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In vitro Propagation of the Endangered Medicinal Plant *Hyoscyamus muticus* L. (Egyptian Henbane)

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ABSTRACT

An efficient plant regeneration protocol has been developed from *in vitro* germinating seeds of *Hyoscyamus muticus* L. (Egyptian henbane), a medicinally important plant in the family Solanaceae. Newly growing stem segment were established on MS media fortified with various concentrations of 6-benzyladenine (BA,1.0- 3.0 mg/l) and kinetin (KN,1.0-3.0 mg/l) alone or in combination with 2iP (0.5 mg/l).The maximum mean number of shoots per explant (8.20) with average length (1.37) cm was achieved with medium containing 1.0 mg/l KN alone. The highest number of shoots (8.200) with average length (1.374cm) was obtained by subculture on MS medium supplemented with 1.0 mg/l KN after four weeks and the number of shoots formed per explant increase gradually by subculturing on the same media up to 12.467 of shoot formed per explant with average length 2.298 cm in the fourth subculture. Rooting of shoots was achieved on MS medium supplemented with 1.0 mg/1 indole butyric acid (IBA). Well-developed plantlets which are transferred to plastic pots containing soil and peat moss (1:1) showed 75-90% survival and were then transplanted in glasshouse and normally transferred to environmental conditions. The described method can be successfully employed for large-scale multiplication and conservation of *H.muticus* (an endangered medicinal plant).

KEYWORDS: *Hyoscyamus muticus* L., Egyptian henbane, *in vitro* propagation, direct organogenesis, endangered medicinal plants.

INTRODUCTION

Hyoscyamus muticus L. (Egyptian henbane) is a member of the family Solanaceae, which is one of the large drug producing families **(1).** It is famous for its tropane alkaloid content; the main alkaloids present are scopolamine and hyoscyamine. The effects of these alkaloids include stimulation of the central nervous system and simultaneous depression of the peripheral nerves typical for a parasympathomimetic. The medicinal uses include spasmolytic, antiasthmatic, anticholinergic, narcotic and anaesthetic properties **(2, 3).** Scopolamine butylbromide is an antispasmodic drug indicated for the treatment of abdominal pain associated with cramps induced by gastrointestinal spasms **(4).** Egyptian henbane is gradually degenerated in Egypt due to the rapid growth of modern industrialization. This requires our efforts to introduce an alternative to *in vivo* culture as a tool to keep the plant and at the same time improve its medicinal quality **(5).** About 500 to 600 tons of *H.muticus* stems and leaves, most of it collected in the wild, are exported annually from Egypt to Germany **(6).**

***H. muticus* L.** Stout succulent perennial herb or shrub, over 1m, glabrous or pubescent; stems long, much branched in the upper part; lower leaves 8-12 × 4.9 cm, ovate to rectangular, acute, cuneate or truncate, usually with several coarse teeth; petiole to 30 cm or more, dense; calyx 2-3 cm, with short triangular blunt teeth, becoming dry, reticulate and many nerved; corolla 2 ×2 cm, white or green, the upper lip heavily flushed purple- violet; anthers 4mm; capsule 6mm. the plant has been used for medicinal remedies since ancient times **(7)**.

The present study aims to conserve the ***H. muticus* L.**plant by establishing a protocol for the *in vitro* propagation of the plant as one of the applied methods of conservation, for obtaining high-yielding individuals, to maintain their diversity.

MATERIALS AND METHODS

Explant Collection and Disinfection*: H. muticus* seeds were obtained from the Faculty of Agriculture, Alazhar University. Surface sterilization was carried out under complete aseptic conditions in the Laminar Air Flow Hood, to avoid the explant contamination with bacteria or fungi after culturing in the media. Firstly, seeds were washed with soap and water then left under running tap water for at

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least one hour before sterilization to remove surface contamination. The explants were subjected to different sterilization treatments using commercial Clorox containing 5.25% sodium hypochlorite (NaOCl) at different concentrations and durations. After each treatment the explants were rinsed 5-6 times in sterile distilled water, and then sterilized seeds were cultured on MS basal medium for primary growth. Each treatment in this experiment consisted of 10 replicates, and the survival percentage was counted for each after 20 days of culture. NaOCl was applied to sterilize *H. Muticus* seeds at different concentrations and durations.

Culture medium and culture conditions: *H. muticus* seeds were planted on solid basal medium of **(8)** salts and vitamins supplemented with 100 mg/l myo-inositol and 30 g/l sucrose with 2.5 g/l phytagel. The pH value of the nutrient media was adjusted to 5.7 ± 0.1 by adding few drops of either 0.1N HCl or NaOH prior to addition of phytagel or agar and autoclaving media into 30 ml volume jars for different stages. All jars were closed with autoclavable polypropylene caps and autoclaved for 15 min. at 121°C under 1.1 kg/cm2 pressures, then left to cool. The sterilized explants were cultured on the media under complete aseptic conditions in the Laminar Air Flow Hood. Tissue culture Jars were then placed in an incubation room at a temperature of 26+2°C under 16 hours photoperiod of 3000 Lux supplied with white cool fluorescent lamps.

Shoot establishment: In this stage initiated shoots were cultured on MS media free of plant growth regulators or fortified with different combinations and concentrations of cytokinins (BA, KN) individually or in combination with (2iP) to evaluate the ability of these different hormonal combinations to initiation the growth of *H. muticus*ex plants *in vitro*. Data were taken after 4-5 weeks.

Shoot multiplication: In this stage shoots were cultured on MS medium supplemented with KN in different concentrations to study the effect of KN on shoot multiplication after four subcultures. Data were taken after 4 weeks.

Rooting of regenerated shoots: Shoots derived from multiplication stage (about 3-5cm long) were transferred to Murashige and Skoog salts and vitamins in addition to 30 g/l sucrose and 2.7 g/l phytagel and different treatments of auxins IBA and NAA or both, in addition to the control (free from plant growth regulators)Cultures were incubated under the same conditions used for shoot proliferation. Data were taken after 5 week.

Acclimatization of Regenerated Plants: Well rooted plantlets (4-7 cm long) were removed from the culture media, washed gently under running water to get rid of residues, then hardened off inside the growth room in black pots 8cm in diameter filled with soil mixture of sand and peat moss (1:1 v/v) for two weeks, pots were covered with transparent polyethylene bags. After two weeks pots transferred and placed in a greenhouse. One week later, the covers were removed gradually within one month. The percentage of survived transplants (%) was recorded.

Statistical analysis: Variance analysis of data was done using **ANOVA** program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by using **(9)**new multiple range tests as described by **(10)**. Means followed by the same letter are not significantly different at $p \leq 0.05$.

RESULTS AND DISCUSSION

Explant Sterilization: Contamination by microorganisms is considered a bottle neck in tissue culture. Therefore, the work with tissue culture should be carried out under effective aseptic conditions. The choice of the sterilization method depends on the relative occurrence of contamination and death. High concentrations and durations of the sterilizing agent prevent contamination and cause higher death percentage and *vice versa*.

H. muticus seeds were surface sterilized to eliminate fungi and bacteria. In this respect, different concentrations of Clorox containing 5.25% sodium hypochlorite (NaOCl) were used to prevent the contamination during growth. From the different sterilization treatments carried out, the following results were obtained.

The highest survival percentage (100%) was achieved by immersing the explants into 1.5% NaOCl solution for 10 min **(Table 1**, **Fig.1)**. Also, 1% NaOCl solution for 20 min and 1% NaOCl solution for 30 can produce the highest survival percentage. Increasing or decreasing the duration reduced the survival percentage significantly to 80 and 60 %, respectively. Lower concentrations of NaOCl solution significantly decreased the survival percentage and increased the contamination of explants even with longer durations, up to 30 min. On the other hand, higher concentrations of NaOCl solution significantly decreased the survival percentage by increasing explant death.

The results in harmony with those obtained by **(11)** who found that Seeds of *H. muticus* were washed in current tap water for 30 min, then surface sterilized in a 70% (v/v) ethyl alcohol for 30 sec. and followed by 30% Clorox solution of household bleach (5.25 % sodium hypochlorite) with a drop of Tween-20 for 15 min. then the seeds were thorough washing in sterile distilled water for several times.

On the other hand **(5)** showed that Hyoscyamus seeds were serialized with 0.1% HgCl2 for 5 min, 50% Chlorex for 30 min and washed three times with distilled water.

Table 1: Effect of different sterilization treatments with different concentrations and durations of sodium hypochlorite solution on the survival percentage of *H. muticus* L. seeds.

Sterilization treatments	% Survived	% Contaminated	% Dead
0.5 NaOCl for 10 min	40%	60%	0%
0.5 NaOCl for 20 min	60%	40%	0%
0.5 NaOCl for 30 min	70%	30%	0%
1.0 NaOCl for 10 min	60%	40%	0%
1.0 NaOCl for 20 min	100%	0%	0%
1.0 NaOCl for 30 min	100%	0%	0%
1.5 NaOCl for 10 min	100%	0%	0%
1.5 NaOCl for 20 min	80%	0%	20%
1.5 NaOCl for 30 min	60%	0%	40%

Fig 1: seed sterilization and germination

Shoot establishment and multiplication: The primary goal of the establishment stage is to obtain a large percentage of explants free from surface pathogens (**12**). Factors that affect the success of this stage include choice of explant, elimination of contamination, and culture conditions which include ingredients, light, temperature and choice of explant support (**13**).

Multiplication is a rapid increase of organs which can ultimately give rise to plant. This increase is achieved by enhancing axillary shoot initiation (**12**). This stage is repeated at regular intervals to produce large-scale shoot multiplication to be commercially useful (**14**). The shoot induction and proliferation depend on plant growth regulators and types of explants **(15 &16).** In many plants; multiple shoots were obtained from the shoot tips or axillary buds by administering BA or KN **(17-21).**

On this experiment survival percentage and the percentage of growth induction of explants not reflect real results because most of tested MS media free or supplemented with plant growth regulators produced high percentage of survived explants and high percentage of growth induction. On the other hand the mean number of axillary shoots/explant and mean length of axillary shoots (cm) clearly reflect the difference between different plant growth regulators with actually affect the plant growth. KN even alone or in combination with 2iP was found to be the best effective for culture establishment of *H. muticus* L. Nodal explants which cultured on MS medium supplemented with 1.0 mg/l KN exhibited about 100% of shoot regeneration with a largest mean number of axillary shoots/ explant (8.20) a with mean length of axillary shoots (1.37 cm). The second largest mean number of axillary shoots/ explant (6.33) with mean length of axillary shoots (1.11 cm) was obtained on MS medium supplemented with 2.0 mg/l KN. At the same time the lowest survival percentage and also the lowest percentage of explant forming shoots with the lowest mean number of axillary shoots/explant (3.73, 2.17, and 0.90) and mean length of axillary shoots (0.46, 0.32 and 0.20 cm) were obtained on MS medium supplemented with

different concentrations of BA (1.0, 2.0 and 3.0 mg/l) respectively. Also MS medium supplemented with BA in combination with 2iP not effective for *in vitro* establishment of *H. muticus* L. explants in compared with MS medium supplemented with KN either alone or in combination with 2iP. Also it is clearly that MS medium supplemented with different concentrations of KN more effective for inducing shoot formation of *H. muticus* L. explants. This may be due to the fact that requirement of cytokinin and auxin depends on the endogenous levels of these substances in the tissues used for the culture (22). But this is in contrary with **(23)** who obtained maximum number of *Solanum nigrum* (family Solanaceae) shoots on MS medium fortified with 13.5 µM BA. Also **(24),** showed that MS medium supplemented with BA was superior in compared to KN for direct shoot regeneration of *Solanum surattense* (family Solanaceae) using internodal explants. The highest mean number of shoots per explant was recorded on MS medium containing 0.5 mg/l BA.

In the present study, different concentrations of BA and KN as cytokinins are used individually or in combination with 2iP in addition to the control medium without plant growth regulators for shoot induction and multiplication of *H. muticus* L.nodal segment which are eliminated from seeds growing on MS media without any plant growth regulatorsas represented in Table 2.

Table 2: Establishment stage of *H. muticus* L. with different concentrations of growth regulators after four weeks. Growth regulators conc. (mg/l)

BA	KN	2iP	% of survived explants	% of growth induction	Mean number of axillary shoots/ explant	Mean length of axillary shoots (cm)
0.0	0.0	0.0	100% a	100% a	1.00 ef	2.58 a
1.0	0.0	0.0	100% a	100% a	3.73 cd	0.46 de
2.0	0.0	0.0	47% cd	80% a	2.17 de	0.32 de
3.0	0.0	0.0	27% d	60% a	0.90 ef	0.20 ef
0.0	1.0	0.0	100% a	100% a	8.20 a	1.37 b
0.0	2.0	0.0	100% a	100% a	6.33 b	1.11 c
0.0	3.0	0.0	100% a	100% a	4.13 c	0.98 c
0.0	1.0	0.5	100% a	100% a	3.80 cd	0.62 d
0.0	2.0	0.5	60% bc	100% a	3.13 cd	0.48 de
0.0	3.0	0.5	100% a	80% a	2.17 de	0.53 de
1.0	0.0	0.5	0% e	0% b	0.00 f	0.00 f
2.0	0.0	0.5	33% cd	80% a	2.50 cde	0.20 ef
3.0	0.0	0.5	80% ab	67% a	0.36 ef	0.36 de

On the other hand **(25)** showed an efficient method for *in vitro* propagation of *Solanum nigrum* L. plants (family Solanaceae). The nodal explants were cultured on MS medium supplemented with different concentrations of BA and KN. Out of the two cytokinin tested, KN was found to respond well in shoot multiplication and number of shoots from nodal explants when compared with BA. Large number of shoots was produced from all the concentrations of both the cytokinins. The highest frequency of 100% shoot induction was observed on MS medium supplemented with 10-15µm KN and BA. The number of shoots produced on the basal medium supplemented with BA ranged between 40-46 and 43-49 with KN 30 days after inoculation.

Fig 2: Establishment of *H. muticus* L. nodal segment explants on MS medium supplemented with 1.0 mg/l KN after four weeks.

The effect of the best KN concentrations from the previous stage was studied on the multiplication of *H. muticus* L. axillary shoots. Axillary shoots produced from the establishment stage were excised and cut into 1-2 bud segments. Shoots produced from the establishment stage gave an increase in the mean number of axillary shoots per explant on MS medium supplemented with different concentrations of KN during the successive subcultures, until reached the fourth subculture. The concentration of 1.0 mg/l KN gave higher mean number of axillary shoots per explant in all successive subcultures. And the effects of MS medium containing 1.0, 2.0 and 3.0 mg/l KN on the multiplication of *H. muticus* L. were examined through four successive subcultures.

Table 3: Effect of MS medium supplemented with different concentrations of KN on multiplication of *H. muticus* L. axillary shoots during 4 successive subcultures

No. 0f subcultures	Treatment	% SE	% EFS	MNS	MLS
1 st subculture	1.0 mg/l KN	100%	100%	8.200	1.374
	2.0 mg/l KN	100%	100%	6.333	1.106
	3.0 mg/l KN	100%	100%	4.133	0.984
2 nd subculture	1.0 mg/l KN	100%	100%	9.333	1.703
	2.0 mg/l KN	100%	100%	7.200	1.387
	3.0 mg/l KN	100%	100%	6.467	1.245
3 rd subculture	1.0 mg/l KN	100%	100%	11.067	2.105
	2.0 mg/l KN	93%	100%	8.833	1.683
	3.0 mg/l KN	87%	100%	7.867	1.486
4 th subculture	1.0 mg/l KN	100%	100%	12.467	2.298
	2.0 mg/l KN	100%	100%	8.467	1.658
	3.0 mg/l KN	100%	100%	7.733	1.219

%SE: percentage of survived explants, **%EFS:** percentage of explant forming shoots, **MNS:** mean number of axillary shoots, **MLS:** mean length of axillary shoots.

Fig 3: Multiplication of *H. muticus* L. nodal segment explants on MS medium supplemented with 1.0 mg/l KN through successive subcultures after four weeks.

From the data represented on **Table 3 & Fig. 3** it is noticed that MS medium supplemented with 1.0 mg/l KN gave significantly higher mean number of shoots per explant with the highest mean length of formed shoots than the other tested media which fortified with 2.0 and 3.0 mg/l KN during four successive sub culture. It is also observed that the mean number of axillary shoots per explant increased with each subculture until reached the fourth subculture on MS medium supplemented with 1.0 mg/l KN to give 12.467 mean number of shoots formed per explant with mean length 2.298 cm oh the fourth sub culture. However MS medium supplemented with 2.0 mg/l KN give an increase in the mean number of shoots per explant (6.333) with mean length of shoot 1.106 cm on the first sub culture which increase gradually up to third sub culture to rich 8.833 mean number of shoots formed per explant with mean length 1.683 cm and decreased in the fourth subculture (8.467 mean number of shoots formed/explant, 1.658 cm mean length of shoot). On the other hand MS medium fortified with 3.0 mg/l KN show minimum activity for shoot formation of *H. muticus* L. plant *in vitro* during sub culture. Which give an increase in mean number of shoots formed per explant 4.133 with mean length

0.984 cm in the first sub culture increase gradually up to third sub culture (7.867 mean number of shoots formed/explant, 1.486 cm mean length of shoot) and began to decrease in the fourth sub culture (7.733 mean number of shoots formed/explant, 1.219 cm mean length of shoot).So, it could be concluded from **Table 2 &3** that MS medium supplemented with 1.0 mg/l KN were the most promising treatments for the establishment and continuous proliferation of *H. muticus* L. explants during successive subcultures **(Fig. 2, 3 & 4)**. It is show increase in shoot number/explant with subsequent cultures. The fact of increasing of shoot number / explant by subculture clarified also in *Holostemma ada-kodien* **(26),** *Ceropegia candelabrum* **(27),** *Leptadenia reticulate* **(28),** and *Ceropegia intermedia* **(29).** Also, it is reviewed that the cytokinin promotes cell division by activating DNA synthesis; promoting the growth of lateral buds and inducing shoot formation **(30).**

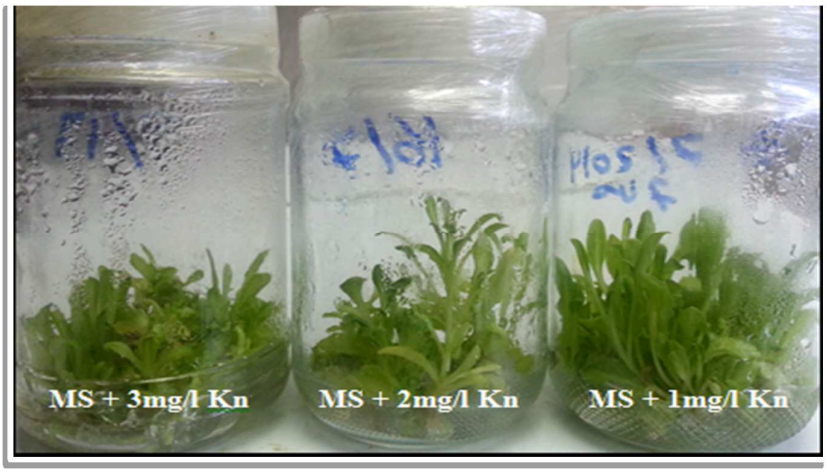

Fig 4: Multiplication of*H. muticus* L. nodal segment explants on MS medium supplemented with 1.0 mg/l KN, 2.0 mg/l KN and 3.0 mg/l KN through successive subcultures after four weeks.

Rooting of regenerated shoots: The function of rooting stage is to prepare the plantlets for transplanting and establishment outside the artificial, closed environment of the culture vessel **(13).** Auxins have several promotive functions in tissue culture and they have the ability to promote root initiation **(31).** Synthetic auxins such as IBA and NAA are frequently used for *in vitro* root initiation and increasing root number and length even present alone or in combination with each other. Regenerated shoots of *H. muticus* L. that were developed from the previous stage with a mean length 1.5- 4 cm are rooted on MS medium fortified with different concentrations and combinations of IBA and NAA.

Table 4: Rooting of *H. muticus* L. on MS nutrient medium supplemented with different auxin concentrations after five weeks.

Auxin conc. mg/l))		% of surv. exp.	% of rooting. exp.	Mean number of roots/ exp.	Mean length of roots cm))
IBA	NAA				
0.0	0.0	100%	67% ab	1.87 ab	12.07 ab
0.5	0.0	100%	67% ab	1.9 ab	4.8 ab
1.0	0.0	100%	100% a	3.3 a	16.6 a
2.0	0.0	100%	73% ab	2.8 a	13.9 ab
0.0	0.5	100%	47% abc	1.9 ab	6.3 ab
0.0	1.0	100%	33% bc	0.9 ab	1.8 ab
0.0	2.0	100%	0% c	0.0 b	0.0 b
0.5	0.5	100%	80% ab	1.6 ab	6.3 ab
1.0	0.5	100%	40% abc	2 ab	5.44 ab
2.0	0.5	100%	% bc	2 ab	3.4 ab

Data represented in **Table 4 & Fig 5** indicated that MS medium supplemented with 1.0 mg/l IBA gave the highest percentage of root formed per explant (100%) and also the best mean number of root per explant (3.3) was obtained on the same media with a maximum mean length of roots (16.6 cm) after five weeks, while, MS medium supplemented with 2.0 mg/l NAA did not show any root formation. NAA had a potential in inducing rhizogenesis of the shoots, however, in our experiment IBA was more effective than NAA with respect to rooting of the regenerated shoots **(32).** These results in agreements with **(33)** that represented that MS medium supplemented with 0.5 mg/l IBA were effective for inducing root formation on *Capsicum annuum* L. plants and also IBA effective for root formation more than NAA even alone or in combination with each other. **(34)**showed that MS medium supplemented with 0.5 mg/l IBA were effective for root formation of *Solanum nigrum* plants *in vitro* more than other auxins (NAA and IAA). On the other hand MS medium free of plant growth regulators show high percentage of root formed per explant (67%) with a mean number of roots per explant1.87 with mean length of roots 12.07 cm. from the results it can be concluded that the highest percentage of root formation of *in vitro* well-developed shoots of *H. muticus* L. were obtained on MS medium supplemented with 1mg/l IBA and its more effective than NAA even alone or in combination with IBA and MS media free of plant growth regulators.

Fig 5: Rooting of *H. muticus* L. on MS medium supplemented with 1.0 mg/l IBA after five weeks.

Acclimatization and Field Establishment: Transferring plants derived from *in vitro* conditions, to the free-living *(ex-vitro)* is considered as a problem that affects the success of such technique **(35).** About 75-90% of *in vitro* regenerated plantlets were successfully established *ex vitro* and finally transferred to the greenhouse.

Well rooted plantlets were removed from the culture media, washed gently under running water to get rid of residues, then hardened off inside the growth room in black pots 8cm in diameter filled with soil mixture of sand and peat moss (1:1 v/v) for two weeks, pots were covered with transparent polyethylene bags. After two weeks pots transferred and placed in a greenhouse. One week later, the covers were removed gradually within one month.

Through these methods of acclimatization the *in vitro* established plantlets were hardened *ex vitro* with a survival rate of 75-90% and were then transplanted in glasshouse. Transplanted plantlets were kept under shade for 15 days and then were transferred to normal environmental conditions. **(36)** Recorded that the *in vitro* regenerated plantlets of *Capsicum annuum* L. showed 80% survival during transplantation **(Fig 6)**. Also, **(37)** describe a protocol for *in vitro* proliferation and plant regeneration of *Physalis minima* L. and showed that the *in vitro* grown plantlets with strong root system were successfully established in normal room temperature for seven days before transplanting in pots here they were reared for three weeks through successive acclimatization. The regenerated plants were successfully transferred to the soil with 90% survival rate**.(38)** Showed that the *in vitro* regenerated plantlets of *Solanum nigrum* L. plants were hardened successfully in green house and transferred in the field. On the other hand, **(39)** showed that *Solanum melongena* L. well developed *in vitro* plantlets were successfully established and finally transferred to the green house with 80% survival percentage in the soil and about 90% of the of *Solanum surattense* Bum *in vitro*-propagated plantlets survived when they were transferred to *ex vitro* conditions for acclimatization as reported by **(24).** Also, the well rooted plantlets of *Solanum nigrum* L. were transplanted to the paper cup for hardening and the well-established plants were transferred to the field for acclimatization as reported by **(25).(40)** Showed that *in vitro* regenerated plantlets of tomato (*Lycopersicon esculentum*) were successfully transferred to the open field. **(41)** Reported that the *in vitro* regenerated plantlets of *Solanum nigrum* L. could be successfully established in soil where they grow normally.

Fig 6: Different acclimatization stages of *H. muticus* L. plantlets.

Overall findings of the present study are significant in obtaining the maximum regeneration with minimum concentrations of growth regulator. In conclusion, we have developed a promising method for an efficient *in vitro* regeneration protocol of *H. muticus* L. (Egyptian henbane) using KN and IBA. The protocol could be useful for large scale production and provides a possible system for plant conservation, and obtaining high-yielding individuals, to maintain their diversity.

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