريت هذة الدراسة بمعمل زراعة الانسجة (النباتات الطبية و العطرية) قسم البساتين، كلية الزراعة، جامعة الازهر، القاهرة، مصر، بهدف زيادة انتاجية الديجوكسين و الديجتوكسين معمليا باستخدام تقنية زراعة الانسجة. تم زراعة البراعم الخضرية معمليا لنبات الديجيتالس لاناتا من البادرات الناتجة من زراعة البذرة تحت ظروف التعقيم الكامل و تم زراعة القمة النامية و العقل الساقية على بيئة موراشيج و سكوج الصلبة و المحتوية على تركيزات مختلفة من البنزايل ادينين، وقد تأثر نمو الافرع الخضرية معنويا بالجزء النباتى المنزرع و كذلك تركيز البنزايل ادينين، وتم الحصول على اكبر كمية من الافرع على بيئة موراشيج و سكوج والمحتوية على ٥ ميكرو مولار من البنزايل ادينين، بينما تأثر سلبيا محتوى الديجوكسين والديجيتوكسين بالتركيزات العالية من البنزايل ادينين (١٧,٥ ميكرو مولار)، كما ادت اعادة زراعة الافرع االخضرية على بيئة موراشيج وسكوج الصلبة والمحتوية على تركيزات عالية من البنزايل ادينين الى زيادة النموات الخضرية وتثبيط المحتوى الجليكوزيدى، وقد تحسن انتاج الديجوكسين والدديجيتوكسين عندما تم زراعة الافرع الخضرية على نصف قوة املاح بيئة موارشيج وسكوج، كما ظهرت على الافرع الظاهرة الزجاجية نتيجة زراعتها على بيئة غنية بالبنزايل ادينين، كما ادى اضافة البروجسترون كمادة اولية الى زيادة محتوى الديجوكسين والديجيتوكسين خاصة مع الافرع التى كونت الجذور