Azhar Int J Pharm Med Sci 2024; Vol 5 (1):109-117
Research Article

Open Acces

Print ISSN: 2735-4598
Online ISSN: 2735-4601

Conservation efforts for the endangered medicinal plant *Hyoscyamus boveanus* (Dun.) Aschers. & Schweinf. (Endemic to Egypt): An *in vitro* propagation protocol

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Article history: Received: 24-07-2023 **Revised:**30-08-2023 **Accepted:** 10-07-2024

Abstract: *Hyoscyamus boveanus*, a rare medicinal herb from the Solanaceae family, is one of seven *Hyoscyamus* species found in Egypt, and it is the only one endemic to the region. It holds immense medicinal and economic value for the local community. However, it is currently endangered. Thus, the primary goal of this study was to develop a viable *in vitro* propagation protocol to preserve this valuable species. Surface-sterilized seeds of *H. boveanus* were germinated on liquid hormone-free Murashige and Skoog (MS) medium. Then, various parts of axenic *in vitro* grown seedlings (leaves, stems, and roots) were used as explants for both direct and indirect organogenesis. The study examined the effects of different concentrations and combinations of plant growth regulators on the maximum callus induction from these explants *in vitro*. The hormonal combination of 1.0 mg/L IAA and 0.5 mg/L KIN proved to be the most effective for callus induction, as well as indirect and direct organogenesis among all tested combinations of plant growth regulators. Interestingly, direct organogenesis was observed only in leaf explants from different types of seedlings, whereas all types of seedling explants cultured together in the same media exhibited callus formation, which subsequently developed into roots, shoots, leaves, and flowers. Following this, the plantlets obtained from direct and indirect organogenesis were transferred to asbestos perlite soil growing media. Notably, only the plantlets derived from indirect organogenesis successfully acclimatized, while those from direct organogenesis failed to adapt to the *ex-vitro* environment.

Keywords: *Hyoscyamus boveanus*, callus initiation, *in vitro* propagation, direct organogenesis, explant, plant growth regulators.

1. INTRODUCTION

The Solanaceae family, known for its taxonomic significance and extensive botanical diversity, encompasses approximately 3000–4000 species grouped into around 90 genera. This family displays remarkable versatility, comprising both perennial trees and herbaceous annual species, and thriving across a wide range of terrestrial environments, from rainforests to deserts. Despite the large size of the family, only a few of its members have achieved prominence in human civilizations as sources of food, ornamental plants, and medicinal compounds [1]. Notably, the Solanaceae family is characterized by its rich content of alkaloids, including glycoalkaloids, tropane, indole, and pyrrolizidine alkaloids [2].

In Egypt, the Solanaceae family is well-represented, with approximately 91 species from 25 genera, including 30-33 wild species [3]. The genus *Hyoscyamus* is particularly significant, with seven species found in Egypt, one of which is common while the remaining six are rare. *Hyoscyamus* species

Cite this article: Saad S.F., Ibrahim M.T., El Kousy R.H., Abo-Elghiet F., and El-Dahmy S.I. Conservation efforts for the endangered medicinal plant *Hyoscyamus boveanus* (Dun.) Aschers. & Schweinf. (Endemic to Egypt): An *in vitro* propagation protocol. Azhar International Journal of Pharmaceutical and Medical Sciences, 2025; 5 (1):109-117. doi: 10.21608/aijpms.2024.224893.1226

DOI : 10.21608/aijpms.2024.224893.1226

are renowned for being natural source of tropane alkaloids, particularly hyoscyamine and scopolamine, with hyoscyamine being the predominant component [4]. These alkaloids possess various medicinal properties, including mydriatic, anti-colic and spasmolytic, local anesthetics, broncho-dilatory, and antiemetic effects [5].

Among the *Hyoscyamus* species in Egypt, *H. boveanus* stands out as a rare perennial herb and the only endemic species. It is restricted to montane wadis within St Catherine protected area in southern Sinai, characterized by sandy to fine gravelly soil. This species holds significant medical and economic value for the local community. However, over the past decade, *H. boveanus* has experienced a critical decline in its population size due to the combined pressures of natural factors such as aridity and climate change, along with human factors including dam construction and unmanaged human development. These detrimental influences have pushed *H. boveanus* to the verge of extinction. To address this pressing issue, several management plans have been proposed. These include limiting population growth, implementing species recovery efforts, employing captive breeding and artificial propagation techniques, as well as establishing a genome resource bank [6].

Plant tissue culture techniques provide valuable tools for the conservation and propagation of plant species, allowing the production of numerous individuals from small tissue explants. These methods actively contribute to the conservation of rare plant species by generating genetically identical plants, preserving their unique genetic makeup, and safeguarding against extinction. Tissue culture also addresses challenges such as low seed germination rates and limited plant material availability, thereby increasing the population size and enhancing species resilience to threats [7]. However, despite these benefits, plant tissue culture is not without difficulties. Challenges such as vitrification, contamination, browning of media, failure to acclimatization, and recalcitrance can impede successful tissue culture.[8]. One important challenge of tissue culture techniques is recalcitrance which refers to the inherent difficulty or resistance of certain plant species or tissues to *in vitro* growth, often due to low regeneration capacities. Overcoming recalcitrance requires trial and error, involving the exploration of different explant sources, optimization of culture media, and modifications in culture conditions such as adjusting environmental factors [9]. Thus, the development of an effective tissue culture protocol necessitates persistent experimentation and refinement.

Therefore, the present study aimed to conserve the *H. boveanus* plant by establishing an effective *in vitro* propagation protocol as an applicable conservation approach to maintain diversity and obtain high-yielding individuals.

2. METHODS

2.1. Plant material, seed germination, and explant preparation:

Seeds of *H. boveanus* Dun. were purchased from Zagazig University, Faculty of Pharmacy, and subsequently underwent authentication by Prof. Dr. Waffa Amer, a professor of plant taxonomy, Faculty of Science at Cairo University. A voucher specimen (Hb-3-2018) has been cataloged and deposited at the herbarium of Pharmacognosy department at the Faculty of Pharmacy, Zagazig University. Following this, the seeds were subjected to a sterilization protocol based on the method described by Abdelmaksood et al. [10] with slight modifications. Initially, the seeds underwent a one-hour wash under running tap water to remove surface contamination. Then, the seeds were treated with 70% ethyl alcohol for varying durations (1, 2, 3, and 4 minutes) and subsequently immersed in a 5% commercial sodium hypochlorite solution (Clorox®) with shaking for different time periods (10, 15, 20, and 25 minutes). Surface sterilization was carried out in a Laminar Air Flow Hood to maintain aseptic conditions. The sterilized seeds were rinsed five times with sterile distilled water using either cold or hot water (50-60°C). Following sterilization, the seeds were cultured on both liquid and solid hormonal-free media for germination. The liquid medium consisted of 4.4 g/L Murashige and Skoog (MS) media obtained from Duchefa, Germany, along with 30 g/L sucrose. The solid medium contained 4.4 g/L MS media, 30 g/L sucrose, and 7 g/L agar. The seeds were incubated at 25 °C with 16-hour light and 8-hour dark cycle for *in vitro* germination. The percentage of seed germination resulting from the different durations of the sterilization technique was calculated using the following formula: Seed germination % = (Number of germinated seeds / Total number of cultured seeds) x 100. After 30 days, the resulting seedlings were dissected into 5-10 mm segments of leaves, stem, and roots using a sterilized sharp blade. These segments, along with the seeds, were used as explants for assessing callogenic potentialities.

2.2. Callus induction:

To induce primary callus formation, different explants (leaves, stems, roots, each measuring 0.5 – 1 cm, and seeds) were cultured on MS medium supplemented with a combination of auxins, either Indol-3-acetic acid (IAA) or 2,4-Dichlorophenoxyacetic acid (2,4-D), at a concentration of 1.0 mg/L, and cytokinins, specifically Kinetin (KIN), at a concentration of 0.5

mg/L. These plant growth regulators, obtained from Sigma Chemical Co, U.S.A, were of analytical grade. The callus induction medium also contained 30 g/L sucrose and 7 g/L agar for solidification. The pH of all media was adjusted to 5.6- 5.8 before autoclaving at 125 °C for 17 minutes. The cultures were maintained at a temperature of 25± 1°C with a 16-hour light and 8-hour dark cycle using cool white fluorescent lamps. The percentage of callus induction and the mean fresh weight of callus per explant were determined. The percentage of callus induction was calculated from the following formula: Callus induction % = (No. of callus formed /No. of explants cultured) X 100.

2.3. Indirect organogenesis:

Morphogenetic response was examined by culturing well-proliferated callus on MS solid medium supplemented with 30 g/L sucrose. The medium was either left unsupplemented with plant growth regulators (PGRs) or fortified with different concentrations of IAA and KIN. The used concentrations of IAA+KIN were (0.5 + 0.5), (1.0 + 0.5), (1.0 + 1.0), (1.0 + 2.0), and (2.0 + 1.0) mg/L. Cultures were transferred to fresh medium every four weeks.

2.4. Direct organogenesis:

Each of the aseptically excised leaves, stems, and roots obtained from seedlings was cultured horizontally on MS medium supplemented with 1.0 mg/L IAA, 0.5 mg/L KIN, 30 g/L sucrose and 7 g/L agar. The culture media were then incubated at a temperature of 25± 1°C with a 16-hour light and 8-hour dark cycle.

2.5. Transplantation of plantlets:

The plantlets were removed from the culture media and then washed multiple times with sterilized water to eliminate any remaining traces of media on their root surfaces. Subsequently, the plantlets were transferred to perforated pots filled with autoclaved asbestos perlite soil. These pots were placed in translucent plastic covers (measuring 50 cm × 30 cm × 3 cm) and positioned in a growing chamber at a temperature of 25°C±1. The photoperiod consisted of 16 hours of light followed by 8 hours of darkness. After two days, the translucent plastic bags were punctured to lower the relative humidity inside the covers. Every two days, a small amount of water was used to irrigate each pot. Finally, after seven days, the clear plastic bags were discarded.

3. RESULTS

3.1. Seed sterilization and germination:

The balance between achieving effective sterilization and minimizing potential damage to the plant material is crucial. Therefore, Table 1 provides information on the effects of different sterilization durations on contamination and seed germination percentage. Optimal results were obtained by subjecting the seeds to a 70% ethanol solution for 1 minute, followed by a 5% commercial sodium hypochlorite solution for 15 minutes. Furthermore, rinsing the seeds with hot water (50-60 ^{o}C) and using liquid hormone-free MS media enhanced seedlings formation (Figure 1). It is worth noting that when the same conditions were applied to solid media, seedlings were observed without the formation of rootlets.

Figure 1. Seedlings of *H. boveanus* after 30 days of cultivation in liquid hormone-free MS media.

3.2. Callus induction:

Callus induction percentage was calculated to determine the optimal culture conditions for inducing callus formation using growth hormone combinations. The results of the experiment showed that the combination of 1.0 mg/L IAA and 0.5 mg/L KIN was the most effective in inducing callus formation from both seedlings and seeds explants, with a 100% callus induction percentage for both types of explants. The combination of 1.0 mg/L 2,4-D and 0.5 mg/L KIN was also effective in inducing callus formation, but to a lesser extent than IAA+KIN, with a 50% callus induction percentage for seeds explants and a 70% callus induction percentage for seedlings explants. Additionally, the experiment aimed to assess the time required for callus initiation and the fresh callus weight at various time intervals (10, 20, and 30 days) for different explant types cultured on media supplemented with different combinations of plant growth regulators. The findings, presented in Table 2, indicated that seedling explants cultured on MS medium fortified with IAA+KIN exhibited the shortest time required for callus initiation, which was 7 days. Furthermore,

this combination of plant growth regulators also yielded the highest fresh callus weight. Moreover, it was observed that callus formed on media fortified with the hormonal combination of 2,4D + KIN gradually changed its color to brown, as illustrated in Figure 2.

Table 1. Effects of sterilization durations on contamination and seed germination percentage of *H. boveanus*.

5% Clorox® ---------- **70% Ethyl alcohol**	**10 min.**	**15 min.**	**20 min.**	**25 min**
1 min.	Contamination 0% Germination	**Sterilized 10% Germination**	Sterilized 0% Germination	Sterilized 0% Germination
2 min.	Contamination 0% Germination	Sterilized Slight Germination	Sterilized 0% Germination	Sterilized 0% Germination
3 min.	Contamination 0% Germination	Sterilized 0% Germination	Sterilized 0% Germination	Sterilized 0% Germination
4 min.	Contamination 0% Germination	Sterilized 0% Germination	Sterilized 0% germination	Sterilized 0% Germination

Table 2. Time required for callus initiation and fresh callus weight at various time intervals for different explant types of *H. boveanus* cultured on media supplemented with different combinations of plant growth regulators.

Explant	**Hormonal combination**	**Callus induction time (days)**	**Mean fresh weight of callus (mg)**		
			After 10 days	After 20 days	After 30 days
Seedlings	1.0 mg/L IAA + 0.5 mg/L KIN	7	111	1330	1970
Seeds	1.0 mg/L IAA + 0.5 mg/L KIN	10	90	975	1440
Seedlings	1.0 mg/L 2,4-D + 0.5 mg/L KIN	10	70	233	976
Seeds	1.0 mg/L 2,4-D + 0.5 mg/L KIN	14	55	194	900

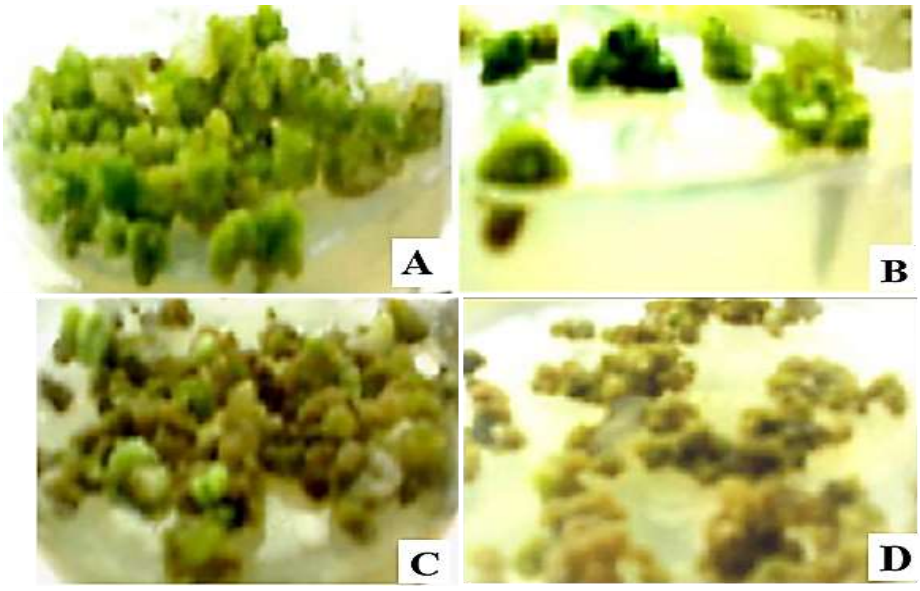

Figure 2. Callus formation from seedling explants of *H. boveanus* on MS medium fortified with **A)** 1.0 mg/L IAA + 0.5 mg/L KIN after 30 days, **B)** 1.0 mg/L IAA + 0.5 mg/L KIN after 60 days, **C)** 1.0 mg/L 2,4-D + 0.5 mg/L KIN after 30 days, and **D)** 1.0 mg/L 2,4-D + 0.5 mg/L KIN after 60 days

3.3. Indirect organogenesis:

In our study, we investigated the potential of various hormonal combinations in promoting plantlet formation through callus sub-culture on MS media. Among the combinations tested, only the specific pairing of 1.0 mg/L IAA and 0.5 mg/L KIN proved effective in inducing plantlet development from the

callus. After 30 days of sub-culturing the callus on the media supplemented with this hormonal combination, the emergence of roots was observed (Figure 2B). Subsequent sub-culturing on the same media components led to the development of small shoots and leaves within 60 days. As the culture continued with a fourth sub-culturing (after 90 days), the plantlets exhibited further growth and differentiation, culminating in the formation of a small flower displaying a violet color (see Figure 3).

Figure 3. Outcomes of callus sub-culturing of *H. boveanus* on MS medium supplemented with a hormonal combination of 1.0 mg/L IAA and 0.5 mg/L KIN. **A)** Differentiation of leaves and shoots after 60 days. **B & C)** Formation of small flowers with a violet color after 90 days.

3.4. Direct organogenesis:

After 30 days of culturing each of leaf, stem, and root explants separately on MS solidified media supplemented with a combination of plant growth regulators (1.0 mg/L IAA + 0.5 mg/L KIN), direct organogenesis was observed exclusively in leaf explants, as illustrated in Figure 4. Leaf explants successfully differentiated into whole plants, while the stem and root explants did not exhibit this capability. However, both the stem and root explants were able to induce callus formation.

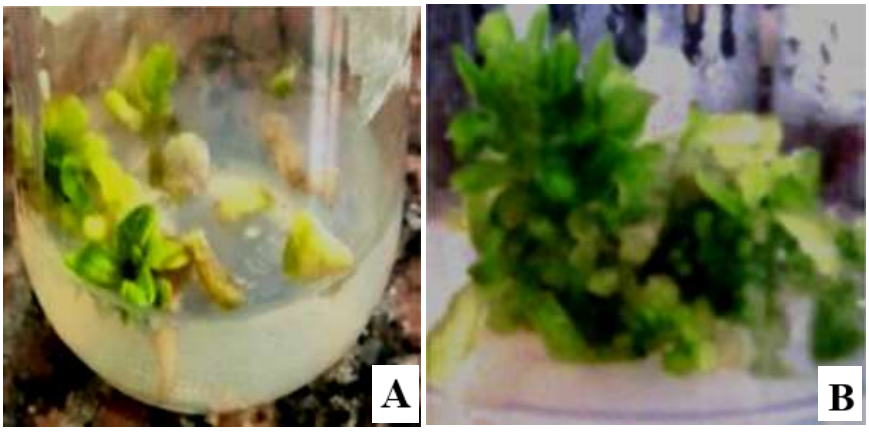

Figure 4. Direct organogenesis from leaf explants of *H. boveanus* on MS medium fortified with a hormonal combination of 1.0 mg/L IAA and 0.5 mg/L KIN after **A)** 30 days and **B)** after 60 days.

3.5. Transplantation:

H. boveanus plantlets produced from callus culture can be successfully acclimated to asbestos perlite soil growing media in a greenhouse environment for one week. After this period, they can be transferred to a normal environment, with a remarkable 100% survival rate. Figure 5 illustrates the different growing stages of these plantlets at various time intervals over a span of seven months. In contrast, plantlets generated through direct organogenesis failed to survive when transferred into soil growing media.

4. DISCUSSION

H. boveanus, a rare endemic medicinal plant species within the *Hyoscyamus* genus in Egypt, is under severe threat and immediate conservation measures are necessary to prevent its extinction. An essential conservation trial involves the establishment of an efficient *in vitro* regeneration protocol. This protocol aims to increase the population size of *H. boveanus* and enhance its resilience against threats. It plays a crucial role in ensuring the long-term survival of this species. In this

study, we aimed to develop an effective *in vitro* regeneration technique to overcome this problem.

Microbial contamination in plant tissue culture adversely affects plant growth, leading to issues such as variable growth, tissue necrosis, and reduced shoot proliferation. Effective decontamination methods include washing in running water and using surface sterilizing agents, but the appropriate concentration, exposure duration, and sequencing of these treatments must be standardized to minimize explant injury and ensure viability [11]. Previous research [12, 13] has indicated the optimal conditions for sterilizing *Hyoscyamus* seeds, involving the use of tap water, followed by a 70% ethanol solution and a commercial hypochlorite solution with varying durations. Building upon these findings, we conducted trials to determine the most effective sterilization durations for *H. boveanus* seeds, considering different combinations of concentration and duration. The best results were achieved by rinsing the seeds with tap water for 60 minutes, followed by a 1-minute treatment with 70% ethanol and a 15-minute treatment with a 5% commercial sodium hypochlorite solution. Additionally, the seeds were rinsed five times with hot water (50-60°C). This sterilization technique resulted in a 10% germination rate. The low germination rate observed is attributed to the double dormancy of *Hyoscyamus* seeds, which involves a hard seed coat and a partially dormant embryo [14].

Figure 5. The successive growth stages of *H. boveanus* plantlets derived from indirect organogenesis and cultivated in asbestos perlite soil at various time intervals. The stages include A) at time of transfer, B) after 2 weeks, C) after 6 weeks, D) after 8 weeks, E) after 15 weeks, F) after 5 months, G) after 6 months, and H) after 7 months.

The use of liquid hormone-free MS media for germinating sterilized *H. boveanus* seeds led to the successful formation of complete seedling, while the solid media resulted in incomplete seedlings without rootlets. This can be attributed to the improved nutrient availability and uptake in the liquid medium, where nutrients are dissolved and easily accessible to the developing seedlings. Additionally, the absence of agar in the liquid medium promotes improved gas exchange, ensuring an adequate oxygen supply for root and shoot development [15].

Utilizing different parts of axenic *in vitro* grown seedlings as explants has attracted considerable attention in enhancing organogenesis across various plant species [16]. The use of axenic conditions ensures that the observed organogenesis is solely attributed to the plant's own genetic and physiological factors, as it eliminates the risk of microbial contamination that can interfere with tissue growth and development. In our study, we focused on seedlings obtained from *H. boveanus* seeds germinated on liquid hormone-free MS media as the source of explants for both direct and indirect organogenesis. For direct organogenesis, we cultured leaf, stem, and root explants separately in SM media supplemented with a combination of 1.0 mg/L IAA and 0.5 mg/L KIN. Among the different types of explants used, we found that only leaf explants successfully yielded plantlets, while the stem and root cultures resulted in callus formation rather than direct organogenesis. This finding is consistent with previous research that has highlighted the advantages of using leaf explants for direct organogenesis [17].

In this study, we also investigated the induction of undifferentiated cell masses, callus, from *H. boveanus* for the purpose of indirect organogenesis. The experiment aimed to optimize callus formation by testing different combinations of plant growth

regulators and utilizing both seeds and axenic *in vitro* grown seedlings as explants. A unique approach was taken by culturing different explant types from seedlings together in the same media, with the intention of gaining insights into the regeneration capacity and interplay between different plant tissues in tissue culture. The specific combinations and concentrations of plant growth regulators, such as auxins and cytokinins, were selected based on previous research conducted on regeneration protocols of related *Hyoscyamus* species [18]. The experiment yielded promising results, demonstrating the effectiveness of the hormonal combination of 1.0 mg/L IAA and 0.5 mg/L KIN in inducing callus formation compared to other combinations. Notably, both seedlings and seed explants showed a 100% callus induction percentage when treated with this hormonal combination. The initiation of callus formation occurred within 7 days for seedling explants and 10 days for seeds, indicating a relatively rapid response to the hormonal treatment. In addition to the successful initiation of callus formation, this hormonal combination also resulted in the highest fresh callus weight. Remarkably, callus derived from seedling explants reached an impressive weight of 1970 mg after 30 days of culture. This significant increase in callus weight might be related to the technique used, where all types of seedling explants were cultured in the same media. As by comparison with previous research conducted on a closely related species, *Hyoscyamus muticus* L., which evaluated the effect of different plant growth regulators on *in vitro* induction and maintenance of callus from various types of seedling explants separately. According to that research, the most effective results were obtained with root explants, which exhibited a fresh callus weight of 31.56 mg/explant after 60 days of culture using the most efficient hormonal combination. Nodal segment explants followed with a fresh callus weight of 21.81 mg/explant, while leaf explants showed 20.11 mg of fresh callus after 60 days [10]. The significant difference in the fresh weight of callus obtained from the two techniques highlights the importance of considering the approach of culturing all seedling explant types in the same media for future trialing and comparison with commonly used explant types in tissue culture techniques.

The subsequent sub-culturing of the callus on the same media components resulted in the development of small shoots and leaves within 60 days. This successful regeneration signifies that the culture conditions and media composition support essential cell division and differentiation processes for plant regrowth. Continuing the culture with a fourth sub-culturing (after 90 days) led to further growth and differentiation of the plantlets, eventually culminating in the formation of a small violet-colored flower. This achievement demonstrates the stability and sustainability of the plant tissue culture system, enabling prolonged growth and development. The appearance of the violet flower is a crucial milestone in tissue culture, indicating successful organogenesis and the potential to obtain fully mature plants capable of sexual reproduction. The violet color suggests that the tissue culture conditions have been carefully optimized to support the plant's normal development and biochemical pathways.

After transplantation into asbestos perlite soil growing media, only the *H. boveanus* plantlets produced from callus culture exhibited a remarkable 100% survival rate. In contrast, plantlets generated through direct organogenesis failed to survive when transferred to the soil growing media. This difference in survival rates can be attributed to distinct physiological and morphological characteristics between the two types of plantlets, including non-functional stomata, weak root systems, and poorly developed cuticles [19]. It indicates that the *H. boveanus* callus-derived plantlets have better adaptability and resilience to the new environmental conditions, supporting their long-term survival and growth in the *ex-vitro* environment.

Finally, our study presents a comprehensive protocol for the successful preservation and propagation of the endangered plant, *H. boveanus*, through *in vitro* propagation. The protocol involves multiple stages, commencing with the induction of callus via indirect organogenesis. Subsequently, the callus is carefully sub-cultured to promote shoot and root development. These well-established plantlets undergo an acclimatization process in asbestos perlite soil growing media. Table 3 provides an overview of the final steps in our protocol, showcasing its potential for conserving and sustainably growing *H. boveanus* through *in vitro* propagation.

5. CONCLUSIONS

This study achieved a successful *in vitro* regeneration protocol for the endangered medicinal plant, *H. boveanus*. This is crucial due to the species' rarity and the urgent need for conservation efforts to prevent extinction. The experiments tested both direct and indirect organogenesis using different parts of axenic *in vitro* grown seedlings as explants. The protocol involved precise sterilization techniques, hormone-free MS media for germination, and optimized combinations of plant growth regulators for callus formation. The results showed that the callus-derived plantlets had better

adaptability and survival rates when transferred to asbestos perlite soil growing media compared to those obtained through direct organogenesis. This indicates the potential of the callus-based approach for successful *in vitro* regeneration of *H. boveanus*.

Table 3. *In vitro* regeneration protocol of *H. boveanus* with optimal conditions.

Step	Description
Seed sterilization	Seeds underwent a one-hour wash under running tap water, followed by surface sterilization with 70% ethanol for 1 minute and 5% commercial sodium hypochlorite solution (Clorox®) for 15 minutes. Finally, they were rinsed five times with hot water (50-60°C).
Germination	Seeds were germinated on hormone-free MS media (4.4 g/L) supplemented with 30 g/L sucrose, at 25°C, under a 16-hour light/8-hour dark photoperiod.
Explant using seedling	After 30 days of germination, seedlings were used as explants for callus induction. The seedlings were cut into 0.5–1 cm segments of leaves, stems, and roots, and then placed together on MS medium supplemented with a combination of plant growth regulators (1.0 mg/L IAA and 0.5 mg/L KIN), sucrose (30 g/L), and agar (7 g/L).
Callus induction	Callus induction is initiated after 7 days of culture. The callus is transferred to fresh medium every 4 weeks.
Indirect organogenesis	After 4 weeks of callus induction, indirect organogenesis is initiated by sub-culturing the callus onto the same MS medium used for callus initiation. Subsequent sub-cultures resulted in the development of roots, shoots, leaves, and small flowers, respectively.
Transplantation	Plantlets successfully acclimated to asbestos perlite soil growing media in a greenhouse for one week, and then transferred to a normal environment, with a remarkable 100% survival rate.

Funding: This work has no funding.

Acknowledgement: The authors sincerely acknowledge Professor Wafaa Amer, Professor of Plant Taxonomy at the Faculty of Science, Cairo University, for her invaluable support and encouragement throughout this research.

Conflict of interest: The authors declare no conflict of interest.

Authors' contributions: Sherin F. Saad, do the practical work; Magda T. Ibrahim, Rawah H. El Kousy, and Samih I. El-Dahmy, design the work and supervision; Fatma Abo-Elghiet, writing, review, and editing; All authors have read and agreed to the published version of the manuscript.

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