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Tropane Alkaloids Production of Immobilized Cells of Egyptian Henbane (*Hyoscyamus muticus* L.)

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THE development of biochemical from plant cells marks the beginning of a new phase in biotechnological research and increasing global demand for scopolamine. From this point, somatic calli of Egyptian henbane (*Hyoscyamus muticus* L.) were produced from leaf segments cultured on Murashige and Skoog (MS) medium containing various concentrations of Kin (0.5, 1, and 2 mg/l) or 2,4-D (1 and 2 mg/l). Immobilisation callus was conducted using various alginate treatments at 1.0, 1.5 and 2.0% combined with glycerol at 2, 3 and 4%. To complete gleization, two sources of calcium ions ($CaCl_2$ and Ca $(NO_3)_2.4H_2O$) were used at three concentrations 10, 20 and 30% or 1.5, 3 and 5%, respectively. The encapsulated somatic cell aggregates were cultured in liquid MS medium with 100, 200 and 300 mg/l of each tryptophan, glutamine and jasmine oil at 0.1, 0.2 and 0.3 % (v/v). The dried and fresh weights of immobilised cells were obtained after every two weeks of incubation for one month.

The highest callus production (100%) was obtained on a medium containing 0.5 mg/l of Kin. While the medium containing 0.5 mg/l Kin plus 1 mg/l 2,4-D produced the highest percentage of somatic embryos (91.67%) with 147 embryos/g, this count produced a regeneration percentage of 39.27% (equivalent to 57.67 embryos/g).

HPLC analysis revealed that the highest values of scopolamine and hyoscyamine (the major alkaloids) were obtained from applied tryptophan. When cells were treated with 100 mg/l of tryptophan, the medium contained 0.008 mg/ml of scopolamine and 0.09 mg/ml of hyoscyamine, respectively.

Keywords: *Hyoscyamus muticus*, Immobilization cells, HPLC, Scopolamine, Hyoscyamine.

Introduction

Immobilized cell systems should receive a lot of attention because the methods employed in industrial processes are similar to those created for microorganisms (Abedaljasim et al., 2012). *Hyoscyamus muticus* L. (Egyptian henbane, family Solanaceae) is one of the most significant medicinal herbs in Asia, Egypt, Turkey, Iran, and the South-West (Madani et al., 2015 and Walla et al., 2016). The principal source of tropane alkaloids, particularly hyoscyamine and scopolamine, which are frequently used in folk medicine, is the hyoscyamus species. These substances act as an antispasmodic, a nervous system stimulant, and a treatment for stomach pain (Tytgat and Guido, 2007).

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Scopolamine and hyoscyamine have complex chemical structures. For this reason, synthetically, the cost of producing them is prohibitive. They are acquired from Solanaceae plants (as *Hyoscyamus niger*, *Datura*, *Mandragora officinarum*). In reality, they are created in root cells and then moved to the aerial parts of plant (Ghorbanpour et al., 2015). According to Demirci et al. (2022), when L-phenylalanine (L-Phe) was present at 0.50 mM for 3 days, the levels of scopolamine and hyoscyamine in cultures of the adventitious root of *Hyoscyamus niger* were highest.

The primary method for tropane alkaloids producing is the extraction from cultivated plant sources. In a field culture, cultivated plants need more effective management, direction, and growth enhancement (Philippe et al., 2013). But, there are number of benefits to employing plant tissue cultures instead of field cultivation, including the ability to shorten the growth period, maintain consistent growth conditions, improve product quality and avoid the use of pesticides and herbicides. (Oksman-Caldentey and Hilturen, 1996).

The medium composition in tissue cultures, which includes plant growth regulators, diverse sources of plant nutritional components, and various growing circumstances, greatly influences the generation of tropane alkaloids (Dixon, 1985; Biondi et al., 1993 and Tone et al., 1997). Meanwhile, the production of tropane alkaloid in vitro culture led to the examination of some variables such as micro and macro elements, exogenic phytohormones, vitamins, glucose and other physical factors. Also, it has been demonstrated that leaf explants, as opposed to peduncle explants, are the primary sources of callus development, and MS culture, which contains NAA at 0.5 mg/l, is the ideal environment for leaf explants (Iranbkhsh and Riazi, 2000).

Demeyer and Dejaegere (1989) found that by raising the nitrite ion levels, *Datora stramonium* produced more hyoscyamine and increased dry weight under greenhouse conditions. Additionally, in cell suspension lines of *D. innoxia*, Gontier et al. (1994) investigated the effects of alginate, calcium and calcium alginate, in MS media, on the concentration of tropane alkaloids, they found that 10 mM calcium led cells to produce alkaloids at a about 10-fold higher than control cells.

Özmen et al. (2022) studied the impact of methyl jasmonate (MeJA) on cell development and production of secondary metabolites in immobilized cells of *Hyoscyamus niger*. Cells immobilized with calcium alginate were treated with MeJA (1.0 mM). The concentration of the tropane alkaloids (scopolamine and hyoscyamine) as well as cell growth index and the fresh and dry cell weights were assessed after the 30-day treatments. They concluded that, employing the right concentrations of MeJA was found to be a viable method for increasing the production of secondary metabolites in immobilized cell cultures of *H. niger*.

Fomerly, miscellaneous strategies have been investigated to increase the production of secondary metabolites from medicinal plants (Sharafi et al. 2013, Mirzaee et al. 2016). These strategies include choosing high-yielding cell lines, adapting the growth media, precursor feeding, elicitation, hairy root culture, plant cell immobilisation, large-scale culture in bioreactor systems, and biotransformation.

Accordingly, the demand for scopolamine is approximately ten times greater than that for (-)-hyoscyamine and atropine put together, making it the most valuable of the tropane alkaloids (Hashimoto et al., 1993). Furthermore, Husain (1983) found that, the leaves contained between 0.01 and 0.1% of the total alkaloids, with about 75% of them being hyoscyamine and 25% being scopolamine (hyoscine). The global scopolamine market is expected to reach USD 15 million by 2028, after recording USD 12 million in 2022 due to the COVID-19 pandemic, with a CAGR of 4.2 percent during the review period (MarketWatch, 2023).

Our study has aimed to produce tropane alkaloids from Egyptian henbane (*Hyoscyamus muticus* L.) plants at the semi-industrial and industrial scales by immobilising aggregate cells in MS medium containing elicitor materials. Also, it was planned to focus callus production, the first material in cell suspension to reach the greatest concentration of alkaloid production.

Materials and Methods

Plant materials

Seed disinfecting and germination

Egyptian henbane, *Hyoscyamus muticus* L. seeds were collected on 17th August of 2021 from the Farm of Pharmacy Faculty, Cairo University. The seeds were washed with tap water and surface sterilized in 70% ethanol for 2 min, and followed by 50% commercial bleach Clorox (6% sodium

hypochlorite) with a drop of tween-20 for 10 min. After the bleach solution was discarded, seeds were washed once with 70% ethanol and 4 times with sterile distilled water (Abedaljasim et al., 2012). The sterilized seeds were placed on basal MS medium (Murashige and Skoog, 1962) supplemented with 7g/l agar and 30 g/l sucrose for germination.

Shootlets production

The preparation of explants was performed with seedlings that were four weeks old. The seedlings were divided into small pieces each one containing (1-2 buds) and cultured on MS medium containing kinetin (Kin) 0.5 mg/l. The obtained shootlets were divided individually and re-cultured on the same medium.

Callus induction

Leaf segments of 0.5 cm^2 were placed on MS media with Kin at concentrations of 0.5, 1.0, and 2.0 mg/l, as well as 2,4-D (dichlorophenoxy acetic acid) at concentrations of 1 and 2 mg/l. The cultures were incubated at 25±1°C with light (2,000 lux, 16 hours per day). The evaluated callus production percentage and fastest-growing plantlets were chosen for further study after six weeks. This callus was subcultured every four weeks on successive callus induction new media and for four months continuously to maintain callus stock (Abedaljasim et al., 2012).

Somatic embryo enhancement

The embryonic calli were cultivated in MS medium containing Kin 0.5, 1.0 and 2.0 mg/l and two concentrations of 2,4-D (1 and 2 mg/l) with coconut water (30 ml/l) for all treatments. Somatic embryos were grown in MS medium and differentiated into somatic embryos with a 16-hour photoperiod under a cool light fluorescent lamp (351.tEm-2s-1) (Giridhar et al., 2004).

Immobilization process

Immobilization of somatic cells

Glycerol was added at 2.0, 3.0 and 4.0 %, to alginate solutions at 1.0, 1.5 and 2.0%, respectively. The somatic cells were added to bead after semi solidity. The beads were formed by dropping with automatic pipet in $CaCl_2$ solution 10, 20 and 30 % or Ca $(NO_3).4H_2O$ at 1.5, 3 and 5%. Solidity, transparency and survival were determined after cultured on MS liquid media. One bead solution in MS medium was adjusted to contain 2.0% (w/v) sodium alginate. After an hour of stirring at 500 rpm, the bead mixture was transferred to a separating funnel and passed through a 1000 uL automated pipette (with cutting tip) into a wide mouth beaker containing a 10, 20 and 30 % (w/v) calcium chloride solution or calcium nitrate solution) Ca $(NO_3)_2.4H_2O$ (at 1.5, 3.0 and 5.0 % (Esyanti et al., 2008). The beads were mingled for 1 hour with gentle agitation (10 rpm) to solidify. After 1 hour, the beads were filtered through cheesecloth and washed with distilled water.

Alginate loading elicitors immobilization

The immobilized of somatic embryos cells, were soaked in MS liquid medium containing three concentrations of tryptophan 100, 200 and 300 mg/l; three concentrations of glutamine 100, 200 and 300 mg/l and finally, jasmine oil was added at 0.1, 0.2 and 0.3 % (v/v). Cultured beads were suspended in 100 ml media in 500 ml flasks (20 beads/flask) and were shaken at 25°C under continuous light (approximately 950 lux). The morphology of alginate-immobilized cells was investigated by a scanning electron microscope (SEM) (Seron Technology AIS2100) before and after encapsulation (Derikv and Etemadifar, 2014).

Fractionation and determination of total alkaloids

After breaking the capsule, 1g of *Hyoscyamus muticus* somatic calli of the elicitor's treatments and an untreated callus (control) were taken and dried in an oven for 2 days at 60°C before being powdered in a mortar. Alkaloids were extracted using the methods outlined in (Yamada and Hashimoto, 1982 and Li et al., 2011). Dried somatic calli and callus (200 mg each) were drenched overnight in a combination of 28% ethanol and 9:1 NH_4OH (v/v), at which point they were centrifuged for 3 min at 1,500 rpm. The extraction with the basic alcohol was performed twice and dried at 45°C. The residue was dissolved in 1.5 ml of 0.1 N HCl, and the resulting acidic aqueous solution was filtered and adjusted to an alkaline pH of 8–9 with diluted NaOH. After adding 6 ml of chloroform, the tube was forcefully shaken for 30 seconds, until centrifuged for 2 min at 1,500 rpm. The alkaloids were pipetted twice into the lower layer with 6 ml of chloroform.

Determination of operating time

A strychnine solution with a concentration of 25µg/ml was poured into a separatory funnel, 5.0 ml of pH 4.7 phosphate buffer and 5.0 ml of bromocresol green (BCG) solution were added, and extracted twice with 5.0 ml of chloroform. The chloroform phase was then taken. The extraction results were collected in a 10.0 mL volumetric flask and chloroform was added to the mark. Then, the stable absorption time at a wavelength of 470

nm was investigated. The data collected from this analysis was utilized to construct a curve showing the relationship between absorbance and time (Li et al., 2011).

Determination of standard curve

Hyoscyamine standard solution at concentrations of 50µg/ml was pipetted into separatory funnels at 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 ml and combined with 5.0 ml of phosphate buffer at pH 4.7 and 5.0 ml of BCG solution. It was extracted twice with 5.0 ml of chloroform. The chloroform phase was then taken. The extraction results were collected in a 10.0 ml volumetric flask and chloroform was supplemented with the mark. Their absorbance was then verified against the maximum wavelength.

HPLC determination of alkaloids

HPLC equipment Young Lin (Young Lin Cooperation, Seoul, South Korea) comprises a Reprosil-Pur Basic C_{18} 5 µm (dimension: 250 × 4.6 mm) column (flow rate = 0.5 ml/min) and UV detector (λmax = 210 nm). Using an isocratic solution of water as the mobile phase: acetonitrile (65: 35 v/v). Standard hyoscine, hyoscyamine, and atropine were dissolved in methanol at a concentration of 1000 ppm. Different concentrations of each standard were used and the calibration graphs were established by the plotting area under the peak of each standard against the corresponding concentration. Linear calibration of the alkaloids in each sample was calculated from linear regression equation of each standard (Al-Ashaal et al., 2013).

Data and parameters

1. Callusing % = [No. of callus formation / total culture explants] × 100
2. Embryo % = [No. of embryo formation / total callus culture] × 100
3. Embryo count = count of embryos/1 g.
4. Regeneration embryo = No of embryos gave shootlets
5. Regeneration embryo % = [No. of regeneration embryos / total embryos culture] × 100
6. Weight1 and weight 2: weight after 15 days of re-culture: weight after 30 days of re-culture callus (W1and W2)
7. Incr 1 and 2 : increment of weight after subculture one and two (incr1and incr2)

Statistical Analysis

All experiments were performed with at least three replicates per treatment. The significance of treatment effects was evaluated using analysis of variance (ANOVA). Application of the CRD method followed by LSD testing ($P \leq 0.05$) determined significant differences between treatment means (Steel and Torrie, 1980). The data were analyzed by Co-State software Version-4 (Co-Stat Graphics, 1999).

Results and Discussion

Immobilization process

Callus initiation and embryos regeneration

The outcomes revealed that growth regulators had a substantial impact on the callus formed from henbane leaf segments cultured under light conditions. The average callus percentages in the two media (0.5 or 1.0 mg/l Kin + 1.0 mg/l 2, 4-D) were 100 and 95%, respectively (Fig. 1b), and both were found to be significantly higher than the other used medium. The hormone-free medium (control) produced the lowest callus percentage of 45%. According to Table 1, the average embryo formation for callus cultured on the same medium (MS) containing 1.0 mg/l 2,4-D + 0.5 mg/l Kin was significantly different (91.67%). followed by 1 mg/l Kin (81.67 %) compared with that cultured on MS free hormone (control) under light condition (11 %). Although 0.5 mg/l Kin scored the highest percentage of callus (100 %), it gave lower percentage of embryos (32.67 %) than those treated with 2,4-D.

The results in Table (1) and Fig. (1e) showed that the highest average of embryos count was 147 embryos/gram in medium containing 0.5 mg/l Kin + 1 mg/l 2,4-D followed by 2 mg/l Kin + 1 mg/l 2,4-D, (138 embryos/gram), this treatment significantly differed from all other treatments. For a 0.5 mg/l Kin+1 mg/l 2,4-D treatment, 57.67 embryos were the maximum number of regenerated embryos (Fig. 1 c,d) compared with control treatment (2.0 embryos) (without 2,4-D and Kin) (fig. 1g). All cultures gave low count of embryos in response to various treatments of growth hormones. The embryos differentiated to explants using MS media and 0.5 mg/l Kin plus 1 mg/l 2, 4-D scoring 39.27% of all cultured embryos.

TABLE 1. The effect of plant growth regulators on the somatic embryos initiation and regeneration of *Hyoscyamus muticus* L.

Treatments MS with CW	Callusing %	Embryo genic callus %	Embryos count/g.	Developed plantlets No.	Developed plantlets %
control	45.00	11.00	13.67	2.00	15.36
0.5 mg/l (Kin)	100.0	32.67	24.67	3.33	13.57
1 mg/l (2,4- D)	50.00	13.77	12.91	3.02	7.66
2 mg/l (2,4-D)	55.00	17.53	10.45	2.10	8.39
0.5 mg/l (Kin) + 1 mg/l (2,4- D)	85.00	91.67	147.0	57.67	39.27
0.5 mg/l (Kin) + 2 mg/l (2,4- D)	91.67	61.67	115.67	22.0	19.03
1 mg/l (Kin)	73.33	81.67	35.00	7.00	19.97
1.0 mg/l (Kin) + 1.0 mg/l (2,4- D)	95.00	61.67	122.33	35.33	28.77
1.0 mg/l (Kin) + 2.0 mg/l (2,4- D)	88.33	25.67	116.67	19.67	16.93
2 mg/l (Kin)	80.00	55.00	95.33	9.00	9.57
2.0 mg/l (Kin) + 1 mg/l (2,4- D)	90.00	80.00	138.0	37.00	26.83
2.0 mg/l (Kin) + 2 mg/l (2,4- D)	80.00	20.00	66.33	13.67	20.63
LSD 5 %	15.93	15.76	14.81	5.71	6.57

According to the Duncan Multiple Range Test, the differences in means of treatments less than LSD 5% are not significantly different at the 5% level.

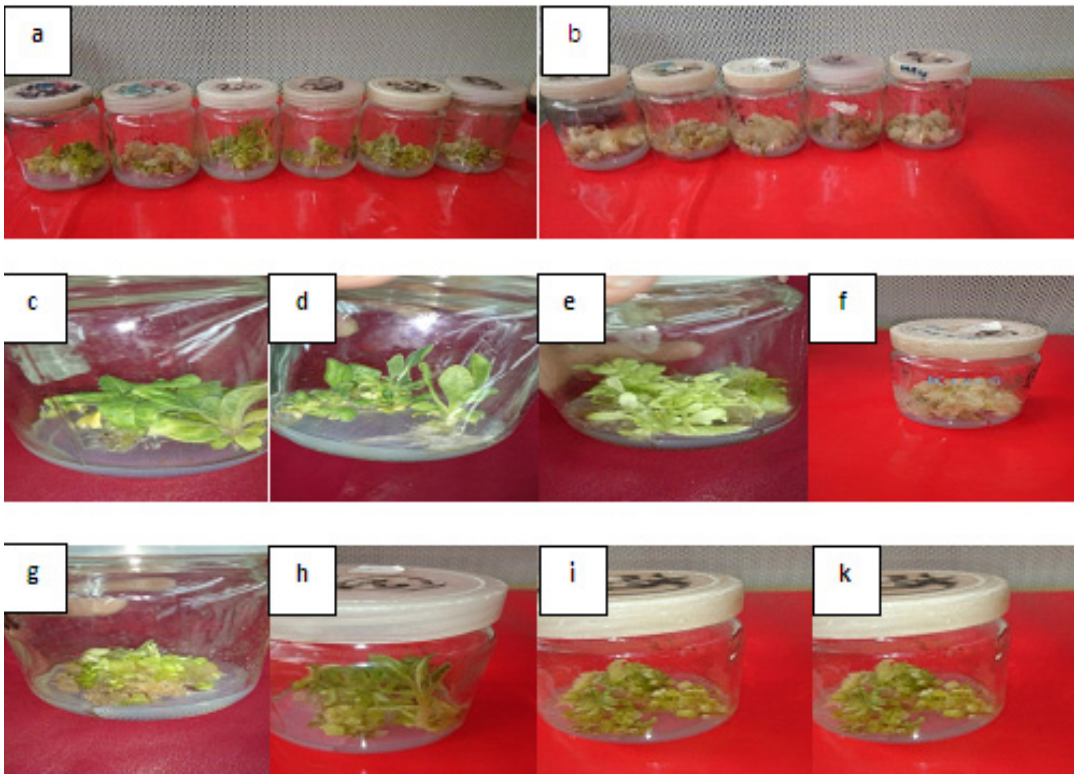


Fig. 1. Hyoscyamus muticus explants propagation; a. proliferative shootlets b: callus induction, c-d-e.: regenerated shootlets, f: callus growth. g-h: somatic embryos and i,k: regenerated callus.

The Effect of alginate concentration

Bead stability increased and became more spherical with increasing alginate concentration; thus, they could be used in somatic cells. Also, the output of hyoscyamine was released out of the cells due to a decrease in mass transfer and leakage in alginate-immobilized cells. In contrast, low-alginate concentration beads had a soft consistency. Srinivasulu et al. (2003) and Zhang et al. (2010) showed that the effectiveness of beads increases as the alginate concentration decreases.

The results recorded in Table 2 and Fig. 2a explained the effect of alginate concentration, glycerol concentration, calcium chloride and nitrite on survival and physical properties of capsules. Increasing concentration of alginate with calcium ion increased solidity without transparency, source of calcium ion had a high effect on transparency. Calcium nitrite was more transparent than calcium chloride which decreased with increasing ion concentration. The optimum concentration of alginate, glycerol and calcium ion was 2 %, 2 % and 20% (calcium chloride), respectively, as they had transparent, solid, cohesive capsules, compared with the other concentrations of all substances.

When the cell immobilization matrix is used again in the production of hyoscyamine, the structural morphology changes that occur after incubation in the elicitation medium had a significant impact on the matrix's efficacy. As for Ca-alginate beads, it was clear that during culture incubation, the beads' structure underwent a number of changes (Fig. 2b).

TABLE 2. Effect of calcium ion source, concentrations, glycerol and alginate on solidity, transparency and survival of immobilized cell after one month culture

$CaCl_2$ Conc.	$CaCl_2$ 10 %			$CaCl_2$ 20 %			$CaCl_2$ 30 %		
Glycerol/alg. Conc.	**S**	**T**	**Surv**	**S**	**T**	**Surv**	**S**	**T**	**Surv**
2.0 Glycerol + 1.0 Alg	-	++	75	+	+	30	+	+	0.0
2.0 Glycerol + 1.5 Alg	+	++	55	++	++	25	++	+	0.0
2.0 Glycerol + 2.0 Alg	+	++	45	+++	+++	75	+++	-	0.0
3.0 Glycerol + 1.0 Alg	-	+	80	+	+	15	++	+	11.0
3.0 Glycerol + 1.5 Alg	-	++	75	++	++	30	++	+	0.0
3.0 Glycerol + 2.0 Alg	+	++	30	+++	+++	0	+++	-	0.0
4.0 Glycerol + 1.0 Alg	-	+	50	+	+	25	++	+	0.0
4.0 Glycerol + 1.5 Alg	-	+	45	++	++	30	++	-	0.0
4.0 Glycerol + 2.0 Alg	+	++	40	+++	+++	0	+++	-	0.0
L.S.D 5 %		6.19			5.92			nc.	
$Ca(NO_3)_2$. $4H_2O$	**$Ca(NO_3)_2$. $4H_2O$ 1.5 %**			**$Ca(NO_3)_2$. $4H_2O$ 3.0 %**			**$Ca(NO_3)_2$. $4H_2O$ 5.0 %**		
Glycerol/alg. Conc.	**S**	**T**	**Surv**	**S**	**T**	**Surv**	**S**	**T**	**Surv**
2.0 Glycerol + 1.0 Alg	-	+++	25.0	-	+++	11.00	+	-	0.0
2.0 Glycerol + 1.5 Alg	-	+++	3.33	-	+++	6.66	++	-	0.0
2.0 Glycerol + 2.0 Alg	+	+++	0.00	-	+++	3.33	++	-	0.0
3.0 Glycerol + 1.0 Alg	-	++	0.00	-	++	25.0	++	-	11.0
3.0 Glycerol + 1.5 Alg	-	+++	0.00	-	++	0.00	++	+	25.0
3.0 Glycerol + 2.0 Alg	+	+++	25.0	+	++	0.00	++	+	0.0
4.0 Glycerol + 1.0 Alg	-	++	6.66	+	++	0.00	++	+	0.0
4.0 Glycerol + 1.5 Alg	+	+++	3.33	+	++	3.33	++	+	0.0
4.0 Glycerol + 2.0 Alg	+	+++	0.00	-	++	3.33	++	+	0.0
L.S.D 5 %		7.07			4.32			NS	

According to the Duncan Multiple Range Test, the differences in means of treatments less than LSD 5% are not significantly different at the 5% level.

Alg.: Alginate; Surv.: survival after 4 weeks; S: solidity; T: transparency.

Transparency: (-) dim; (+) low; (++) moderate; (+++) high transparency

Solidity: (-) soft; (+) low solid; (++) moderate.

Several different textures were seen, ranging from smooth to swelling with a dark spongy texture (Fig. 2a). At a 10x magnification, the effects of the bead size were also seen (Fig. 2b), in contrast to Fig. 2b, where the beads' surface texture showed some folds (furrows).

The imbibition in bead size is possibly due to the imbibition of moisture and dim due to the hyoscyamine production that return to change pH led to more cross linkage between alginate matrix protruding structures were present on the beads before incubation with pores that were congregated (Fig.2b); however, after incubation (Fig. 6 a,b and c), the beads appeared to have a smooth surface with undulated bumps. At 10× magnification (Fig. 2a), an undulated surface was observed on the beads before incubation, while the beads after incubation had a smooth surface with an undulated crust (Fig. 2b).

The performance of the beads' immobilized consortia during hyoscyamine synthesis was impacted by variations in bead size and structure. In this concern, Rao et al. (2008) found that reducing the loss of structural integrity and the loss of cells were achieved by increasing the alginate concentration during the preparation of the beads. In the growth medium, release a high percentage of cells with low concentrations of alginate. Mahmoud and Rehm (1987) demonstrated that an increase in bead efficiency was correlated with a decrease in alginate concentration. Using beads with a 1% alginate concentration resulted in 1.75 times more alkaloids being produced after 24 hours than using beads with a 3% alginate concentration. Additionally, it was discovered that the ideal sodium alginate concentration for effective bioconversion was 2% (w/v).

Effect of calcium ion source and concentration

The requirement of alginate immobilization is cultivation in a medium containing calcium ion so, the effects of this ion supply were examined. Using calcium as chloride and/or nitrate had a significant impact on growth (Fig. 3). As observed for the cells cultivated in standard condition (2% alginate + 2% glycerol + 20 % calcium chloride). Scopolamine (hyoscine) and Hyoscyamine were presented in the cells during one month (the culture period). The chemical compounds (hyoscyamine and scopolamine) were released from the cells to the medium. The maximum production occurred between 10 and 15 days and was nearly ten times that of control cells.

Somatic embryos regeneration

Shootlets formation

In the liquid MS medium, cell growth may be influenced by the alginate matrix that covers the aggregate cells. Fig. 4a displayed cells growth pattern that suggested cells trapped in an alginate matrix grew slowly. The results of some experiments suggested that the alginate matrix could obstruct oxygen and nutrition diffusion. As a result, the alginate-bound cells grew more slowly than the free cells.

Data in Table (3) and Fig. (5 a) showed somatic embryos growth after incubation inside alginate matrix four weeks and release hyoscyamine from matrix outside to the medium by secretion glands (Fig. 4a and b). The regenerated somatic embryos formed after re-culture on MS medium containing 30 ml/l coconut water (CW) with 1 mg/l of 2,4-D plus 0.5 mg/l of Kin gave the highest average of shootlets length (0.9 cm) and formed small leaves at average 5.33 leaves at the end of culture period

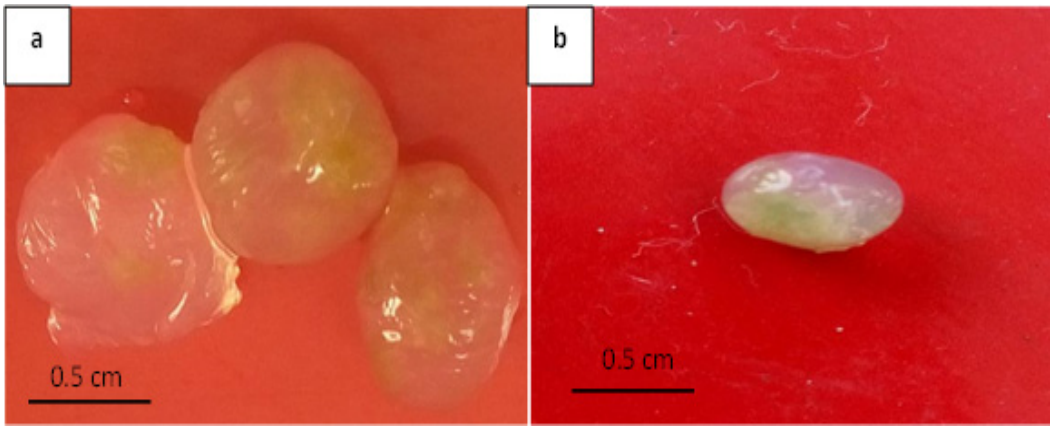


Fig. 2. (a) Immobilization cells before incubation and production hyoscyamine (b) immobilization cells after incubation in MS medium. The first is transparency, semisolid, smooth and the second is dim after hyoscyamine production.

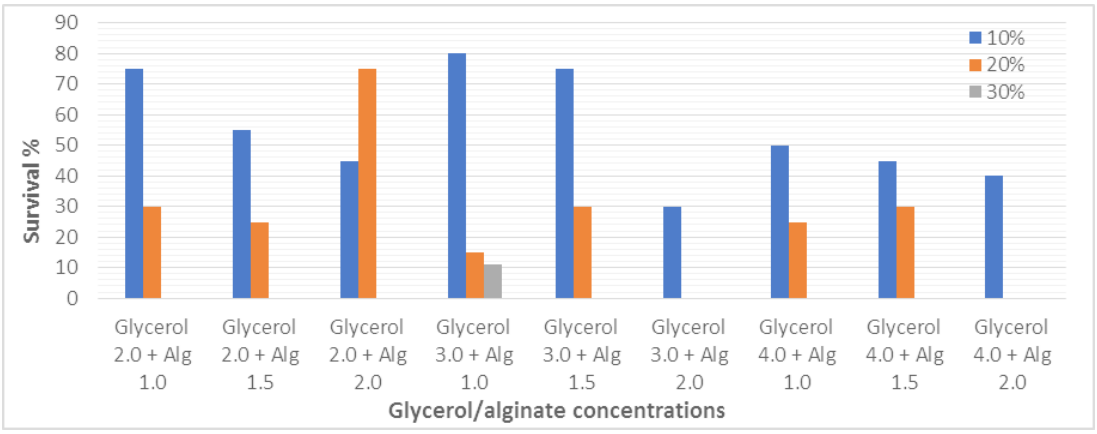


Fig. 3. Effect of glycerol concentrations, alginate concentrations and calcium chloride concentrations on survival percentage of immobilizing cells after incubation in MS liquid media.

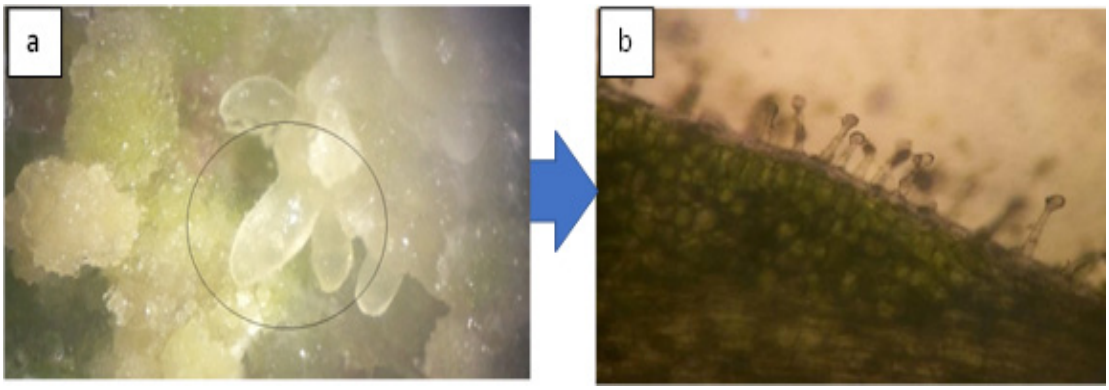


Fig. 4. (a) Somatic embryo cells containing secretion glands on the service of somatic embryos (b) high power (x100) shows service of cells containing secretion glands.

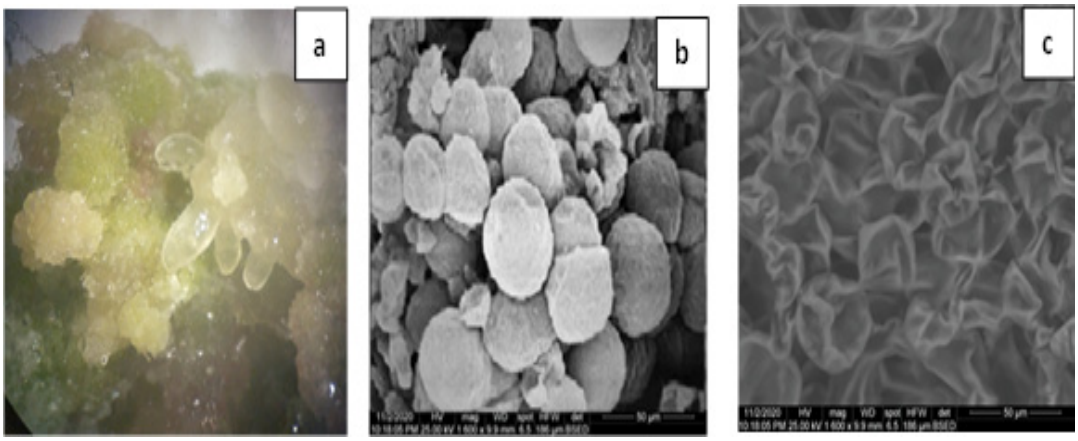


Fig. 5. (a) Greening somatic embryos before immobilization (b) SEM shows homogenization of somatic cells immobilized before incubation (c) somatic cells after incubation showed decreased in size by dehydration.

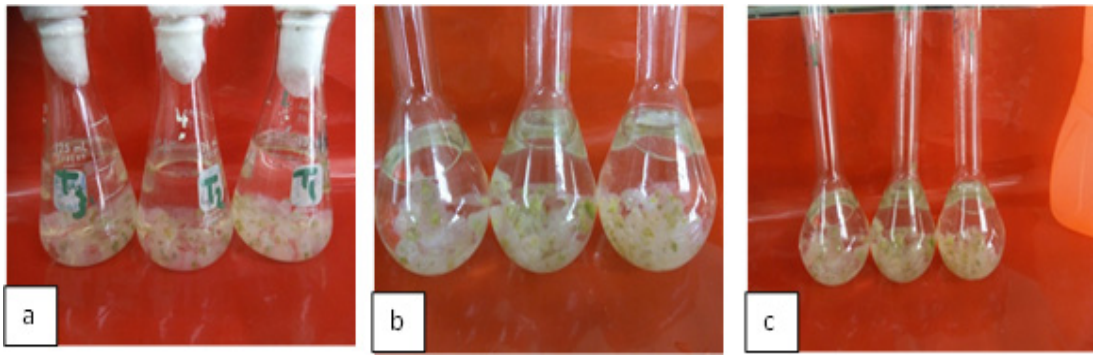


Fig. 6. (a): Glutamine treatments 100, 200 and 300 mg/l liquid media containing alginate matrix coating somatic cells, (b) tryptophan 100, 200 and 300 liquid media (c) Jasmine oil treatments 0.1, 0.2 and 0.3 ml/l

(four weeks). On the other hand, the somatic embryos growth after 15 days culture inside matrix in shape (Fig. 5 a, b and c). The shrinkage somatic obtained after encapsulation as shown in Fig. 5c by SEM which showed somatic embryos before encapsulation Fig. 5 b. . After the first 15 days of cultured on MS media with 2 mg/l kin and 2, 4-D the immobilized embryos gave the highest dry weight 3.67 g. followed by 1.0 mg/l of Kin plus 2 mg/l of 2, 4-D which scored 3.34 g.

At the second 15 days, the same treatment gave 5.58 g. for embryo dry weight with highest growth increment rate 4.58 compared with the first subculture which gave the highest increment rate 2.67. In this regard, catharanthine was seen being synthesized in aggregate cells of vinca beginning on the first day and reaching the highest concentration on the ninth day (39.18 g./g.). Here, because there was an abundance of enhancers available at the time, the cells produced catharanthine at their peak levels. Furthermore, nutrition was used to produce secondary metabolites in addition to helping the cell grow (Jolicoeur et al., 2002). Catharanthine produced in the cells was secreted into the medium. Where catharanthine began to be secreted on the third day and increased over the following days, peaking at 5.69 g/l on the 12th day by ion transport mechanisms and passive diffusion (Dornenburg and Knorr, 1995).

Total alkaloid contents

Spectrophotometric: After encapsulated embryos inside alginate matrix and incubated in liquid medium containing tryptophan, glutamine and jasmine oil, the contents of endogenous and exogenous alkaloids were determined (Table, 4). The data showed the encapsulated somatic embryos after destroyed and re-cultured gave the highest survival 100% for those incubated in liquid medium containing tryptophan 300 mg/l; glutamine 100 mg/l and 0.3 % (v/v) jasmine oil. The alkaloid production in cells released outside the matrix for cells coated with alginate matrix under jasmine oil 0.2 % (v/v) reached to (0.058 g./100 g. DW); but inside the cell was (0.1078 g./100 g. DW) compared with the untreated cells (control) synthesis endogenous alkaloids 0.055 g./100 g. DW and released outside cells in media (0.0255 g./100 g. DW).

Previous studies have shown that adding biotic and abiotic elicitors to culture medium could result in either negative or positive development of cells or tissues, depending on the concentration and type of elicitors used, the type and source of the plant part being cultured, and the incubation conditions. According to Ajungla et al. (2009) the amount of tropane alkaloids (Hyoscyamine and Scopolamine) generated by *Datura* increased when abiotic elicitors like Na_2SO_4, salicylic acid, $CaCl_2$, NaCl, and $AlCl_3$ were applied at various concentrations. Our findings are consistent with those of Ibrahim et al. (2009), who reported that increasing sucrose content resulted in a decrease in alkaloids accumulated by the callus of *H. muticus*. Yamada and Hashimoto (1982) observed that incubation of *H. niger* callus in darkness led to an increase in the quantity of scopolamine levels and a decrease in the amount of hyoscyamine compound by 32.9% compared to callus incubated in light. The above-mentioned results showed that the increased levels of scopolamine produced by *H. niger* callus were due to the influence of abiotic elicitors added to the medium, in addition to the dark environment employed for callus incubation.

Numerous studies revealed that the alginate matrix caused stress in the immobilized cell. In addition to increasing cell contact, it could

TABLE 3. Shootlet formation and growth increment of somatic embryos after two and four weeks of re -culture

Treatments MS with Coconut Water	Embryo morphology		Dry weight (g.) and weight increment			
	Embryo length cm	Leaves No.	DW (g.) 1st 15 days	DW (g.) 2nd 15 days	Increment. 1st 15 days	Increment. 2nd 15 days
Control	0.20	1.66	1.61	2.42	0.61	1.42
1 mg/l 2,4-D	0.19	1.32	1.23	1.32	0.32	0.79
2 mg/l 2,4-D	0.09	1.24	1.04	1.11	0.29	0.77
0.5 mg/l Kin	0.36	2.33	1.69	2.51	0.69	1.51
0.5 mg/l Kin + 1 mg/l 2,4- D	0.90	5.33	2.89	3.84	1.29	2.84
0.5 mg/l Kin + 2 mg/l 2, 4- D	0.23	2.66	2.26	2.36	1.26	1.36
1 mg/l Kin	0.30	1.66	2.75	3.19	1.75	2.19
1.0 mg/l Kin + 1.0 mg/l 2, 4- D	0.40	3.66	2.55	2.93	1.55	1.93
1.0 mg/l Kin + 2.0 mg/l 2, 4- D	0.36	2.33	3.34	3.96	2.34	2.96
2 mg/l Kin	0.20	3.33	1.31	1.80	0.31	0.80
2.0 mg/l Kin + 1 mg/l 2, 4- D	0.43	4.33	2.31	3.25	1.31	2.25
2.0 mg/l Kin + 2 mg/l 2 ,4- D	0.20	2.00	3.67	5.58	2.67	4.58
LSD 5 %	0.19	1.08	0.83	1.85	0.835	1.86

According to the Duncan Multiple Range Test, the differences in means of treatments less than LSD 5% are not significantly different at the 5% level.

TABLE 4. Effect of tryptophan, glutamine and jasmine oil in liquid medium on endogenous and exogenous total alkaloids of somatic embryos

Treatments MS with glycerol 2ml/l		Survival after encapsulation %	Total alkaloids	
			Endogenous alkaloids (Explant) g/100 g Dw	Exogenous alkaloids (Media) g/ 100 ml
Control		22.22	0.055	0.0255
Tryptophan (mg/l)	**100**	95.00	0.0365	0.05648
	200	88.88	0.0674	0.04608
	300	100.0	0.0486	0.04813
Glutamine (mg/l)	**100**	100.0	0.0677	0.04261
	200	90.00	0.1000	0.04743
	300	75.00	0.0718	0.04587
Jasmine oil (%) (v/v)	**0.1**	90.00	0.0630	0.05214
	0.2	80.00	0.1078	0.05825
	0.3	100.0	0.0644	0.04261
LSD 5 %		12.90	0.0011	0.00792

According to the Duncan Multiple Range Test, the differences in means of treatments less than LSD 5% are not significantly different at the 5% level.

prevent cell growth, which reduces cell growth in immobilized cells. Thus, substrate consumption and energy flux were directed toward the desired secondary metabolite pathway, improving process product yields (Rizkita et al., 2008). The combination of growth restriction and stress resulted in increased metabolite synthesis. A similar effect was observed in an immobilised *Datura innoxia* culture; immobilisation may enhance the synthesis of scopolamine (Robins et al. 1991). Anthraquinone production was also boosted when *Vitis vinifera* was immobilized using pectin capsulation (Dornenburg, 2004). However, it was discovered that catharanthine was still retained in the alginate matrix, indicating that the secretion process was not optimal. The

concentration and size of the alginate pores used to make the beads had a significant impact on the secretion of catharanthine into the medium (Facchini and Dicosmo, 1991). So, in order to maximize the secretion of catharanthine into the medium, more research is still required.

HPLC determination:

The results shown in Tables 4 and Fig. 7 and 8 showed that the concentrations of tropane alkaloids (hyoscyamine, scopolamine and atropine) varied significantly based on the type of elicitor which induced tropane alkaloids production inside somatic calli and released outside the cells in culture media (Fig. 8). Concentrations of scopolamine (mg/ml) released from callus to media through matrix gel were 0.008, 0.005 and 0.004 mg/ml for media supplemented with tryptophan 100 ppm; glutamine 200 ppm and jasmine oil 0.2 %, respectively. Moreover, callus produced hyoscyamine (mg/100 ml) in media supplemented the same treatments were 0.09, 0.02 and 0.03 mg/ml, respectively compared with control which scored 0.007 mg/ml. Finally, atropine did not find in the media for any treatment.

Callus immobilized by matrix and immersion in the medium supplemented with tryptophan 300 ppm, glutamine 200 ppm and jasmine oil 0.2 % induced alkaloids production especially scopolamine (mg/ml-media) which reached to level of 0.020, 0.022 and 0.029 mg/ml for the previous treatments, respectively. On the other hand, both of atropine and hyoscyamine disappeared from the cells treated with previous elicitors except with those treated with jasmine oil 0.2 % which scored 0.003 mg/ml atropine in the cells compared with control (0.002 mg/ml).

These results were in harmony with Ajungla et al. (2009) they found that the amount of tropane alkaloids (scopolamine and hyoscyamine) obtained by *Datura metel* increased after the addition of abiotic elicitors like salicylic acid, Na_2SO_4, NaCl, $CaCl_2$ and $AlCl_3$ at various concentrations. *Ammi majus* callus cultures created more secondary metabolites when they were supported with abiotic elicitors such as jasmonic acid and silicon oxide (Królicka et al., 2001). Also, Yamada and Hashimoto (1982) stated that incubation of *H. niger* callus in darkness caused an increment in the amount of scopolamine but a decrease in the hyoscyamine compound of the callus that incubated in light was found.

As shown in Scheme 1, indicated that immobilization amount of hyoscyamine, scopolamine and atropine released into the medium. These results were in agreement with these which obtained by Jordin et al. (1991), Their experiment revealed that, immobilisation increased the release of alkaloids into the medium by 50%, notably ajmalicine, as compared to control cells, which released just 10-25% of alkaloids into the medium. However, the secretion process was inefficient, it was discovered that atropine, hyoscyamine and scopolamine were still retained in the alginate matrix. The secretion of atropine, hyoscyamine, and scopolamine into the medium was strongly impacted by the alginate pore size and the concentration of alginate used to create the beads. As a result, additional research is required to optimise the secretion of atropine, hyoscyamine and scopolamine into the medium. As discussed in the previous studies, an essential prerequisite for the development of a differentiating culture would be the cells' slow

TABLE 5. Effect of tryptophan, glutamine and jasmine oil in liquid medium on scopolamine and hyoscyamine existed in media and somatic embryos

Treatments		Atropine mg/ml	Scopolamine mg/ml	Hyoscyamine mg/ml
Tryptophan 100 ppm	Media	nd	0.008	0.09
Glutamine 200 ppm		nd	0.005	0.02
Jasmine oil 0.2 % v/v		nd	0.004	0.03
Tryptophan 300 ppm	Somatic embryos	nd	0.020	nd
Glutamine 200 ppm		nd	0.022	nd
Jasmine oil 0. 2 % v/v		0.003	0.029	nd
Control (dry plant)	Plantlets	0.002	0.015	0.007

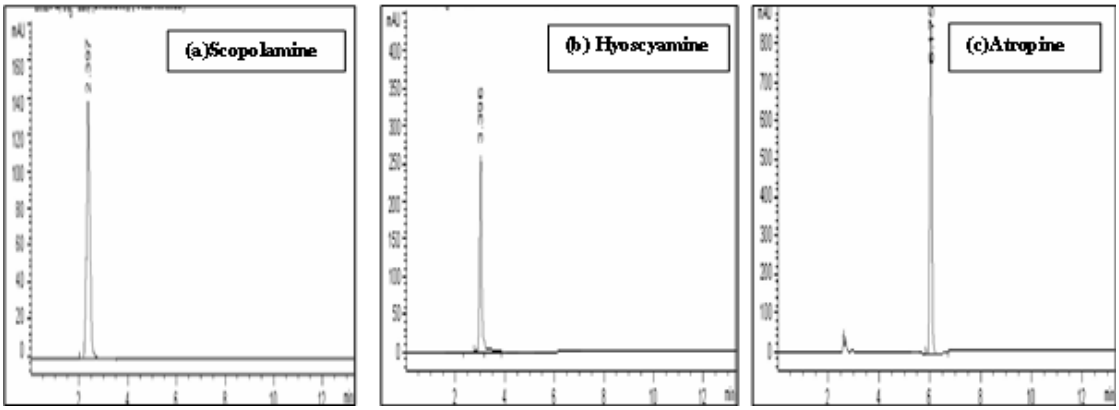


Fig. 7. Chromatogram of HPLC analysis of tropane alkaloids standard: Scopolamine (a), Hyoscyamine (b) and Atropine (c).

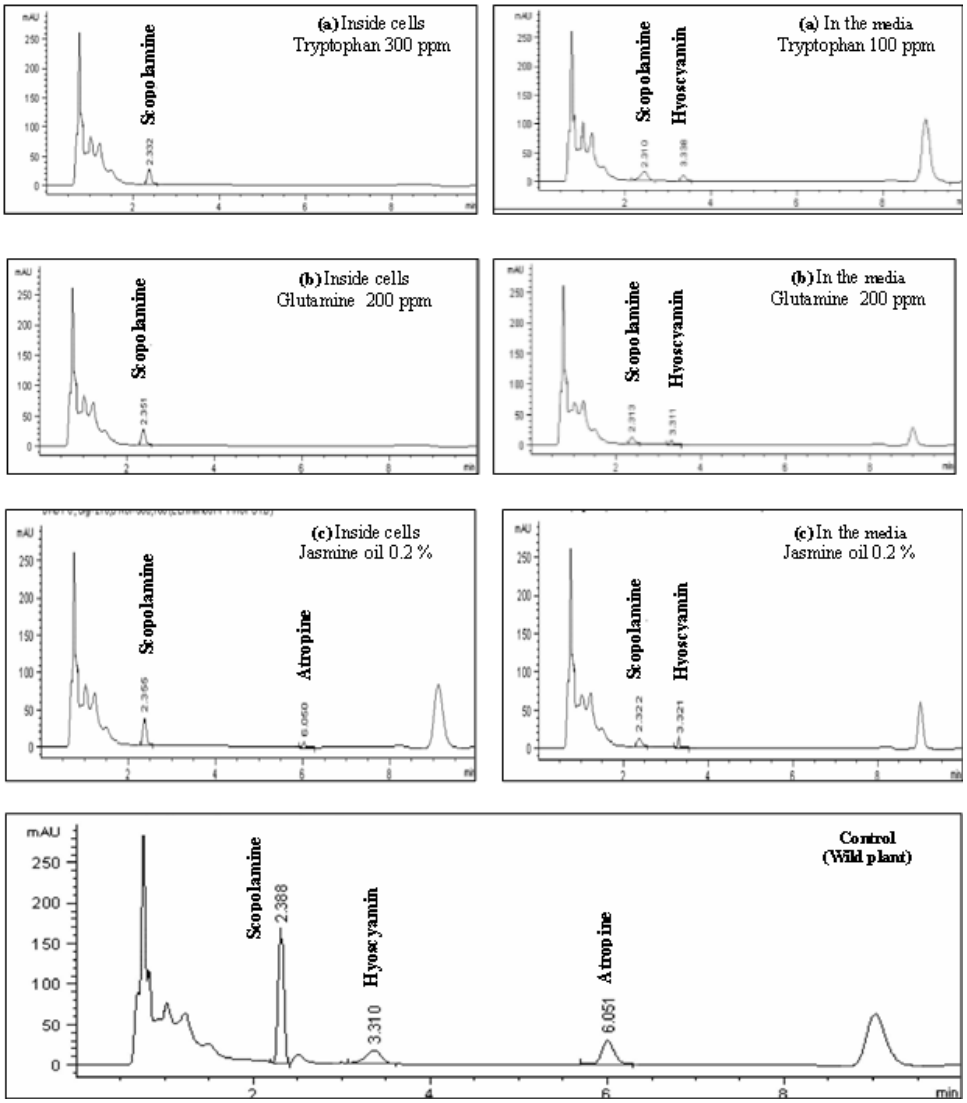
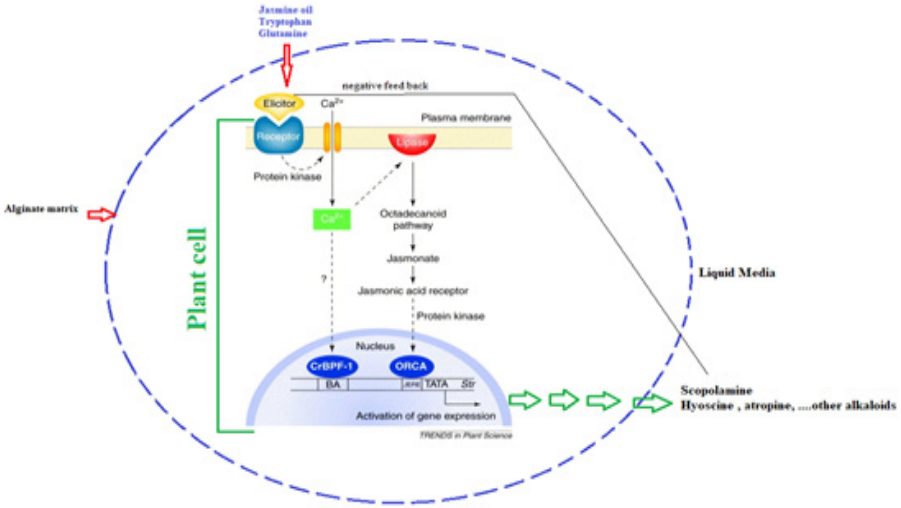


Fig. 8. HPLC chromatogram analysis of atropine, scopolamine and hyoscyamine of encapsulated embryos (inside cells) and in media for encapsulated embryos treated with tryptophan, glutamine, jasmine oil and control (wild plant).



Scheme 1. Showed elicitors (tryptophan, glutamine, jasmine oil) passing from pores of alginate beads and binding with cell membrane by protein kinase receptors. The signal transducer from the nucleus and activation CrBPF-1 and OCRA gene to produce alkaloids outside the granules and work as negative feedback.

growth and high cell-cell contact, and some morphological differentiation is in fact visible in the immobilized cells. As a result, it is assumed that cells immobilised in the way described above are metabolically closer to cells 'immobilised' throughout the plant than to the liquid-suspended cells from which they were derived. This could explain why immobilized henbane cells appear to have a higher capacity for producing tropane alkaloids than suspended henbane cells, a finding that suggests this method may be useful for the large-scale production of this and other secondary metabolites.

On the other hand, Ahmed et al. (1989) determined tropine and hyoscyamine by TLC method in *Hyoscyamus muticus* in three parts of plant, they found that leaves contain 2.43 and 4.3 mg/g. DW, respectively; stem contain 0.84 and 1.22 mg/g. DW, respectively and root contain 0.19 and 0.87 mg/g. DW, respectively. One kilo of dry matter yielded 2.4, 4.3 g. from leaves; 0.84 and 1.22 g. from stem and 0.19 g. and 0.87 g. from root, respectively. In Egypt *Hyoscyamus muticus* yields about 480 k/feddan of dry plant material and 8.4–12.6 kg/feddan of alkaloids after 16 weeks.

In our results we calculated the hyoscyamine and scopolamine (hyoscine) obtained from 20 capsules each one contained 0.2 – 0.4g./somatic embryos produce 0.8 and 9 mg/100 ml media after 4 weeks.

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Conflict of interest

The authors affirm that they do not have any conflicting interests.

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إنتاج قلويدات التروبان من الخلايا المغلفه للسكران المصرى

(*Hyoscyamus muticus* L.)

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مما لا شك فيه ان إستخدام الخلايا النباتيه في مجالات الكمياء الحيويه يمثل حقبه جديده نحو استكشاف مجالات التكنولوجيا الحيويه. وزيادة الطلب العالمي على السكوبولامين الذي يقدر ب ١٢ مليون دولار أمريكي في عام ٢٠٢٢

وإنطلاقا من هذا تم استخدام آجنه جسميه ناتجه من اوراق نبات السكران حيث تم زراعه اجزاء صغيره من الاوراق على بيئه موراشيج واسكوج محتوي على هرمون ٢و٤ داى كلوروفينوكسى اسيتك اسيد بتركيز ١ و ٢ مجم/لتر بالتبادل مع الكينيتين بتركيز ٠,٥ و١ و ٢ مجم/لتر لمده اربع اسابيع حيث تم الحصول علي اكبر نسبة من الكالس (١٠٠٪) بعد الزاعة علي بيئه تحتوي علي ٠,٥ مجم/لتر كينيتين و تم استحداث اجنه جسميه بعد الزراعة علي بيئه موراشيج واسكوج تحتوي ٠,٥ مجم/لتر كينيتين + ١ مجم/لتر٢ و٤ داى كلوروفينوكسى اسيتك اسيد حيث اعطى الجرام من الاجنه عدد ١٤٧جنين جسمى لكل جرام فى مرحله التطور الجنينى.

ثم تم وضع الاجنه الجسميه داخل كبسولات مصنوعة من الجينات الصوديوم بتركيز ١ او ١,٥ او٢ ٪ و الجلسرين بتركيز ٢ و ٣ و ٤٪. ومصدرين لآيون الكالسيوم ($CaCl_2$) و ($Ca(NO_3)_2.4H_2O$) بثلاث تركيزات لكل منهم

ثم تم وضع الخلايا المغلفه فى بيئه موراشيج واسكوج تحتويه علي محفزات لإنتاج القلويدات وخاصه السكوبولامين مثل الجلوتامين او التربتوفان بتركيزات ١٠٠ او ٢٠٠ او ٣٠٠ مجم/لتر ثم تم وضع الخلايا فى انابيب على هزاز و الحصول علي الأوزان الطازجة والجافة للخلايا المغلفة مع ملاحظه انتاج الاسكوبولامين والهيوسين.

و أظهر تحليل HPLC أن أعلى قيم للسكوبولامين والهيوسيامين (المركبات الرئيسية للقلويدات) تم الحصول عليها عند وضع الخلايا المغلفه فى بيئه محتويه علي تربتوفان بتركيز١٠٠مجم/لتر وكان تركيز كل من السكوبولامين والهيوسيامين في البيئه ٠,٠٠٨ مجم / مل و ٠,٠٩ مجم / مل على التوالي.