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The Impact of Some Auxins on the Concentration of Alkaloids in Datura Stramonium (In vitro)

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ABSTRACT

This study was conducted for studying this plant (*Daturastramonium*) and procreating it with tissues by biotic techniques, with well-defining of quantity of Alkaloids contained in it and the impact of the combination of Auxin and Cytokinin upon this plant productivity of Alkaloids and studying the impact of nutrient medium combination upon quantitative production ofTropane Alkaloids in the experimentally propagateddatura plantlets, where quantitative and qualitative determination of Tropane Alkaloids was done by using High-Performance-Liquid-Chromatography (using column C18). The results showed significant differences in average alkaloids concentration inside leaves according to the variation in the added concentration of Auxins and Cytokinin to the nutrient medium, where treatment MS_{14} was superior, and alkaloids quantity average in leaves amounted to 61.32 mg/ml. Alkaloids production inhibition was recorded in two treatments, MS_8 and MS_{12}. In the stalks, treatment MS_{14} was also superior, the average of alkaloids quantity was 52.727 mg/ml, and alkaloids production inhibition was recorded in two treatments MS_8 and MS_{12} as well. The tissue culturing of *DaturaStramonium*is considered as an effective mean in producing Tropane Alkaloids, known for its importance in the global codes of medication, by using the hormonal combination that increases the concentrations of those alkaloids.

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INTRODUCTION

Plants are the main source of drugs, which began to be used by traditional medicine, and then its medical usage evolved after studying its efficiency and ensuring its therapeutic ability. Since the discovery of medicinal plants, pharmaceuticals were made of it, as aspirin which produced as a natural product from plants by Buchner (1826). In a study conducted by the WHO which reported that there are 21,000 species of plants can be used as medical plants, and about 5,000 of them have been studied and at least 121 botanical drug were knowingly composed, but none of them were produced artificially [7].

There was a growth in interest in tissue culture of plants' cells and tissues to be approved as an alternative for traditional cultivation in plants procreating, and in producing medical substances from its plant sources apart from environmental conditions, and plants growth in particular season exclusively, as well as the instability of these environmental factors, and plants being exposed in their natural environments to the risk of pests and insects, which leads to the difficulty of getting these important medically useful substances in appropriate useful quantities and constant quality [12].Tissue culture Technology allows attaining to the same valuable medical compounds with high purity, and its production is rapid without relying on the season in which plants usually grow, and without the need to allocate large areas for the purpose of cultivation and exploitation of these areas for the cultivation of economically important crops [2].[8], found that adding 2.4-D and 1% sucrose enhances the production of Tropane alkaloids where the amount of alkaloids was 0.75% higher in the cell cultivation than it was in the original plant 0.352%.. Objectives

Objectives:

Datura plant is one of the most important medical plants in the world for its medical importance, either in the traditional or constitutional medicine as a main resource of Tropanealkaloids;inparticularhyoscyamine,

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scopolamine and atropine. Thisplant was examined and propagated in tissues culture by biotic techniques, with well-defining of alkaloids quantity and the effect of Auxins and Cytokininscombinationsonthis plant productivity of alkaloids, in addition to studying the effect of nutrients Medium combination upon the quantitative production of Tropane alkaloids in experimentally propagatedDatura plants. where quantitative and qualitative estimation of Tropane Alkaloids using High-Performance-Liquid-Chromatography (HLPC).

MATERIAL AND METHODS

Site of Research:

This research was conducted in the laboratory of medicinal and aromatic plants and the laboratory of industrial and food technologies at the National commission for Biotechnology /Damascus/ during the period from 2012- 2014.

Plant Material:

*DaturaStramonium*seedsinthe Biotechnology of Medicinal and Aromatic PlantsLaboratory in the National commission for Biotechnologywere used in these experiments. These seed were originally collected from Kalamoon area in Damascus Countryside during field tours in 2012.

Tissue culture:

Preparation and sterilization of Nutrients Medium:

Initial Culture Medium:

Nutrient medium MS (Murashige&Skooge, 1962)was prepared and divided into 150x 25 mm Pyrex glass tubes; 15 ml / tube in average. Tubes were closed with cotton plugs, sterilized with autoclave in 121^{o}C for 20 min. and was left for cooling until they become ready for planting.

Table 1: MS Medium Components

MS Components	Concentration per 1 liter
Macronutrients	50 ml
Micronutrients	1 ml
Thiamine	1 ml
Mono potassium phosphate	50 ml
Chelated Iron	5 ml
Sucrose	30 g
Agar	9 g

Vegetative MultiplicationMedium of Datura:

Several hormonal combinations including Auxins (IBA, NAA) and Cytokinin (BAP, kin) with studied concentrations, were used in order to determine the best hormonal combination to create the best growth and number of *Daturastramonium* growing, as shown in the table below:

Table 2: Hormonal combinationsused in experimentally propagated *Daturastramonium*.

Medium Code	Growth Regulators (Concentrationμm)			
	Cytokinins		Auxins	
	BAP	Kin	IBA	NAA
MS0	0	0	0	0
MS1	0,5		1	
MS2	1		1	
MS3	1,5		1	
MS4	2		1	
MS5	0,5			1
MS6	1			1
MS7	1,5			1
MS8	2			1
MS9		0,5	1	
MS10		1	1	
MS11		1,5	1	
MS12		2	1	
MS13		0,5		1
MS14		1		1
MS15		1,5		1
MS16		2		1

Preparation and Surface disinfection of Explants:

Initial Culture Phase:

Initial Culture Phase or so-called Foundational culture is considered as the first stages of the tissue culture, which aims to obtain pollute-free samples and able to grow.Surfacesterilization for plants parts used in tissue culture (Explants) is the first and most important step on which success of tissues culture depends. It means the sterilization of plant samples in order to eliminate microorganisms that grow on the external surface of plant part used in the tissue culture.

Seeds of Daturawere washed by running water for 30 min. then were rinsed in Sulfuric Acid 0.4N for 10 min. to irritate the seeds' casing which is considered as one of the most important obstacles to germination. Afterward, seeds were immersed in Ethylalcohol 70% for one minute, and then were transferred into Sodium Hypochlorite NaOClwhich has been used as an antiseptic substance. They were washed thrice with sterile distilled water; once each five minutes in average. Then, they were exposed for 30 min to get dry by air and become ready for cultivation. Final wash and cultivationwere conducted in strict isolation conditions under bacterial isolation device from JSCR-1200 SB.

Cultivation Method:

21replicates of each treatment (concentration X duration) distributed as one seed per replicate. They were incubated in 24±2^{o}C until germination of seeds. Then germination and pollution percentage was calculated after one week of cultivation. Growing plants were transferred after a month of cultivation to new medium of same combination of Initial Culture Medium, supported with GA3 (200 µl/ L) to ensure elongation of plants to provide required and sufficient quantity for studying. Resultant plants were cultivated in Gibberellin-Free Initial Culture Medium twice consecutively to get rid of its impacts in plants when cultivated in MultiplicationMedia.

Multiplication and Elongation Stage:

This phase aims to obtain the highest possible number of well-growth shoots. Therefore, all samples (shoots) resultant from Initial Culture Phase were movedto MultiplicationMedium to increase the resulting shoots, and obtaining sufficient number of shoots for Multiplication and studying some of effecting factors.

Effect of various hormonal combinations have been studied in terms of the type of hormone used and its concentration in the number and length of resulting shoots, and the number of resulting leaves after one month of cultivation. For this purpose, hormonal combinations shown in table (2) were used:

- Benzyl Amino Purine and Indole Butyric Acid (BAP + IBA)
- Benzyl Amino Purine and Naphthalene Acetic Acid (BAP + NAA)
- Kinetin and Indole Butyric Acid (Kin + IBA)
- Kinetin and Naphthalene Acetic Acid (Kin + NAA)

Results were analyzed with SPSS to determine least significant difference at level of significance 5%.

Chemical Study:

Chemical analysis of the leaves and stalks of the *Daturastramonium*plantletswas conducted, where Alkaloids concentration (hyoscyamine and scopolamine) in the leaves and stalks of growing plantlets of two months age according to the following steps:

Alkaloids Extraction:

Alkaloids were extracted according the following step (Kamada*etal*, 1986):

- 1 gram of dry plant powder was retted in a mixture of (60 ml Methanol + 4 ml Chloroform + 20 ml Ammonium hydroxide). Samples were put in Ultrasonic device for 15 min.

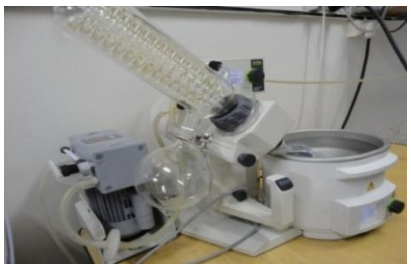

Fig. 1: Ultrasonic Device.

- The remnants of the powder were separated from the extract by filtration under vacuum as in fig. (2). The remnants were washed with 4 ml of chloroform, in order to extract all Alkaline salts from the powder.

Fig. 2: Filtration under vacuum.

- The sample was vaporized in a rotary evaporator until it is completely dry.

Fig. 3: Rotary evaporator.

- 5 ml chloroform + 10 ml sulfuric acid were added. Extract was put then in decantation tube to separate the acid phase from chloroform. Chloroform layer was taken and pH level was adjusted at 10 by Ammonium hydroxide 28%, then 4 ml chloroform was added.
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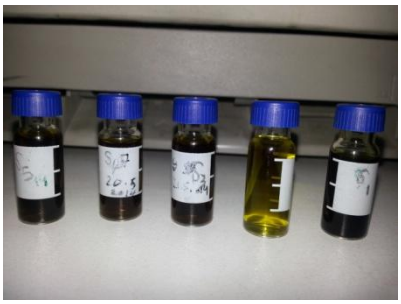

Fig. 4: Separation of organic phases in decantation tube

- Sample was dried by adding 1 gram of anhydrous sodium sulfate Na_2So_4. Remnants of powder were washed with 4 ml chloroform. Chloroform was released then by rotary evaporator at 40 ^oC until it is dry. Final extract was solved in 3 ml of Methanol, specific for chromatographic analysis.1 µL of solved extract was taken and injected in HPLC.

Quantitative and qualitative determination of Tropane Alkaloids:
High-Performance-Liquid-Chromatography (HPLC):

Separation principle in HPLC relies upon sample being distributed between two phases. One of them consists of a fixed layer with large area. The second is a liquid that moves within the fixed phase. Mostly, HPLC is used in Biochemistry and analytical chemistry to separate, determine and measure the compounds within one mixture.(Central Laboratory - Ministry of Science and Communications). It is used also in the quantitative and qualitative of medicines, amino acids, botanical extracts and pesticides. (Environmental Sciences Research Division).

Fig. 5: HPLC Device.

Terms of analysis:

Column C18 or what so-called L1, (25 cm × 4 mm i.d., RP).
Flow Rate: (1.5) ml/min.
Thermal program:

- Primary temperature: 70 ^{o}C for one min.
- Then it should be raised from 70-250 ^{o}C (9 degrees/min. in average).
- Temperature must be maintained at 250 ^{o}C for 13 min.

Temperature of Detector: 250 ^{o}C
Temperature of Injector: 250 ^{o}C
Sample analysis duration: 42 min.
Injected quantity: 1µl.
Holding phase (moving phase) is a mixture of Methanol and water (50:50).
Length of wave: 210 nm.

RESULTS AND DISCUSSION

a. Effect of growth regulators upon Alkaloids concentration in leaves of D. stramonium:

Results shown in table (3) demonstrates the existence of significant differences in the average of alkaloids concentration in leaves by variations in added concentration of Auxins and Cytokinins to nutrient medium (Test F significance value ≤ 0.05). MS_{14} was superior, and the average of alkaloids concentration in leaves reached 61.32 mg/ml, followed by MS_{15} with average of alkaloids concentration in leaves 40.73 mg/ml, then MS_9 with average 38.76 mg/ml. These three treatments were superior to the control MS_0 in the quantity of Alkaloids. That means that interaction of concentrations of Auxins and Cytokinins in these treatments has an inducing effect for increasing the concentrations of alkaloids in leaves. Alkaloids percentage varied in the other treatments in significant differences, but the percentage was less than the control. Alkaloids inhibition was noted in two treatments; MS_8 and MS_{12}. Average of alkaloids concentration in these two treatments was 0, as shown in table below and Fig.(6).

Number of researchers confirmed that Auxins added to the nutrient medium prepared for the cultivation of plant cells is making those cells lose their ability to assemble alkaloids (Rhodes et al., 1989).Iranbakhsh and Riazi (2000) showed that the best environment to optimize the production conditions and the accumulation of Tropane alkaloids in plantations of cellular pendants of *D. stramonium* is to cultivate the leaves part in MS medium supported with NAA hormone (0.5 g/l).While roots cultivation in MS medium with (IBA IndoleButyric Acid) of 0.5 mg/liter concentration has resulted in a high concentrations of alkaloids of about eight times more than its concentration in growth regulators-free MS medium (Natasha et al., 1993).Iranbakhshetal.(2007) have worked on optimization of production conditions and the accumulation of Tropane alkaloids in plantations of cellular pendants of *D. stramonium*created from callus created in turn from leaves in MS medium supported with K (0.5 mg) + (2) NAA. It turned out that Increase the concentration of nitrates led to a reduction of alkaloids.

Existence of AuxinsandCytokinins is necessary for strengthening the role of each other's role in the process of organic formation and improving the quality of formed shoots.Christison and Warnick (1988) explained that the organic formation is under the control of Auxin-Cytokininratio.

Some Cytokinins, and in the presence of Auxins, are increasing the response of plant to other hormones more than other. However, the presence of both Auxins and Cytokinins in necessary to motivate each other

function, as of the stimulant effect of one of them is weak, if found alone.This is explained by the basis of difference in their respective functioning sites; Auxin works on the level of replication in DNA during the prophase, while Cytokinin works in a late phase, at the cytoplasm division during telophase.The significant role played by growth regulators when added to the cultural Medium as Auxins working to supply the cells with water and help to activate the production of amino acids that helps in the elongation of the cells and enlarging their size. While Cytokinins are increasing the synthesis of nucleic acids, especially DNA, mRNA, and are also working on increasing the size of the cells by increasing its width, leading ultimately to an increase in the mass of stalks of the plant and increasing its humid weight (AbouZaid, 2006).The superiority of treatments MS_{14} and MS_{15} supported with 1 mg / l NAA and 1.5 - 2 mg / K, respectively, in the accumulation of Tropane alkaloids in all of the leaves and the stalks is attributed to the presence of each of Auxins (NAA) and Cytokinins (K) in balanced proportions, and inducing of Tropane alkaloids production significantly by stimulating the production of amino acids, especially acid ornithine, which is the initiator of building and accumulation of Tropane alkaloids (Mann, 1987).

Christen et. al. (1992) confirmed that plant growth regulators, if used in proper concentrations, will enhance the absorption of mineral elements;cations, particularly. They may induce the gene responsible for the production of Hyosyamine-6β Hydroxylase (H6H), inducing ultimately the increasing in production and accumulation of Tropane alkaloids (Rafael et al.2004; Zhang et al. 2004, Nabil et al., 2009). This corresponds with the results of (KadietYahia, 2007) on *Hyoscyamusalbus*.

Table 3: The effect of the mutual interaction between the different concentrations of AuxinsandCytokininsontheaverage of alkaloidsconcentration in the leavesof*D. stramonium*.

Treatment	scopolamine (mg/ml)	
	Mean	±SE
MS0	26.84 d*	.06
MS1	7.13 h	.06
MS2	4.85 i	.06
MS3	6.87 h	.06
MS4	5.28 i	.06
MS5	5.89 hi	.06
MS6	1.90 j	.06
MS7	14.22 f	.06
MS8	0.00 k	.00
MS9	38.76 c	.06
MS10	2.28 j	.06
MS11	16.66 e	.06
MS12	0.00 k	.00
MS13	10.23 g	.06
MS14	61.32 a	.06
MS15	40.73 b	.06
MS16	3.14 j	.06
F	107.606	
Sig.	0.000	
LSD	0.156	

*Different letters indicate the significant differences at statistical significance level of 0.05

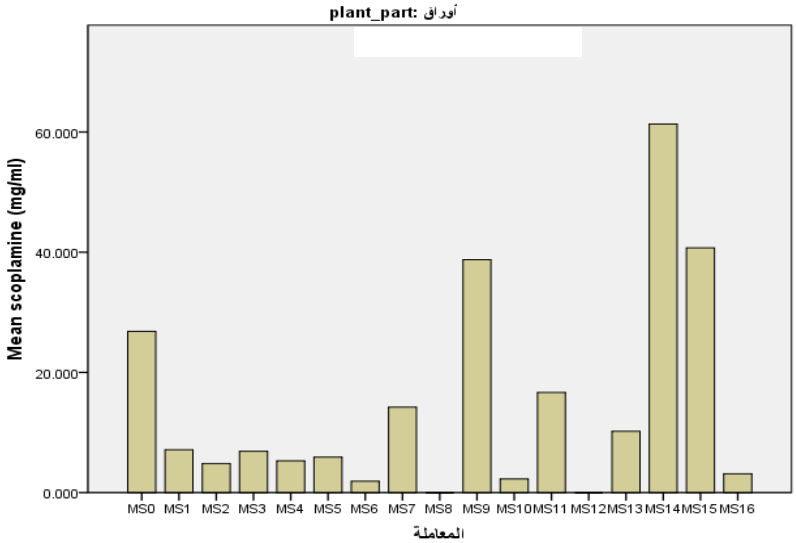

Fig. 6: The average concentration of alkaloids (scopolamine) in leaves by differences among the Medium of tissue culture used.

b. The effect of growth regulators on the concentration of alkaloids in the stalks of D. stramonium:

The results show the existence of significant differences in the average concentration of alkaloids in the stalks by the differences in the added concentration of Auxins and Cytokinins in the nutrient medium (F test significance value ≤ 0.05). Treatment MS_{14} surpassed with average of alkaloids in the stalks of 52.727 mg / ml, followed by treatment MS_{15} with average of alkaloids of 40.458 mg/ml, then treatment MS_9 with average of alkaloids of 38.732 mg/ml, where these three treatments surpassed the control MS_0 in alkaloids quantity. That means that interaction of Cytokinins and Auxins concentrations in these transactions had a provocative effect to increase the concentration of these substances in the stalks. It also had a provocative effect in the increase in alkaloids concentration in the leaves and its superiority on the control previously. Concentration of alkaloids differed in the rest of the transactions with significant differences for the control, but the percentage was less. Alkaloids production inhibition was noted in two treatments; MS_8 and MS_{12}. Average alkaloids concentration in these two treatments is 0, as shown in table (4) below and Fig. (7).The cultivating of *D.innoxia* in growth regulators-free medium (MS) leads to lower concentrations of hyoscyamine and hyoscinealkaloids.

Table 4: The effect of the mutual interaction between the different concentrations of Auxins and Cytokinins on the average concentration of alkaloids in the stalks.

Treatment	scopolamine (mg/ml)	
	Mean	±SE
MS0	23.340 d*	.950
MS1	7.426 h	.058
MS2	8.241 g	.058
MS3	4.462 j	.058
MS4	5.412 i	.058
MS5	3.826 k	.058
MS6	1.112 l	.058
MS7	12.925 f	.033
MS8	0.000 m	.000
MS9	36.732 c	.058
MS10	1.426 l	.058
MS11	14.873 e	.033
MS12	0.000 m	.000
MS13	8.217 g	.058
MS14	52.727 a	.058
MS15	40.458 b	.058
MS16	3.181 k	.058
F	451.542	
Sig.	0.000	
LSD	0.678	

*Different letters indicate for significant differences at statistical significance level of 0.05

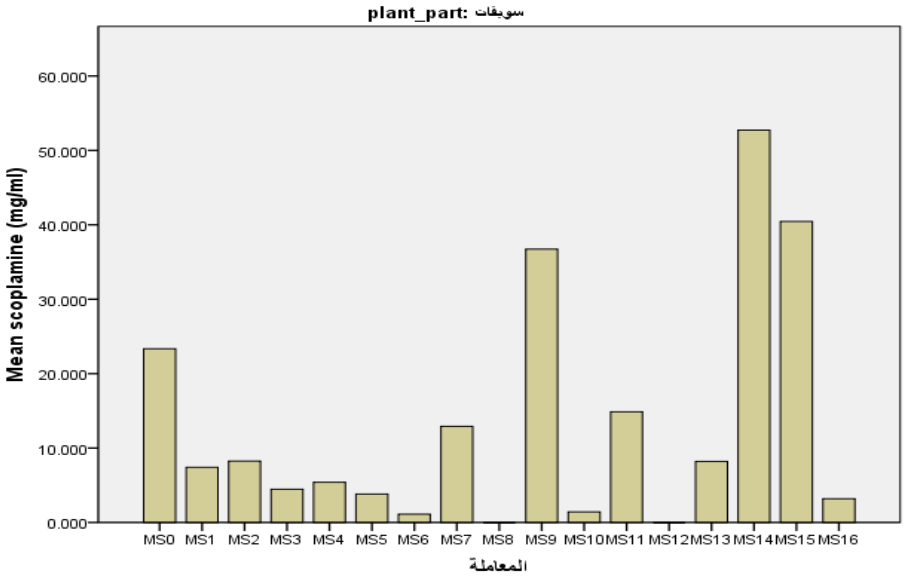

Fig. 7: The average concentration of alkaloids (scopolamine) in stalks by differences in percentages of added Auxins and Cytokinins.

The superiority of treatments MS_{14} and MS_{15} supported with 1 mg / l NAA and 1.5 - 2 mg / K, respectively, in the accumulation of Tropane alkaloids in all of the leaves and the stalks is attributed to the presence of each

of Auxins (NAA) and Cytokinins (K) in balanced proportions, and inducing of Tropane alkaloids production significantly by stimulating the production of amino acids, especially acid ornithine, which is the initiator of building and accumulation of Tropane alkaloids (Mann, 1987).Christen et. al. (1992) had confirmed that plant growth regulators, if used in proper concentrations, will enhance the absorption of mineral elements;cations particularly. They may induce the gene responsible for the production of Hyosyamine-6β Hydroxylase (H6H), inducing ultimately the increasing in production and accumulation of Tropane alkaloids (Rafael et al.2004; Zhang et al. 2004, Nabil et al., 2009). This corresponds with the results of (KadietYahia, 2007) on *Hyoscyamusalbus*.

The failure of both treatments MS_8 (supported with 1 mg / l IBA and 2 mg / l BAP) and MS_{12} (supported with 1 mg / l IBA and 2 mg /l K) in the synthesis of Tropane alkaloids is attributed to the use of Cytokinin in high concentration. While planting roots in the(MS) medium equipped with IBA Indole Butyric acid)) with concentration of 0.5 mg / L had resulted in high concentrations of alkaloids of what estimated at more than eight times the concentration in the growth regulators-free medium (MS) (Natashet al., 1993).

2. Study of the effect of Auxin type on the concentration of alkaloids in D. stramonium:

Results showed that Auxin NAA surpassed significantly over IBA in terms of alkaloids concentration in leaves in average of 17.179 mg/ml and 10.228 mg/ml respectively, while differences were not significant between them in terms of alkaloids concentration in stalks with average of concentration is 15.306 mg/ml for NAA and 9.822 mg/ml for IBA. Generally, it is noted that adding Auxins resulted in a significant decrease in quantity of alkaloids in both leaves and stalks in comparison with the control, where control surpassed alkaloids in an average of 28.838 mg/ml for leaves and 23.340 mg/ml for stalks, as shown in table (5) and figures (8 and 9).

There was an increase in the quantity of hyoscyamine and hyoscine with an increase in IBA in the callus growing medium (Al-abdaly, 1975). Auxin NAA surpass over IBA in this feature may be attributed to the high susceptibility of the cells to absorb NAA by cell in better way. It may be attributed to the length of its duration of effectiveness inside plant cells in comparison with the rest of Auxins (Hartmann et al., 2002).

Iranbakhsh and Riazi (2000) argued that the best environment to obtain examples of production conditions and accumulation of Tropane alkaloids in plantations of cellular pendants of *D. stramonium* is MS includes NAA hormone (0.5 g/l).

Table 5: Effect of Auxin type on the average concentration of alkaloids in the leaves and stalks of *D. stramonium*.

	Leaves		Stalks	
	Mean	±SE	Mean	±SE
Control	26.838 a*	.058	23.340 a*	.950
IBA	10.228 c	2.443	9.822 b	2.300
NAA	17.179 b	4.307	15.306 b	3.903
F	1.892		1.458	
Sig.	0.016		0.024	
LSD	6,746		5.584	

*Different letters indicate for significant differences at statistical significance level of 0.05

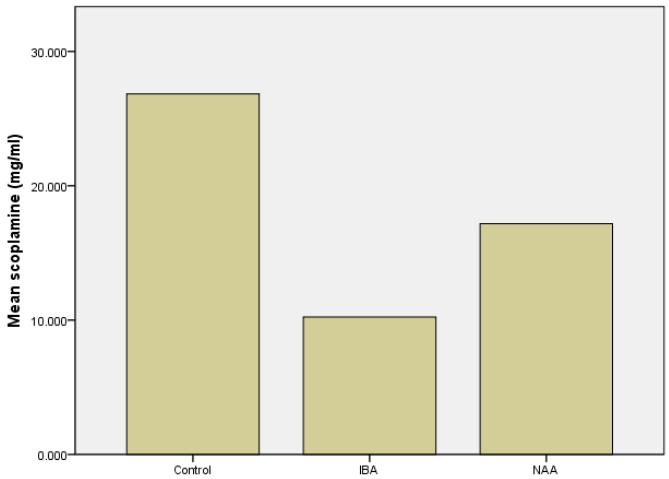

Fig. 8: The average concentration of alkaloids (scopolamine) in leaves according to the type of Auxin compared with the control.

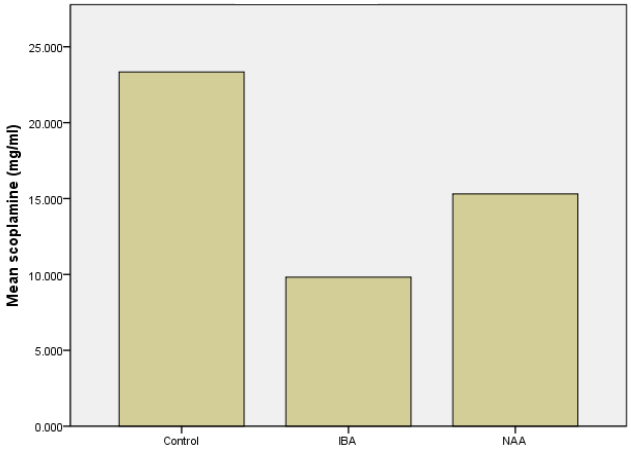

Fig. 8: shows the average concentration of alkaloids (scopolamine) in stalks according to the type of Auxin compared with the control.

3. Effect of Cytokinintype on the concentration of alkaloids in the leaves and stalks of D. stramonium:

Results shown in table (6) stated that Cytokinin K surpass significantly over BAP in terms of Alkaloids concentration in leaves with an average of 21.640 mg/ml and 5.767 mg/ml respectively. Furthermore, differences were significant between them in terms of Alkaloids concentration in stalks with an average of 19.702 mg/ml and 5.426 mg/ml respectively. Generally, it is noted that adding Cytokinins resulted in a significant decrease in quantity of alkaloids in both leaves and stalks in comparison with the control, where control surpassed alkaloids in an average of 28.838 mg/ml for leaves and 23.340 mg/ml for stalks, as shown in table (6) and figures (10 and 11). Al-Khaledy (2005) noted that presence of Cytokinins, Benzyl adenine BA in particular with concentration of 0.5 mg/liter, in callus growing medium of *D.stramonium* and *D.Innoxia*, caused an increase in production of hyoscyamine and hyoscine alkaloids compared in its quantity in the origin plant. The superiority of K over BAP can be attributed in general to the fact that Kinetin increases the absorption of mineral elements and the accumulation of carbohydrates (Richter, 1993) and these materials in the beginning of growth are consumed in the formation of the shoot, while successive phases are accumulating alkaloids that are considered as final metabolism outcomes and a Nitrogen storage in plant (Maa and Majie, 1992). This was noted in treatments that includes Kinetin (Al-Shahhat, 2006). It corresponds with the results of (Trease and Evans, 1996) study when treating Hyoscyamusmuticus with Kinetin. That was considered as significant increase in alkali yield.

Table 6: Effect of Cytokinins on the average concentration of alkaloids (scopolamine) in the leaves.

Cytokinin	Leaves		Stalks	
	Mean	±SE	Mean	±SE
Control	26.838 a*	.058	23.340 a*	.950
BAP	5.767 b	.821	5.426 b	.807
Kin	21.640 a	4.407	19.702 a	4.012
F	7.597		7.196	
Sig.	.001		.002	
LSD	8.823		8.057	

*Different letters indicate for significant differences at statistical significance level of 0.05

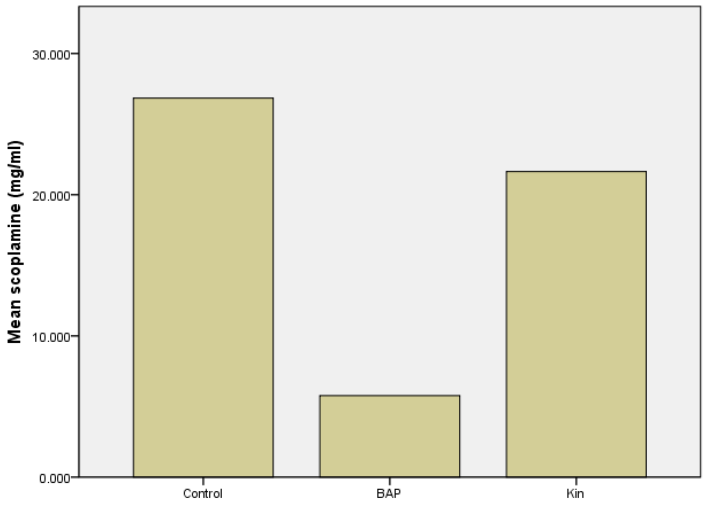

Fig. 10: The average concentration of alkaloids in leaves according to the type of Cytokinin compared with the control.

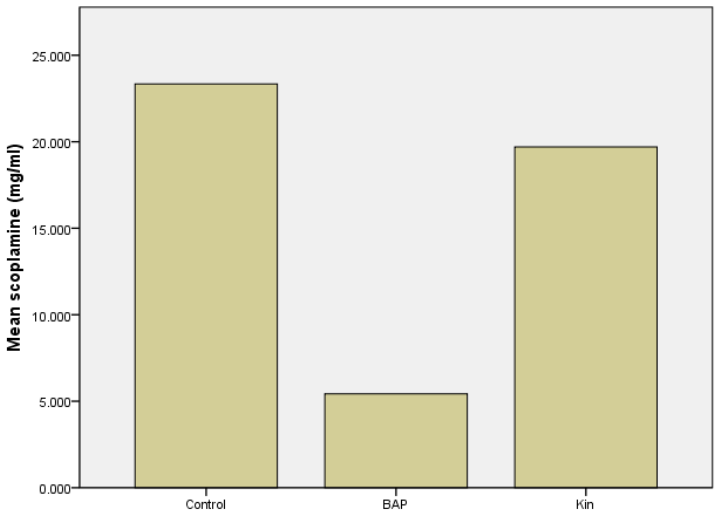

Fig. 11: The average concentration of alkaloids in stalks according to the type of Cytokinin compared with the control.

Conclusion:

Growth regulators added to tissue culture medium, especially Auxins and Cytokinins, have an effect on the process of growth and differentiation of plantations. It affects also the process of formation ofsecondary metabolism products. In addition, the type and concentration of growth regulator affects the formation of secondary metabolism products might result in increasing the formation of certain substances or reducing the formation of others according the balance between Auxins and Cytokinins added to growth medium; high concentrations motivates formation of certain substances, while low concentrations motivates formation of others.

Growth regulators affect the formation of secondary metabolism products, because they have effect the effectiveness enzymatic responsible for formation of secondary metabolism products. Thus, the tissue culture technique for the medical plants provides possibility of producing medically effective compounds in oneway or another. The Totipotency of plant cells *in vitro* provided access to secondary compounds which are formed in the normal plant cells. Outcomes of this study find out that tissue culture of *DaturaStarmonium*consideredas an effective tool in producing Tropane alkaloids known for its importance in the constitutions of the global pharmaceutical by using a hormonal combination that increases the concentartions of these alkaloids.

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