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Regeneration and Genetic Transformation by pAB6 Plasmid of *Hyoscyamus muticus* L., (Egyptian Henbane) Using Particle Bombardment

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ABSTRACT

Media chemical composition effect on production of embryogenic calli and production of transformed plants from *Hyoscyamus muticus* L (Egyptian henbane) were assessed using gene gun. *Hyoscyamus muticus* L., (Egyptian Henbane) is medically and economically important plant as it contains widely used atropine alkaloids, scopolamine, hyoscyamine. Callus induction and regeneration ability from leaf of *Hyoscyamus muticus* L were examined. Effects of 7 media were evaluated on type II callus production and regeneration. T3 medium showed greater positive response in embryogenic calli formation frequency (84.95%), reflected on shoot formation frequency (7.65/explant) and produced a high root yields after transfer on the rooting medium. Transformed henbane plants were achieved by particle bombardment using plasmid pAB-6 harboring the *gus* and *bar* genes with transformation efficiency 13.4%. Total alkaloid contents were reached to 6.05% in transformed plants. It compared with untransformed plants which contained 2.95%.

Keywords: *Hyoscyamus muticus* L, regeneration, genetic transformation, particle bombardment.

INTRODUCTION

High interests in plant research concerning potentials of medicinal use has been progressively increased during the last decade.

The WHO (1999) stated that medicinal plants are used as medical herbs, pharmaceuticals, cosmetics and other uses and around fifteen thousand species representing one tenth of all species are used in medical purposes. Among substances and materials found in medicinal plants there are anticancer compounds, antibiotics, and others (Hai-Hang., 2012).

Hyoscyamus muticus L., commonly known as Egyptian henbane (Family Solanaceae), is a winter crop which is medicinally and economically important as it contains widely used tropane alkaloids, scopolamine, hyoscyamine and atropine. The crude drug contains hyoscyamine, hyoscyine (scopolamine) and atropine, is used in against nervous disorders, asthma and whooping cough. Scopolamine and atropine (a racemic mixture of L-hyoscyamine) are used in ophthalmology and in combination as analgesics or narcotics; the former is used in controlling motion sickness (Berlin., 1988). Anthocyanin, which is a biologically and economically important natural pigment, which is found in the plant and also in its *in vitro* grown callus cultures (Bekheet *et al*., 2013).

In vitro regeneration and genetic transformations are used to multiply and enhance and produce medicinal plants and their metabolites (Abd El-Motaleb, *et al*., 2015)

Particle bombardment has been widely used to transform exogenous genes into plant tissues and has a major impact on basic plant biotechnology (Ahmad *et al*., 2017). Purkayastha *et al*., (2010) stated that transforming *Hyoscyamus muticus* L. through genetic means is not effective in comparison with the use of medical plants.

The present investigation was aimed at (1) attaining an effective regeneration *Hyoscyamus muticus* L. (2) assessing the most effective of composition of media composition for callus formation taking in consideration contents of alkaloids (hyoscyamines) (3) Transforming *Hyoscyamus muticus* L. calli systems with plasmid-DNA having *Gus* gene and herbicide resistant *bar* gene as a marker (4) confirming gene integration in the putatively transformed plants using PCR.

MATERIALS AND METHODS

Plant Genotype of plant and preparation of media

Seeds of *Hyoscyamus muticus* L were obtained from Gene bank, Desert Research Center, Egypt. Seeds were immersed for 10 min into 0.1% mercuric chloride solution, then rinsed 4 times with d.w. ; then left to germinate in jars containing 10 mL MS (half-strength) (Murashige and Skoog, 1962) supplemented with myoinositol (100 mgL^{-1}),thiamine-HCl (2 mgL^{-1}) pyridoxine-HCl (0.5 mgL^{-1})nicotinic acid (0.5 mgL^{-1}) and sucrose (30 g L^{-1}) .

Media solidification was done using 3 g agar L^{-1} agar and cultures were left for 2 days in dark under temperature of 18 ±1°C then in light for 16 h with 50 µmol m^{-2} s^{-1} (25°C) and 8 h dark (20°C).

Callus induction and regeneration

Leaves were shredded in pieces and transferred to the callus induction media to obtain a sufficient number of *in vitro* embryogenic callus as shown in Table 1, then left in dark (25 °C) for 2 months and sub-cultured for two weeks onto fresh medium of same composition. Selection was done by discarding the slow growing and dark calli during the next subcultures. The number of embryogenic calli was calculated 8 weeks kater and pieces were transferred to regeneration media.

All calli initiated on different callus induction media were moved to a medium containing 2.0 mg L^{-1} kin for shoot development. Callus cultures were incubated for 4 weeks at 25°C with 8 to 16 h photoperiod from cool-white fluorescent lights with an intensity of 2000 lux. Germinated embryoids having small and large shoots were moved to a rooting medium (MS+ 4.0 mg L^{-1} IBA). Shoots developed into plantlets which were transferred to an aquarium having modified Hoagland solution (Johnson *et al*., 1957).

Healthy plantlets taken from the nutrient solution and put into pots of peat moss: soil: sand mixture (1:1:1 by volume) in a greenhouse under ambient temperature of 22 to 25 °C, 16h photoperiod and 50% humidity. Pots were watered with half strength Hoagland solution.

Total alkaloids (Hyoscyamine Alkaloids) were determined spectrophotometry according to methods of Ganga *et al*. (2011).

Table 1. Components of media used for callus induction, regeneration and rooting of *Hyoscyamus muticus* L.

Medium number	Callus induction medium	Shoot induction medium	Rooting medium
T1	MS+ 0.5 mg/L kin (kinten)+ 1mg/L IBA (indol butyric acid)	MS+ 2.0 mg/L kin	MS+ 4.0 mg/L IBA
T2	MS+ 0.5 mg/L kin (kinten)+ 1mg/L NAA (naphthalene acetic acid)	MS+ 2.0 mg/L kin	MS+ 4.0 mg/L IBA
T3	MS+ 2.0 mg/L kin (kinten)+ 1mg/L 2,4 –D (2,4- Di chloro phenoxy acetic acid)	MS+ 2.0 mg/L kin	MS+ 4.0 mg/L IBA
T4	MS+ 1.0 mg/L kin+ 2.0mg/L 2,4 –D	MS+ 2.0 mg/L kin	MS+ 4.0 mg/L IBA
T5	MS+ 2.0 mg/L kin+ 2.0mg/L 2,4 –D	MS+ 2.0 mg/L kin	MS+ 4.0 mg/L IBA
T6	MS- sharkol+ 0.5 mg/L BA (Benzyl adenine) + 1.0 mg/L 2,4 –D	MS+ 2.0 mg/L kin	MS+ 4.0 mg/L IBA
T7	MS- sharkol+ 1.0 mg/L BA+ 0.25 mg/LGA (Gebrelic acid)	MS+ 2.0 mg/L kin	MS+ 4.0 mg/L IBA

Plant Expression Vector

The plasmid pAB6 (kindly provided by Dr. Bassem Abd Elgwad, Agricultural Genetic Engineering Research Institute) has been used for the transformation of *Hyoscyamus muticus* L callus. Plasmid map of transformation vector pAB6 is shown in Figure 1. The plasmid contains the *Gus* gene (with 1st exon & intron) and terminated by *Nos* gene 3' non-translated region. It also contained *Bar* gene (controlled by 35S promoter and *Nos* terminator) as a selective marker. The *bar* gene encodes phosphinothricin acetyl transferase (PAT) enzyme that inactivates the active ingredient phosphinothricin, of the bialaphos herbicide.

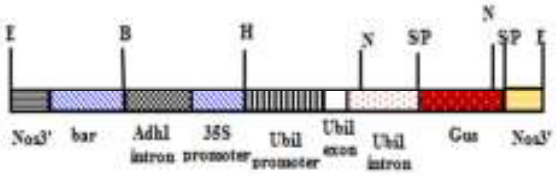

Figure 1. Plasmid map of the transformation vector pAB6.

Transformation was done by a biolistic particle acceleration device (PDS 1000/He, Bio-Rad). For microprojectile bombardment, plasmid DNA (1µg μL^{-1}) was precipitated onto gold particles (0.7 µm in diameter) following the procedure described in the Bio-Rad instruction Manual. Henbane callus plates were bombarded twice at a pressure of 1300 and 1800 psi with 10µL of particle suspension mixture per bombardment.

Transformation experiments were performed on friable callus only exposed to an osmotic treatment of sorbitol (45.4 gL^{-1}) of and mannitol 45.4 gL^{-1} for 4 h before and 16 h after bombardment(Kikkert *et al*., 2005).

Selection and recovery of transformed calli

Bombarded callus was incubated on the best selected callus induction medium for one week after bombardment. The recovery period after bombardment allows the bombarded cells to recover from mechanical damage caused by accelerated gold particles. Also, this period gives the transformed cells enough time to begin the expression of introduced selectable marker genes which allows reliable selection (Weeks *et al*., 1993). Then, transformed calli were transferred for 2 weeks to same medium supplied with bialaphos (1.0 mg L^{-1}) then to fresh selective bialaphos medium (2 mg L^{-1}), with continued sub-culturing every 2 weeks; with cultures being in a dark growth chamber at 28°C

Bialaphos-resistant calli having uniform growth were taken to regeneration bialaphos (3mg L^{-1}) medium. Somatic embryogenic calli developed within 2 to 4 weeks into green shoots were considered putative transformants and transferred to a shooting media. Plantlets were transferred to a 1 mgL^{-1}bialaphos rooting medium the developed putatively transformed were transferred into Hoagland solution for 4 to 6 days, then transferred to soil in growth chambers (Cem *et.al*,2003)

Evaluation of transformed plants

Herbicide testing

PAT activity was done through indirect means, by assaying the resistance of transformed plants to the herbicide (product of Hoechst, Germany) containing 200 gL^{-1}glufosinateammoniuim. The basta was painted on leaves of the putatively transformed plants. Application was done on 5 to 10 cm leaf sectors near the tip of the youngest fully-extended leaf at the four-and eight-leaf stage with a 1% solution of the herbicide containing 0.1% (v/v) Tween 20. (Roger and Bendich ,1985)

Molecular Analysis

Genomic DNA extraction

Isolation of genomic DNA from leaf tissues of each putatively transformed plant was done on untransformed and control plants using a modified CTAB method (Rogers and Bendich, 1985).

Polymerase chain reaction analysis

Detecting the bar and HVAI genes was done by PCR on 2 primer sets. Specific primer sequences bar genes of *Nos-Gus* and 35S- are shown in Table 2.

Table 2. Sequence of specific primers for the *Nos-Gus* and 35S- *Bar* genes.

Gene code	Forward primer	Reverse primer
Nos- Gus gene	5'- GGT GAT CTA ACC ATG GCC TCC AAC CAG AAC CAG GGG -3'	5'- GGC ATA TCT ATT GAT TCC TGG TGGTGGTGG TG -3'
35 S- *Bar* gene	5'-TGG CAC CGA GGA GAC ATG CCG GC-3	5'-CGT GAG GTG GAG GCC ATG GGG-3'

The PCR technique was done on 25uL reaction volume having the followings: - 25 ng of genomic DNA, 25 pmol mL-1 primers, 200 umol of each of dATP, dCTP,dGTP and DTTP,50 µM KCl, 10 mM Tris-HCl, 0.2 mM MgCl2 and one unit of Taq polymerase enzyme.

For the amplification, temperature profile of 94°C for 5 min for the initial denaturation cycle followed by 35 cycles

of 1 min 94 °C, 2 min 60°C 2 min72 °C and a terminal extension 10 min cycle 72 °C for the *gus* and *bar* genes. The PCR products were resolved by electrophoresis on 30 g agarose gel per liter (Dellaporta *et al.,* 1983). Data were analyzed statistically (Gomez and Gomez, 1984)

RESULTS AND DISCUSSION

The results presented in Table 3 indicate that the highest callus induction frequency (22.69) was recorded by maintaining the leaf explants on callus induction medium T3. However, the lowest callus induction frequency (6.65) was shown by maintaining on medium T7.

All calli maintained on medium T3 showed more positive response in embryogenic calli formation frequency (84.95%) in comparison with other media. These results reveal that components of medium T3 (specially the 2.0 mg L^{1} kin + 1 mg L^{-1} 2,4 –D) caused marked effects on the quality and quantity of embryogenic calli formation (Figure 2).

Transfer of the embryogenic calli obtained on callus induction media induced regeneration using media containing 2.0 mg L^{1} kin. Green shoot formation was evident within two weeks. Data obtained in Table 3 and Figure 2 indicate that the highest shoot formation frequency was recorded by maintaining the calli on callus induction medium T3 (7.65 per explant).

On the other hand, the lowest shoot formation frequency (0.18 per explant) occurred by calli maintained on medium T7. This indicates an improvement of callus induction and maintenance of medium T3 caused enhancement of shoot formation ability of Hyoscyamus muticus L genotype, as well as, production of high root yield after transfer onto rooting medium contained 4.0 mg IBA L^{-1}.

Table 3. Callus parameters and embryogenesis frequency of *Hyoscyamus muticus* L maintained on different media

Media callus induction	Callus uniformity	Explant weight before culturing g/explant	callus weight after culturing g/explant	Growth rate g/explant	Embryogenesis frequency %	Mean number of shoots/ explants
T1	Compact	1.87	12.32	10.45	16.65	0.39
T2	Compact	2.53	11.41	8.88	18.03	0.47
T3	Friable	3.79	26.48	22.69	84.95	7.65
T4	Friable	2.03	15.45	13.42	39.16	2.11
T5	Friable	2.83	11.73	8.9	36.89	1.59
T6	Watery	2.11	9.98	7.87	4.12	0.21
T7	Watery	2.67	6.65	3.98	2.34	0.18
LSD 5%		2.29	1.48	1.45	1.24	0.86

Figure 2. *Hyoscyamus muticus* L regeneration of callus induction on medium T3 (A, B and C), somatic embryogenesis (D, E and F) and shoots proliferation (G and H) plantlets on regeneration medium.
Note: (I) watery calli on T6 medium. (J) watery calli on T7 medium. (K) compact calli on T2 medium.

Although, more embryogenic calli ranging between 2.34 (T7) and 84.95 % (T3) were formed by *Hyoscyamus muticus* L and maintained on different media, most of these calli failed to form healthy root system on the regeneration medium, while calli maintained on T3 medium were able to produce very efficient rooting systems, as shown in Figure 3.

Therefore, the 2.0 mg L^{-1} kin + 1mg L^{-1} 2,4 –D promoted fast differentiation that led to increased number of shoots, and more regeneration suitable for rooting shoots than other media. These results indicate that the presence of such components in callus induction medium is recommended for initiating friable embryogenic callus. These results agree with those of Abd-Elmaksood *et al*., (2016), who proposed MS medium supplemented with 1.0 mg L^{-1} KN as the most promising treatment for establishment and continuous proliferation of *H. muticus* L. explants during successive subcultures. Ali *et al.*, (2010) studied media types, strengths, components and combinations of auxin and cytokinin (NAA and BA) and found that the light and inoculum density increased total alkaloid which accumulated in suspension cultures of *Hyoscyamus muticus* L. The same pattern was reported by Bhardwaj *et al.* (2018), who observed high percentage of callus induction in medium containing 10 to 15 µM NAA from *Rhodiolaimbricata* genotypes with highest percentage of shoot given by leaf derived callus medium of 5 µM NAA and 2.5 µM BAP as well as1.0 µM NAA , 5 µM BAP, 5 µM (38.88% and 37.49%); rooting of regenerated shoots as effective when a lower concentration of NAA (0.5 µM) was used alone. However, a maximum number of roots (22.0) and higher length (0.6 cm) was observed.

On the other hand, Ngetich *et al.* (2018) used MS medium components of α-naphthaleneacetic acid (NAA), 2,4- dichlorophenoxy acetic acid (2,4-D), benzyl aminopurine (BAP) and kinetin for callus induction and somatic embryogenesis in millets for shoot and root inductions

Figure 3. Regenerated plantlets (A, B) on rooting medium T3 and T7. Plant root profile (C, D), regenerated *Hyoscyamus muticus* L plants in Hoagland solution (E, F and G) and fertile regenerated plants (H) appeared, respectively.

***Hyoscyamus muticus* L transformation**

Transformed henbane plants were achieved by particle bombardment using plasmid pAB-6 harboring the *gus* and *bar* genes. Transformation experiments were performed on the most effective calli of medium T3 achieved in the regeneration protocol. The putatively transformed calli were selected on the same medium supplemented with bialaphos as a selective agent (Figure 4).

Data presented in Table 4 show three transformation experiments performed using 300 callus pieces, which produced 256 embryogenic calli with a high average number of embryogenic calli (85.3%). These results reflect the average regeneration frequency of plant genotype which produced 27.3% of shoots on selective medium.

Table 4. The average number of embryogenic calli formed by *Hyoscyamus muticus* L on selective medium and their regeneration efficiency

No. of experiment	No. of treated calli	Average No. of embryogenic calli on free media	Average No. of survival calli on selective media	Average No. of shoots on selective media	Regeneration Frequency %
1	100	89	56	31	31
2	100	81	44	24	24
3	100	86	51	27	27
Total	300	256	151	82	82
Mean	100	85.3	50.3	27.3	27.3

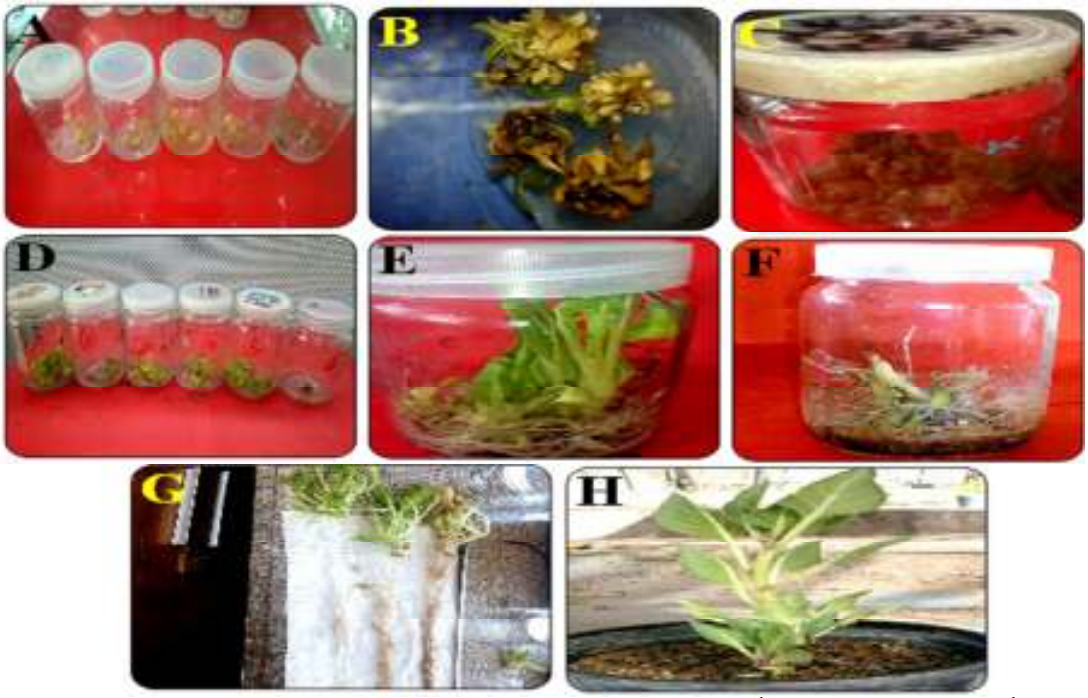

Figure 4. Transformed Calli (A, B) on T3 medium containing 1.5 mgL^{-1}bialaphos and 3 mgL^{-1}bialaphos, respectively. Non-transformed calli (C), regenerated transformed plantlets(D, E) on rooting medium containing 1.0 mgL^{-1}bialaphos, non-transformed plantlets (F), transformed plant root profile (G) and regenerated transformed plants (H) appeared, respectively.

Success of transformation was correlated with the integration, level of expression and functionality of transgenes in transgenic plants (Zarata and Verpoorte ,2007). The PCR confirmed the presence of both *gus* and *bar* genes in the DNA extracted from transgenic plants as shown in Table 5 and Figure 5.

Eighty two putative regenerated plants recovered from the selection step were obtained. A number of 11 from 82 plants succeeded in producing a positive result with PCR for the *gus* and *bar* genes with transformation efficiency of 13.4 %. Such efficiency is greater than the 2.2% in maize obtained by Assem *et al*. (2008).

Or the 1.5 to 4.0% obtained by Altpeter*et al.* (2005) or 1.3% in *paspalumnotatumflugge* obtained by Agharkar *et al*. (2007) or the 1.6% in *Eleusinecoracana* obtained by Jagga-Chugh *et al*. (2012). Song *et al.* (2006) obtained transformation efficiency of 13% was on *Populus trichocarpa*.

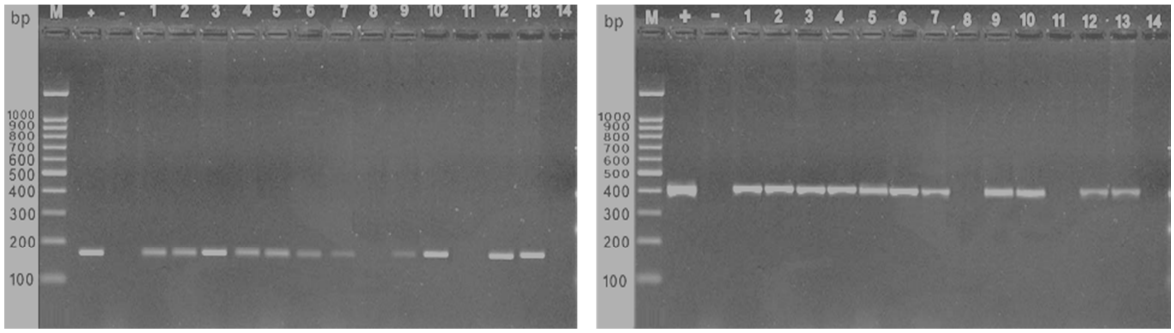

Figure 5. PCR amplified DNA using *Gus* gene specific primers (A) and the *Bar* gene primers (B).
Note: positive control from pAB6 plasmid (+), untransformed plant (-), putatively transformed *Hyoscyamus muticus* L plants (1-14) and (M) 100bp ladder marker.

Table 5. Molecular analysis and alkaloid content of the transformed plants.

No. of plants	PCR Assay		Leaf painting	Total alkaloids mg/g dry weight		
	Gus gene	*Bar gene*		Control	Regenerated plants	Transformed plants
1	+	+	++	2.95	5.45	5.94
2	+	+	++	2.95	5.79	5.59
3	+	+	+++	2.95	4.97	7.1
4	+	+	++	2.95	5.88	6.11
5	+	+	++	2.95	5.11	6.45
6	+	+	++	2.95	5.98	6.43
7	+	+	+	2.95	6.01	5.06
8	-	-	-	2.95	4.97	4.91
9	+	+	+	2.95	4.93	5.94
10	+	+	+++	2.95	5.91	6.88
11	-	-	-	2.95	5.67	4.88
12	+	+	+++	2.95	5.33	7.39
13	+	+	+++	2.95	4.18	7.31
14	-	-	-	2.95	4.94	4.67
Mean				2.95	5.36	6.05
LSD5%				0.35	0.35	0.35

Painting plant leaves with herbicide basta was effective in evaluating the production of transformed plants harboring the *bar* gene, (Yadava *et al.,* 2017). Herbicide resistance of two transformed tested by such technique enabled plant resistance after one week of painting. Transformed leaves resisted the herbicide and maintained their green color, while the non-transformed leaves became yellow to white following 5 days after painting (Figure 6).

Results demonstrate the positive results for total alkaloid content maintained on tissue culture media particularly the T3 medium (5.36 mg g^{-1} dry weight in comparison with field plants which recorded mean value of 2.95 mgg^{1} dry weight. On the other hand, there was greater effect of transformation on the transformed plants which increased the content of hyoscyamine alkaloid (6.05 mg g^{-1} dry weight) more than the untreated plants.

Transformation studies on plants using particle bombardment-mediated transformation carried out by Clapham *et al*. (2003) on Norway spruce (*Piceaabies*) and Malabadi and Nataraja, (2007) on Himalayan blue pine (*Pinus wallichiana*) are in agreement with results in the current study.

Figure 6. Leaves of transformed plant (T) and non-transformed control plant (C) painted with herbicide Basta solution.

In conclusion, the method described in the current study seems promising for an efficient *in vitro* regeneration of *H. muticus* L. (Egyptian henbane) using KN and 2,4-D. This could be useful for large scale production and provides a possible technique for plant conservation system to obtain a high-yielding trans-formant maintaining their diversity.

Transformed plants appeared over expression of *Gus* and *Bar* genes. The metabolite detection showed that alkaloids content (hyoscyamine) underwent marked increase.

Transformation was effective increasing gene products and can be used for characterizing the function of genes. Tissue culture and genetic transformation could be used as a factory to produce many important medical products on a large scale, as well as, to study gene expression. Hence, it is a suitable time to replace tobacco with Egyptian Henbane for its medicinal importance.

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إعادة التمايز والتحول الوراثي عن طريق البلازميد pAB6 باستخدام قاذف الجينات للسكران المصرى *Hyoscyamus muticus* L

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تم تقييم تأثير التركيب الكيميائي للبيئات المسئولة عن استحداث الكالس الجنينى واعادة التمايز للنباتات المحولة من نبات السكران المصرى باستخدام مسدس الجينات . ويعد نبات (*Hyoscyamus muticus* L.) المعروف باسم السكران المصرى ذو أهمية طبية واقتصادية وذلك لاحتوائة على قلويدات الأتروبين كثيرة الاستخدام و

كذلك الاسكوبولامين والهسكومين. تم إجراء استحداث للكالس وإعداد نظام لإعادة التمايز من الأوراق وذلك باستخدام سبعة أنواع من البيئات من أجل دراسة تأثير مكونات البيئة على استحداث كالس من النوع الثاني . أظهرت البيئة T3 أعلى استجابة لاستحداث الكالس بنسبة (٨٤,٩٥%) والذي انعكس على معدل تكوين النموات الخضرية (٧,٦٥ لكل قطعة من الكالس) وكذلك أعطت معدلا عاليا لتكوين الجذور وذلك بعد نقلها على بيئة التجذير . وكذلك تم إجراء عدد من تجارب التحول الوراثي باستخدام البلازميد ح pAB6 والذي يحتوى على جينى *gus, bar* لتقدير كفاءة معدل التحول الوراثي والتي قدرت بنسبة (١٣,٤%) وكذلك تم تقدير المحتوى الكلى للقلويدات بنسبة (٦,٠٥%) للنباتات المحوله وراثيا الناتجة من تجارب زراعة الأنسجة مقارنه بالنباتات الغير محوله وراثيا والتي تحتوي علي ٢.٩٥%.