In vitro propagation of *Paphiopedilum helenea* Aver.

Cao Thi Viet Nga*, Nguyen Thi Nhu Quynh, Vu Quang Nam
Vietnam National University of Forestry

Nghiên cứu nhân giống Lan hài Helen (*Paphiopedilum helenea* Aver.) bằng phương pháp nuôi cấy *in vitro*

Cao Thị Việt Nga*, Nguyễn Thị Như Quỳnh, Vũ Quang Nam
Trường Đại học Lâm nghiệp
**Corresponding author: ngactv@vnuf.edu.vn*

https://doi.org/10.55250/jo.vnuf.10.1.2025.003-010

Article info:
Received: 14/02/2025
Revised: 18/03/2025
Accepted: 07/04/2025

Keywords:
Conservation, in vitro, Paphiopedilum, Paphiopedilum helenae.

ABSTRACT

Paphiopedilum helenae Aver. (Orchidaceae), commonly known as Helen's Lady Slipper, is a native species of Vietnam and predominantly found in limestone mountain areas of the Northern regions such as Bac Kan and Cao Bang provinces. It is also distributed in Guangxi province of China. The species typically grows at elevations ranging from 600 to 1000 m above sea level, in a tropical monsoon climate with high humidity and low light conditions due to canopy coverage. Paphiopedilum helenae is classified as Critically Endangered (CR) in the Vietnam Red Data Book (2007) and listed under Appendix I of the CITES. The study demonstrated that sterilizing the seeds of Paphiopedilum helenae using alcohol in three successive steps is an effective method, achieving 94.4% sterilization rate and 88.9% germination rate of the sterilized seeds. The highest shoot proliferation of Paphiopedilum helenae is obtained on MS (Murashige and Skoog, 1962) medium supplemented with 3 mg/L BAP and 1 mg/L Kinetin, 30 g/L sucrose, and 7 g/L agar. Supplementing the MS medium with 2 mg/L NAA and 1 mg/L IBA resulted in the best rooting of Paphiopedilum helenae shoots. Preliminary results suggest the successful application of in vitro culture techniques for micropropagating Paphiopedilum helenae, offering a large-scale production of high-quality plantlets to meet the demand for cultivating this valuable species.

Từ khóa:
Bảo tồn, in vitro, Paphiopedilum, Paphiopedilum helenae.

TÓM TẮT

Paphiopedilum helenae Aver. (Orchidaceae), hay còn gọi là Lan hài Helen, là một loài bản địa của Việt Nam, chủ yếu phân bố ở các khu vực núi đá vôi của các tỉnh miền Bắc như Bắc Kạn và Cao Bằng, cũng như ở tỉnh Quảng Tây, Trung Quốc. Loài này thường mọc ở độ cao từ 600 đến 1000 m so với mực nước biển, trong điều kiện khí hậu nhiệt đới gió mùa, với độ ẩm cao và ánh sáng yếu do có tán che phủ. Paphiopedilum helenae được xếp hạng là loài nguy cấp (CR) trong Sách Đỏ Việt Nam (2007) và được liệt kê trong Phụ lục I của CITES. Nghiên cứu đã chỉ ra rằng việc tiệt trùng hạt của Paphiopedilum helenae bằng cồn qua ba lần liên tiếp là một phương pháp hiệu quả, đạt tỷ lệ mẫu sạch 94,4% và tỷ lệ nảy mầm của mẫu sạch đạt 88,9%. Công thức nhân nhanh chồi của Paphiopedilum helenae trên môi trường MS (Murashige và Skoog, 1962) bổ sung 3 mg/L BAP, 1 mg/L Kinetin, 30 g/L sucrose và 7 g/L agar đạt kết quả tốt nhất. Việc bổ sung 2 mg/L NAA và 1 mg/L IBA vào môi trường MS mang lại hiệu quả tốt nhất trong việc tạo rễ cho chồi Paphiopedilum helenae. Kết quả ban đầu cho thấy việc ứng dụng thành công các kỹ thuật nuôi cấy in vitro trong nhân giống Paphiopedilum helenae, mở ra tiềm năng sản xuất cây giống chất lượng cao quy mô lớn, đáp ứng nhu cầu trồng loài cây quý giá này.

1. INTRODUCTION

The genus *Paphiopedilum helenae* (Orchidaceae), commonly known as Lady Slipper orchids, is recognized for its unique morphological characteristics. These orchids are easily distinguishable from other species within the family due to their distinctive flower structure, which features a single pouch-like petal resembling a small slipper. This unique floral morphology is a defining characteristic of this group of orchids [1].

Paphiopedilum helenae Aver., commonly known as Helen's Lady Slipper, is an endemic species of Vietnam and is considered the smallest among slipper orchids. It was first discovered in 1995 in Cao Bang province and has since been recorded in Bac Kan and several limestone mountains in Thai Nguyen province.

Unfortunately, Paphiopedilum helenae is currently facing a severe risk of extinction due to habitat loss, illegal exploitation, and climate change. It has been classified as Critically Endangered (CR, A1a, cd, B1+2b, c, e) in the Vietnam Red Data Book (2007) and is listed under Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Despite its significant ecological and scientific value, the conservation of Paphiopedilum helenae encounters numerous challenges [2].

Paphiopedilum orchids are characterized by slow growth, and in the wild, their seeds have a low germination rate due to the slow germination process, which also requires symbiosis with mycorrhizal fungi [3]. Various propagation methods, such as seed sowing and shoot division, have been employed; however, these methods generally yield low multiplication rates and inconsistent seedling quality [4]. The Paphiopedilum species is considered difficult to propagate via in vitro techniques, particularly during the regeneration phase from tissue cultures. While some hybrid varieties of Paphiopedilum have been successfully propagated from callus, shoots, and seeds [5-9] there is currently no published research specifically addressing the in vitro propagation of *Paphiopedilum helenae*. Most existing studies have focused on its morphological characteristics, distribution, or, more recently, on morphological and molecular markers for species identification. This article presents preliminary results from a study on the in vitro propagation process of *Paphiopedilum helenae*, aiming to conserve and sustainably develop this rare genetic resource while providing high-quality seedlings for market demand.

2. RESEARCH METHODS

Materials

The research material consists of mature Paphiopedilum helenae fruits (6 months old, with firm and shiny skins) collected from the Xuan Mai Orchid Garden in Xuan Mai town, Chuong My, Ha Noi.

Methods

Sterile sample preparation: *Paphiopedilum helenae* fruits were washed with soap, followed by rinsing under running water. They were then sterilized in a laminar flow hood by immersing the fruits in 96% ethanol and flaming them until the alcohol evaporated completely. This procedure was repeated for 1, 2, 3, and 4 times. After sterilization, the fruits were peeled, and the seeds were placed on a germination medium (MS medium supplemented with 20 g/L sucrose and 7 g/L agar). Data were collected after 12 weeks based on the following criteria: survival rate of samples, contamination rate, and the time it took for germination to occur and initiate shoot formation.

Shoot propagation: The shoots of *Paphiopedilum helenae* from the sterile culture medium, with a length of 1 cm, were transferred to MS medium supplemented with varying concentrations of 6-Benzylaminopurine (BAP) ranging from 0-4 mg/L, or 3 mg/L BAP combined with 6-furfurylaminopurine (Kinetin) at concentrations of 0.25–1 mg/L, 30 g/L sucrose, and 7 g/L agar. Data were collected after 8

weeks of cultivation, with the following parameters: number of regenerated shoots, number of shoots per sample, and shoot size.

Complete plant formation: The shoots meeting the criteria (length of 2.5 cm, with 2–3 leaves and dark green color) were transferred to a rooting medium consisting of MS with 0.5 mg/L IBA combined with 0.5–2 mg/L NAA, 30 g/L sucrose, and 7 g/L agar. MS medium without IBA and NAA was used as the control in this experiment. Data were collected after 6 weeks, based on the following criteria: rooting rate (%), average root length (cm), average number of roots per sample, and root quality.

All culture media were adjusted to a pH of 5.8±0.1 using 1N NaOH. The experiments were carried out under conditions of 25°C ± 2°C temperature, light intensity of 2000 lux, and a 12-hour light cycle per day. All experiments were repeated three times, with 30 samples per repetition. To handle the data, the mean value of the samples in each repetition was calculated.

Data Analysis: The collected data were processed using Excel and SPSS version 20 software (post-hoc One-way Anova, Bonferroni Test and Duncan Test).

3. RESULTS AND DISCUSSION

3.1. Results of *in vitro* sterile sample preparation

Sterile sample preparation is a critical first step and plays a key role in the success of tissue culture propagation. A key factor in this process is selecting an appropriate sterilization method that ensures a high sterilization rate and promotes good shoot regeneration. In this study, 96% ethanol was selected as the sterilizing agent for *Paphiopedilum helenae* fruits, with varying flaming durations applied. The effectiveness of the sterilization methods is presented in Table 1.

Table 1. Effect of sterilization method on the ability to create clean samples of Paphiopedilum helenae

Experimental procedure	Number of times burning the fruit peel with 96° alcohol	Evaluation criteria	
		Percentage of clean samples	Percentage of clean samples germinated
KT1	1	43.3%	42.2%
KT2	2	66.7%	63.3%
KT3	3	94.4%	88.9%
KT4	4	96.7%	76.7%
Sig		*0.0001*	*0.0001*

The results presented in Table 1 indicate that the number of times Paphiopedilum helenae is treated with alcohol significantly affects the ability to produce clean cultures (Sig = 0.0001 < 0.05). Specifically, as the number of alcohol treatments increased from 1 to 3, the rate of clean cultures rose from 43.3% to 94.4%, while the rate of germinating clean cultures also increased from 42.2% to 88.9%. This suggests that enhancing the frequency of alcohol disinfection is effective in sterilizing the seed capsules of the slipper orchid. However, when the number of alcohol treatments was increased to 4, the rate of clean cultures only increased to 96.7% (an increase of 2.3% compared to 3 treatments), while the rate of germinating clean cultures significantly decreased from 88.9% to 76.7% (a decrease of 12.2%). This decline may be attributed to the increased frequency of alcohol treatment, which, while enhancing surface sterilization of the seed capsules, simultaneously causes damage to the seed embryos, leading to a reduced germination capacity. Therefore, the optimal method for sterilizing slipper orchid seed capsules is to apply alcohol treatment three times, resulting in 94.4% clean cultures and 88.9% germinating clean cultures.

When compared to the results of Tinh et al. (2022), who used 0.1% $HgCl_2$ for 5 minutes to

create clean samples from fruit on Purple *Paphiopedilum* orchids, resulting in a clean sample rate of 64.66%, the alcohol burning method shows clear effectiveness without causing toxicity to the user. The results are shown in Figure 1.

Figure 1. *In vitro* culture of Helen's *Paphiopedilum* orchid

(a) Helen's Paphiopedilum orchid seeds observed under a ×10 objective microscope and 150x magnification; (b) Seeds of Helen's Paphiopedilum orchid begin to germinate on the starter culture medium; (c) Protobuds of Helen's Paphiopedilum orchid forming on the starter culture medium.

3.2. Results of the effect of growth regulators on shoot multiplication ability

In *in vitro* culture, growth regulators play a crucial role in promoting the growth and development of explants. Depending on the species and the specific objectives of the culture, both the type and concentration of growth regulators used may vary. Cytokinins, a group of plant hormones, are known to enhance cell division and the differentiation of adventitious buds. Identifying the most suitable type and concentration of cytokinin is critical for the success of the culture process. In this experiment, explants were cultured on media containing 0-4 mg/l BAP and on media containing 3 mg/l BAP combined with 0.25-1 mg/l kinetin. The results of shoot multiplication ability were evaluated after 8 weeks of culture.

3.2.1. Results of the impact of BAP on the ability to multiply Helen's Paphiopedilum orchid

To evaluate the effect of different concentrations of BAP on shoot cluster formation in *Paphiopedilum helenae*, the results obtained from the experiments after 8 weeks of culture are presented in Table 2.

Table 2. Results of Helen's *Paphiopedilum* shoot cluster formation at different BAP concentrations

Experimental procedure	BAP (mg/l)	Percentage of samples with multiple shoots (%)	Shoot multiplication rate (times)	Average shoot length (cm)	Quality of shoot buds
BA1	0	23.33	1.1^{a}	1.3^{a}	+
BA2	1	36.67	1.3^{a}	1.7^{b}	+
BA3	2	52.59	1.73^{b}	2^{c}	+++
BA4	3	76.30	2.6^{c}	3.2^{d}	+++
BA5	4	81.51	1.82^{b}	2.3^{e}	++
Sig		*0.0001*	*0.001*	*0.0001*	

+++ Good shoot quality (tall, plump, and uniform shoots, dark green); ++ Average quality (tall, thin shoots, uneven shoots in clusters, green); + Poor shoot quality (short, uneven shoots, light green).

In the same column, mean values followed by the same letter are not statistically different at a confidence level of P = 0.05 (Duncan's test).

The results obtained from the analysis in Table 2 and Figure 2 demonstrate a clear difference in the effect of BAP concentrations across the experimental formulas. *In vitro* shoots of Helen's *Paphiopedilum*, with an initial height of approximately 1.0 cm, were cultured

on MS medium supplemented with 30 g/l sucrose and 7 g/l agar, combined with different BAP concentrations (0; 0.1; 0.25; 0.5; 0.75 mg/l). The results indicate that the shoots exhibited different responses to each medium formulation. As shown in table 2, adjusting the BAP concentration significantly affected the multiplication rate and growth of the *in vitro* Helen's *Paphiopedilum* shoots. The multiplication rate and shoot height increased progressively when cultured on media supplemented with BAP at concentrations ranging from 1.0 to 4.0 mg/l. The highest shoot multiplication rates were observed in formulas BA3, BA4, and BA5 (supplemented with 2.3 and 4 mg/l BAP, respectively), with rates ranging from 1.73 to 2.6 times. The greatest shoot height (2.0-3.2 cm) was found in BA2 and BA3, and these formulas showed statistically significant differences compared to the other treatments. Formulas with BAP concentrations of 2.0 and 3.0 mg/l produced the best shoot quality. Therefore, formula BA4, with a shoot multiplication rate of 76.3%, a multiplication coefficient of 2.6 times, and an average shoot height of 3.2 cm, was the most suitable formulation for rapid multiplication of Helen's *Paphiopedilum* buds when using BAP as a growth regulator Figure 2 below illustrates the growth of *Paphiopedilum helenae* shoots on MS medium supplemented with BAP, highlighting the differences in shoot development under the experimental conditions.

Figure 2. *Paphiopedilum helenae* shoots growing on MS medium supplemented with BAP

3.2.2. Effect of BAP and Kinetin on the shoot multiplication ability of Helen's Paphiopedilum

Table 3 below presents the results of shoot cluster formation in *Paphiopedilum helenae* when BAP and Kinetin are combined after 6 weeks of culture. This information provides valuable insight into the effects of this combination of growth regulators on shoot growth and overall development in *Paphiopedilum helenae*.

Table 3. Results of cluster formation in Helen's P*aphiopedilum* buds with BAP and Kinetin combination

Experimental procedure	Kinetin (mg/l)	Percentage of samples with multiple shoots (%)	Shoot multiplication rate (times)	Average shoot length (cm)	Quality of shoot buds
BAK1	0.25	66.67	3.6^{a}	3.2^{a}	++
BAK2	0.5	80	4.9^{b}	3^{b}	++
BAK3	0.75	88.9	7.1^{c}	2.8^{c}	+++
BAK4	1	93.33	9.2^{d}	2.6^{d}	+++
Sig		*0.0001*	*0.0001*	*0.0001*	

+++ Good shoot quality (tall, plump, and uniform shoots, dark green); ++ Average quality (tall, thin shoots, uneven shoots in clusters, green); + Poor shoot quality (short, uneven shoots, light green).

In the same column, mean values followed by the same letter are not statistically different at a confidence level of P = 0.05 (Duncan's test).

The results presented in Table 3 indicate that the combination of 3 mg/l BAP and Kinetin at different concentrations significantly influences the multiplication rate, growth, and development of Paphiopedilum helen orchids in vitro, with all results being superior to those obtained when BAP was used alone. Specifically, in the BAK1 formulation supplemented with 0.25 mg/l Kinetin, the multiplication coefficient was 3.6 times, representing a 1.38-fold increase compared to the best result achieved with BAP alone. Although the average shoot height tended to decrease compared to the treatments with only BAP, the highest shoot multiplication rate was observed in the BAK4 formulation (supplemented with 3 mg/l BAP and 1 mg/l Kinetin), achieving a multiplication rate of 9.2 times, which is 3.5 times higher than the best formulation using BAP, with an average shoot height of 2.6 cm. This can be explained by the fact that the addition of Kinetin reduces apical dominance, a common phenomenon when only BAP is used, and promotes the development of axillary shoots. This leads to enhanced shoot multiplication capacity as well as improved uniformity in the quality of shoot clusters.

Based on these results, it can be concluded that the combination of BAP and Kinetin has a significant impact on the formation of shoot clusters in the studied tissue cultures. The BAK4 formulation, consisting of MS medium supplemented with 3 mg/l BAP and 1 mg/l Kinetin, is the most suitable for rapid shoot multiplication of Paphiopedilum helen orchids. In comparison with the studies by Cuc et al. on Paphiopedilum delenatii, which achieved a multiplication rate of 4 shoots/sample after 3 months, and the research by Tinh et al. on Paphiopedilum tranlienianum, which reached a multiplication rate of 2.63 times with an average shoot height of 2.73 cm after 90 days, the BAK4 formulation yields superior results.

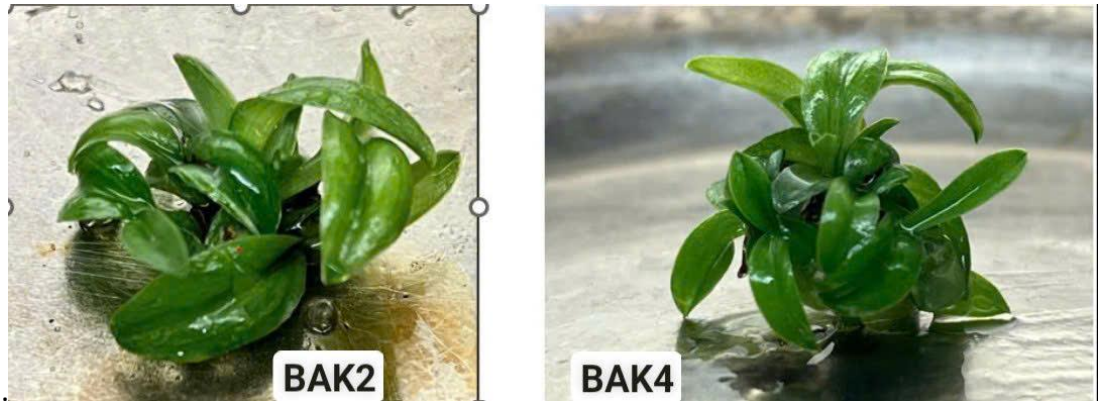

Figure 3*. Paphiopedilum helenae* orchid shoots growing on MS medium supplemented with BAP and Kinetin

3.3. Effects of IBA and NAA concentrations on the rooting ability of Helen's Paphiopedilum orchid shoots

The survival rate, growth, and development of in vitro seedlings during the nursery stage largely depend on the quality and vitality of the shoots formed during the in vitro shoot induction and rooting stages. Each species responds differently to root induction agents at varying concentrations, but ensuring optimal shoot development is essential for achieving the best root quality and quantity. Studies on *in vitro* culture of *Paphiopedilum* species [4, 8, 10-13] have shown that NAA and IBA are the most effective agents for shoot and root induction among the various formulas proposed. Therefore, the experiment on the rooting ability of Helen's *Paphiopedilum* shoots was conducted on MS medium supplemented with IBA at a concentration of 1 mg/l and NAA at concentrations ranging from 0.5 to 2.5 mg/l. The results, obtained after 6 weeks of culture, are summarized in Table 4.

Table 4. Effect of IBA and NAA concentrations on the rooting ability of Helen's Paphiopedilum buds

Experimental procedure	IBA (mg/l)	NAA (mg/l)	Rooting success rate	Mean root length (cm)	Average number of roots per sample	Root quality
RBA1	1	0,5	23,33	1,8[a]	2,73[a]	+
RBA2	1	1	40,00	2,1[b]	3,3[b]	++
RBA3	1	1,5	63,33	2,7[c]	3,9[c]	++
RBA4	1	2	86,67	3,8[d]	5,6[d]	+++
RBA5	1	2,5	87,67	2,7[c]	3,4[e]	++
Sig			*0,0001*	*0,0001*	*0,0001*	

+++ Good quality (Thick, long roots); ++ Average quality (Small, short roots); + Poor quality (Weak, thin, short roots).

In the same column, mean values followed by the same letter are not statistically different at a confidence level of P = 0.05 (Duncan's test).

In plant tissue culture, most in vitro shoots are unable to synthesize auxins like natural seedlings, making the addition of appropriate auxins essential for root formation. In this study, two types of auxins, NAA and IBA, were utilized. After six weeks of culture on a rooting medium supplemented with 1.0 mg/l IBA and varying concentrations of NAA, increasing the NAA concentration from 0.5 to 2.0 mg/l resulted in improved rooting success rate, including mean root length, average number of roots per sample. The optimal results were achieved with a combination of 1.0 mg/l IBA and 2.0 mg/l NAA, yielding a rooting percentage of 86.67%, an average root length of 3.8 cm, and an average of 4.6 roots per sample. However, when the NAA concentration was further increased to 2.5 mg/l, although the rooting percentage increased by 1%, both the average root length and the average number of roots per sample significantly decreased. According to the results presented in Table 4, the formulation RBA4, which included 1.0 mg/l IBA and 2.0 mg/l NAA, achieved a rooting percentage of 86.67%, an average root length of 3.8 cm, and an average of 4.6 roots per sample, making it the most suitable formulation for stimulating the complete root formation of Helen's slipper orchid (Figure 4).

Figure 4. *Paphiopedilum helenae* orchid shoots rooting on media supplemented with IBA and NAA

4. CONCLUSION

The suitable disinfection formula for physiologically mature *Helen's Paphiopedilum* orchids involves burning with 96^{o} alcohol three times to reach a concentration of 94.4%, resulting in a seed germination rate of 88.9%. The optimal environment for rapid multiplication of *Helen's Paphiopedilum* orchid

shoots is BAK4: MS medium + 3 mg/l BAP + 1 mg/l Kinetin, 30 g/l sucrose, and 7 g/l agar. Under these conditions, 99.33% of samples produced multiple shoots, with an average shoot multiplication coefficient of 9.2 times and an average shoot height of 2.6 cm after 8 weeks of culture. The best culture medium to stimulate rooting of in vitro buds for *Helen's Paphiopedilum* after 6 weeks is MS medium + 1.0 mg/l IBA + 2.0 mg/l NAA + 25 g/l sucrose + 7.0 g/l agar. After 6 weeks of culture, the rooting rate of buds reached 86.67%, with an average of 2.7 roots per plant and an average root length of 3.4 cm. The roots were thick, large, and strong.

Acknowledgements:

This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.03–2021.05.

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