Using Tissue Culture Technique for the Production of Cardiac Glycosides From Roots of *Digitalis purpurea* L Plantlets (Var .Excelsior Mixed)

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ABSTRACT

The present study was performed using the tissue culture technique , where by roots formation was stimulated shoot tips of ***Digitalis purpurea* L** plant using auxins in this study ,shoot tips (1cm length) were excised from sterilized seedlings of *Digitalis purpurea* L.Var Excelsior mixed , then they were cultured on MS medium with the addition of one of the auxins IAA, IBA ,NAA of different concentration (0.0 ,0.05 ,0.1 ,0.5 and 1.0 mg / L).

After 45 days of starting the culture results showed that the treatment 0.5 mg/ L of IBA had a high significant effect on the dry weight of roots formation and containing Digitoxin and Gitoxin whose quantities as a rate of (2.96 gm , 30.01 and 11.05 µg/gm dry weight respectively .Also this treatment has given highest values with the other studied characteristics where the percent of the soluble sugars and starch and they are (2.48 % , 2.82%) respectively

Abbreviation

(Indole Butyric Acid)IBA , (Indole Acetic Acid)IAA , (Murashige and skoog medium , 1962) MS , (Naphthalen acetic Acetic) NAA , (Milligram) Mg , (Microgram)ug , (Litter) L , (Gram) gm .

الخلاصة

اجريت هذه الدراسة في مجال زراعة الانسجة النباتية , والتي تم فيها تحفيز انشاء الجذور من اطراف الافرع (shoot tips) المزروعة لنبات *Digitalis purpurea* بواسطة استخدام الاوكسينات , حيث في هذه الدراسة تم اخذ اطراف الافرع بطول 1 سم من البادرات المعقمة لنبات Digitalis purpurea صنف Excelsior Mixed وزرعت على الوسط الغذائي MS مضافا اليه الاوكسينات IAA, IBA, NAA وبالتراكيز (0.0, 0.01, 0.1. 0.5, 1.0) ملغم / لتر لكل منهم على حده , وبعد 45 يوما من بدء الزراعه , كانت المعاملة المتالفة من وجود الاوكسين IBA وبتركيز 0.5 ملغم /لتر هي الافضل مقارنة بالمعاملات الاخرى

(30.01 , من حيث الصفات التي درست , حيث اعطت هذه المعاملة مجموع جذري ذات حاصلا جافا بلغ 2.96 غم محتويا على Gitoxin و 11.05Digitoxin) مايكروغم / غم وزن جاف

ايضا اعطت هذه المعاملة معدلا عاليا بالصفات الاخرى التي درست وهي النسبة المئوية للسكريات الذائبة والنشا والتي بلغت وعلى التوالي (2.48%, 2.82 %) .

INTRODUCTION

Digitalis purpurea L is one of the herbal plants, belonging to the scrophularaceae family. This plant is considered to be one of horticultural plants and it is planted as an ornamental in the garden for its beautiful flowers and coordination as well as its various colours [1] .

Digitalis purpurea has a great benefit in the medical and pharmacy fields because it contains cardiac glycosides which are found in all of its parts especially the leaves [2]. These compounds are used to heal some of the important heart diseases like congestive heart failure [3]. Because of their interest and demand in the medical field and due to the possibility of the

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industrializing them in chemically or microbiologically, so the only method available to get these compounds is in the agriculture production [4] . From this importance, many pharmacological, chemical and agriculture researches were carried out to study this plant in order to raise its contents for these compounds via using modern technologies like cell , tissue and plant organ culture [5], via the usage of this technology different explants can be cultured and stimulated to different growth in order to be a permanent source for these important medical compounds without being restricted to the environmental conditions as well as cleans of the pharmaco which could be obtained [6]. So the aim of the present study performed in the tissue culture field is to :-

stimulate the shoot tips of *Digitalis purpurea* plant to form roots by adding different kinds and concentrations of auxins to MS medium, and then we can benefit from the formed roots to be a source for demanded cardiac glycosides Digitoxin and Gitoxin .

MATERIALS and METHODS

The present study contained the following :-

First - Tissue Culture of the Plant *Digitalis purpurea.*

The work went through the following steps -: :

1.Initiation a tissue culture:

That is, initiating a specific culture empty of any contamination, so that it would be a source for shoot tips which are desired to be cultured and that was performed by preparing a sterile medium for culturing the seeds which contain merely distal water and agar [7], whereby difco – bacto –agar kind was used with 6 gm/L, which was added to distilled water.

Then the heating process was performed till boiling with the use of magnetic stirrer hot plate machine, after that the medium was distributed in glass test tubes with a capacity of 25 x 180 ml and a quantity of l0 ml for every tube then they were covered with special coverlet and autoclaved in autoclave at a temperature of 121°C, pressure 1.04kg/cm^2, for 20 minutes , after disposing , then it was left to be cooled until the time of its culture. Next the seeds of the plant *Digitalis purpurea* variety excelsior mixed which was demanded for the culture is sterilized inside a laminar-air-flow cabinet with the 70% ethyl alcohol , for 30 sec ,then rinsed with distilled sterilized water three times for 5 min for every time [8]. Finishing this process the seeds were cultured and distributed upon the prepared medium surface. Then they were transmitted to the growth room under controlled - environmental conditions, 25 ± 2°C temperature, and photo period 16 hour/day with the severity amounting to 40 – 60 micro inshtain /m^2/ sec. After 14 day , there were seedlings in which the demanded shoot tips in them were ready to be amputated and cultured .

2 - Formation the roots

This process was demande :-

A-Preparation the Nutrient Medium MS (table 1) .

This was performed with the stock solution preparation, then the content was merged with the requested size and to which one of the auxins IAA, IBA, NAA were added in different, concentrations (0.0, 0.05, 0.1 ,.0.5 and 1.0 mg/L). The size is completed with distal water and the pH was adjusted to 5.5 with the used of 1 N NaOH and HCI. Next agar was added in a concentration of 8gm/L and solution it as in 1 .These media were distributed into glass test tubes with capacity of 25 x 180 ml and quantity of 25 ml for every tube. The tubes were covered with special coverlet and sterilized in an autoclave at 121 °C, and a pressure of 1.04 kg/cm^2 , for 25 min period of time. After disposing them ,they were left to be cooled at room temperature until the nutrient media were solid ready to be used in culture.

B- Shoot tips from seedlings were isolated and cultured with one tip in every tube and cultures were incubated under controlled environmental conditions as in I for 45 days for the shoot tips to roots .

Table (1): Composition of the MS nutrient medium [9]

Compound	Concentrate (mg /L)
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KNO_3	1900
NH_4NO_3	1650
KH_2PO_4	170
$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25
$MnSO_4.4H_2O$	22.3
$ZnSO_4.4H_2O$	8.6
$Cu\ SO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
KI	0.83
H_3BO_4	6.2
$Na_2MoO_4.2H_2O$	0.25
Inositol	100
Nicotinic acid	0.5
Pyridoxine – HCl	0.5
Thiamine – HCl	0.1
Glycine	2
Sucrose	3000

Second - Preparation the roots for chemical analysis.

After finishing the assumed incubation period necessary for rooting in the culture media, the formed roots had the following characteristics:

1- Percent of the rooting.

2- Estimation of the dry weight, The roots mass was disposed into the culture tubes and washed with flowing water to get rid of the agar residues conglutinated to it, then wiped with clean cloth to get rid of the washing water, and then the root samples were differentiated and spread out on the filter paper, to be dried in the oven at 40°C until the weight affirmation [10] , Later samples were grinder to a hard powder and kept in paper sacks, in dry condition (closed disicator) and dark place

3- The ratio percent for the soluble sugars and starch were estimated according to the Joslyn [11] method .

4- Extracting and purifying the cardiac glycosides, here used the Fujii et al , [12] method .

5-The qualitative and quantitative assay for the studied cardiac glycosides, were done by using high performance liquid chromatography (HPLC) following Braga et al , [13] method .

The data were arranged with the above Studied characteristics (1 , 2 ,3 ,5) following the complete randomize design in a factorial experiment (14) .

RESULTSand DISCUSSION

1- The Roots formation.

Table-2 showed that stimulated the shoot tips of *Digitalis purpurea* plant to form roots for the control treatment (0,0 mg/L) and for all the auxins concentrations which were used except 1 mg/L NAA concentration which had lead after 25 day of starting the culture to the death of the cultured shoot tips, where in this table showed should that the rooting ratio was 100 % for the control treatment and for all the IAA concentrations and (0.01, 0.1, 0.5) mg/L concentrations for IBA, and (0.05,0.1) mg/L for NAA, whereas 1 mg/L concentration for IBA and 0.5 mg/L concentration for NAA the ratio of rooting was decreased and significantly different from the other studied treatments .

Table (2): The effect of the interference between IAA, IBA, NAA and their concentrations on the rooting ratio for the shoot tips of *Digitalis purpurea* plantlets

Concentration mg / L	Rooting %		
	Auxins		
	IAA	IBA	NAA
0.0	100	100	100
0.05	100	100	100
0.1	100	100	100
0.5	100	100	30
1.0	100	70	0.0***
L.S.D 5%	20.03		

***All the shoot tips are dead

***Digitalis purpurea* plantlets**

stimulating root formation in tissue culture is usually controlled by many factors such as:- Presence of growth regulators auxins, whether it could be a natural auxin, that was, naturally found inside the plant tissue or added to the nutrient medium [15]. Its physiological effect then, would be increase in cell division and transformion to meristem cells. In this case adventitious root meristem will have cell division to form the adventitious roots [16]. In the results of our study mentioned in table 2, it was noticed that :

In the control treatment (non - addition of auxins to the nutrient medium), the rooting process happened this might be due to existence of the natural auxin inside the shoot tips used for culture, and it might be that, this natural auxin was present in a quantity that helped and stimulate root formation, or otherwise, to the nutrient medium MS used, which probably helped to made root growth and development like P element, which in turn will have a role in stimulating root formation, and N element which will help to increase internal auxin concentration because this element involved in auxin composition [17]. So, naturally internal auxin concentration increase will affect root formation. More over sucrose has a role in root formation because it will be a source of necessary energy for this process [18]. All what is mentioned above would be interlinked to affect stimulation of root formation. Similar to the result of root formation stimulation without any external additions for the nutrient medium appeared in previous studies upon Digitalis plant like the study performed by Awad et al. [19], Brisa and Segura [20].

As for auxins types and concentrations added to the MS nutrient medium, affecting to root formation, the reason might be due to what is mentioned above about control treatment as well as to the known role of these auxins in stimulating root formation.

In comparison to the results found by schoner and Reinhard [21],
the difference considerably in this study lies in :-

The number of the roots formed as they were very numerous and slim, and greatly attached to one another as in figure 1, that's why it was not possible to count them and measure their lengths, which may involve other characteristics , and when their data are taken, they will help us to maintain more clarification for the difference between effect of types and concentrations of auxins upon root formation , growth and development. Hence, some differences appeared in other characteristics which were cleared and would be mentioned in 2.

As for the high concentrations at which rooting ratios were decreased or cultured shoot tips were dead, the reason might be due to that these high auxin concentrations were encouraging to made an increase in ethylene construction in cultured explants tissue, consequently it will lead to the frustration in their growth and development [22].

*** All the shoot tips are dead

Figure (1) :- Effect of different auxins on root formation from shoot tips of *Digitalis purpurea* L .Result were obtained after 45 day in culture

2. The production of cardiac glycosides , soluble sugar and starch .

Table 3 shows that the auxins concentrations which were used to stimulate root formation, had a positive and significant effect on the production of cardiac glycosides compared, with the control treatment,. Which could be due to increase the auxin level inside the cultured explants .This might help to make more development in most of the cell components like vacuoles and mitochondria , which have a role in cardiac glycosides formation [23].

As for the other characteristics especially soluble sugars and starch
(table 4), it was studied because of the references made by many other workers , showed a positive effect on production of the cardiac glycosides in tissue culture [24]:-

They have shown that these characteristics have a positive relation with the quantities formed from the cardiac glycosides.

The results of our study in table 4, showed that the auxins concentrations which stimulated root formation, had significant effect upon increase of the percent for the soluble sugar, starch and total dry weight compared with the control treatment. The general reason is due to the role of the known auxin in forming these compounds [7], so far the increase in auxin concentration inside cultured explant tissue, with the effect of added auxins, to the nutrient medium will lead consequently to make an increase in the results of the different metabolic processes inside cell , like sugars and starch formation and this will be reflected positively
upon cardiac glycosides formation [23], All this made an increase in the dry weight of the roots formed.

Table 3 and 4 clarify that the lBA had a greater effect upon the studied characteristics in comparison to IAA and NAA , the reason might be due to the strong effect of IBA caused by its slow .
decomposition by the oxidative enzymes which had little poisnous , slow transference and then it stays near its place of addition. As for the IAA ,
it was distinguished for its weakness because of its high oxidation by light and some of the oxidative enzymes for auxin inside plant and more than IBA and NAA [25]. As for 1mg/L of NAA which led to the death of cultured shoot tips , the reason might be due to the high poison which was known for NAA appearing within little concentration compared with IAA and IBA [22], The poisonous concentrations of auxins usually lead to release high quantities of ethylene which lead to quick and un regulated cell division and then to release the destructive enzymes for the cell components which consequently lead to their death [22].

Accordingly, in our experiment the treatment which consisted of IBA auxin in 0.5 mg/L concentration was the best regarding formation of dry weight of roots besides containing the studied cardiac glycosides (Digitoxin and Gitoxin) and soluble sugars and starch..

Table (3) : The effect of the interference between the auxins IAA , IBA , NAA and their different concentrations on production of the cardiac glycosides in roots of *Digitalis purpurea* plantlets .

CONCENTRATIO MG/ L	CARDIAC GLYCOSIDES (μG / G DRY WEIGHT)					
	Digitoxin			Gitoxin		
	Auxins			Auxins		
	IAA	IBA	NAA	IAA	IBA	NAA
0.0	0.87	0.87	0.87	0.31	0.31	0.31
0.05	4.37	10.31	3.77	0.51	6.44	0.58
0.1	4.57	18.92	1.77	1.60	5.77	0.16
0.5	7.97	30.01	0.54	2.36	11.05	0.07
1.0	7.41	11.89	*** 0.0	5.09	8.81	*** 0.0
L.S.D. 5%	2.11			1.92		

*** All the shoot tips are dead

Table (4) : The effect of the interference between the auxins IAA , IBA , NAA and their different concentrations on the roots of *Digital purpurea* plantlets according to :- the contents (soluble sugars and starch) and total dry weight .

CONCENTRATION MG/ L	SOLUBLE SUGARS %			STARCH %			DRY WEIGHT (G)		
	Auxins			Auxins			Auxins		
	IAA	IBA	NAA	IAA	IBA	NAA	IAA	IBA	NAA
0.0	1.10	1.10	1.10	1.54	1.54	1.54	0.19	0.19	0.19
0.05	1.28	1.41	1.34	1.61	1.66	1.34	1.07	1.18	0.83
0.1	1.32	1.82	0.71	2.11	2.13	0.80	1.27	1.38	1.31
0.5	1.36	2.48	0.14	2.07	2.82	0.54	1.81	2.96	0.59
1.0	1.91	2.15	*** 0. 0	2.21	2.34	*** 0.0	1.72	1.93	*** 0.0
L.S.D	0.07			0.12			0.17		

*** All the shoot tips are dead

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