Conservation of *Hyoscyamus boveanus* Asch. & Schweinf. as a rare plant endemic to Egypt

(Received: 03. 04.2019; Accepted: 02.05.2019)

Heba Elsayed Ghareb
Tissue Culture Unit, Genetic Resources Dept., Desert Research Center El-Matareya, Cairo, Egypt

ABSTRACT

With increasing world population and potential climate changes, there is a growing awareness for conservation and sustainable use of the world natural resources for maintaining biological diversity. Advances in biotechnology have generated new opportunities for the conservation of genetic resources. A preliminary investigation to study the genetic diversity among wild individuals of Hyoscyamus boveanus collected from different sites of Saint Katherine was estimated for obtaining primary information to protect our patency and quality assurance of this endemic plant. The estimated level of polymorphism using 19 inter-simple sequence repeat (ISSR) markers was 20.9%. DNA barcode analysis was used for characterization, identification, and registration of H. boveanus. The matK, rbcL and rpoC1 sequences in H. boveanus were recorded in NCBI GenBank with accession numbers of MK189190, MK189191, and MK189192, respectively. Micropropagation of this plant was also another objective of the present research to find an alternative way to conserve and produce large numbers of this rare medicinal plant. Shoot tips and stem nodal segments from seedlings were used as explants for micropropagation. Murashige and Skoog (MS) medium fortified with 0.5 mg l^{-1} kinetin (Kn) and 0.5 mg l^{-1} α-naphthalene acetic acid (NAA) was the most promising medium for both explants for shoot initiation and multiplication. The highest rooting percentage (80%) and the highest number of roots were obtained on half strength MS medium fortified with 1 mg l^{-1} each of NAA and indole-3-butyric acid (IBA). The rooted plantlets were successfully transferred to the greenhouse and exhibited a normal development.

Keywords: *Sakaran, DNA fingerprinting, ISSR, DNA barcoding, GenBank, micropropagation, Saint Katherine.*

INTRODUCTION

There are about 65 species endemic to Egyptian flora of the total, 2145 species. About 52% of them are endemic to Sinai (Boulos, 2009 and Zahran *et al*., 2015). Most of the endemic plants are categorized as: very rare, endangered and severely threatened affected by several natural and human factors. Saint Katherine Mountains consider a center of endemism (Zahran *et al*., 2015). *Hyoscyamus boveanus* Asch. and Schweinf. is a rare plant, found in Saint Katherine protectorate in a mono regional element. It is a member of family Solanaceae. This family contains nine genera. *Hyoscyamus* is one of the most important genera in this family. In Egypt, this genus is known as "Sakaran" and contains one common and six rare species, where *Hyoscyamus boveanus* is the only endemic

species (Boulos, 2009). Species of the Solanaceae are famous for their medicinal values. The genus *Hyoscyamus* is known for its high content of tropane alkaloids. It contains 23 alkaloids, where the main constituents are hyoscyamine and scopolamine with an average ratio of 13: 1 (El-Shazly *et al*., 1997).

Tropane alkaloids are widely used in medicine for their antispasmodic, anticholinergic, mydriatic, analgesic and sedative properties (Zolala *et al*., 2007). They also affect the parasympathetic nervous system, are useful in the treatment of Parkinson's disease and are used to increase heart rate (Sevon *et al*., 2001). Scopolamine has high commercial importance because of its lower side effects on the nervous system. Scopolamine is only obtained from natural plant sources due to the complexity of its chemical structure, thus it is very expensive to synthesize industrially (Ghorbanpour *et al*., 2013). The International Union for Conservation of Nature (IUCN) reported that many of the medicinal plants are facing an extinction risk due to the shortage in plant propagation systems (Anis and Faisal, 2005). According to Food and Agricultural Organization (FAO), there is an incredible increase in human population, which will be 9.1 billion by 2050; about 80% of them depend on medicinal plants for their disease treatment (Ramakrishnan *et al*., 2017). Climatic changes, overgrazing and over collection by natives and pharmaceutical companies, which depend on naturally growing plants, lead to plant declining (Gantait *et al*., 2014). Therefore, there is an urgent need to find alternative methods for plant propagation and conservation to fulfill the growing human demand.

Genetic diversity is important to conserve species because it affects the population ability for adapting to environmental changes. DNA fingerprints, based on DNA markers are efficient tools to discriminate, characterize, identify and conserve different medicinal plants than the visual based assessment by morphological and phonological methods (Ahmad *et al*., 2017). The usefulness of DNA fingerprints rely on the protection of the patency and the quality assurance of various plants for industry and estimation of genetic diversity. Also, the molecular methods can be applied at any developmental stage and are reliable, easy, and quick (Mishra *et al*., 2016). Inter Simple Sequence Repeats (ISSR) technique is easy, quick, reproducible, cost-efficient, requires low amounts of DNA and polymorphous than the other popular DNA markers such as RFLP and RAPD. ISSR can amplify the inter sequences of the microsatellite at the multiple loci in the genome. ISSR markers don't require previous knowledge about the DNA sequence (Rajasekharan *et al*., 2017). DNA barcoding technique is used for identifying and characterizing species within an organism utilizing a short sequence of DNA of a standard position in the genome. Because of the low evolutionary rates of chloroplast loci, it is widely used for DNA barcoding technology when compared with nuclear loci (Dong *et al*., 2012). The plastid barcoding markers; *matK*, *rbcL* and *rpoC1* are widely used and depend on a single locus (Li *et al*., 2015). Tissue culture technology has opened the way to researchers for biodiversity conservation and propagation of rare wild and medicinal plants (Chandana *et al*., 2018). It is a preferable technique than the conventional propagation method because only a small piece of tissues is needed to produce a large number of clonal plants, production of virus- free plants, regeneration can be achieved at any time of the year, plant germplasm can be stored for a long time and high amounts of secondary metabolites can be produced (Oseni *et al*., 2018).

Therefore, tissue culture could be an

efficient protocol for micropropagation and conservation of the rare *H. boveanus*. There are few researches on the micropropagation of other *Hyoscyamus* species, such as *Hyoscyamus niger* (Prabhakar *et al*., 2001) and *Hyoscyamus reticulatus* (Madani *et al*., 2015) from shoot tip explants and *Hyoscyamus muticus* from stem segments (Abed Elmaksood *et al*., 2016). This work aimed to conserve the rare medicinal *H. boveanus* plant, endemic to Egypt, by studying the genetic diversity across the naturally growing individuals of *H. boveanus* in Saint Katherine region, and identifying the plant using DNA barcoding. It also, aimed to establish an efficient protocol for the micropropagation of this species to support our intellectual property rights.

MATERIALS AND METHODS

Estimation of the genetic diversity among wild individuals of *H. boveanus* using ISSR markers

Plant collection

Plant materials were collected from three different sites in Saint Katherine; site 1 (Alroses), site 2 (Shak Groceah) and site 3 (Abo-Cela). The plant materials were identified by Dr. Ibrahim Abdelrafee Elgamal, Nature Conservation Sector, Egyptian Environmental Affairs Agency, South Sinai, Egypt. The collected samples were kept into silica gel powder for preservation of leaf samples until the isolation of DNA.

Genomic DNA isolation and PCR amplification

Plant leaf tissues (0.1 g) were ground to a fine powder in liquid nitrogen using a mortar and a pestle. This powder was used for total genomic DNA isolation using DNeasy Genomic DNA Extraction Kit (Qiagen, Santa Clarita, CA). The isolated DNA was then used for PCR amplification.

ISSR PCR reaction

A total of 20 ISSR primers (Table 1) were tested for *H. boveanus* identification and detection of polymorphism between the wild individuals of *H. boveanus*. amplification reaction was carried out in 25 µl reaction mixture containing 1X PCR buffer (Promega, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1µM primers, 1U Taq DNA polymerase (Go Taq, Promega, USA) and 30 ng template DNA. PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems, PCR 9700, USA) programmed to fulfill 35 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 1 min, an annealing step at 50ºC for 1 min, and an elongation step at 72ºC for 1.5 min. The primer extension segment was extended to 7 min at 72ºC in the final cycle. The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 ug/ml) in 1X TBE buffer at 95 volts. 1 kbp DNA ladder was used as a molecular size standard. PCR products were visualized on UV light and photographed using a Gel Documentation System (BIO-RAD 2000, Hercules, CA, USA).

Data Analysis

The banding patterns generated by ISSR marker analysis were compared to determine the genetic relatedness of the samples under study. Clear and distinct amplification products were scored as '1' for the presence and '0' for the absence of bands. Bands of the same mobility were scored as identical. The genetic similarity coefficient (GS) between samples was estimated according to the Dice coefficient as follows:

Dice formula: GSij = 2a/(2a+b+c)

Table (1): The sequence of ISSR primers used for testing the genetic diversity among Hyoscyamus boveanus individuals.

Primer	Sequence 5'-3'	Primer	Sequence 5'-3'
ISSR- 1	(AG)8YC	ISSR-11	(AC)8YA
ISSR- 2	(AG)8YG	ISSR-12	(AC)8YC
ISSR- 3	(AC)8YT	ISSR-13	(AG)8YT
ISSR- 4	(AC)8YG	ISSR-14	(CTC)5TT
ISSR- 5	(GT)8YG	ISSR-15	(CT)8YG
ISSR- 6	CGC(GAT)6	ISSR- 16	(TC)8CA
ISSR-7	GAC(GATA)4	ISSR- 17	(TC)8TG
ISSR-8	(AGAC)4GC	ISSR- 18	HVH(CA)7T
ISSR-9	(GATA)4GC	ISSR- 18	HVH(TCC)5
ISSR- 10	(GACA)4AT	ISSR- 20	HVH(TG)7T

A: Adenine, T: Thymine, G: Guanine and C: Cytosine Y: (C or T), V: (A or C or G), H: (A or C or T).

Where GSij is the measure of genetic similarity between individuals i and j, a is the number of bands shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i. The similarity matrix was used in the cluster analysis. Unweighed Pair Group Method using Arithmetic Average (UPGMA) was carried out for cluster analysis (Sneath and Sokal, 1973).

DNA barcoding

Barcode PCR reaction

Short fragments of specific regions of the DNA (*matK*, *rpcL* and *rpoC1*) were amplified using universal primers for the three DNA loci. The reaction conditions were conducted according to Song *et al*. (2009). The primers used for the amplification of these three regions are listed in Table (2). The amplification reaction, reaction mixture and PCR conditions for DNA barcode was carried out as in ISSR reaction but in 50 µl reaction volume using ultra-pure water and was programmed to fulfill 40 cycles.

Purification of PCR Products

Amplified products for the three PCR fragments were purified using Qiagen PCR purification Kit (Qiagen, Santa Clarita, CA).

Table (2): List of PCR primers used for amplification of plastid DNA for barcoding of Hyoscyamus boveanus plants.

Primer Code	Sequence
matKF	5'-CGATCTATTCATTCAATATTTC-3'
matKR	5'-TCTAGCACACGAAAGTCGAAGT-3'
rbcl-F	5'-ATGTCACCACAAACAGAGACTAAAGC-3'
rbcl-R	5'-TCGCATGTACCTGCAGTAGC-3'
rpoC1F	5'-GGCAAAGAGGGAAGATTTCG-3'
rpoC1R	5'-CCATAAGCATATCTTGAGTTGG-3'

Sequencing analysis

The sequencing of the PCR products was carried out through an automatic sequencer ABI PRISM 3730XL Analyzer (Microgen Company, USA) using BigDyeTM Terminator Cycle Sequencing Kits, following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using *matK*, *rbcL* and *rpoC1* primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer.

Computational analysis (BLASTn)

The sequences of *matK, rbcL and rpoC1* were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The sequences were aligned using Align Sequences Nucleotide BLAST.

Micropropagation of *H. boveanus*

Plant material and sterilization

Seeds of *H. boveanus* were collected from mature plants grown naturally in Alroses site, Saint Katherine, Sinai, (Fig. 6a). They were cleaned by washing under tap water and sterilized by soaking into 1.25% NaOCl for 10 min, and then rinsed 5 – 6 times in sterile distilled water. Seeds were cultured on plant growth regulators (PGRs) free-Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The percentage of seed germination was calculated after four weeks of culture. Shoot tips and stem nodal segments from the seedlings were tested for propagation.

Culture medium and conditions

Shoot tip and stem nodal segment explants about 3 cm long were cultured on MS basal medium fortified with 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol and solidified with 3 g l^{-1} phytagel. Auxins [β – naphthalene acetic acid (NAA) and indole – 3 – butyric acid (IBA)] and cytokinins [6 – benzyl amino purine (BA), kinitin (Kn) and N^6 – (2 – isopentenyl) adenine (2iP)], at different concentrations, were added individually or in combinations to the medium to obtain the best requirements for each growth stage. The MS medium free from PGRs served as control. The pH of the medium was adjusted to 5.7 – 5.8 before autoclaving for 15 min at 121^{o}C under a pressure of 1.1 kg cm^{-2}.The cultures were incubated at a temperature of 26 ± 2^{o}C and were exposed to a 16 h photoperiod under cool white fluorescent tubes of 2 k lux light intensity.

Initiation and multiplication stages

Two different experiments were carried out for the *in vitro* initiation and multiplication of shoot tips and stem nodal segments of *H. boveanus*.The effect of various cytokinins (BA, Kn, and 2iP) with different concentrations (0.1, 0.25 and 0.5 mg l^{-1}) was evaluated when added individually on MS medium. In the second experiment, explants were cultured on MS medium fortified with BA or Kn at the same concentrations (0.1, 0.25 and 0.5 mg l^{-1}) in combination with NAA (0.1 and 0.5 mg l^{-1}) to evaluate the effect of cytokinin/auxin combination on shoot initiation and multiplication. MS medium free from PGRs was used as a control. For each experiment, growth percentage (%) (percentage of explant forming growth to survived explants), the average number of shoots/explant and the average length of shoots (cm) were recorded after six weeks.

Effect of auxins on root induction

Multiplied shoots were transferred to half strength MS medium fortified with four combinations of IBA and NAA at 1.0 and 2.0 mg l^{-1} to stimulate rooting of the shoots. Half

strength MS medium without auxins was used as a control. Rooting percentage (%), the average number of roots/explant and the average length of roots (cm) were recorded after six weeks of transfer.

Acclimatization of plantlets to *ex vitro* conditions

Rooted shoots were taken out from the culture medium and washed in sterile distilled water. The plantlets were then transferred to plastic pots containing a mixture of peat moss and sand (1:1), and covered with plastic bags in the greenhouse (28 ± 2°C, 70 – 80% humidity). The plants were irrigated regularly by tap water. After two weeks, the covers were removed gradually to complete acclimatization. The percentage of survived plants (%) was recorded.

Data analysis

The micropropagation experiments were repeated twice and the treatments had at least 10 replicates. All experiments were carried out by the completely randomized design. Analysis of Variance of the data was carried out using ANOVA program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level using Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Genetic diversity among wild individuals of *H. boveanus*

High genetic variation gives protection for the wild population to adapt to any changes in the environmental conditions. Rare plants always suffer from low genetic diversity because of limited gene flow (Szczecinska *et al*., 2016). This preliminary investigation was conducted to study the genetic diversity of the rare endemic *H. boveanus* species, to obtain primary information about this spesies. Due to the reduced number of individuals; genetic diversity estimation of *H. boveanus* was conducted for only three wild individuals found in Saint Katherine region, as a mono-regional element (Zahran *et al*., 2015). A total of 20 ISSR primers (Fig. 1 and Table 3) were employed to scan and estimate the genetic variation at the molecular level across three wild individuals of *H. boveanus*, which were collected from three different population sites of Saint Katherine. From the twenty tested ISSR primers, 19 primers generated bands. ISSR primer number 14 didn't give any result.

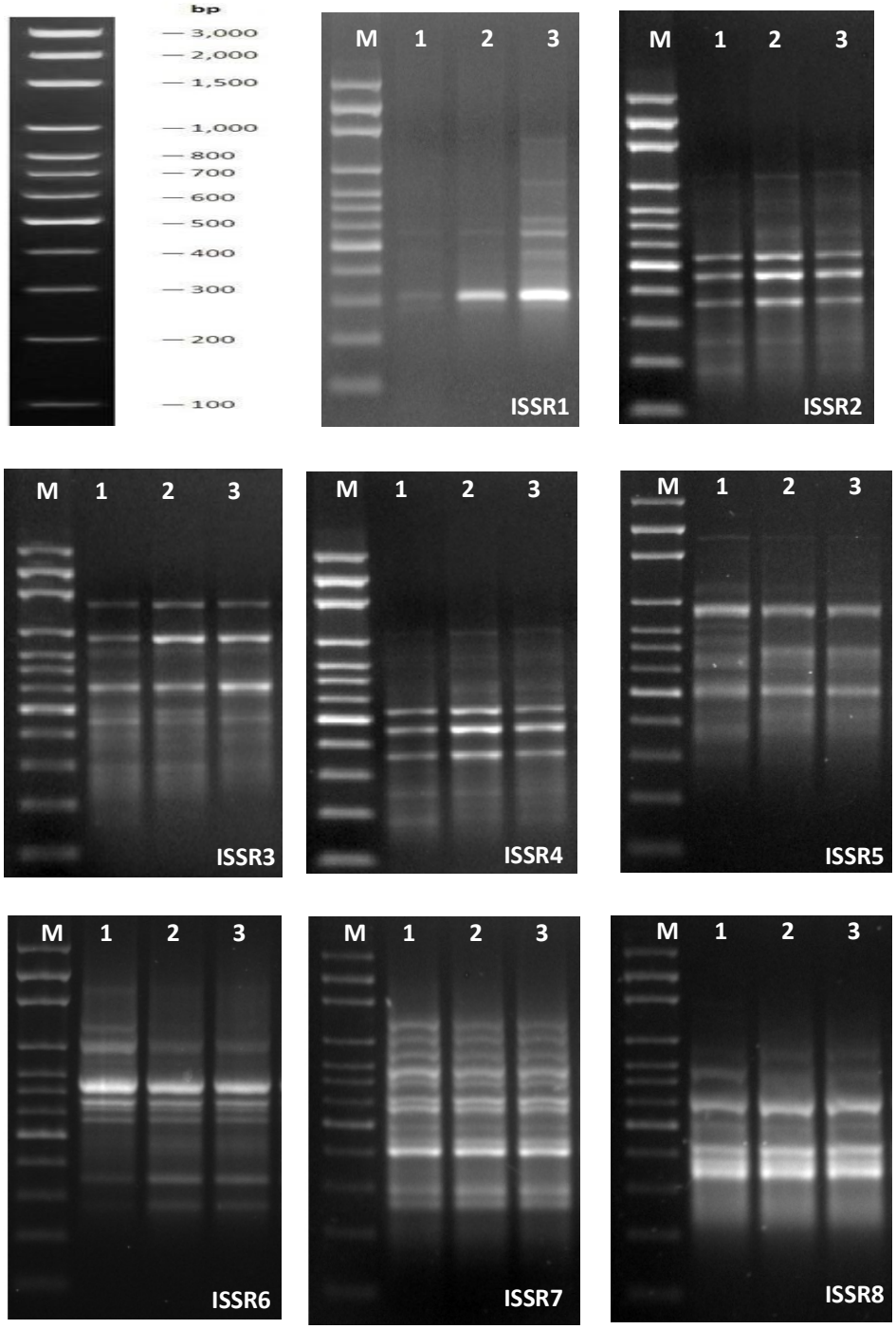


Fig. (1): ISSR profiles of three individuals of Hyoscyamus boveanus from different sites at Saint Katherine, Sinai via 19 primers. Lane M- 1-kb DNA ladder; lane 1, 2, and 3 individuals collected from site 1, 2, and 3, respectively.

Fig. (1): Continued

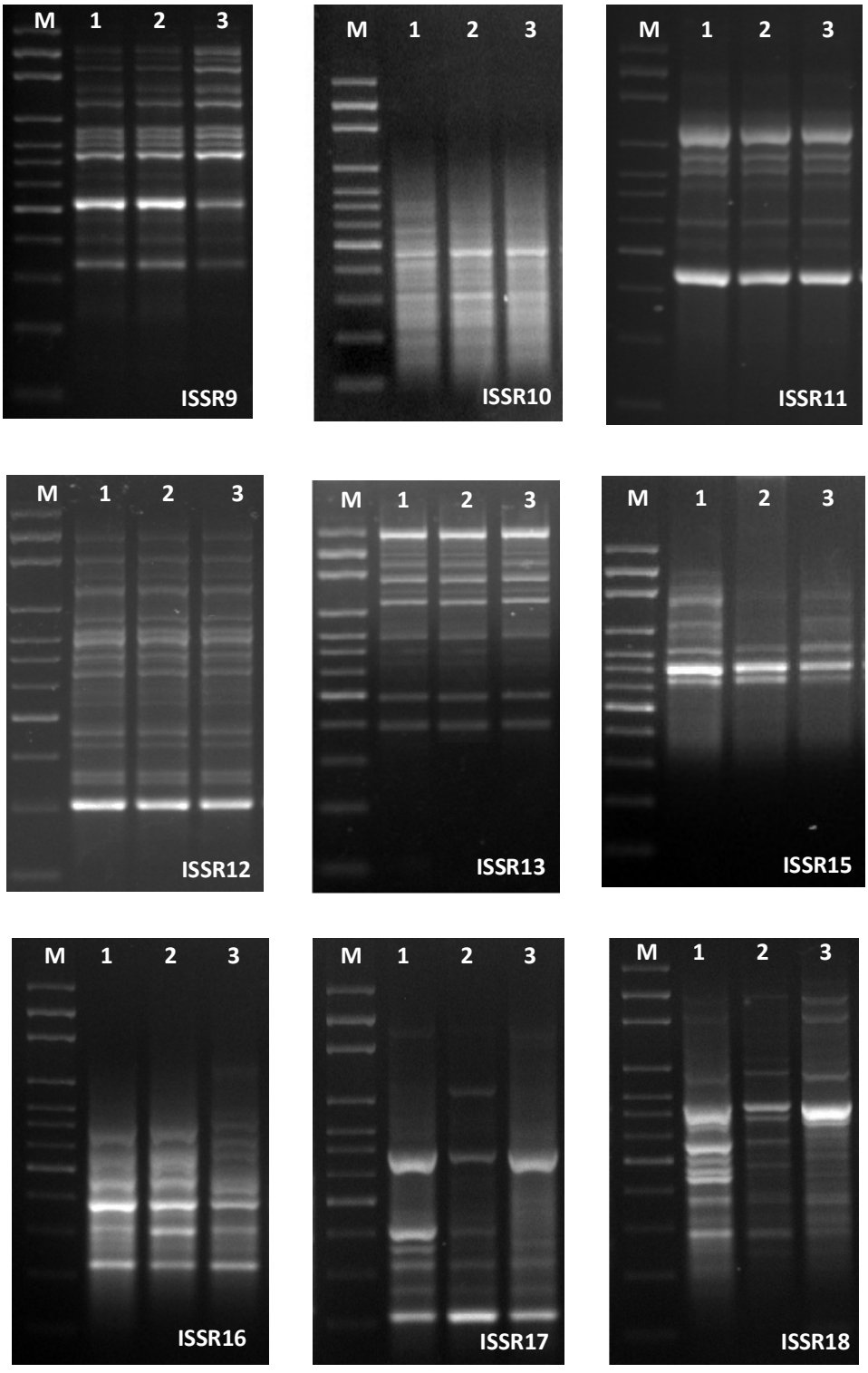
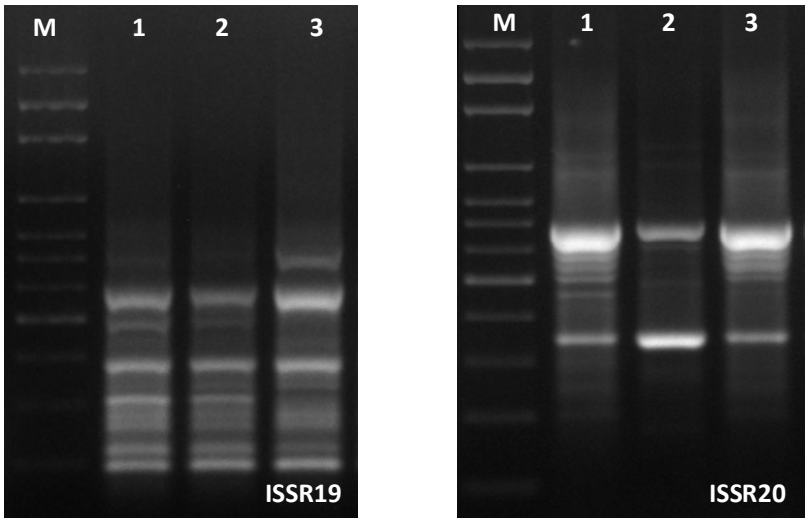


Fig.(1): Continued



A total of 225 amplicons were generated when the 19 ISSR primers were employed among the three wild individuals of *H. boveanus* (Fig. 1 and Table 3) (lanes 1, 2, 3). Only 47 amplicons were polymorphic (20.9%). The number of amplicons scored for each primer ranged from 7 - 18. The size of these amplicons was between 150-3000 bp. The ISSR primers 1 and 16 recorded the highest percentage of polymorphism (42.9%).

Despite of the limited geographical region, self-pollination in family Solanaceae and low chances of gene flow; the level of genetic diversity among the naturally occurring *H. boveanus* in Saint Katherine was 20.9%. This percentage may be changed if more geographical regions were compared. That ensures the strong need to study the genetic diversity of *H. boveanus* in a wider geographical range to protect and conserve the plant from the environmental changes. Similar results were obtained by Rajasekharan *et al*. (2017), who found a high level of genetic diversity among 39 accessions of *Oroxylum indicum*, an important threatened traditional medicinal plant native to India, using 17 ISSR markers primers. On the other hand, Xiao *et al*. (2004) studied the genetic variation in the endemic and endangered *Cycas guizhouensis* plant using 11 ISSR primers and obtained low genetic diversity at the population and the species level, they referred that to the geographical range within cycads than other seed plants. Also,Torre *et al*. (2012) evaluated the genetic diversity among 35 accessions of *Calibrachoa caesia* using 13 ISSR primers and detected no significant differences among the populations. Fig. 2 represents the UPGMA dendrogram obtained from the data of ISSR analysis showing the relationship between the three individuals of *H. boveanus*, which were collected from the three sites at Saint Katherine region.

The dendrogram analysis showed two clusters, where the two plants from site 1 and 2 in one cluster, and the second cluster represents the plant from site 3. The individual located in site 1 is closely related to the individual from site 2. While, the individual of site 3 is occupying a distinct place and related to the individuals of site 1 and 2.

Table (3): ISSR analysis for the genetic diversity among three individuals of Hyoscyamus boveanus plant from three different population sites at Saint Katherine.

Primer No.	No. of amplified fragments			Amplicon range (bp)	Total no. of amplicons	Polymorphic amplicons	Polymorphism (%)
	Site 1	Site 2	Site 3				
ISSR1	4	5	7	280-1500	7	3	42.9
ISSR2	8	8	9	180-1100	9	2	22.2
ISSR3	12	11	10	220-1400	12	2	16.7
ISSR4	8	10	9	180-1100	10	3	30.0
ISSR5	10	8	8	300-1800	11	4	36.4
ISSR6	10	9	9	270-1700	10	1	10.0
ISSR7	13	13	13	270-1300	13	0	00.0
ISSR8	8	7	7	330-1500	8	1	12.5
ISSR9	17	17	15	230-3000	17	2	11.8
ISSR10	13	12	11	150-700	13	2	15.4
ISSR11	9	9	9	450-1800	9	0	00.0
ISSR12	18	18	18	300-2000	18	0	00.0
ISSR13	12	13	11	370-3000	13	2	15.4
ISSR14	-	-	-	-	-	-	-
ISSR15	8	5	8	630-1800	8	3	37.5
ISSR16	9	8	13	180-1100	14	6	42.9
ISSR17	10	10	9	210-1800	13	5	38.5
ISSR18	14	14	17	220-1900	17	5	29.4
ISSR19	10	10	10	200-800	12	5	41.7
ISSR20	11	10	10	200-1400	11	1	9.1
Total	204	197	203		225	47	20.9

This study concentrated on estimating the genetic diversity of *H. boveanus* plants growing naturally within Saint Katherine region. So, more studies are needed to cover other regions in Egypt, Arabian Desert (east of the Nile), Red Sea Coastal region and Oasis of Libyan desert to obtain a complete knowledge about the genetic variation in this plant.

DNA barcoding

DNA barcoding is currently a widely used and effective tool that enables rapid and accurate identification of plant species. *MatK* , *rbcL* and *rpoC1* are portions of plastid genes and widely used in phylogenetic investigations (Li *et al*., 2015). *MatK* (maturase K) has a high evolutionary rate, suitable length and obvious interspecific divergence as well as a low transition/transversion rate (Li *et al*., 2015). *RbcL* (Ribulose-1, 5–bisphosphate carboxylase/ oxygenase large subunit gene) is responsible for the production of the large subunit of the enzyme RuBisCo (important for carbon fixation). *RbcL* is still suggested as one of the best potential candidate plant barcodes based on the straightforward recovery of the gene sequence and providing a useful backbone to the barcode dataset (Mohamed, 2016). *RpoC1* is a portion of protein coding gene in the plastid and has universality and sequence quality, but had low discriminatory power (CBOL Plant Working Group, 2009). Sequences of *matK*, *rbcL* and *rpoC1* barcode regions of *H. boveanus* were 953,714 and 531 bp, respectively. These sequences were BLASTed against GenBank accessions. The alignments of the three barcodes yielded a query cover from 87 to 92%, 72 to 75% and 91 to 94% for *matK*, *rbcL* and *rpoC1*, respectively.The phylogenetic analysis of *H.*

boveanus based on the *matK* nucleotide sequences region generally discriminate *Hyoscyamus* species with 98% similarity (Fig. 3). Similarly, *rbcL* phylogenetic analysis of *H. boveanus* showed that its sequence has 99% identity to the species of *Hyoscyamus* genus; *H. niger, H. albus*, *H. muticus* and *H. turcoman* (Fig. 4) and highly supported to *H. boveanus*. In contrast with *rbcL* and *matK*, *rpoC1* poorly belonged to genus *Hyoscyamus*, except for *H. niger* (97% similarity), which may be due to the lower availability of *rpoC1* sequences in the Genbank database for *H. boveanus* than *matK* and *rbcL*. However, the *rpoC1* sequence supports its inclusion in the family Solanaceae with 97% similarity (Fig. 5). In this research the *matK*, *rbcL* and *rpoC1* barcode sequence regions were submitted for *H. boveanus* in Genbank database with accession numbers MK189190, MK189191 and MK189192 for *matK*, *rbcL* and *rpoC1*, respectively.

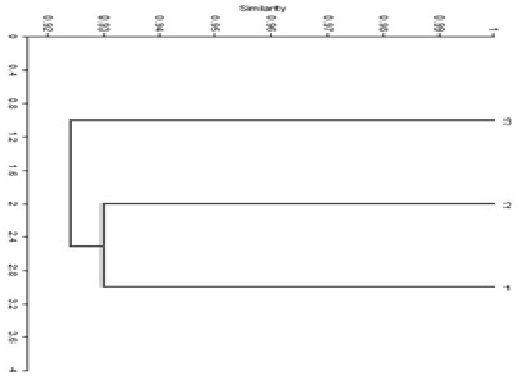


Fig. (2): UPGMA dendrogram of three Hyoscyamus boveanus individuals collected from sites 1, 2, and 3 at Saint Katherine, Sinai which developed from ISSR primers.

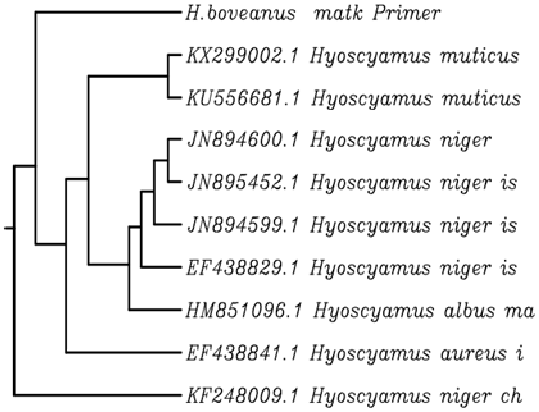


Fig. (3): matK based maximum likelihood tree for Hyoscyamus boveanus plants.

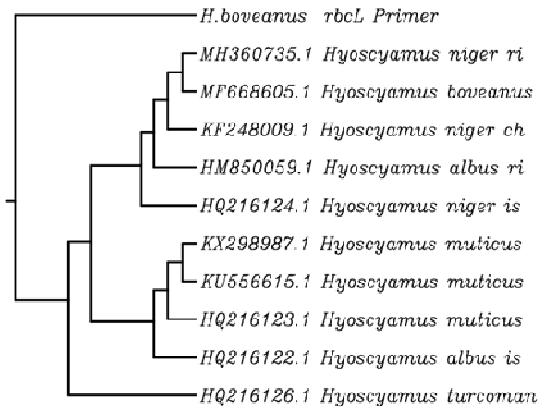


Fig. (4): rbcL based maximum likelihood tree for Hyoscyamus boveanus plants.

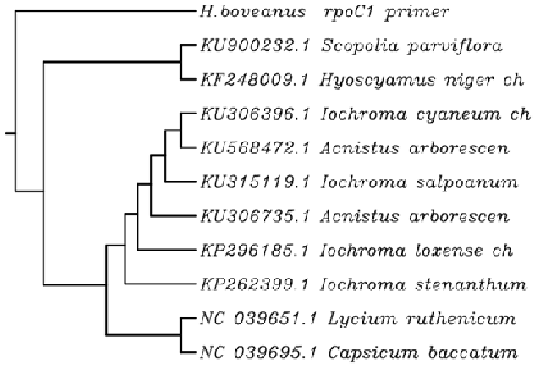


Fig. (5): rpoC1 based maximum likelihood tree for Hyoscyamus boveanus plants.

Micropropagation of *H. boveanus*

Initiation and multiplication stages

About 80% germination percentage was obtained after four weeks of culture on PGRs free-MS medium used for seeds germination.

Influence of cytokinin and explant type

An experiment was conducted to test the effect of different cytokinins (BA, Kn, and 2iP), added individually in various concentrations (0.1, 0.25 and 0.5 mg l^{-1}) on shoot initiation and multiplication of two types of explants (shoot tips and stem nodal segments) obtained from the germinated seedlings. Table (4) clears that the growth percentage and the average number of shoots per explant increased with the increase in any cytokinin concentration for both explants. Also, BA and Kn were more effective than 2iP in shoot initiation and multiplication, since both of them gave the highest percentage of growth for both types of explants.Kn at 0.5 mg l^{-1} gave the highest percentage of explants forming growth (100%) and the highest average number of shoots/explant for both explants. This number was 6.7 and 6.8 shoots/explant for shoot tips and stem nodal segments, respectively (Fig. 6b and c). The second best concentration of Kn was 0.25 mg l^{-1}, which gave 6.0 and 6.1 shoots/explant for

shoot tips and stem nodal segments, respectively. BA at 0.5 mg l^{-1} gave also a high number of shoots/explant for shoot tips (6.3 shoots/explant) and 6.7 shoots/explant for stem nodal segments. For both types of explants, there were no significant differences between each of 0.5 or 0.25 mg l^{-1} Kn or 0.5 mg l^{-1} BA in the growth percentage and the average number of shoots/explant. With respect to shoot length, the longest shoot for shoot tips explants was obtained with 0.5 mg l^{-1} Kn (3.4 cm), followed by 0.25 and 0.5 mg l^{-1} 2iP, which gave 3.2 cm for each of them, although these treatments were insignificantly different regarding shoot length. For stem node segment, 0.1 mg l^{-1} BA gave the best length of shoot (2.9 cm). There were no significant differences between all used cytokinin in shoot length. These results clarify that the type and the concentration of the cytokinin is the key that leads the establishment. This is in agreement with Schuelter *et al*. (2009) and Tahtamouni *et al*. (2017), who examined the *in vitro* propagation in *Solanum villosum* (family *Solanaceae*), using BA, 2iP, Kn and Thidiazuron (TDZ) and reported that the response of the *in vitro* grown nodal segments of the plant varied with the type and concentration of the added cytokinin. The best cytokinin in this experiment was Kn followed by BA. Thus they were used in addition to NAA in the second experiment to study their effect in improving shoot multiplication.

Influence of cytokinin/auxin combination and explant type

This experiment was conducted to study the effect of adding 0.1 or 0.5 mg l^{-1} NAA to each of BA or K, individually at concentrations of 0.1, 0.25 and 0.5 mg l^{-1} (Tables 5 and 6). From Table 5, it is clear that the addition of NAA either at 0.1 or 0.5 to all concentrations of BA led to the increase in the average number of shoots in both types of explants. There is also a gradual increase in this number as the concentration of BA and NAA were increased. Concerning the shoot tip explants, the highest average number of shoots was gained with medium fortified with 0.5 mg l^{-1} BA with either 0.1 mg l^{-1} or 0.5 mg l^{-1} NAA, which gave 16.0 and 17.0 shoots/explant, respectively.

With respect to stem nodal segments, the maximum average number of shoots were 12.0 and 14.5 with the media fortified with 0.5 mg l^{-1} BA with either 1.0 or 0.5 mg l^{-1} NAA, respectively. By taking the shoot length into consideration, it was noticed that the PGRs free MS medium gave the highest average length of shoots, 2.4 and 1.8 cm for shoot tips and stem nodal segments, respectively.

Table (4): Effect of cytokinin type in MS medium and explant on Hyoscyamus boveanus in vitro initiation and multiplication after six weeks.

Cytokinin concentration (mg l^{-1})			Explant type					
			Shoot tip			Stem nodal segment		
BA	Kn	2iP	Growth percentage (%)	Average no. of shoots/ explant	Average shoot length (cm)	Growth percentage (%)	Average no. of shoots/ explant	Average shoot length (cm)
0.00	0.00	0.00	75^{e}	3.0^{d}	2.1^{b}	70^{d}	3.0^{c}	2.5^{ab}
0.10	0.00	0.00	85^{c}	3.7^{d}	1.3^{b}	95^{b}	4.0^{bc}	2.9^{a}
0.25	0.00	0.00	95^{b}	4.3^{bcd}	1.2^{b}	100^{a}	4.7^{abc}	1.7^{ab}
0.50	0.00	0.00	100^{a}	6.3^{ab}	1.3^{b}	100^{a}	6.7^{a}	2.1^{ab}
0.00	0.10	0.00	95^{b}	4.0^{cd}	1.2^{b}	95^{b}	3.7^{c}	1.7^{ab}
0.00	0.25	0.00	100^{a}	6.0^{abc}	3.0^{a}	100^{a}	6.1^{ab}	2.3^{ab}
0.00	0.50	0.00	100^{a}	6.7^{a}	3.4^{a}	100^{a}	6.8^{a}	2.4^{ab}
0.00	0.00	0.10	75^{e}	3.7^{d}	2.1^{b}	70^{d}	3.3^{c}	1.3^{b}
0.00	0.00	0.25	80^{d}	4.3^{bcd}	3.2^{a}	85^{c}	3.7^{c}	2.2^{ab}
0.00	0.00	0.50	85^{c}	4.7^{bcd}	3.2^{a}	85^{c}	4.0^{bc}	2.3^{ab}

Means in the same column followed by different letter(s) are significantly different at $p \leq 0.05$.

Table (5): Effect of PGRs (BA and NAA) in MS medium and explant type on Hyoscyamus boveanus in vitro initiation and multiplication after six weeks.

Growth regulators conc. (mg l^{-1})		Explant type			
		Shoot tip		Stem nodal segment	
BA	NAA	Average no. of shoots/ explant	Average shoot length (cm)	Average no. of shoots/ explant	Average shoot length (cm)
0.00	0.00	2.8^{c}	2.4^{a}	3.3^{b}	1.8^{a}
0.10	0.10	5.3^{c}	1.8^{b}	8.3^{ab}	0.70^{c}
0.25	0.10	10.5^{b}	0.8^{cd}	10.0^{ab}	0.70^{c}
0.50	0.10	16.0^{a}	1.0^{c}	12.0^{a}	0.83^{c}
0.10	0.50	7.3^{b}	0.3^{e}	10.0^{ab}	1.30^{abc}
0.25	0.50	11.5^{b}	0.5^{de}	14.3^{a}	1.00^{bc}
0.50	0.50	17.0^{a}	0.7^{cd}	14.5^{a}	1.60^{ab}

Means in the same column followed by different letter(s) are significantly different at $p \leq 0.05$.

It is obvious that, the addition of the auxin to the cytokinin resulted in a high frequency of multiplied shoots. Kocot *et al*. (2011) reported that the interaction between auxin/cytokinin promotes lateral bud formation, controls and induces cell division and leads to slow aging or causes juvenility. The cytokinin/auxin crosstalk has proved to be effective in shoot regeneration in *Hyoscyamus niger* (Ghorbanpour *et al*., 2013), *Hyoscyamus reticulatus* (Aminnejad *et al*., 2015) and *Solanum lycopersicum* (Alatar *et al*., 2017). Replacing BA with Kn, revealed that, when Kn was used there was also a positive correlation between the average number of shoots/explant and the increasing in each of NAA and Kn concentrations (Table 6). The medium fortified with 0.5 mg l^{-1} Kn and 0.5 mg l^{-1}NAA gave the highest shoot number of 19.5 shoots/explant in addition to the longest shoot (1.5 cm) for shoot tip explants (Fig. 6d). Also, the same medium gave 17.9 shoots/explant with 1.3 cm length for stem nodal segments. However, the medium

fortified with 0.25 l^{-1} Kn and 0.1 mg l^{-1} NAA gave the highest length of shoots (1.5 cm) with stem nodal segments.

By comparing the obtained data in Tables (5) and (6), it could be concluded that Kn was more effective than BA in stimulating bud initiation and shoot multiplication in *H. boveanus*. Kocot *et al*. (2011) reported that Kn plays an important role in living organisms. The low concentrations of Kn positively influenced plants, but high concentrations caused toxicity. In several studies, Kn proved its efficiency in shoot multiplication than BA as in *Solanum nigrum* (Padmapriya *et al*., 2011), *Cucumis sativus* (Abu-Romman *et al*., 2015) and *H. muticus* (Abed Elmaksood *et al*., 2016).

Effect of auxins on rooting and acclimatization stages

Half strength MS medium fortified with different concentrations of IBA alone was used in preliminary experiments, but it did not stimulate rooting. Hence, combinations of NAA with IBA were used. Table (7) clears media fortified with 1.0 mg l^{-1} IBA with either 1.0 or 2.0 mg l^{-1} NAA gave the highest rooting percentage (80.0%). Only the medium containing IBA and NAA each at 1.0 mg l^{-1} gave the highest mean number of roots per explant (19.7) with the highest average length of roots/shoot (4.7 cm) (Fig. 6e and f). Using a combination of NAA and IBA to stimulate *in vitro* rooting in plants is supported by Cheng *et al*. (1992), who used a combination of 2.5 µM IBA and 2.5 µM NAA for rooting of *Eucalyptus sideroxylon* on a modified half-strength MS medium. Hegazi *et al*. (2011) observed that the rooting in *Capparis* species produced *in vitro* was superior when a combination of the two auxins (IBA and NAA) was used.

Half strength MS medium free from PGRs was not effective in root induction. This indicates the effect of auxins in stimulating root formation. This result is in line with Hegazi *et al*. (2011), who obtained a negative response with the control medium during their experiments to induce rooting in *Capparis orientalis* and *Capparis leucophylla* shoots. Rooted plantlets were successfully acclimatized in greenhouses (Fig. 6g and h). A percentage of 70% of acclimatized plantlets were survived after six weeks of transferring into the greenhouse.

Table (6): Effect of PGRs (Kn and NAA) in MS medium and explant type on Hyoscyamus boveanus in vitro initiation and multiplication after six weeks.

Growth regulators conc. (mg l^{-1})		Explant type			
		Shoot tip		Stem nodal segment	
Kn	NAA	Average no. of shoots/ explant	Average shoot length (cm)	Average no. of shoots/ explant	Average shoot length (cm)
0.00	0.00	3.5^{c}	1.3^{ab}	5.0^{c}	1.2^{ab}
0.10	0.10	7.0^{bc}	0.9^{b}	6.0^{c}	0.9^{ab}
0.25	0.10	10.0^{b}	0.9^{b}	8.9^{bc}	1.5^{a}
0.50	0.10	12.0^{b}	0.9^{b}	12.3^{b}	1.3^{ab}
0.10	0.50	7.5^{bc}	1.0^{b}	7.0^{bc}	0.7^{b}
0.25	0.50	13.0^{b}	1.1^{ab}	10.6^{bc}	1.3^{ab}
0.50	0.50	19.5^{a}	1.5^{a}	17.9^{a}	1.3^{ab}

Means in the same column followed by different letter(s) are significantly different at $p \leq 0.05$.

Fig. (6): Micropropagation of Hyoscyamus boveanus; a. H. boveanus grown in Saint Katherine, Sinai; b. Shoot tip initiation with 0.5 mg l^{-1} Kn; c. Stem nodal segment initiation with 0.5 mg l^{-1} Kn; d. Shoot tip multiplication with 0.5 mg l^{-1} Kn and 0.5 mg l^{-1} NAA; e. In vitro rooting; f. Preparation for acclimatization; g. acclimatized in vitro derived plantlets; h. In vitro derived plantlet after six weeks of acclimatization.

Table (7): Effect of ½ MS medium and auxins (IBA and NAA) on the in vitro rooting of Hyoscyamus boveanus after six weeks of culture.

Auxins conc. (mg l^{-1})		Rooting (%)	Average no. of rootlets/ explant	Average rootlet length (cm)
IBA	**NAA**			
0.0	0.0	00.0	0.0	0.0
1.0	1.0	80.0[a]	19.7[a]	4.7[a]
1.0	2.0	80.0[a]	1.3[b]	3.7[b]
2.0	1.0	66.6[b]	9.7[bc]	2.8[c]
2.0	2.0	33.3[c]	5.7[c]	2.4[c]

Means in the same column followed by different letter(s) are significantly different at $p \leq 0.05$.

In conclusion, to the best of our knowledge, this is the first study that estimates the genetic diversity of *H. boveanus*, a rare medicinal plant endemic to Egypt, at Saint Katherine. This estimation produced about 21% genetic diversity, suggesting more scanning in other occurring regions to make a complete vision about all the possible genetic diversity available of this species. Also, the molecular identification of *H. boveanus* plant was done using DNA barcodes to classify, identify the plant for its conservation and protect our intellectual property rights of this plant. Finally, an efficient and successful protocol for micropropagation of *H. boveanus* plant was conducted.

ACKNOWLEDGMENTS

The author acknowledge the environmental researcher Dr. Ibrahim Abdelrafee Elgamal, Nature Conservation Sector, Egyptian Environmental Affairs Agency, South Sinai, Egypt, for his help in identifying and collecting the plant material. Also, the author is thankful to Dr. Shafik Darwish Ibrahim, Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center, Giza, Egypt, for his help in molecular data analysis. Also, the author is grateful to Professor Dr. Ghada Hegazi, Head of Tissue Culture Unit, Genetic Resources Department, Desert Research Center, for reviewing this paper.

REFERENCES

Abed Elmaksood, W.M.; Ebad, F.A. and Bosila, H.A. (2016). *In vitro* propagation of the endangered medicinal plant *Hyoscyamus muticus* L. (Egyptian Henbane). J. Appl. Environ. Biol. Sci., 6(4): 25-34.

Abu-Romman, S.M.; Al-Hadid, K.A. and Arabiyyat, A.R. (2015). Kinetin is the most effective cytokinin on shoot multiplication from cucumber. Journal of Agricultural Science, 7(10):159-165.

Ahmad, W.; Muhammad, K.; Hussain, A.; Ahmad, H.; Kahn, K.; Qarshi, I.A.; Shinwari, K.I.; Nadeem, M.S.; Que, Y.; Khan, A. and Iqbal, J. (2017). DNA fingerprinting of essential commercialized medicinal plants from Pakistan. J. Plant Sci., 8:2119- 2132.

Alatar, A.A.; Faisal, M.; Abdel-Salam, E.M.; Canto, T.; Saquib, Q.; Javed, S.B.; El-Sheikh, M.A. and Al-Khedhairy, A.A. (2017). Efficient and reproducible *in vitro* regeneration of *Solanum lycopersicum* and assessment genetic uniformity using flow cytometry and SPAR methods. Saudi J. Biol. Sci., 24:1430–1436.

Aminnejad, M.; Hosseini, B. and Qaderi, A.

(2015). Effect of plant growth regulators and explant types on *in vitro* direct plant regeneration of *Hyoscyamus reticulatus* L. Inte'l. J. Adv. Res., 3:457-462.

Anis, M. and Faisal, **M. (2005).** *In vitro* regeneration and mass multiplication of *Psoralea corylifolia*-An endangered medicinal plant. Indian J. Biotech., 4:261-264.

Boulos, L. (2009). Flora of Egypt Cheklist: Alhadara Publishing, Egypt.

CBOL Plant Working Group (2009). A DNA barcode for land plants. Proceedings of the National Academy of Sciences of the United States of America 106, 12794–12797.

Chandana, B.C.; Kumari, N.H.C.; Heena, M.S.; Shashikala, S.K. and Lakshmana, D. (2018). Role of plant tissue culture in micropropagation, secondary metabolites production and conservation of some endangered medicinal crops. J. Pharmacogn. Phytochem., SP3: 246-251.

Cheng, B.C.; Peterson, M.R. and Mitchell, J. (1992). The role of sucrose, auxin and explant source on *in vitro* rooting of seedling explants of *Eucalyptus sideroxylon*. Plant Sci., 87(2):207-214.

Dong, W.; Liu, J.; Yu, J.; Wang, L. and Zhou, S. (2012). Highly variable chloroplast markers for evaluating plant phylogeny at low taxonomic levels and for DNA barcoding. PLoS ONE, 7 (4): e35071.

Duncan, B.D. (1955). Multiple range and multiple F tests. Biometrics, 11:1-42.

El-Shazly, A.; Tei, A.; Witte, L.; El-Domiaty, M. and Wink, M. (1997). Tropane alkaloids of *Hyoscyamus boveanus, H. desertorum*, *H. muticus* and *H. albus* from Egypt. Zeitschrift für Naturforschung C, 52:729–739.

Gantait, S.; Debnath, S. and Nasim, A.M. (2014). Genomic profile of the plants with pharmaceutical value. 3 Biotech, 4: 563–578.

Ghorbanpour, M.; Omidi, M.; Etminan, A.; Hatami, M. and Shooshtari, L. (2013). *In vitro* hyoscyamine and scopolamine production of black henbane (*Hyoscyamus niger*) from shoot tip culture under various plant growth regulators and culture media. Trakia J. Sci., 2:125- 134.

Hegazi, G.A.; Eid, S.R. and Sharaf, A.M. (2011). Micropropagation for conservation of two rare Capparis species from Egypt. CATRINA, 6(1):29–39.

Kocot, K.P.; Kita, A.; Haduch, A. (2011). The effect of kinetin on the chlorophyll pigments content in leaves of *Zea mays* L. seedlings and accumulation of some metal ions**.** Inzynieria i Ochrona Srodowiska, 14(4):397-409.

Li, X.; Yang, Y.; Henry, R.J.; Rossetto, M.; Wang, Y. and Chen, S. (2015). Plant DNA barcoding: from gene to genome. Biol. Rev. Camb. Philos. Soc., 90(1):157-166.

Madani, H.; Hosseini, B.; Dehghan, E. and Rezaei-Chiyaneh, E. (2015). Enhanced production of scopolamine in induced autotetraploid plants of *Hyoscyamus reticulatus* L. Acta Physiol. Plant., 37(3):53-55.

Mishra, P.; Kumar, A.; Nagireddy, A.; Mani, D.N.; Shukla, A.K.; Tiwari, and Sundaresan, V. (2016). DNA Barcoding: an efficient tool to overcome authentication challenges in the herbal market. Plant Biotech. J., 14:8-21.

Mohamed, A. (2016). DNA barcoding of five medicinal plants from Siwa Oasis Egypt. KMITL Sci. Tech. J., 16(2):89-96.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant., 15:473- 479.

Oseni, O.M., Pande, V. and Nailwal, T.K. (2018). A Review on Plant Tissue Culture, A technique for propagation and conservation of endangered plant species. Inte'l. J.Cur. Microbiol.Appll. Sci., 7(7):3778-3786.

Padmapriya, H.; Karthikeyan, A.V.P.; Jahir Hussain, G.; Karthi, C. and Velayutham, P. (2011). An efficient protocol for *in vitro* propagation of *Solanum nigrum* L. from nodal explants. J. Agric. Tech., 7(4):1063-1073.

Prabhakar, T.N.; Kautikrao, P.P.; Ramkrishna, S.V.; Preeti, A.; Rohidas, B.S. and Maheshwari, V.L. (2001). *In vitro* propagation of *Hyoscyamus niger* through tip culture. J. Med. Aromat. Plant Sci., 23(4):597-599.

Rajasekharan, P.E.; Abdul Kareem, V.K.; Ravish, B.S. and Mini, S. (2017a). Genetic diversity in *Oroxylum indicum* (L.) Vent., a threatened medicinal plant from India by ISSR analysis. Indian J. Biotech., 16:357-365.

Ramakrishnan, R.; Periyasamy, R.; Lakkakula, S.; Subramani, P.; Arockiam, R. and Manikandan, R. (2017b). *In vitro* propagation and conservation of useful endangered medicinal plants with anticancer activity. J. Mol. Biol. Biotech., 2(3:8):1-8.

Schuelter, A.R.; Grunvald, A.K.; Amaral Junior, A.L.; Luz, C.L.; Gonçalves, L.M.; Stefanello, S. and Scapim, C.A. (2009). *In vitro* regeneration of cocona (*Solanum sessiliflorum*, Solanaceae) cultivars for commercial production. Genet. Mol. Res., 8(3):963- 975.

Sevon, N.; Biondi, S.; Bagni, N. and Oksman Caldentey, K.M. (2001). Transgenic *Hyoscyamus muticus* (Egyptian henbane), p. 171-200. In: Bajaj, Y.P.S. (ed.). Biotechnology in Agriculture and Forestry Transgenic Crops III, (48). Springer Verlag, Berlin, Heidelberg, Germany.

Sneath, P.H.A and Sokal, R.R. (1973). Numerical taxonomy: the principles and practice of numerical classification. 1st Edition, W. H. Freeman, San Francisco, USA. 573 p.

Song, J.; Yao, H.; Li, Y.; Li, X.; Lin, Y.; Liu, C.; Han, J.; Xie, C. and Chen, S. (2009). Authentication of the family Polygonaceae in Chinese pharmacopoeia by DNA barcoding technique. J. Ethnopharmacol., 124(3):434-439.

Szczecinska, M.; Sramko, G.; Wołosz, K. and Sawicki, J. (2016). Genetic diversity and population structure of the rare and endangered plant species *Pulsatilla patens* (L.) Mill in East Central Europe. PLoS ONE, 11(3): e0151730.

Tahtamouni, R.W.; Shibli, R.A.; Younes, L.S.; Al-Qudah, T.S.; Al Hawmdeh, F. and AL- Kiyyam, M. (2017). *In vitro* propagation, direct regeneration and acclimatization of *Solanum villosum* (L.) Mill.: a promising medicinal plant that grows wild in Jordan. Jordan J. Agric. Sci., 13(1):65-78.

Torre, M.P.; Garcia, M.; Heinz, R. and Escandon, A. (2012). Analysis of genetic variability by ISSR markers in *Calibrachoa caesia*. Elect. J. Biotech., 717-3458.

Xiao, L.Q.; Gee, X.J.; Gong, X.; Halo, G. and Sheng, S.X. (2004). ISSR variation in the endemic and endangered plant *Cycas guizhouensis*
(Cycadaceae). Ann. Bot., 94(1):133-138.

Zahran, M.A.; Amer, M.W.; Afiah, A.S. and Ghaly, N.O. (2015). Endemic species in Sinai Peninsula, Egypt, with particular reference to Saint Katherine Protectorate: I-Ecological features. J. Environ. Sci., 44(4):589-609.

Zolala, J.; Farsi M.; Gordan H.R. and Mahmoodnia M. (2007). Producing a High scopolamine hairy root clone in *Hyoscyamus muticus* through transformation by *Agrobacterium rhizogenes.* J. Agric. Sci. Tech., 9:327-339.

.

الملخص العربي

الحفاظ على البنـج الثوري كنبـات نـادر متوطن في مصر

هبه السيد غريب
وحدة زراعة الأنسجة، قسم الأصول الوراثية، مركز بحوث الصحراء، المطرية، القاهرة، مصر

مع تزايد عدد سكان العالم، والتغيرات المناخية المحتملة، فإن الوعي بالإستخدام المستدام والمحافظة على الموارد الطبيعية في العالم يزداد، وذلك للحفاظ على التنوع البيولوجي. التقدم في مجال التكنولوجيا الحيوية أدى إلى إيجاد فرص جديدة للحفاظ على الأصول الوراثية. تم تقييم التنوع الوراثي بين ثلاثة أفراد برية لنبات البنج الثوري (السكران) والمجمعة من مناطق مختلفة في سانت كاترين، وذلك لحماية حقوق الملكية لهذا النبات الموجود بمصر فقط. وقد وصل مستوى التباين الوراثي إلى ٢٠.٩٪ بإستخدام ١٩ من معلمات الـ ISSR الجزيئية. تم إستخدام باركود الحامض النووي لتوصيف وتعريف وتسجيل النبات. تم تسجيل التتابعات النيوكليتيدية الخاصة بجينات الـ *mat*K ، *rbc*L، *rpo*C1 في بنك الجينات تحت أرقام MK189190، MK189191 وMK189192، على الترتيب. إكثار هذا النبات معمليًا كان هدف آخر في هذا البحث كي يتم التوصل إلى طريقة أخرى للحفاظ على هذا النبات الطبي النادر وإنتاج أعداد كبيرة منه. تم إستخدام القمم النامية والأجزاء الساقية البرعمية كمنفصلات نباتية. وكانت بيئة مراشيجي وسكوج المزودة بـ ٥. من كلاً من Kn و NAA هي أفضل بيئة على الإطلاق لإحداث نمو وتضاعف للأفرع. أعلى نسبة تجذيركانت ٨٠٪ وأكبر عدد من الجذور تم الحصول عليه على نصف قوة بيئة موراشيجي وسكوج المزودة بـ ١ مجم/لتر لكلا من NAA و IBA وقد نقلت النباتات المحتوية على جذور إلى الصوبة وأظهرت نمو طبيعى.