Effects of adding digitoxigenin and gitoxigenin to the nutrient medium on cardiac glycosides production from digitalis purpurea (Var. Excelsior Mixed) by using tissue culture technique

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الخلاصة

اجريت هذه الدراسة في مجال زراعة الانسجة ، وذلك لغرض معرفة تاثير استخدام اثنين من البوادئ وبوجود مادة شبيه السايتوكاينين TDAوسكر المالتوز على محتوى الافرع المتضاعفة لنبات Digitalis purpurea L من الكلايكوسيدات القلبية , Digoxin Gitoxin , Digitoxin ، حيث تم اضافة البادئين Digitoxigenin ، Gitoxigenin الى الوسط الغذائي MS وبالتراكيز (2.0, 1.0, 0.5, 0.1, 0.0) ملغم/لتر ولكل منهما على حدة ، ايضا اضيف الى الوسط الغذائي ولكافة المعاملات مادة TDZ بالتركيز 0.5 ملغم/لتر وسكر المالتوز بالتركيز 30 غم/لتر ، اظهرت نتائج هذه الدراسة وبعد 45 يوما من بدء الزراعة بان المعاملة المحتوية على البادىء Digitoxigenin وبتركيز 0.5 ملغم /لتر هي الافضل مقارنة ببقية المعاملات من حيث الصفات التي درست ، حيث اعطت الافرع المتضاعفة في هذه المعاملة حاصلا جافا بلغ 6.52 غم محتويا على Digitoxin ، Gitoxin ،Digoxin بمقدار بلغ (203.58, 192.18, 382.48) مايكروغم/غم وزن جاف وعلى التوالي ، كما اعطت هذه المعاملة اعلى القيم بالصفات الاخرى التي درست وهي محتوى الافرع المتضاعفة من الكلورفيل الكلي ،النسبة المئوية للسكريات الذائبة والنشا وبلغت (4.18 ملغم/غم وزن طري ، 5.59% ،8.56%) وعلى التوالي .

الرموز المستخدمة:- Dg (Digitoxigenin) ; Gg (Gitoxigenin) ; MS (Murashige and Skook Medium) ; TDZ (Thidiazuron) ; Mg (Milligram) ; µg (Microgram) ; L (Litter) ; gm (gram) .

ABSTRACT

The present study was performed using tissue culture technique purpose of knowing the effect of using precursors in the presence of TDZ semi–cytokinin and maltos sugar on the cardiac glycosides constituents shoots of Digitalis purpurea digitoxin, gitoxin and digoxin, in this study , shoot tips were taken from seedlings of Digitalis purpurea (var . Excelsior Mixed) with 1 cm lenth , then they were cultured on nutrient medium adding with two kind of precursorres digitoxigenin (Dg) and gitoxigenin (Gg) with the concentrations 0.0, 0.1, 0.5, 1.0 and 2.0 mg/L for each one, as well as adding for all the treatments ,the TDZ with the 0.5 mg/L concentration and the maltose sugar with the 30 gm/L concentration for induction the multiplication shoot tips processe .

The results showed that this study and after 45 day of starting the culture, the treatment which contained digitoxigenin precursors in the concentration 0.5 mg/L was the best compared of the other treatment in respect to the characteristics studied.In this treatment, the multiplication shoots have given sum dry with the amount weight of 6.52 gm containing gitoxin, ditoxin, digoxin with the amount of 382.48, 192.18 and 203.58 µg/gm dry weight respectively, beoide the inceased amount of dry weight and cardiac glycosides , other characteristics component of the shoots was increased as the containing of total chlorophyll and percent ratio of soluted sugar and starch in the amount 4.81 mg/g fresh weight, 5.59% and 8.56% respectively.

Abbreviations :- Dg (Digitoxigenin); Gg (Gitoxigenin); MS (Murashige and Skoog Medium); TDZ (Thidiazuron); Mg (Milligram);µg (Microgram); L (Litter); gm (gram).

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INTRODUCTION :

Digitalis purpurea L. belongs to the scrophularaceae family[1] .The arabic name for this plant is the purpule alkishtiban flower[2]; the plant is biennial and herbacious plant used as ornamental because of the beauty of its flowers . It is considered one of the medical plants because it contains highy quantities and various types of cardiac glycosides, like digitoxin, gitoxin[1].

The dry powder or the extracted the glycosides are used to heal some of the important heart diseases especially congestive heart failure, as well as healing some other diseases like cardiac systems confusion and heart throbing[3].

Because of the importance of these compounds from the medical viewpoint and the impossibility of its preparation chemically or microbiologically hence until now extracted from plant[4]. then, many agriculturing studies were carried out upon this plant, aiming to increase their important medical contents and benefit from them in the fields of medicine and pharmaceutics[5],one of these studies is the use of modern agricultural techniques to get these compounds in a trading method, including the cell, tissue and organ culturing technique[6], while in this technique different explants can be cultured and developed so that they become a source for different growths like the vegetative shoots considering it a place of forming and storing these compounds[7], which eventually could be a permanent and natural source for these medical compounds.

The concept of our present study is condensed by adding two kinds of precursors digitoxigenin and gitoxigenin in different concentrations to the MS nutrient medium[8] in the presence of maltose sugar and TDZ and then notice their effects upon the cardiac glycosides production digitoxin, gitoxin and digoxin .

MATERIALS AND METHODS :

The present study contained the following :-

First – Tissue culture of Digitalis purpurea .

The work went through the following steps :-

1- Initiation a tissue culture :

That was, initiated a specific culture empty of any contamination, so that it would be a source for shoot tips which are used for subsequent to be tissue culturing . This was performed by preparing a sterile medium for culturing the seeds which contain merely distal water and agar[9], 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 ; after that 10 ml of the medium was distributed in each glass test tube capacity (25 × 180 ml) then they were covered with special coverlet and autoclaved in a temperature of 121°C, pressure 1.04 kg/cm^2, for 20 min , then was left to be cooled until the time of its culture. Next the seeds of the plant *Digitalis purpurea* variety excelsior mixed which is demanded for the culture was sterilized inside laminar–air–flow cabinet with 70% ethyl alcohol for a period of 30 sec, then rinsed with distilled sterilized water three times for a period of 5 min each time[10]. 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. Seedling obtained after 14 days the demanded shoot tips are ready to be amputated and cultured.

2- Preparation of murashige and skoog (MS) medium (table 1) . This was performed with the stock solution preparation, The content was merged with the requested volum and addition to it 30g/L maltose sugar ,0.5mg/L TDZ[11] and precursors Dg ,Gg, in which each one is put a side with the concentration (0.0, 0.1, 0.5, 1.0, 2.0) mg/L each of them, then the volum was completed with distalled water . The pH was adjusted to 5.5 with the use of one normality (1N) of NaOH and HCl, next agar was added (difco–bacto–agar kind) to a 8 gm/L and solution as in **1. ,** then this media distributed into glass flasks with capacity of 250 ml and quantity of 50 ml for every flask, flaskes were covered with alluminum foil and sterilized in an autoclave in a temperature of 121°C, and pressure 1.04 kg/cm^2, for 25 min period . After disposing them ,they were left to be cooled at room temperature until the nutrient media are solid to be used in culture.

Table 1 . The in organic and organic components for the MS nutrient medium.

Compounds	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_2 EDTA	37.25
$MnSO_4.4H_2O$	22.3
$ZnSO_4.4H_2O$	8.6
$CuSO_4.5H_2O$	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

3- The isolated shoot tips from the seedlings in 1 were culture and incubated under controlled environmental conditions as in 1 for 45 days .

Second – Prepare the samples plant for chemical analysis .

After finishing the incubation period for the multiplication of shoots in culture, the following studies were done :-

1:- Dry Weight, here the multiplication shoots were taken out the flasks and separated from the agar residue by washing with flowing water, then wiped with clean cloth to get rid of the washing water,then the shoots were differentiated and spread out on the filter paper, to be dried in an oven at 40°C until the weight affirmation[12]. Later , samples were grinded in the form of powder kept in paper sacks, in a dissector containing calcium chloride material under it, in a dark place.

2:-The ratio percent for the soluted sugars and starch were estimated according to the Joslyn method[13].
3:- Extracting and purifying the cardiac glycosides, have used the Fujii et al. method[14] .
4:- The qualitative and quantitative assay for the studied cardiac glycosides, were done by using high performance liquid chromatography (HPLC) following the Braga et al. method[7].
The data were arranged for the above studied characteristics (1,2,4) following the complete randomize design in a factorial experiment[15].

RESULTS AND DISCUSSION :

The effect of the interference between the precursores Dg , Gg and their different concentration with the presence of 0.5 TDZ and 30 g / L maltose sugar upon the shoot tips multiplication for the Digitalis purpurea plant
It was noticed in table (2) ,that was presence to the processe of multiplication shoots and the general reason may lie to :
1) The presence of TDZ material (semi cytokinine) in the nutrient medium which has a vital strong role in stimulating lateral shoots for the cultured shoot tips and to get rid of the apical dominance[16].
2) As well as the presence of maltose sugar which was disaccharide and it is disassembled during nutrient medium sterilization process to two units of glucose that is considered of the most widely used sugars which interfere in the biointeraction to produce necessary energy for different growth and development processes[15]. Consequently, the increase of the glucose unites will give via the maltose sugar an increase in producing power.
3) Also the making of multiplication shoots process may be because of the presence the precursors materials Digitoxigenin and Gitoxigenin, the reason might be that the materials and their great carbonic structure consisting of (21, 21) carbon atom respectively[17], have added to the nutrient media (MS) in the state of its analysis some nutrient elements like carbon, these elements have an important role in the different growth and development processes[18].

Table 2 . The effect of the interference between the precursors Dg, Gg and their different concentrations with the presence of 0.5 mg/L TDZ and 30 g/L maltose sugar upon the shoot tips multiplication for the *Digitalis purpurea* plant.

Concentration Mg/L	Shoot tips multiplication Precursors	
	Dg	Gg
0.0	+ +	+ +
0.1	+ +	+ +
0.5	+ +	+ +
7.0	+ +	+ +
2.0	+ +	+ +
thick leaf group ++		

Then the interference of the effect of these nutrient elements with the effect of TDZ and the sugar maltose may be make the induction of the multiplication shoot tips process.
But the matter differs considerably in this study . It lies in :-
The number of the shoots formed which were very numerous as well as the very small and great interaction to one another as in figur (1) , so it was not possible to count them , which involve other characteristics and when the data is taken that will help us to take more clarification for the difference between effect of precursors upon multiplication shoots process, but that are some difference appeared in other characteristics which were clear and will be mentioned in 2 .
The effect of the interference between the precursors Dg, Gg and their different concentrations with the presence of 0.5 mg/L TDZ and 30 g/L maltose sugar upon multiplication shoots for the Digitalis purpurea plant according to the :- Contain (cardiac glycosides, total chlorophyll, soluted sugars and starch), total dry weight.

Figure 1. Effect of addition of 0.5mg/L TDZ + 30g/L maltose Sugar + 0.5mg/L Digitoxigenin on multiplication of Shoot tips of Digitalis purpurea L. , after 45 days in Culture .

It was noticed in table (3), that the addition of the two materials Dg, Gg to the nutrient media will have a significant effect upon the quantity increase of the studied cardiac glycosides (Digitoxin, Gitoxin, Digoxin) in the multiplication shoots for the Digitalis purpurea plant in comparison with the control treatment, because these materials are precursors forming which studied cardiac glycosides, that is kinds of precursors initiating the final steps to form the studied cardiac glycosides[19]; hence , adding them to the nutrient medium cause makes an increase in the internal level for these precursors inside the tissue of explants cultured. This eventually was reflected in the formed studying cardiac glycosides quantity during shoots (leafs) drying process, and that was clarified by many researchers in their studies like Milek et. al.[20] .

Table 3 . The effect of the interference between the precursors Dg, Gg and their different concentrations With the presence of 0.5 mg/L TDZ and 30 g/L maltose sugar upon the containing of multiplication shoots for the *Digitalis purpurea* plant from the cardiac glycosides.

Concentration mg/L	Cardiac glycosides (ug /g dry weight)					
	Digitoxin Precursors		Gitoxin Precursors		Digoxin Precursors	
	Dg	Gg	Dg	Gg	Dg	Gg
0.0	71.0.5	71.05	34.96	34.96	19.11	19.11
0.05	99.34	71.56	66.31	37.18	77.44	34.33
0.1	382.48	86.18	192.18	70.27	103.58	31.12
0.5	321.61	191.33	74.27	83.43	83.21	51.23
1.0	344.19	197.13	53.52	192.98	87.11	72.51
L.S.D. 5%	21.17		23.88		12.61	

As for the other characteristics especially total chlorophyll, soluted sugars and starch whose results are clarrified in the table (4), it was studied because many researchers have pointed in their studies that these characteristics have a positive effect upon the quantities formed from the cardiac glycosides like Ohlsson[18].

Table 4 . The effect of the interference between the precursors Dg, Gg and their different concentrations with the presence of 0.5 mg/L TDZ and 30 g/L maltose sugar upon the multiplication shoots for the Digitalis purpurea plant according to : containing (total chlorophyll, soluted sugars and starch), total dry weight.

Concentration mg/L	Chlorophyll Precursors		Soluted sugars % Precursors	
	Dg	Gg	Dg	Gg
0.0	4.47	4.47	4.22	4.22
0.1	4.41	4.41	4.13	4.23
0.5	4.81	4.66	5.59	4.18
7.0	4.51	4.44	5.31	4.33
2.0	4.61	4.31	4.88	4.41
L.S.D. 5%	0.13		1.22	
Concentration mg/L	Starch % Precursors		Dry weight (g) Precursors	
	Dg	Gg	Dg	Gg
0.0	7.18	7.18	3.41	3.41
0.1	7.09	7.11	5.77	4.64
0.5	8.56	7.77	6.52	4.35
7.0	8.11	7.19	6.13	4.71
2.0	8.19	7.23	6.21	5.06
L.S.D. 5%	0.22		0.17	

Result of our study in table (4), show that the studying characteristics showed a significant increase within certain concentration for the two precursors in comparison with the control treatment, especially at 0.5 mg/L for Dg . The reason may be due to the quantity of analysis which in these precursors, may be helped to increasing in the content of chlorophyll, sugars and starch and that is reflected positively upon the metabolite products like the studied cardiac glycosides[18] .

As for the dry weight characteristic for the multiplicated shoots, they reached highest value in the 0.5 mg/L concentration for the Digitoxigenin and the reason may be due to raise in the multiplication shoots content from the metabolite products, which could be the reason in increasing the dry weight for these shoots[11,12].

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