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Effect of growth regulators on *in vitro* production of *Hyoscyamus aureus* L. and tropane alkaloids

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Abstract: Hyoscyamus is an herbal plant belonging to family *solanaceae* that consider a natural pool of secondary metabolites called alkaloids. This study aimed to determination the optimum artificial conditions for rapid, mass production of hyoscyamus and tropane alkaloids *in vitro* under different growth regulators (2, 4-D, NAA and IAA) with different concentrations. By adding 2, 4-D with 1mg/l and NAA with 2 mg/l, to MS media gave the highest callus induction frequencies (%) (88.6 and 72.5 %) respectively. The maximum callus fresh weight archived after 40 days only on MS media containing 2 mg/l NAA (1.817 gm) and after 100 days from culture on MS media containing 1mg/l 2, 4-D (1.41gm). However, the amount of alkaloid yielding of callus was decreased by increasing 2,4-D concentration, moreover, these alkaloid amounts increased by increasing days of culture on MS media with NAA and IAA.
Key words: *Solanaceae, Hyoscyamus aureus* L., *Hyoscyamus, alkaloids, in-vitro, callus.*

Introduction

Family *solanaceae* is one of the largest families in plant Kingdome. There are about 33 species out of 8 genera from this family indigenous to Egypt, distribution different localities[1]**.** Many plants from the Solanaceae family are considered as medicinal plants. The tropane alkaloids, found mainly in the plants of this family, contain the anticholinergic drugs hyoscyamine and scopolamine. Solanaceous plants, used traditionally for their medicinal, hallucinogenic and poisonous properties, were due to these tropane alkaloids[2].

The majority of this family that is consider as a natural pool of secondary metabolites that is called alkaloids, however, it contains a wide range of these alkaloids, ie, tropane alkaloids, indole alkaloids, iso quinoline, purine, pyrazole, pyridine etc.

Hyoscyamus is a highly diversified genus in the Solanaceae family and comprises twelve to fifteen species, Hyoscyamus is one of the important plants in this family, and however, all plant parts contain tropane alkaloids (hyoscyamine, scopolamine, and atropine) and are toxic to humans and animals when they are ingested in large amount. Moreover, these alkaloids have many medicinal importances, ie, action on Autonomic Nervous System (ANS), Ophtholmic properties, action on the Central Nervous System (CNS), Parkison's disease, action on Respiratory system and Anti inflammatory.

Plant tissue culture techniques offer a valuable tool for mass propagation, biomass production, plant pathology, plant tissue preservation and scientific research[2,4,5]**.** Application of tissue culture in medicinal plants has proven to be useful in the production of therapeutic compounds, so that, it has been suggested as an

alternative method for the production of these phytochemicals. LAI E.M.[6] had proven that tropane alkaloids could be obtained via micropropagated plantlets and the root cultures of *Hyoscyamus niger*.

From previous notes, it was clear that Hyoscyamus is rich in antispasmodic tropane alkaloids which characterized by their medicinal values, so that, this study aimed to:

1. Rapid and mass production of secondary products by plant tissue culture.
2. Determination the optimum conditions for *in vitro* production of secondary metabolites.
3. Studying the factors that effects on the yield of tropane alkaloids under *in vitro* conditions.

Materials and Methods

Hyoscyamus aureus L. seeds were kindly obtained from Pharmacology Dept, Faculty of Pharmacy, Zagazig University. The present investigation was carried out during two successive seasons, 2013/2014 and 2014/2015 at the Greenhouse Genetics Dept. Agric. Fac. Zagazig Univ., and Tissue culture laboratory, Botany Department, National Research Centre.

Methods

The work in this study divided into two main experiments (*In vivo* and *In vitro*), the first one (*In vivo*) was at the greenhouse to obtain seedling and leaves explants. The second one (*In vitro*) was at the laboratory to obtain callus tissues and determine the optimum conditions to produce good and healthy callus of Hyoscyamus and also studying the factors that effect on callus growth and alkaloids production.

Callus induction:

The leaves of sown plant planted on a solid MS medium[7] for callus induction. Three types of growth regulators with different concentrations were applied in the present study (Table 1).

Leaves were divided into small segments and two segments per jar were cultured on the media with a replication of 10 jars per different hormonal concentration, and all jars were incubated at 25 ± 2ºC in artificial light (16 hrs and 8 hrs darkness).

Table 1: Composition of different media with different growth regulators at different concentrations for callus induction from leaf explants of *Hyoscyamus aureus* L.

No	MS-media composition	Growth regulators	Growth regulator concentration
1	**MS** (4.4g/l) + **Sucrose** (30g/l) + **Agar** (7.5 g/l)	**2,4 -D**	**Free (zero mg/l)**
			1 mg/l + 0.5mg/l (Kin)
			2 mg/l + 0.5mg/l (Kin)
			3 mg/l + 0.5mg/l (Kin)
2	**MS** (4.4g/l) + **Sucrose** (30g/l) + **Agar** (7.5 g/l)	**NAA**	**Free (zero mg/l)**
			1 mg/l + 0.5mg/l (Kin)
			2 mg/l + 0.5mg/l (Kin)
			4 mg/l + 0.5mg/l (Kin)
3	**MS** (4.4g/l) + **Sucrose** (30g/l) + **Agar** (7.5 g/l)	**IAA**	**Free (zero mg/l)**
			1 mg/l + 1 mg/l (Kin)
			2 mg/l + 1 mg/l (Kin)
			3 mg/l + 1 mg/l (Kin)

Results and Discussion

Effect of different hormonal concentrations on callus induction of *Hyoscyamus aureus* L.

There were a markedly differences in callus induction frequency between different growth regulators Table (1) and Fig. (1). However, the growth regulator 2,4- D with all concentrations recorded the highest percentage in hyoscyamus callus induction in comparable with other used growth regulators (NAA and IAA) and control (free medium) which recorded the lowest percentage (0%). There were highly increasement in callus induction frequency (**65.43%**) by using 1 mg/l. 2, 4-D, on the other hand, there were a markedly decreasement with increasing 2, 4-D concentration up to 3 mg/l. 2, 4-D that scored the lowest value (30.15%). Moreover, the callus induction frequency (%) increased regularly by increasing days (age of callus) till 60 days which found the maximum percentage (88.6, 74.2 and 40.9 %) with 1, 2 and 3 mg/l. 2,4-D concentrations respectively, and after that this percentage was decreased Table (1) and Fig. (1).

Moreover, by using other growth regulators (NAA and IAA), callus induction frequency increased in response to the increase of both NAA and IAA concentrations up to 2 mg/l. which possessed the highest (59.83 and 38.47 %) value in both NAA and IAA, respectively. Table (1) and Fig. (1). With regard to previous data, 2,4-D with 1 mg/l consider the best growth regulator for inducing high percentage of callus induction frequency in hyoscyamus as reported in previous studies [8-13].

Table 2: Combinations of different growth regulators at different concentrations for callus production from leaf explants of *Hyoscyamus aureus* L.

Growth regulators	Conc.	Age of callus (days)						Mean
		10	20	30	40	60	100	
Control	**Free (zero mg/l)**	-	-	-	-	-	-	-
2,4 -D	**1 mg/l + 0.5 mg/l**	**28.7**	**50.2**	**65.5**	**78.7**	**88.6**	**80.9**	**65.43**
	2 mg/l + 0.5mg/l (Kin)	26.3	41.6	53.9	66.1	74.2	80.0	**52.67**
	3 mg/l + 0.5mg/l (Kin)	11.0	25.9	29.4	34.5	40.9	39.2	**30.15**
NAA	**1 mg/l** + 0.5 mg/l	14.9	25.8	34.9	41.1	54.4	52.3	**37.23**
	2 mg/l + 0.5mg/l	**33.8**	**49.7**	**61.5**	**69.2**	**72.5**	**72.3**	**59.83**
	4 mg/l + 0.5mg/l (Kin)	30.6	44.4	47.9	58.2	68.0	68.0	**52.85**
IAA	**1 mg/l** + 1 mg/l (Kin)	22.4	26.2	33.0	39.4	43.2	43.0	**34.53**
	2 mg/l + 1 mg/l (Kin)	**26.6**	**31.3**	**35.6**	**41.0**	**49.8**	**46.5**	**38.47**
	3 mg/l + 1 mg/l (Kin)	20.7	24.9	31.8	35.0	40.0	38.05	**31.75**

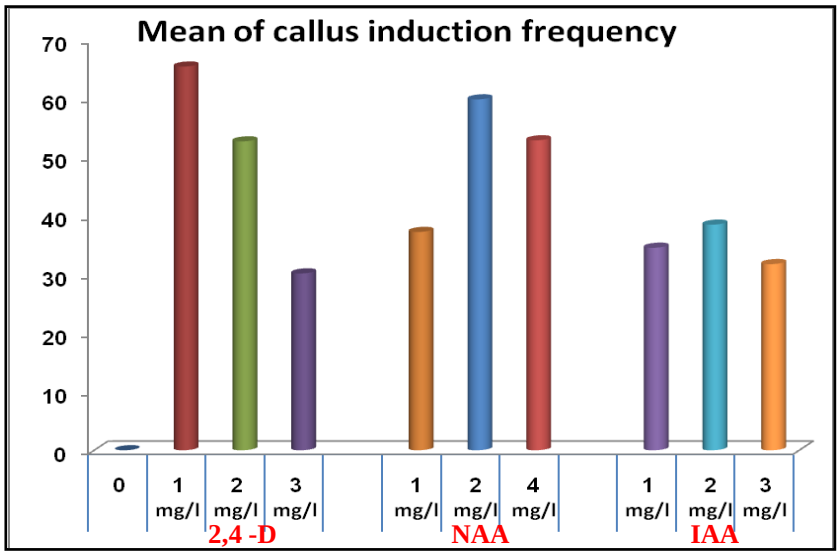


Fig. 1: Average mean of callus induction frequency of *Hyoscyamus aureus* L. under different hormones and concentrations.

Callus Growth Criteria:

The growth characteristics and pattern of calli derived from leaf explants of *Hyoscyamus aureus* L. growing in MS media containing 1,2 and 3 mg/l of 2,4-D and 0.5 Kin. were investigated (Table 3). Calli growth patterns were expressed as callus fresh weight (mg). Callus grown on MS media containing 1 mg/l. 2,4-D reached to the maximum value callus fresh weight after 100 days (1.41 gm), and the lowest amount (1.01 gm) was in the media that contained 3 mg/l. 2,4-D. These results were in disagreement with that obtained by El-Bahr M.K.[14] recorded that the best growth as expressed on callus fresh weight was obtained at 2.0 mg/l 2,4-D in fenugreek, and[13] on *Atropa belladonna* L. Moreover, Table (4) illustrates the effect of both NAA and IAA on callus growth dynamics (callus fresh weight) in *Hyoscyamus aureus* L. With regard of both NAA and IAA concentrations and callus fresh weight, MS media containing 2 mg/l. of both NAA and IAA possessed higher values of callus fresh weight (1.817 and 0.998 gm, respectively).

Table 3: Effect of different hormonal concentrations of 2, 4- D on callus growth (fresh weight (gm) of *Hyoscyamus aureus* L.

Growth regulators	Conc.	Callus fresh weight (gm)		
		30 days	60 days	100 days
Control	Free (zero mg/l)	-	-	-
2,4 -D	**1 mg/l + 0.5 mg/l (Kin)**	**0.70**	**1.00**	**1.41**
	2 mg/l + 0.5mg/l (Kin)	0.91	0.98	1.19
	3 mg/l + 0.5mg/l (Kin)	0.71	0.86	1.01

Table 4: Effect of NAA and 2 mg/l IAA in combination with 1 mg/l Kin. on callus growth (fresh weight (gm) of *Hyoscyamus aureus* L.

Growth regulators	Conc.	Callus fresh weight (gm)			
		10 days	20 days	30 days	40 days
Control	Free (zero mg/l)	-	-	-	-
NAA	**1 mg/l** + 0.5 mg/l (Kin)	0.136	0.760	1.037	1.776
	2 mg/l + 0.5 mg/l (Kin)	**0.480**	**0.885**	**1.587**	**1.817**
	4 mg/l + 0.5 mg/l (Kin)	0.069	0.089	0.278	0.364
IAA	**2 mg/l + 1 mg/l (Kin)**	**0.587**	**0.699**	**0.907**	**0.998**

Alkaloid productivity:

Data presented in Fig.2 show the total alkaloids (mg/gm), as well as, hyoscyamine amounts (mg/gm) in callus decreased by increasing 2,4-D concentrations, whereas at the first concentration (1 mg/l) recorded the high amount of alkaloids. On the other hand, high concentrations of total alkaloids were recorded in callus tissues that obtained from MS media with 2 mg/l. of both NAA and IAA and with an age 30 days, Fig. (3).

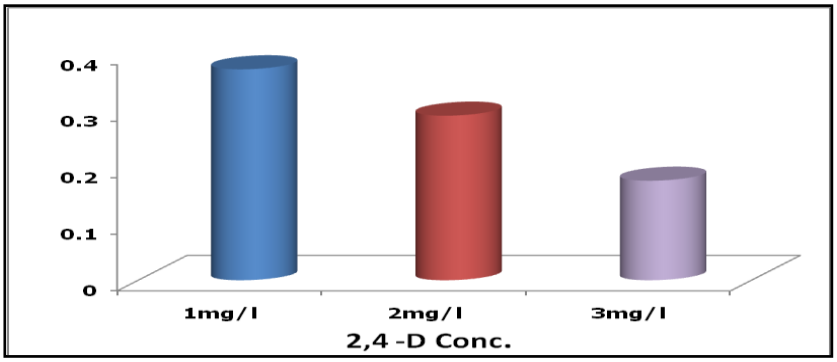


Fig. 2: Effect of 2, 4- D concentrations on alkaloid yielding in callus of *Hyoscyamus aureus* L.

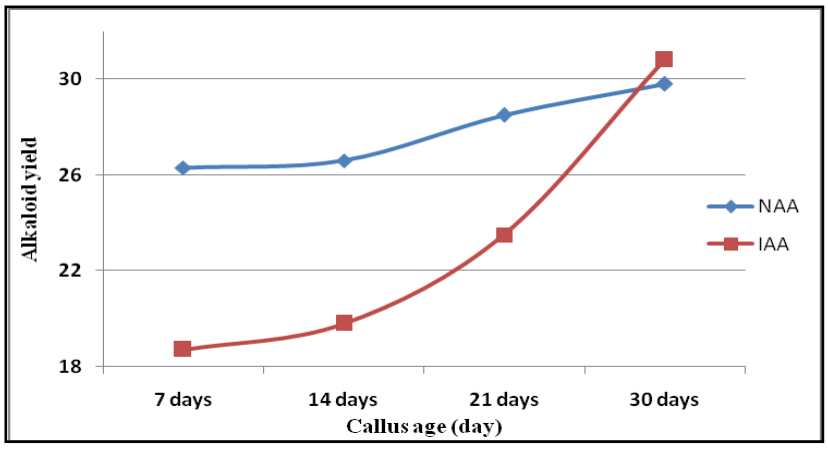
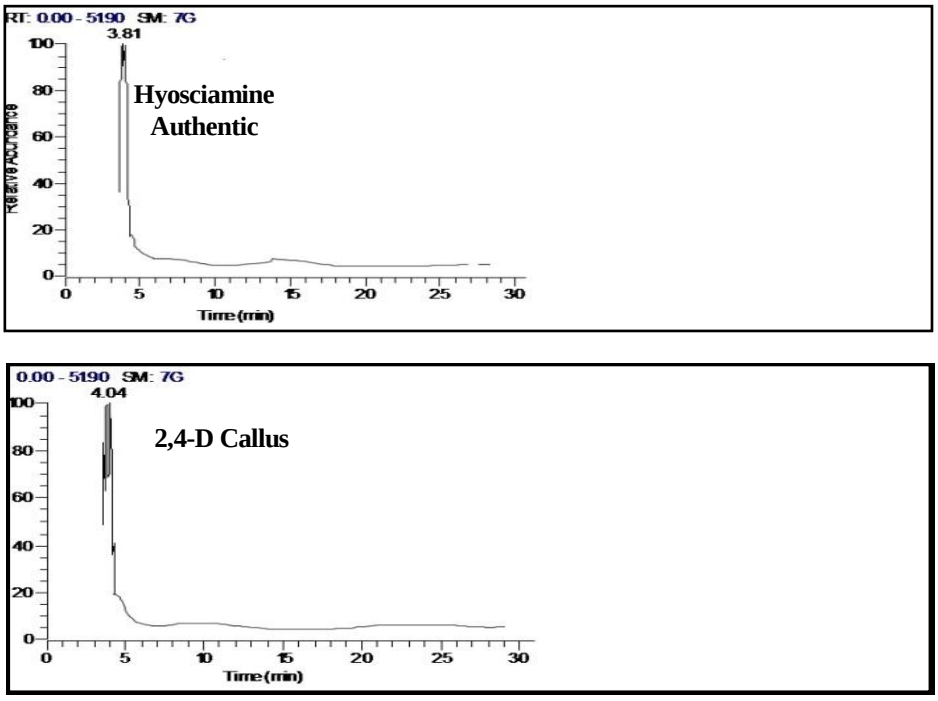


Fig. 3: Effect of NAA and IAA on alkaloid yielding in callus of *Hyoscyamus aureus* L.



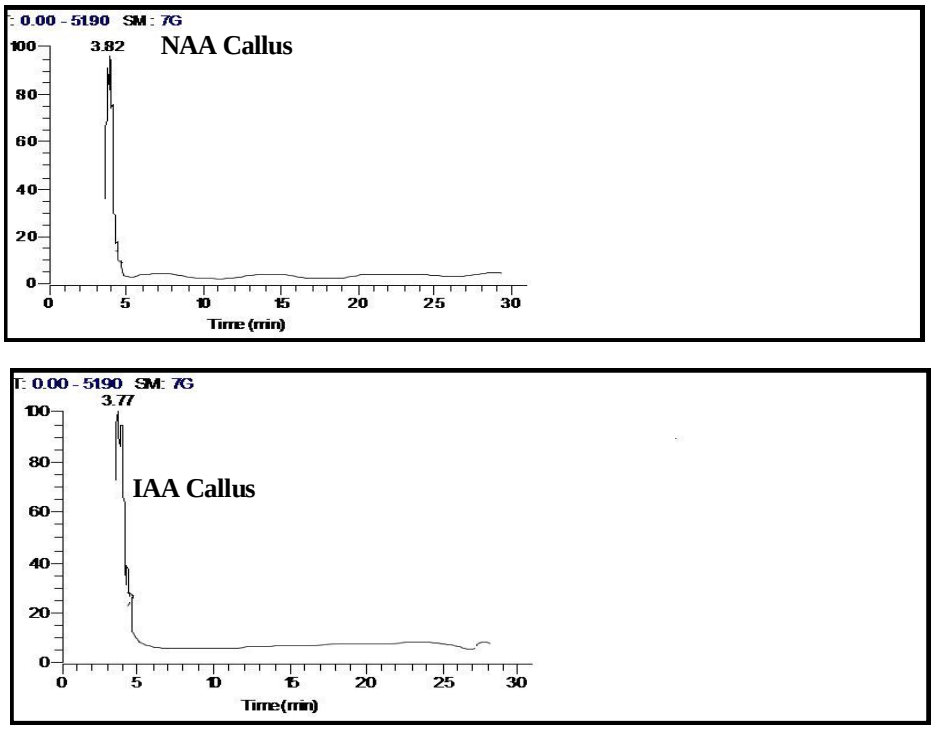


Fig. 4: HPLC chromatograms of Hyoscyamine as a standard and callus produced on MS supplemented with 2 mg/l of 2,4-D, NAA and IAA of *Hyoscyamus aureus* L.

The above figure (Fig.4) draws the attention to the secondary metabolites (**Hyoscyamine**) produced in callus that grown on MS media supplemented with 2,4-D, NAA and IAA..

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