Scientia Horticulturae 256 (2019) 108648

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Effects of shoot tip removal, wounding manipulation, and plant growth regulators on shoot regeneration and plantlet development in *Paphiopedilum* species

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ARTICLE INFO

Keywords:
P. callosum
P. delenatii
P. gratrixianum
Shoot apex decapitation
Shoot regeneration
Wounding method

ABSTRACT

The micropropagation procedure of three endangered *Paphiopedilum* species (*Paphiopedilum callosum*, *Paphiopedilum gratrixianum*, and *Paphiopedilum delenatii*) by removing shoot apex and wounding method is reported in this study. The results showed that shoot regeneration of *P. callosum*, *P. gratrixianum*, and *P. delenatii* was at high frequency (5.61; 5.48; and 6.00 shoot/explant, respectively) when decapitated shoot apex was cultured on liquid Schenk and Hildebrandt (SH) medium added with thidiazuron (TDZ, 0.4–0.6 mg L^{-1}). Multiple shoots were also induced in vitro shoots by wounding method, when cultured on medium containing TDZ (0.4–0.6 mg L^{-1}) which were 4.48; 5.37; and 5.31 shoots/explant, respectively in each species. The shoots regenerated via both shoot apex decapitation and wounding method were well-developed to plantlets on SH medium supplement with 1.0 mg L^{-1} Naphthaleneacetic acid (NAA) after 90 days of culture. High-quality plants of *P. callosum*, *P. gratrixianum*, and *P. delenatii*. were observed when the plantlets were transferred onto peat moss with 100% survival rate after 24 months in greenhouse.

1. Introduction

Species from the genus *Paphiopedilum*, Orchidaceae, popularly known as “slipper orchids”, are one of the most beautiful plants in the world due to their unique and exotic flowers. *Paphiopedilum callosum*, *Paphiopedilum gratrixianum*, and *Paphiopedilum delenatii*, native species of Vietnam, are listed under the appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2013). While *P. callosum* distributed in Center and South Annamese floristic province, *P. delanatii* and *P. gratrixianum* are distributed only in South Annamese and Chinese floristic province of Vietnam, respectively. The wild forms of *P. delanatii* and *P. gratrixianum* are terrestrial and lithophytic, while *P. callosum* is terrestrial. *P. delanatii* and *P. callosum* grow mainly in 0–1500 m, while *P. gratrixianum* grows in 800–1500 m above sea level of silicate (commonly acidic) rocks (Averjanov and Averyanova, 2003). *Paphiopedilum* orchid seeds are known to be very difficult to germinate (Kauth et al., 2008), therefore, it is very important to conserve these plants before they become extinct.

The conventional method of propagation *Paphiopedilum* species are conducted through the separation of axillary buds from the mother plant, which is time consuming and unproductive. Zeng et al. (2016) reported that 58 protocols for in vitro seed germination have been described which occupied 80.6% of all *Paphiopedilum* in vitro studies. Nevertheless, the germination rate of many *Paphiopedilum* orchids are extremely low and affected by various unknown factors (Arditti, 2009; Zeng et al., 2016). Although commercial scale of in vitro production of *Paphiopedilum* species has been achieved for several species (Liao and Chen, 2006; Zeng et al., 2006, 2010, 2012, 2013; Liao et al., 2011; Chen et al., 2015), strategies for enhancing mass productivity of in vitro shoots, especially for the endangered orchids is a challenge for the researchers.

In a previous study, our results showed a high shoot regeneration rate of *P. delenatii* by wounding method and stem node culture (Nhut et al., 2007, 2005). The present study therefore aimed (1) to improve the shoot regeneration rate of three endangered *Paphiopedilum* orchids by applying the innovation methods, (2) to compare the effect between shoot apex decapitation and wounding methods on adventitious shoots formation, (3) to establish the suitable in vitro and ex vitro condition for three endangered *P. callosum*, *P. gratrixianum*, and *P. delenatii* species.

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https://doi.org/10.1016/j.scienta.2019.108648
Received 8 May 2019; Received in revised form 9 July 2019; Accepted 10 July 2019

2. Materials and methods

2.1. Plant materials

Paphiopedilum delenatii, *P. callosum*, and *P. gratrixianum* were collected from wild populations and planted in greenhouse of Tay Nguyen Institute for Scientific Research, Vietnam. The 3-month-old shoots (3–7 cm in length) with 3–4 leaves were collected and cleaned the surface with liquid soap (Unilever Co., Ltd., Vietnam), and placed under running tap water 2–3 h. The shoots were soaked with Streptomycin (Pharmaceutical Co., Ltd., China) 1.0 ppm for 30 min. Then, the shoots of *Paphiopedilum* orchids were dipped into 70% (v/v) ethanol 30 s, followed by agitation for 10 min in sodium hypochlorite and rinsed five times with sterilized distilled water. The shoots of *P. delenatii*, *P. callosum*, and *P. gratrixianum* were cultured on Schenk and Hildebrandt, 1972 (SH) medium, added with Thidiazuron (TDZ, 1.0 mg L^{-1}), Naphthaleneacetic acid (NAA, 0.3 mg L^{-1}), 30 g L^{-1} sucrose, and 9 g L^{-1} agar for 90 days. Vigorous and uniform adventitious shoots of *Paphiopedilum* species formation after 90 days of culture were used for shoot apex decapitation and wounding method.

2.2. In vitro shoot apex decapitation method

The 3-month-old shoots with well-developed 4–5 leaves and 5 cm leaf length of three *Paphiopedilum* species were placed onto autoclaved petri dishes for removing the under medium parts. Then, first forceps was used to keep the last leaf of shoot and the shoot apex was removed out by the second forceps (Fig. 1). The rest of shoots after removing the apex were placed on liquid SH medium with difference concentration of TDZ (0.2–1.0 mg L^{-1}) or 6-Benzylaminopurine (BA, 0.5–2.5 mg L^{-1}), 30 g L^{-1} sucrose. The decapitated shoots were placed on 250 mL bottle with 40 mL of medium and the absorbent cotton (7 × 7 cm; 2.02 ± 0.03 g) (Baothach JSC., Vietnam) was used as the substrate in the culture for shoot regeneration.

2.3. Wounding method on in vitro shoot

After detaching the ground medium parts, 3-month-old shoots were pierced 3–4 times at bases (0.4-0.6 cm in diameter) using a sterilized sharp needle. The injury diameter was approximately 0.3 mm (Fig. 1). The wounding shoots were cultured on liquid SH medium with difference concentration of TDZ (0.2–1.0 mg L^{-1}) or BA (0.5–2.5 mg L^{-1}), 30 g L^{-1} sucrose for shoot regeneration. The wounding shoots were placed on 250 mL bottle with 40 mL of medium and the absorbent cotton (7 × 7 cm; 2.02 ± 0.03 g) (Baothach JSC., Vietnam) was used as the substrate in the culture for shoot regeneration.

2.4. In vitro and ex vitro conditions

The medium was adjusted pH to 5.8 before autoclaving at 121 °C, 1 atm for 30 min. The culture condition was set up under 16-h photoperiod with light intensity 15–20 μmol $m^{-2}{\cdot}s^{-1}$ of cool white fluorescent tubes at 25 ± 1 °C.

The plantlets were transplanted into plastic pots (9 cm diameter) containing rice hush ash (RHA), coconut fiber (CF) (Eco Source Co. Ltd., Vietnam), 2 kinds of fern fibers including *Cibolium barometz* fiber (CFF), grind fern (GFF), which is native to Vietnam, and peat moss (PM) (Eco Source Co. Ltd., Vietnam). Transplanted plantlets were grown in greenhouse at 16–25 °C ambient temperature, 60–90% relative humidity, and natural sunlight with PPFD less than 200 μmol $m^{-2}{\cdot}s^{-1}$. Watering was one time per day from November to April (dry season) and twice per day from May to October (rainy season). Fertilized once per month with slow-release Hi-control 13-11-11 + ME (Arysta Health and Nutrition Science Co., Ltd., Japan).

2.5. Statistical analysis

In shoot regeneration and root induction experiment, 30 explants (3 shoots/flask) and 25 explants (5 shoots/flask), respectively of each *Paphiopedilum* species were utilized per replication with 4 replications. In greenhouse acclimation experiment, each treatment was conducted with 20 plantlets/replication (1 plantlet/vessel) with 5 replications were set in each experiment. Data were analyzed using SPSS version 16.0 (SPSS Inc., USA). Where a significant difference ($P \leq 0.05$) was observed for a measured parameter, means were separated using Duncan's multiple range test at 5% level of significance (Duncan, 1995).

3. Results and discussion

3.1. Combination of shoot apex decapitation method and PGRs on shoot regeneration of P. callosum, P. gratrixianum, and P. delenatii

The regeneration and proliferation of adventitious shoots are substantially influenced by external supplement of PGRs such as BA and TDZ. In this study, supplement of external PGRs in high concentrations promoted shoot regeneration regardless species of *Paphiopedilum* in liquid SH medium after 90 days of culture (Fig. 2). The combination of removing shoot apex method and BA (1.0–2.5 mg L^{-1}) stimulated shoot multiplication (4.37; 4.24; 4.75 shoots/explant, respectively) of *P. callosum*, *P. gratrixianum*, and *P. delenatii* (Fig. 2A-C). The study of Huang et al. (2001) showed that the highest number of shoot regeneration (3 shoots/explant) of *Paphiopedilum* orchids when cultured on modified Murashige and Skoog, 1962 (MS) medium adding 3 mg L^{-1} BA. Wattanawikkit et al., 2011 cultured shoots of *P. callosum* on medium had 2.25 mg L^{-1} BA produced 2.3 shoots/explant after 3 months of culture.

The shoots of *P. callosum*, *P. gratrixianum*, and *P. delenatii*, where shoot apex was decapitated, and cultured on SH liquid medium supplemented with TDZ (0.4-0.6 mg L^{-1}) showed higher regeneration frequency (5.61; 5.48; 6.00 shoots/explant, respectively) than BA (Fig. 2D-F). Amoo et al. (2011) reported that the regeneration of multiple adventitious shoots is associated with the type and amounts of cytokinins used. Although BA has proved to be an effective cytokinin in case of orchid micropropagation, in this study however, it was observed to be comparatively less efficient than TDZ. The results may be caused by the chemical properties, because TDZ has been determined to be 50-100-folds more efficient than other cytokinin compounds (Genkov and Ivanova, 1995). On other hand, Mok et al. (1982); Hare and Van Staden (1994) reported that the application of TDZ enhanced the internal

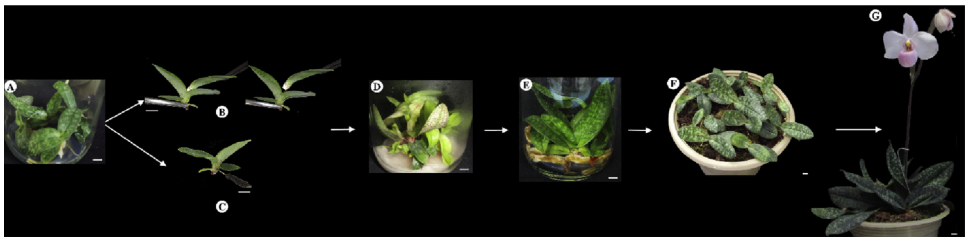

Fig. 1. Scheme of shoot regeneration of three endangered *Paphiopedilum* species via shoot apex decapitation and wounding method. A, Initial in vitro shoot; B, shoot apex decapitation method; C, wounding method; D, Adventitious shoot induction; E, Root formation; F, Acclimatization in greenhouse; E, Flowering of *Paphiopedilum* species. Bar: 1 cm.

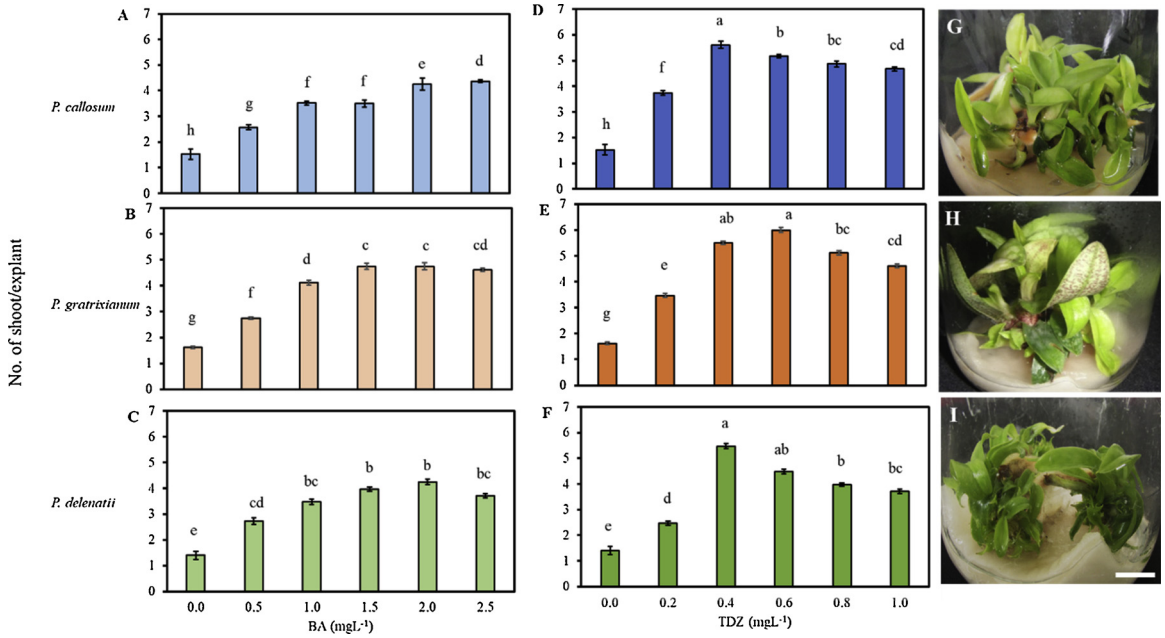

Fig. 2. Combination of shoot apex decapitation method and PGRs (BA: A, B, C; TDZ: D, E, F) on shoot multiplication and morphology (G, H, I) of *P. callosum*, *P. gratrixianum*, and *P. delenatii* after 90 days of culture. Data represent mean ± SE from four replications. Different letters indicate significant difference in Duncan's test (p value P ≤ 0.05). Bar: 1 cm.

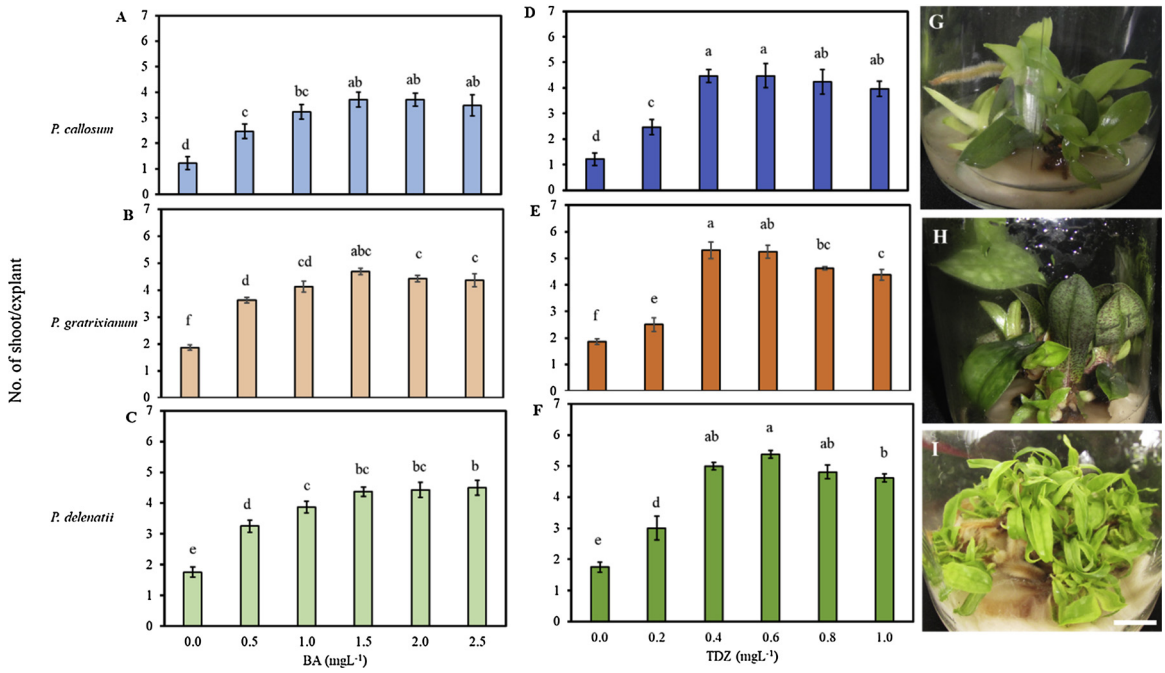

Fig. 3. Combination of wounding method and PGRs (BA: A, B, C; TDZ: D, E, F) on shoot multiplication and morphology (G, H, I) of *P. callosum*, *P. gratrixianum*, and *P. delenatii* after 90 days of culture. Data represent mean ± SE from four replications. Different letters indicate significant difference in Duncan's test (p value P ≤ 0.05). Bar: 1 cm.

cytokinin and perhaps it had reducing effect of cytokinin oxidase activation. Therefore, TDZ is more stable than all cytokinin hormones.

Morphology of regenerated shoots were different on medium supplemented with difference concentration of TDZ in *P. callosum*, *P. gratrixianum*, and *P. delenatii* (Fig. 1G-I). Adding high concentration of TDZ (0.8–1.0 mg L^{-1}) on medium produced dwarf and thicker shoots, abnormal leaves which hardly induced roots. Our report is supported by other previous studies (Norhanizan and Aziz, 2018; Vasudevan and Van Staden, 2011; Vogel and Macedo, 2011). Cytokinins ordinarily stimulate the regeneration and inhibit the elongation of adventitious shoots, therefore; stunted and dwarf shoot formation by TDZ was consistent with its high cytokinin activity.

The combination between shoot apex decapitation method and appropriate concentrations of (0.4-0.6 mg L^{-1}) TDZ are important for the formation of more shoots in *Paphiopedilum* orchids. It is a well-established phenomenon of apical dominance, and is also known that removing the shoot apex results in the activation of axillary buds (Domagalska and Leyser, 2011). Domagalska and Leyser (2011) reported that cytokinins is one of candidates to serve as second messengers, which directly promote bud outgrowth regardless of the presence

Table 1
Roots induction of *P. callosum*, *P. gratrixianum*, and *P. delenatii* after 90 days of culture.

Species	NAA ($mg{\cdot}L^{-1}$)	No. of root/shoot	Root length (cm)	Fresh weight (g)
P. calosum	0.0	1.76b*	2.35d	1.20c
	0.5	3.56ab	4.12c	1.37b
	1.0	**4.15a**	**5.10a**	**1.65a**
	1.5	3.85a	4.56b	1.40b
	2.0	3.98a	4.45b	1.33b
P. graxtrixianum	0.0	2.43d*	2.76c	1.24c
	0.5	3.50c	4.56b	1.56b
	1.0	**4.33a**	**5.29a**	**1.85a**
	1.5	3.89ab	4.57b	1.47b
	2.0	3.69b	4.45b	1.40b
P. delenatii	0.0	3.20c	3.20c	2.10c
	0.5	3.79b	4.57b	2.67b
	1.0	**4.50a**	**5.10a**	**3.13a**
	1.5	4.24ab	5.00a	2.79b
	2.0	4.00b	4.68b	2.68b

* Different letters in the same column indicate significant difference in Duncan's test (p value $P \leq 0.05$).

of auxin. Although, cytokinins are the only PGRs that are known to promote bud out growth, the mechanism of action still remains unclear (Domagalska and Leyser, 2011; Rameau et al., 2015).

In other hand, the process of shoot apex decapitation also wounded the explants leading to the stimulation of ethylene, which often known as an inducement for the formation of adventitious shoots (George, 1993). Chatfield and Raizada (2008) reported that the increased ethylene biosynthesis can enhance rates of shoot regeneration in *Arabidopsis thaliana*. Huang et al. (2014) shown that the physical wounding and ethylene stimulated the embryogenic stem cell proliferation and plantlet regeneration in protocorm-like bodies of *Phalaenopsis* orchids. Therefore, ethylene also can have a significant impact on regeneration of *Paphiopedilum* orchids.

3.2. Combination of wounding method and PGRs on shoot regeneration of P. callosum, P. gratrixianum, and P. delenatii

To establish which method was the most suitable for adventitious shoot proliferation of *Paphiopedilum* orchids, the effect of wounding method combined with difference concentration of TDZ (0.2–1.0 mg L^{-1}) or BA (0.5–2.5 mg L^{-1}) were studied (Fig. 3A-F). The results showed that the combination of wounding method and PGRs determined the positive effected on shoot regeneration of *Paphiopedilum* species (Fig. 3). The highest number of shoot induction (4.48; 5.37; 5.31 shoot/explant) was found at SH liquid medium adding with 0.4-0.6 mg L^{-1} TDZ on *P. callosum*, *P. gratrixianum*, and *P. delenatii*, respectively (Fig. 3D-I). They had vigorous shoot growth and verdurous leaves (Fig. 3G-I).

Correspondingly with shoot apex decapitation method, the results in Fig. 2 shows that when supplemented with high concentration of TDZ it presented a negative effect on shoot proliferation of *Paphiopedilum* orchids. We observed decrease in shoot regeneration and visual abnormalities in shoot formation.

In the previous study, the wounding method was first successful applied for regenerating shoot of *P. delenatii* with 5.2 shoot/explant (Nhut et al., 2005). The results of this present study implicated that wounding method, which penetrated explants bases by sharp needle, can be potential method for shoot multiplication of *Paphiopedilum* orchids. The removing shoot apex method was then established for enhancing the shoot proliferation frequency. The results implicated that there were no significant differences between the two methods of shoot apex decapitation and wounding on shoot regeneration of *P. callosum*, *P. gratrixianum*, and *P. delenatii*. However, the removing of shoot apex

Table 2
Effect of substrates on survival rate, growth and development of *P. callosum*, *P. gratrixianum*, and *P. delenatii* plantlets after 24 months transportation in greenhouse.

	Substrate	Survival rate (%)	No. of new leaf/plant	Leaf width (cm)	Leaf length (cm)	No. of root/plant	Root length (cm)	Fresh weight (g)	SPAD	Morphology
P. calosum	CF	100	4.75a*	2.40d	7.00d	4.12b	7.25e	10.75d	40.85b	Short leaves, chartreuse green, stunted plants
	RHA	100	4.75a	2.50cd	8.62c	4.25b	8.75d	12.00d	41.45b	Short leaves, dark green, stunted plants
	GFF	100	4.87a	2.60c	9.25bc	4.50ab	10.50c	14.00c	41.35b	Short leaves, lime green, stunted plants
	CFF	100	5.00a	2.80b	10.12b	5.00a	12.25b	16.00b	41.70b	Long leaves, dark green, vigorous plants
	PM	**100**	**5.00a**	**3.05a**	**12.00a**	**5.00a**	**15.00a**	**19.50a**	**44.67a**	Long leaves, green, vigorous plants
	CF	100	4.56a	2.00c	7.50d	4.75b	6.75c	9.00d	38.67d	Short leaves, lime green, stunted plants
	RHA	100	4.68a	2.12c	8.62c	4.75b	7.00bc	11.00c	40.50bc	Short leaves, dark green, stunted plants
P. graxtrixianum	GFF	100	4.87a	2.27b	8.75c	5.00b	8.0abc	12.37b	39.25cd	Short leaves, chartreuse green, stunted plants
	CFF	100	4.93a	2.42a	10.75b	5.50b	8.25ab	13.25ab	40.92ab	Long leaves, dark green, vigorous plants
	PM	**100**	**5.00a**	**2.55a**	**12.50a**	**6.75a**	**9.00a**	**14.00a**	**42.00a**	Long leaves, green, vigorous plants
P. delenatii	CF	100	5.00b	2.25e	6.00b	4.00c	6.00c	8.75c	38.82c	Short leaves, chartreuse green, stunted plants
	RHA	100	5.00b	2.40d	7.25b	4.25c	7.00c	10.25c	40.37b	Short leaves, dark green, stunted plants
	GFF	100	5.12b	2.50c	7.62b	4.37bc	8.50b	12.75b	39.50bc	Short leaves, lime green, stunted plants
	CFF	100	6.00a	2.60b	8.00ab	5.00ab	8.87b	13.12ab	40.32b	Long leaves, dark green, vigorous plants
	PM	100	6.00a	2.82a	9.00a	5.25a	10.25a	14.75a	41.47a	Long leaves, green, vigorous plants

* Different letters in the same column indicate significant difference in Duncan's test (p value $P \leq 0.05$). CF, Coconut fiber; RHA, Rice hush ash; GFF, Grind *Cibolium barometz* fern; CFF, *Cibolium barometz* fern fiber; PM, peat moss.

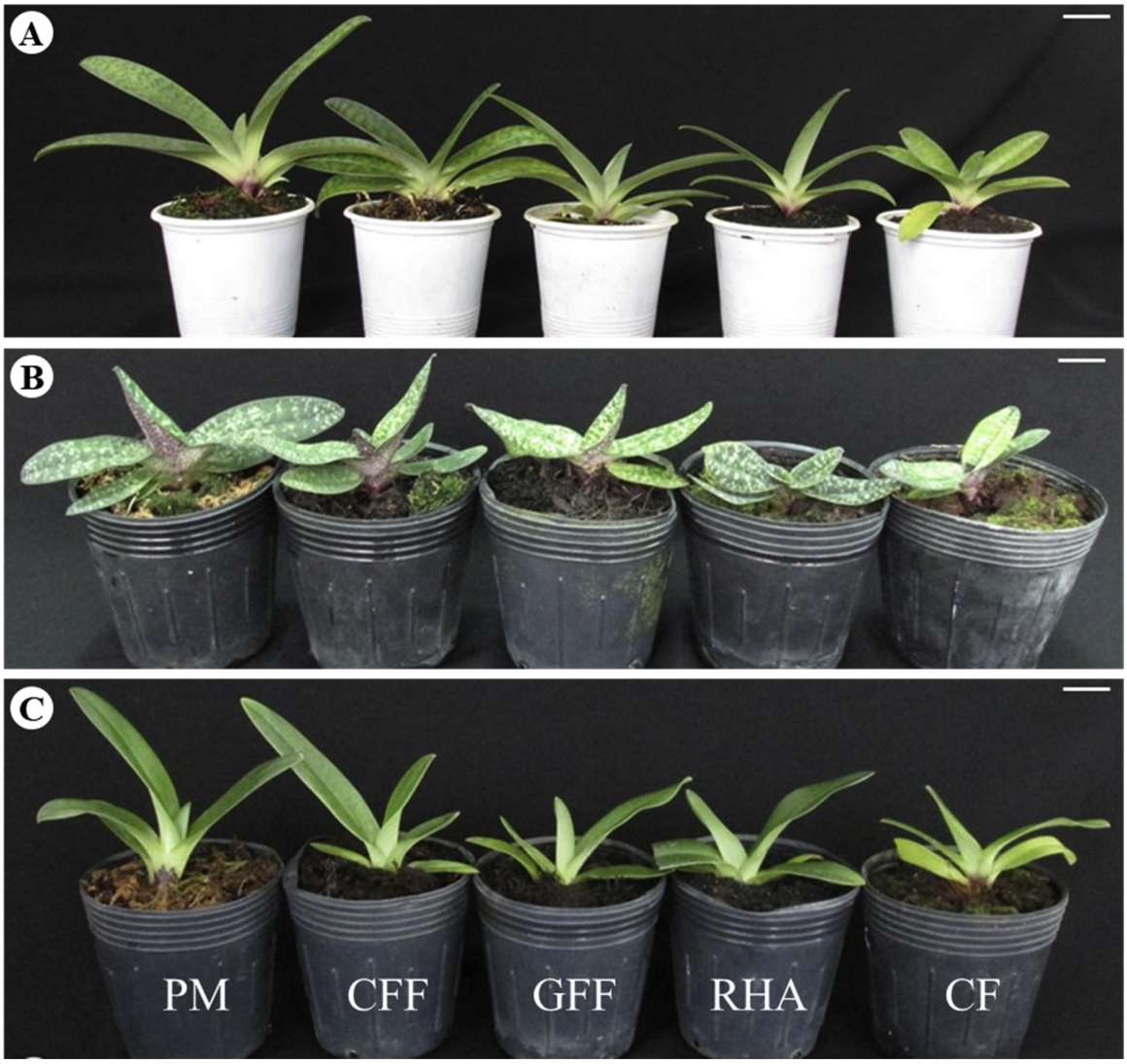

Fig. 4. Morphology of *P. callosum*, *P. gratrixianum*, and *P. delenatii* plantlets under difference substrates after 24 months transportation in greenhouse. Bar: 1 cm.

Fig. 5. Flowering plants from *P. callosum*, *P. gratrixianum*, and *P. delenatii* plantlets after 36 months in greenhouse.

was less time consuming and easier to conduct than wounding method alone in *Paphiopedilum* orchids.

3.3. Rooting and acclimatization of P. callosum, P. gratrixianum, and P. delenatii

For root induction, the shoots of three *Paphiopedilum* species were transferred to medium containing NAA (0.5–2.0 mg L^{-1}) (Table 1). In the PGR-free medium (control), regenerated shoots could form 1.76–3.2 roots per shoot after 90 days of culture (Table 1). Maximum number of roots in *P. callosum*, *P. gratrixianum*, and *P. delenatii* (4.15; 4.33; 4.5 root/explant, respectively) were achieved in SH medium supplemented with 1.0 mg L^{-1} NAA (Table 1). In this medium, an average of root length (5.10–5.29 cm) was obtained after 90 days of culture. Total fresh weight of plantlets was also recorded the highest value when adding 1.0 mg L^{-1} NAA into culture medium of *Paphiopedilum* orchids. Auxins can play an important role on the early occurrence of root differentiation from cells (Bielach et al., 2012). The study of Hong et al. (2008) and Jen-Tsung (2018) of *P.* 'Alma Gavaert' rooting was showed the highest root number (2.7 and 3.6 roots/shoot, respectively) in the presence of 5 mg L^{-1} NAA on the culture medium.

The full-grown plantlets were transferred to pots in greenhouse with

different substrates resulting in 100% survival (Table 2). For understanding the physical characteristics of potting substrates on plantlet growth, five substrates including CF, RHA, GFF, CFF, and PM were used for acclimatization of the endangered *Paphiopedilum* orchids (Table 2, Fig. 4). The plantlets of *P. callosum*, *P. gratrixianum*, and *P. delenatii* showed extreme adaptability and high ability for acclimatization with 100% survival rate, regardless of the substrates after 24 months of transportation in greenhouse (Table 2). Although, the number of new leaves formation were constant, the growth of plantlets depended on difference substrates (Table 2, Fig. 4). Plantlets of *P. callosum*, *P. gratrixianum*, and *P. delenatii* exhibited slow growth with short and thin leaves, few roots, short roots, and light weight when they were growth on CF substrate. The different substrates on plantlets development of *Paphiopedilum* orchids are: PM, CFF, GFF, RHA and CF (Table 2, Fig. 4A-C). The plantlets of *P. callosum*, *P. gratrixianum*, and *P. delenatii* which were placed on PM substrate also showed the highest fresh weight (19.50; 14.00; and 14.75 g, respectively) and chlorophyll content (44.67; 42.00; and 41.47 SPAD unit, respectively) (Table 2). Generally, the micropropagated plants transferred to ex vitro condition are affected by various stresses. One of the most important factors influencing the success during the greenhouse acclimatization of plantlets is prevention of desiccation. PM can store water via empty cells which prevent dehydration and retain water in drier condition leading to high survival rate and good growth of plantlets in *Paphiopedilum* orchids. The studies on *Epidendrum nocturnum* (Zettler et al., 2007), *P. rothschildianum* (Chyuam-Yih et al., 2010), *P. callosum* var. sublaeve (Wattanapan et al., 2018) also reported that high survival rate (80–96%) of plantlets was observed in sphagnum moss.

The plantlets of *P. callosum*, *P. gratrixianum*, and *P. delenatii* were transferred into bigger pots after 3 months. The flowering of three *Paphiopedilum* orchids was observed after 36 months transportation into greenhouse (Fig. 5). *Paphilopedilum* orchids on the market practically come from in vitro germinated seeds of hybrid species (Liao et al. 2011; Zeng et al., 2016). However, the seedings are highly variable, uncertain, and it takes a long time to maturate before flowering (Liao et al. 2011; Zeng et al., 2016). Besides, clonal plantlets are expected to have the same genetic traits as their mother plants, which are influential for mass production. The present study shown that high shoot frequency was induced by applied shoot apex decapitation and wounding method, and the high survival rate of plantlets regeneration was observed of three endangered *Paphilopedilum* orchids.

3.4. Conclusion

In this study, the high shoot multiplication of *Paphiopedilum* species was recorded when shoots were obtained first and then the shoot apex was removed or subjected to wounding of shoot apex and cultured in SH liquid medium, adding TDZ. The highest quality and quantity of roots in *P. callosum*, *P. gratrixianum*, and *P. delenatii* were formed in medium supplemented with 1.0 mg L^{-1} NAA. The plantlets were well-developed in PM substrate and bloomed after 36 months of transformation in greenhouse. Hence the present study was successful in developing rapid method of high shoot regeneration along with root induction, complete plant development and flowering of three endangered species of *Paphiopedilum* orchids. The production of large number uniform plantlets that has high horticulture potential and the possibility to develop integrated conservation approaches for *P. callosum*, *P. gratrixianum*, and *P. delenatii*.

Author contributions

Vu Quoc Luan, Le Kim Cuong acquired data wrote the manuscript. Hoang Thanh Tung, Vu Thi Hien, Tran Hieu participated in interpretation of data and revision for intellectual content. Duong Tan Nhut conceptualized and designed the study.

Acknowledgments

This work was financially supported by the National Foundation for Science and Technology Development (NAFOSTED), Vietnam, under Project No 106-NN.01-2015.02 and Vietnam Academy of Science and Technology under Project No 2470/QĐ-VHL (NVCC22.01/19-19).

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