Effect of gamma rays and sodium chloride on increasing the concentration of cardiac glycoside compounds of *Digitalis lanata In vitro*

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Abstract

The Explants that was used in the experiment to stimulate the production of glycoside compounds is the tips of the vegetative branches with a length of 2 cm obtained from the stage of vegetative multiplication growing from seeds treated with gamma radiation with a dose of (0, 30) gray, the tips of the branches were planted on MS nutrient medium prepared with different concentrations Of NaCl (3,0, 6, 9, 12) g.l-1 to know the effect of irradiation, sucrose and maltose in stimulating the production of cardiac glycoside compounds, chlorophyll and carbohydrates, the data showed that the concentration of 12 g.l-1 was better in increasing the active compounds: Digoxin, Digitoxin, and Gitoxin (129.46, 171.70, 145.59) µg.g-1 dry weight, respectively, while the neutral treatment was superior in increasing the rate of chlorophyll, which amounted to 2.10 µg.g-1, while the concentration of 3 g.l-1 gave the highest rate of carbohydrates amounting to 2.94 µg.g-1, and the irradiation treatment was significantly superior in The increase in the production of glycoside compounds reached (127.95, 193.95, 161.31) µg.g-1 dry weight, respectively, and the rate of chlorophyll and carbohydrates reached (2.38 and 3.11) µg.g-1, respectively.

Keywords: Cardiac glycosides, Digitalis, Gamma ray, NaCl, Plant tissue culture.

Introduction

The science of plant tissue culture is the science of growing plant cells, tissues or organs isolated from the mother plant or the cell protoplast on food media [1]. The field of propagating many types of plants because of the advantages of this method, perhaps the most important of which is obtaining huge numbers of plants free of pathogens and similar to the mother plant in a relatively short time and at any time of the year, as well as the use of this technology in research and applied fields, including Plant breeding and improvement, the production of medicinal drugs and medicines, and rapid breeding, which is one of the applications of great importance that is carried out by following different methods of differentiation and morphology such as the formation of transverse buds, stimulating the growth of axillary buds and the development of asexual embryos (somatic embryos), and the study of the basic aspects of

plant growth and development, and secondary metabolism [2]. Medicinal plants are a renewable source of medicine and food, and at the present time there is a significant decrease in the sources of medicinal plants arising from human intervention in the natural environment. Therefore, scientists turned to a new method to overcome this difficulty by using tissue culture technology to produce important plants through the technique of micropropagation of plants that It is based on the principle of the potential energy of the cell Totipotancy [3], and medicinal plants are distinguished from others by containing compounds and chemical elements that have a beneficial medical and functional effect within the human body in case of illness and in the scientifically determined and medically prescribed amounts. Active substances (secondary metabolites) are classified on the basis of their properties. Chemical and their natural properties to alkaloids, glycosides, phenols and others [4], Tissue culture techniques have shown in many cases a high production of secondary metabolites compared to the original plant, and this production can be regulated by the use of plant growth regulators [5], and scientists have increased interest in the production of high-value natural plant materials using the tissue culture technology that It can overcome many problems associated with the production of plants in the field or laboratory, as the role of tissue cultures has emerged, which can be described as bioconversion plants from low-value compounds to high-value products [6]. *Digitalis lanata* L. is a herbaceous annual or biennial flowering plant that grows naturally in eastern or western Europe, western and central Asia, and northwest Africa. It is cultivated as a garden ornamental plant for the beauty of its leaves in the first season, and for the beauty of its flowers in the second season, or for medicinal use In the first year, the plant forms long, dense basal leaves in the form of a green bouquet that is arranged in a spiral [7], Previously, the genus Digitalis was classified within the plant family Scrophulariaceae, but recently, from a review of phylogenetic research, this species was classified in the plant family Plantaginaceae [8]. From the general follow-up to the classification of digitalis, we find that it belongs to the plant kingdom within the domain of eukaryotic plants, the division Magnoliophyta, the taxon Magnoliopsida, the phylum Angiosperms, the order Lamiales, the family Plantaginaceae, and the genus Digitalis, which includes twenty plant species of perennials, herbaceous annuals and shrubs, the lanata type is the most important one. It has common local names, including the Virgin's finger or the chimpanzee flower. The English name for it is Fox glove. The digitalis plants are distinguished by their content of glycosides called cardiac glycosides [9]. Glycosides are the second largest group in terms of importance and prevalence after alkaloids, and they are defined as those compounds resulting from secondary metabolism in plants and consist of two parts linked to each other, one of which does not contain sugar and is called (aglycone), and the second part is a sugar, which is called (glycone), And the part that does not contain sugar molecules is a protein, which is the active part, and it represents the active substance and activates the work of enzymes. There are two main types of glycosides, namely cyanide and steroid glycosides, the second type contains 8 types of glycosides, the most important of which are

cardiac glycosides, which are found in 11 plant families and more than 34 genera, the most important plant families that contain cardiac glycosides are Plantaginaceae, which contains Digitalis lanata L., which contains cardiac glycosides [10]. Cardiac glycoside compounds extracted from the digitalis mystic plant have been widely used as a drug for the treatment of various heart diseases, due to the high efficacy of these substances and because the failure of the heart muscle sometimes cannot be controlled by other drugs [11], as well as the indirect effects on the heart system, such as regulating the heartbeat and increasing the strength and speed of contraction of the heart muscle, which has a positive effect [12]. From the foregoing and the importance of the plant, the study aimed to know the effect of low doses of gamma rays and different concentrations of sodium chloride on increasing the production of cardiac glycoside compounds in the cultures of the vegetative branches of the digitalis plant *In vitro*.

Materials and Methods

The explants that was cultivated in the experiment to stimulate the production of glycosides is the ends of shoots with a length of 2 cm obtained from the stage of vegetative multiplication growing from seeds that were treated with gamma rays with a dose of (0, 30) gray, the tips of the branches were planted on the surface of the MS medium prepared with NaCl at concentrations (0,3,6,9,12) $g.l^{-1}$ with ten repetitions, the cultures were incubated in the growth room at a temperature of 25°C +_2 and a light intensity of 1000 lux for 16 hours/day. The study indicators, which included measuring the average concentration of glycoside compounds, chlorophyll and carbohydrates, were taken from the shoots of the seedlings after a month of planting.

Preparation of alcoholic extracts of shoots produced by stimulating treatments

The process of extraction and quantitative and qualitative estimation of the glycoside compounds (Digitoxin, Digoxin, Gitoxin) was carried out by analyzing the samples by HPLC as follows:-

The shoots obtained from the stimulation stage were dried at laboratory temperature and extracted by the ultrasonic frequencies effect according to what was mentioned by [13], 1 gm of dry matter was taken from each sample and crushed in ceramic mortar, then 40 ml of methanol at concentration of 70% was added to it, then it was placed in the ultrasonic frequency device in a water bath at a frequency of 100 Hz and at room temperature for 20 minutes, after that the samples were filtered With 0.13 mm filter paper, the process was repeated again, then the samples were ready for reading in the HPLC device.

Chlorophyll measurement

The chlorophyll pigment was estimated using the method [14] and my agencies:-

1 gm of vegetative growths grown in tissue culture was taken and placed in a ceramic mortar and 9 ml of 85% acetone was added to it, then the plant tissues were crushed until a colorless residue was obtained. A ml was withdrawn from the solution and the

appropriate dilution was made for it. The optical absorption of the solution was read at wavelengths 663 and 645 nm using a spectrophotometer, according to the total content of chlorophyll and according to the following equation:

Total chlorophyll mg/l = 20.2D(645)+ 8.02D(663)

Carbohydrate measurement

The method of [15] called the method of phenol sulfuric acid was followed. Where 0.1 g of the crushed samples was taken and placed in a dry test tube, 10 ml of ethyl alcohol was added to it, 70% concentration. 1 ml of 5% phenol reagent was added to it with 5 ml of 99% sulfuric acid. The mixture was mixed well and incubated in a water bath at a temperature of (25-30) C for 20 minutes. The tubes were left to cool. The concentration of carbohydrates was estimated by measuring the intensity of the color by means of a spectrophotometer at the wavelength 488 nm, with three replicates for each treatment and concentration, and then compared with the standard curve of carbohydrates.

Statistical analysis

The experiments included in the study were carried out using Completely Randomized Design (CRD) and factorial experiments, and the results were analyzed using the statistical program (SAS, 2004) and the averages were compared according to the Least Significant Difference (LSD) test at a probability level of 0.05 [16].

Results and Discussion

The effect of irradiation and NaCl and the interaction between them on the rate of cardiac glycoside production.The results shown in Table (1) and Figures (1 and 2) showed a significant increase in the average concentrations of the cardiac glycoside compounds Digoxin, Digitoxin, and Gitoxin by increasing the concentrations of NaCl added to the MS food medium. Cardiac glycoside compounds amounted to (129.46, 171.70, 145.59) µg.g^{-1} dry weight, respectively, while the NaCl-free treatment gave the lowest rate of glycoside compounds amounted to (67.62, 106.66, 88.14) µg.g^{-1} dry weight, respectively. The results also showed the superiority of the irradiated treatment significantly in the average concentrations of the same cardiac glycoside compounds, as it achieved a rate of (127.95, 193.95, 161.31 µg.g^{-1} dry weight, respectively, compared to the non-irradiated treatment, which achieved a rate of (68.36, 81.03, 73.79) µg.g^{-1} Dry weight straight. As for the results of the bilateral interaction, the results showed the superiority of the irradiation treatment and the interaction with the 12 g.l^{-1} substrate in giving the highest rate of glycoside compounds (164.09, 231.27, 194.11) µg.g^{-1} dry weight, respectively, while the lowest rate of cardiac glycosides concentrations was achieved when treating The non-irradiated and NaCl-free ones gave averages of (42.12, 58.18, 48.09) µg.g^{-1} dry weight, respectively.

Table (1): Effect of irradiation and NaCl and the interaction between them on the rate of concentration of cardiac glycosides compounds from the vegetative branches of digitalis after four weeks of cultivation on MS medium.

NaCl con $g.l^{-1}$	cardiac glycosides con $\mu g.g^{-1}$		
	Digoxin	Digitoxin	Gitoxin
0	67.62	106.66	88.14
3	81.69	118.38	102.08
6	98.17	135.48	117.78
9	113.85	155.22	134.16
12	129.46	171.7	145.59
L.S.D.(0.05)	1.21	1.22	1.21
irradiation			
radiant (30)gray	127.95	193.95	161.31
not irradiated	68.36	81.03	73.79
L.S.D.(0.05)	0.76	0.77	0.76
overlap			
0- radiant	93.11	155.14	128.18
3- radiant	108.15	174.13	145.06
6- radiant	128.20	191.07	160.18
9- radiant	146.22	218.13	179.02
12- radiant	164.09	231.27	194.11
0- not irradiated	42.12	58.18	48.09
3- not irradiated	55.22	62.63	59.09
6- not irradiated	68.14	79.89	75.38
9- not irradiated	81.48	92.31	89.30
12- not irradiated	94.82	112.13	97.07
L.S.D.(0.05)	1.71	1.72	1.71

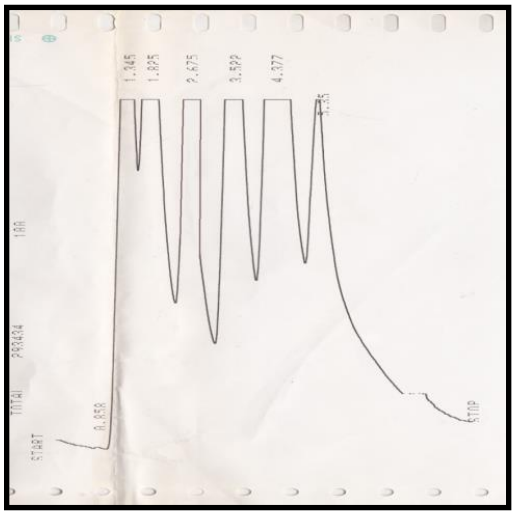

Figure (1): Effect of irradiation 30 gray and NaCl 12 g.l-1on glycoside production

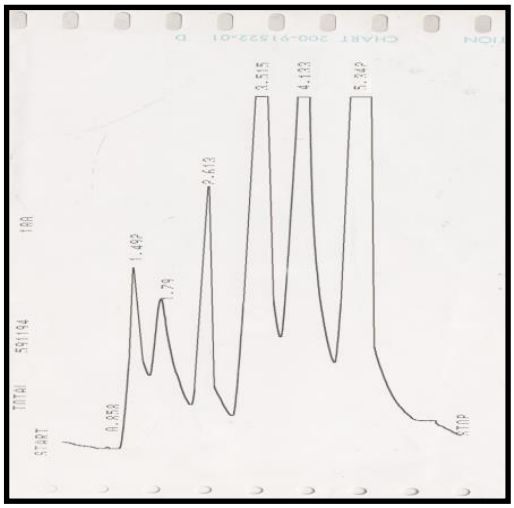

Figure (2): Effect of NaCl 120 g.l-1 on glycoside production

Seq	Compound	Retention time	Area
1	Digoxin	2.775	367619.66
2	Digitoxin	3.522	608078.21
3	Gitoxin	4.377	455181.47

Seq	Compound	Retention time	Area
1	Digoxin	2.613	212546.93
2	Digitoxin	3.515	294823.40
3	Gitoxin	4.133	227625.91

The effect of irradiation and NaCl and the interaction between them on the average concentrations of chlorophyll and carbohydrates.

Table (2) shows a decrease in the rate of formation of chlorophyll and carbohydrates by increasing the concentrations of NaCl added to the MS food medium. The highest rate of chlorophyll was achieved when the neutral treatment was 2.10 mg. g^{-1}, and the concentration 3 g .l^{-1} gave the highest rate of carbohydrates amounting to 2.94 mg. g^{-1}, while the concentration achieved 12 g .l^{-1} The lowest average for chlorophyll and carbohydrates was (1.81 and 2.49) mg. g^{-1}, respectively. The irradiation treatment also had a significant effect on the rate of formation of chlorophyll and carbohydrates, as it achieved a rate of (2.38, 3.11) mg. g^{-1}, respectively, compared to the non-irradiated treatment, which achieved a rate of (1.49, 2.46) mg. g^{-1} respectively. As for the interaction effect, the irradiation treatment excelled at a concentration of 0.0 NaCl and gave the highest rate of chlorophyll which reached 2.51 mg. g^{-1}, and the same treatment was superior at concentration 3 g .l^{-1} NaCl and gave the highest rate of carbohydrates amounting to 3.32 mg. g^{-1}, while the lowest rate of chlorophyll and carbohydrates was achieved. When the non-irradiated treatment and at the concentration of 12 g .l^{-1} of NaCl reached (1.35 and 2.32) mg. g^{-1} respectively.

Table (2): The effect of irradiation and NaCl and the interaction between them on the average concentration of chlorophyll and carbohydrates from the vegetative branches of the digitalis plant after four weeks of cultivation on MS medium

NaCl con $g.l^{-1}$	Chlorophyll con $mg.g^{-1}$	Carbohydrate con $mg.g^{-1}$
0	2.10	2.91
3	1.97	2.94
6	1.92	2.83
9	1.89	2.76
12	1.81	2.49
L.S.D. (0.05)	0.10	0.22
irradiation	2.38	3.11
radiant (30) gray	1.49	2.46
not irradiated	0.06	0.01
L.S.D. (0.05)	2.51	3.28
overlap	2.40	3.32
0- radiant	2.37	3.17
3- radiant	2.35	3.11
6- radiant	2.26	2.65
9- radiant	1.68	2.54
12- radiant	1.53	2.56
0- not irradiated	1.46	2.49
3- not irradiated	1.43	2.41
6- not irradiated	1.35	2.32
9- not irradiated	0.15	0.03

On the basis of the results above, it was found that there is a significant effect of salt tension on the amount of cardiac glycoside compounds whose quantity increased by increasing the levels of salinity in the food medium. It leads to an increase in the process of photosynthesis and then an increase in the manufacture of secondary metabolites, including cardiac glycosides [17]. The reason for the increase in cardiac glycoside compounds may be due to the fact that cells growing under conditions of salt stress have increased levels of amino acids such as arginine, alanine, leucine, serine, glycine, valine as well as proline and amides such as glutamine, asparagines [18] and an increase in amino acids under stress. Salt can synthesize polyamines [19] which are key to the synthesis of many secondary metabolites. The positive role of sodium chloride may be due to the role of chlorine in the photophosphorylation processes that contribute to the construction of sugars [20], and these conditions may be considered the best for increasing the efficiency of secondary metabolism processes. This agreed with what was found by [21] in the increase of purine to five times when the medium of suspended cells of *Coffea Arabica* was treated with 9 $g.l^{-1}$ NaCl. And what was found by [22] that the concentration of 12 $g.l^{-1}$ NaCl was the best in achieving the highest concentration of Digitoxin which reached 40 $\mu g.g^{-1}$ dry weight in the

vegetative branches cultures of the plant *Digitalis purpurea*, it was found [23] that there is an increase in Digitoxin concentration by increasing the concentration of NaCl added to the nutrient medium of digitalis lanata cultured vegetative branches to reach 2.20 mg.g^{-1} dry weight in the medium prepared with 12 g.l^{-1} NaCl. With what [24] mentioned in the study that targeted the effect of NaCl concentrations on the rate of doubling of the peppermint plant and some biochemical characteristics of in vivo cultures, the results showed that the concentration of 2 g.l^{-1} led to a significant increase in the plant's content of chlorophyll and soluble sugars, while it achieved Concentration of 8 g .l^{-1}, the highest rate of menthol compound was 0.99 mg. g^{-1} compared to the neutral treatment. Likewise, it has been mentioned [25] that there was an increase in the accumulation of cardiac glycoside compounds (Digitoxin and Gitoxin) that reached three times in the content of the suspended cells of *Digitalis thapsi* in response to the addition of calcium chloride to the growth medium.

Through the study, it was concluded that irradiation and adding different concentrations of sodium chloride to the MS medium had a positive effect in stimulating an increase in the rate of cardiac glycoside production.

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