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Study on asymbiotic germination and acclimatization of *Paphiopedilum purpureum*

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Abstract

Paphiopedilum purpuratum is a nationally class I protected plant species in China, and its seed germination is extremely challenging. To establish an efficient asymbiotic germination system for *P. purpureum*, capsules were used to investigate the impact of various ages on seed germination, as well as the influence of different organic additives and light intensities on seedling vigor and root development. Additionally, optimal cultivation substrates were screened. The results revealed that seeds with a capsule age of 230 days after pollination (DAP) exhibited the highest germination rate of 80.58% on 1/4 MS medium supplemented with 100 mL of coconut water. In seedling cultivation, the addition of banana was found to effectively enhance crown breadth and root growth, coconut water could effectively facilitate the enlargement of root diameter, whereas the addition of potato significantly promoted the accumulation of soluble sugars and chlorophyll in leaves. High photosynthetic photon flux density (PPFD) of 66.13 $\mu M \cdot M^{-2} \cdot S^{-1}$ facilitated crown enlargement, while low PPFD of 4.67 $\mu M \cdot M^{-2} \cdot S^{-1}$ was beneficial for root elongation. The optimal cultivation substrate was a 1:1(v/v) mixture of *in-situ* substrate and limestone, achieving a transplanting survival rate of 75.93%. These findings provide critical insights into establishing an efficient asymbiotic germination system for *P. purpuratum*, offering technical support for its artificial propagation, and the restoration of the wild population. This study lays a foundation for the conservation and sustainable utilization of this endangered species.

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Introduction

Orchidaceae is one of the largest and most diverse groups of flowering plants in the world, with over 730 genera and more than 29,000 species[1−3]. The genus *Paphiopedilum*, one of the most primitive lineages in the Orchidaceae family, is commonly known as the 'lady's slipper orchid' or 'slipper orchid', due to its distinctive pouch-shaped or sac-shaped labellum, exhibiting both significant scientific research value and high ornamental value[4]. The genus *Paphiopedilum* comprises 109 species and 35 natural hybrids worldwide, with 33 species currently documented in China[3,5,6]. Plants of this genus typically grow in clustered populations, making them highly vulnerable to complete extraction through illegal wild harvesting, which can lead to localized extinction[7]. The genus *Paphiopedilum* primarily reproduces through two methods: seed propagation, and division, both of which have significant limitations. In natural environments, the seeds must establish a symbiotic relationship with fungi to obtain nutrients for germination. However, even with additional nutrient supply, most mature *Paphiopedilum* seeds still exhibit poor germination rates[8]. Vegetative propagation through division is limited by its long cycle and low propagation rate, making it inadequate for meeting large-scale production demands[9]. The reproductive characteristics of *Paphiopedilum* have resulted in narrow wild population distributions, and low individual counts. Coupled with habitat degradation and excessive wild harvesting, these orchids have become one of the most endangered groups within the Orchidaceae family[10]. Currently, all wild *Paphiopedilum* species are listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), receiving international protection[11].

Asexual propagation methods for *Paphiopedilum* species, including axillary bud induction and shoot tip regeneration have been reported in existing literature[12]. However, the *in-vitro* propagation of *Paphiopedilum* is significantly constrained by several technical challenges associated with explant materials, including sterilization difficulties, low induction efficiency, and limited multiplication rates, resulting in inefficient asexual reproduction systems[12,13]. Most *Paphiopedilum* species cannot achieve industrialized tissue cloning production like *Phalaenopsis* orchids[14]. Seeds represent the optimal source for preserving plant genetic diversity, with their inherent heterogeneity making them particularly suitable for conserving the genetic diversity of wild populations[15]. Consequently, asymbiotic germination is recognized as the most efficacious method for *Paphiopedilum* propagation.

Current studies have documented successful *in-vitro* seedling production via asymbiotic germination in multiple *Paphiopedilum* species. However, seed germination and protocorm development in these orchids are significantly influenced by multiple factors, including capsule maturity, seed pretreatment protocols, medium composition, and culture conditions[16]. Studies have shown that different *Paphiopedilum* species have a relatively optimal embryonic stage after pollination, during which seed germination rate is highest, while fully mature seeds are often difficult to germinate[17]. In *P. bellatulum* and *P. niveum*, the seeds collected 120 to 150 days after pollination (DAP) were the optimum for culture *in vitro*, while for *P. godefroyae*, it was 90 to 120 DAP, and for *P. henryanum* and

P. spicerianum, it was 120 to 180 DAP[18]. The optimal germination period for *P. emersonii* was approximately 180 DAP[19], while for *P. concolor*, the germination rate was relatively higher at around 210 DAP[20]. Results from different studies on the same species also show variation. For example, the peak germination period for *P. bellatulum* ranged from 95 to 120 DAP[13,21]. Furthermore, while immature seeds germinate readily, they were often associated with low seedling establishment rates[22]. In contrast, mature seeds, despite being more suitable for long-term storage, present the practical challenge of being difficult to germinate. Therefore, precisely determining the optimal germination period during seed development and balancing the trade-off between germination rate and seedling establishment rate remain the core challenges to be addressed in the asymbiotic propagation of *Paphiopedilum*. Furthermore, due to genotype-specific requirements, optimal asymbiotic germination conditions vary significantly among *Paphiopedilum* species. Notably, propagation protocols remain underdeveloped for several taxa, including *P. purpuratum*[23].

Paphiopedilum purpuratum is a perennial herbaceous species belonging to the genus *Paphiopedilum*, primarily distributed in southern China, including Guangdong, Guangxi, Hainan, and Yunnan provinces, and is also found in Vietnam[24]. Through investigation and research, it has been found that due to habitat destruction and human excavation, the wild population of *P. purpuratum* is extremely scarce, placing the species in an endangered state. There is an urgent need for artificial assistance in its propagation and the reconstruction of its wild populations. Currently, *P. purpuratum* is classified as a Class I protected plant in China[25]. Although studies on its conservation biology, chloroplast genome, taxonomy and community ecology have been reported in recent years[26–29], systematic research on its artificial propagation and cultivation techniques remains inadequate. To address the inadequacies in aseptic germination techniques for *P. purpuratum*, this study focuses on investigating its seed asymbiotic germination. The research aims to elucidate critical stages and influencing factors in its reproductive process, thereby providing theoretical foundations for optimizing sterile propagation protocols. Furthermore, this work contributes to the conservation and utilization of *P. purpuratum* resources, holding significant implications for the preservation of its germplasm.

Materials and methods

Experimental materials

The specimens of *P. purpuratum* used in this study were derived from individuals originally introduced from southwestern China approximately two decades ago and are currently maintained at the Orchid Conservation Research Center of Shenzhen, Guangdong Province. Capsules obtained from artificial cross-pollination in November 2023 were collected as experimental materials.

Methods

Capsule collection and sterilization

Capsules were randomly collected at 140, 170, 200, 210, 220, 230, 240, 250, 260, and 270 DAP (three capsules per time point; 10 sampling stages total). Under sterile conditions, capsules were surface-disinfected by wiping with 75% ethanol-soaked gauze, followed by immersion in a 20% sodium hypochlorite (NaClO) solution for 30 min. After five rinses with sterile water, surface moisture was removed using autoclaved filter paper prior to capsule dissection.

Aseptic sowing

The sterilized seeds were evenly distributed onto a germination medium composed of 1/4 MS basal salts (macronutrients at 1/4 strength) supplemented with 100 mL coconut water. Each capsule was sown in five bottles. Cultures were maintained under dark conditions at 25 ± 2 °C. Germination status was monitored periodically. Upon germination, the cultures were transferred to light conditions, and formal assessments of germination rate and mortality conducted after 3 months of cultivation. At each stage, nine 1 × 1 cm squares were randomly selected, and germination within each square was observed and recorded. The germination rate was determined based on protocorm formation (embryo emergence through the seed coat)[30], while the mortality rate was calculated according to the incidence of protocorm browning or necrosis post-germination initiation. Germination initiation time was recorded at the first observed embryo protrusion through the seed coat.

Protocorm differentiation induction culture

To induce the differentiation of protocorms into leaves and roots, germinated protocorms were transferred to a medium consisting of 1/2 MS + 0.5 mg/L 6-BA + 0.2 mg/L NAA + 0.5 g/L activated charcoal (AC) and cultured for three months. The culture conditions included a temperature of 25 ± 2 °C, photosynthetic photon flux density (PPFD) 66.13 $\mu M \cdot M^{-2} \cdot S^{-1}$, and a photoperiod of 14 h light/10 h dark.

Rooting and strengthening of seedlings

When the seedlings of *P. purpuratum* had differentiated 2–3 leaves and reached a height of 1 cm, they were transferred to a culture medium consisting of 1/2 MS + 0.2 mg/L 6-BA + 0.5 mg/L NAA + 1 g/L AC supplemented with 50 mg/L (mL/L) of potato, banana, or coconut water, respectively. The control group (CK) was cultured in 1/2 MS + 0.2 mg/L 6-BA + 0.5 mg/L NAA + 1 g/L AC without any organic additives. This setup aimed to investigate the effects of different organic additives on the rooting and strengthening of *P. purpuratum* seedlings. Seven culture bottles were inoculated for each formulation, with three seedlings per bottle, and the experiment was repeated three times. The culture conditions included a temperature of 25 ± 2 °C, PPFD of 66.13 $\mu M \cdot M^{-2} \cdot S^{-1}$, and a photoperiod of 14 h light/10 h dark.

To investigate the impact of different light intensities on the rooting and strengthening of *P. purpuratum* seedlings, the seedlings were cultured in a medium consisting of 1/2 MS + 0.2 mg/L 6-BA + 0.5 mg/L NAA + 1 g/L AC + 50 mg/L potato used as the rooting and strengthening medium under varying PPFD (4.67 $\mu M \cdot M^{-2} \cdot S^{-1}$ and 66.13 $\mu M \cdot M^{-2} \cdot S^{-1}$). Potato extract was added as an organic supplement to promote rooting and seedling strengthening[31]. Six culture bottles were inoculated for each light intensity treatment, with three seedlings per bottle, and the experiment was repeated three times. The culture temperature was maintained at 25 ± 2 °C, with a photoperiod of 14 h light/10 h dark.

After 90 d of culture, observations and records were made on the rooting and strengthening of the seedlings, and measurements were taken for chlorophyll content, soluble sugar content, and root activity. Chlorophyll content was quantified through ethanol extraction[32], while soluble sugar content was determined by anthrone-sulfuric acid colorimetry[33]. Root system vitality was assessed via the 2,3,5- triphenyltetrazolium chloride (TTC) reduction assay[34].

Acclimatization and transplantation

The *in vitro* seedlings of *P. purpuratum*, approximately 2 cm in height with three fully developed leaves and 3–5 robust roots, were transferred to a greenhouse for 7 d. Subsequently, the bottle caps were opened for an additional 7-d acclimatization period to complete the hardening process. During transplantation, the culture medium adhering to the roots was rinsed off with clean water, and the seedlings were air-dried before being transplanted into three distinct substrate mixtures with varying volume ratios: bark: limestone (1:1), *in-situ* substrate: limestone (1:1), and bark: volcanic rock (1:1). Eight plants were transplanted into each substrate, with the experiment repeated three times. The culture environment parameters were as follows: PPFD $120.6–136.7 \mu M·M^{-2}·S^{-1}$, humidity 70%, daytime temperature 24–28 °C, and nighttime temperature 20–24 °C. The characteristics of these substrates are detailed in Table 1. After 90 d, parameters including survival rate, crown width increment, chlorophyll content, and soluble sugar content were measured.

Data statistics and analysis

Experimental data were organized using Microsoft Excel 2019 and subjected to analysis of variance (ANOVA) and *t*-tests with SPSS Statistics 27.0.

Results

Longitudinal section observation of *P. purpuratum* seeds at different developmental stages

As shown in Fig. 1, seeds at different developmental stages exhibited distinct color variations in their longitudinal sections. At 140 d after pollination (DAP), the seeds displayed white coloration and were tightly attached to the placenta, making it difficult to separate individual seeds from the placental tissue (Fig. 1a). The 170 DAP seeds retained their white pigmentation while exhibiting reduced placental adhesion compared to 140 DAP specimens, facilitating easier detachment of individual seeds (Fig. 1b). At 200 DAP, the capsule apex developed light brown pigmentation while maintaining white coloration in remaining regions, with seeds exhibiting significantly enhanced dispersibility (Fig. 1c). The 210 DAP capsules exhibited pronounced darkening of brown pigmentation at the apex, with progressive extension of browning towards the median capsule region, while seeds adjacent to the pedicel maintained a characteristic yellowish-white coloration (Fig. 1d). Longitudinal sections revealed a gradual browning of seeds from the ovarian apex to the base. At 220 DAP, the capsule seeds exhibited more extensive browning compared to the 210 DAP specimens, with only a few remaining yellowish-white seeds (Fig. 1e). At 230 DAP, the seeds had turned tan with occasional dark brown specimens (Fig. 1f). From 240 to 270 DAP, the seeds developed uniform brown coloration and became loosely attached, easily dislodged from the capsules (Fig. 1g–j).

Table 1. Characteristics of cultivation substrates.

Cultivation substrate	Characteristics
Bark	The fermented pine bark, with a particle size of 3–6 mm.
In-situ substrate	The substrate collected from natural *P. purpuratum* populations in Zhongshan city, Guangdong Province, China, primarily consists of humus and decomposed plant litter.
Limestone	With a particle size of 3–6 mm.
Volcanic rock	Particle size 3–6 mm, featuring a rough surface with honeycomb-like porosity.

Effects of capsule maturity on seed germination in *P. purpuratum*

Statistical analysis of initial germination time and germination rate was conducted for *P. purpuratum* seeds at different developmental stages (Table 2). The results showed that 140-DAP seeds turned brown 20 d after sowing without germination. Seeds at 170 DAP achieved a relatively high germination rate of 65.77%, but 68.39% of the resulting protocorms browned and failed to develop into seedlings. For seeds at 200–210 DAP, the germination rates ranged from 42.64% to 48.27%, with corresponding protocorm mortality rates between 33.93% and 47.71%. The highest germination rate of 80.58% was observed in 230-DAP seeds, and all germinated protocorms developed normally into seedlings. Germination rates gradually declined with increasing capsule age, decreasing to 45.28% at 240 DAP and 42.25% at 250 DAP. Complete loss of germinability occurred by 260 DAP. The germination initiation time remained consistent at 51–53 d across all developmental stages, showing no discernible pattern.

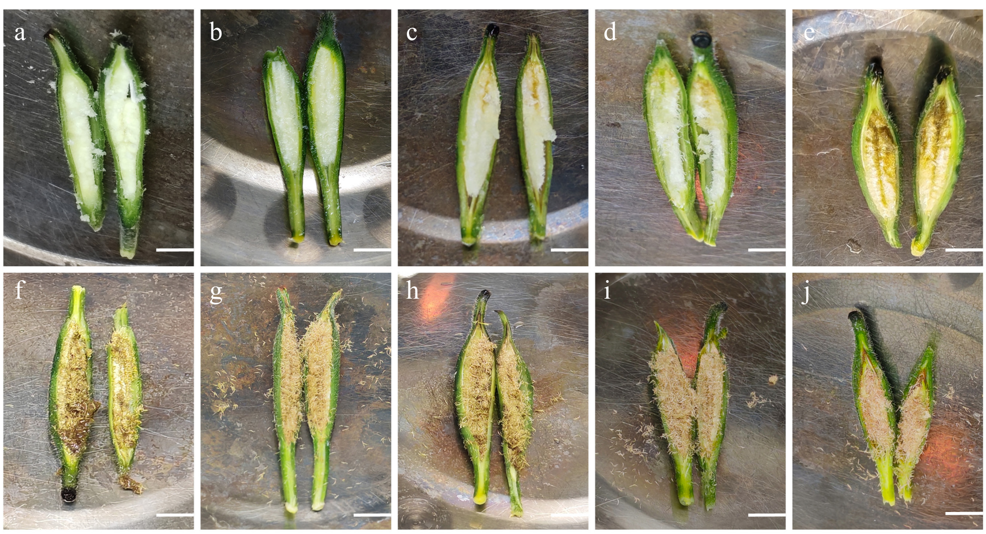

Fig. 1 Longitudinal sections of *P. purpuratum* capsules at different developmental stages (scale bars = 1 cm). (a) 140 DAP, (b) 170 DAP, (c) 200 DAP, (d) 210 DAP, (e) 220 DAP, (f) 230 DAP, (g) 240 DAP, (h) 250 DAP, (i) 260 DAP, and (j) 270 DAP.

Table 2. Effects of capsule maturity on germination rate in *P. purpuratum*.

DAP	Germination rate (%)	Mortality rate (%)	Germination initiation time (d)
140	0d	–	–
170	65.77 ± 3.31ab	68.39 ± 4.01a	52
200	42.64 ± 3.68c	47.71 ± 8.72b	51
210	40.90 ± 3.37c	33.93 ± 2.46c	52
220	48.27 ± 14.03bc	42.00 ± 1.45bc	53
230	80.58 ± 9.17a	0d	52
240	45.28 ± 5.61c	0d	52
250	42.25 ± 5.82c	0d	51
260	0d	–	–

The data are mean ± standard deviation, and different letters after the same column of numbers indicate significant differences between different treatments ($p < 0.05$). '–' indicates no statistically available data.

Effects of different organic supplements on seedling strengthening and rooting in *P. purpuratum*

Seedlings with 2–3 leaves and approximately 1 cm in height were transferred to culture media containing 1/2 MS + 0.2 mg/L 6-BA + 0.5 mg/L NAA supplemented with either 50 g/L potato, coconut water, or banana. The results demonstrated significant variations in the effects of different organic supplements on seedling strengthening and root development in *P. purpuratum*. As presented in Table 3, marked differences were observed across multiple growth parameters among the treatment groups. Regarding crown width expansion, all organic supplement treatments demonstrated statistically superior performance compared to the control group (CK), with the banana-supplemented group exhibiting optimal results. The banana treatment achieved a crown width increment of 4.53 cm, showing a significant advantage over both the potato and coconut water treatments. In terms of root proliferation, the banana treatment group demonstrated optimal performance, reaching 4.78 roots per seedling, which was significantly higher than other treatment groups. The coconut water and potato treatments showed intermediate results with 3.65 and 3.61 roots, respectively, though no statistically significant difference was observed between these two groups. Regarding total root length increment, both banana and coconut water treatment groups showed statistically significant differences compared to the CK, with increases of 31.85 and 27.52 cm, respectively. Although the coconut water treatment group performed slightly inferior to the banana group in terms of both root proliferation and elongation, it induced the development of more robust root systems (Fig. 2). Physiological index analysis (Table 4) revealed that the potato treatment group achieved optimal performance in total soluble sugar content, reaching a maximum value of 89.44 mg/g, which was significantly higher than all other groups, while no significant differences were observed among other physiological indices. In contrast, the coconut water treatment group showed lower values in total soluble sugar content compared to other treatments. In summary, in the cultivation of robust *P. purpuratum* seedlings, banana effectively promotes the enlargement of crown width and root growth, coconut water significantly enhances root diameter, while potato facilitates the accumulation of soluble sugars in the leaves.

Effects of light intensity on seedling vigor of *P. purpuratum*

Plantlets with 2–3 leaves and approximately 1 cm in height were transferred to a seedling-strengthening medium composed of 1/2 MS basal salts supplemented with 0.2 mg/L 6-BA, 0.5 mg/L NAA, and 50 g/L potato extract. The cultures were then subjected to distinct light conditions: PPFD 4.67 $\mu M{\cdot}M^{-2}{\cdot}S^{-1}$ (representing indoor diffused light) and 66.13 $\mu M{\cdot}M^{-2}{\cdot}S^{-1}$ (achieved using dual 18W LED lamps) for cultivation. The results presented in Table 5 demonstrate

Table 3. Effects of different organic additives on the robust seedling growth of *P. purpuratum*.

Treatment	Crown width increment (cm)	Root proliferation number	Total root elongation (cm)
CK	1.99 ± 0.21c	3.29 ± 0.27b	16.52 ± 1.76b
Potato	3.91 ± 0.21b	3.61 ± 0.41ab	20.76 ± 1.73b
Banana	4.53 ± 0.21a	4.78 ± 0.51a	31.85 ± 3.22a
Coconut water	3.62 ± 0.18b	3.65 ± 0.33ab	27.52 ± 1.72a

The data are mean ± standard deviation, and different letters after the same column of numbers indicate significant differences between different treatments ($p < 0.05$).

Table 4. Effects of different organic additives on physiological characteristics of *P. purpuratum*.

Physiological parameters	CK	Potato	Banana	Coconut water
Chlorophyll a content (mg/g.FW)	0.09 ± 0.02ab	0.13 ± 0.02a	0.10 ± 0.01ab	0.08 ± 0.01b
Chlorophyll b content (mg/g.FW)	0.20 ± 0.05ab	0.26 ± 0.04a	0.19 ± 0.02ab	0.15 ± 0.02b
Total chlorophyll content (mg/g.FW)	0.29 ± 0.07ab	0.39 ± 0.06a	0.29 ± 0.03ab	0.23 ± 0.03b
Root activity [mg TTF/(g.h)]	0.03 ± 0.01a	0.03 ± 0a	0.04 ± 0a	0.03 ± 0a
Total soluble sugar content (mg/g)	64.37 ± 5.23b	89.44 ± 3.47a	26.30 ± 2.61b	59.21 ± 5.39c

The data are mean ± standard deviation, and different letters after the same line of numbers indicate significant differences between different treatments ($p < 0.05$).

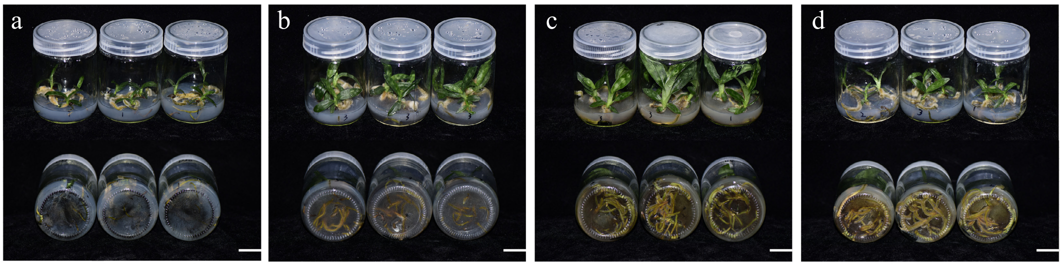

Fig. 2 Effects of different organic additives on robust seedling growth of *P. purpuratum* (scale bars = 2 cm). (a) CK, (b) potato, (c) banana, (d) coconut water.

significant differences in canopy expansion and root elongation of *P. purpuratum* under different light conditions, while no effect was observed on root proliferation. Under high PPFD (66.13 μM·M^{-2}·S^{-1}), the crown width growth rate was significantly greater than that under low PPFD (4.67 μM·M^{-2}·S^{-1}). Conversely, root elongation was significantly enhanced under low light conditions compared to high light intensity. Neither light treatment showed significant effects on chlorophyll content, root activity, or soluble sugar content (Table 6). Therefore, high light conditions promote canopy expansion, whereas low light favors root elongation growth.

Effects of different cultivation substrates on transplant survival of *P. purpuratum*

In-vitro seedlings after robust growth cultivation were acclimatized and then transplanted into various growth media, followed by cultivation in greenhouse conditions. The results demonstrated that the *in-situ* substrate mixture of limestone (1:1) achieved the highest survival rate of 75.93%, followed by bark: volcanicrock (1:1) at 63.89%, while bark: limestone (1:1) showed the lowest survival rate of merely 36.11% (Table 7). In addition, the results showed no statistically significant differences in crown width (Fig. 3), soluble sugar content, and chlorophyll content among the different cultivation substrate treatments.

Table 5. Effects of different light intensities on robust seedling growth of *P. purpuratum*.

Growth parameters	Treatment (μM·M^{-2}·S^{-1})		t	*p*
	66.13	4.67		
Crown width increment (cm)	3.91 ± 0.21	3.15 ± 0.19	2.652	0.012*
Root proliferation count	3.61 ± 0.41	3.12 ± 0.51	0.759	0.453
Total root growth increment	20.76 ± 1.73	27.02 ± 1.082	−2.492	0.018*

The data are mean ± standard deviation, and * indicates significant differences between the two treatments (* $p < 0.05$).

Table 6. Effects of different light intensities on physiological indices of *P. purpuratum*.

Physiological parameters	Treatment (μM·M^{-2}·S^{-1})		t	*p*
	66.13 ($n = 18$)	4.67 ($n = 17$)		
Chlorophyll a content (mg/g.FW)	0.13 ± 0.08	0.14 ± 0.05	−0.217	0.829
Chlorophyll b content (mg/g.FW)	0.26 ± 0.16	0.27 ± 0.11	−0.211	0.834
Total chlorophyll content (mg/g.FW)	0.39 ± 0.24	0.41 ± 0.16	−0.213	0.832
Root activity [mg TTF/(g.h)]	0.03 ± 0.02	0.02 ± 0.01	0.331	0.743
Total soluble sugar content (mg/g)	89.44 ± 14.73	82.94 ± 21.06	1.063	0.295

The data are mean ± standard deviation, and * indicates significant differences between the two treatments (* $p < 0.05$).

Discussion

Effects of capsule maturity on seed germination efficiency in *P. purpuratum*

Germination rate follows a unimodal pattern with capsule maturity

Seeds, as commonly used explants for *in vitro* culture in *Paphiopedilum* species exhibit a germination rate that was directly influenced by capsule maturity and generally follows a dynamic unimodal pattern, initially increasing before declining[18]. Among terrestrial orchids in the family Orchidaceae, near-mature seeds typically exhibited higher germination rates compared to fully mature seeds[22]. The study found that *P. purpuratum* took about 280 DAP from pollination to capsule dehiscence, but achieved optimal germination and seedling establishment at 230 DAP. This phenomenon was not an isolated case. Similar germination patterns have been reported in multiple other *Paphiopedilum* species, including *P. armeniacum*[8], *P. spicerianum*[35], and *P. delenatii*[36]. The consistent germination trend observed in *P. purpuratum*, as a member of this genus, suggests it may reflect a sophisticated and evolutionarily conserved reproductive strategy within *Paphiopedilum*.

Correlation between the optimal germination period and embryo developmental stage

The optimal germination period for seeds of the *Paphiopedilum* genus often corresponds to specific stages of embryonic development. For *P. spicerianum*, the highest germination rates occurred 180–210 DAP, when the embryo reached the late globular stage with initial suspensor degeneration[35]. Research on *P. barbigerum* similarly confirmed that seeds collected 255 DAP achieved the highest germination rate of 90.71%, at which stage the embryos were in the globular phase, and the suspensors had not yet fully degenerated[37]. In this study, while *P. purpuratum* seeds at 170 DAP were capable of germination, they failed to develop into seedlings, suggesting that the embryos might not have completed their morphological and physiological construction at this stage. In contrast, seeds at the near-maturity stage of 230 DAP exhibited the highest germination vigor and seedling potential, which may be related to the complete development of their embryos.

Seed coat maturation leads to a decline in germination rate

Germination rates declined rapidly after reaching a peak, which may be attributed to the maturation and strengthening of the seed coat structure. In *P. armeniacum*, the seed coats of fully mature seeds became increasingly dense, which impedes the absorption of water and nutrients, thereby suppressing germination[13]. In *P. appletonianum* and *P. hirsutissimum*, the decline in germination rates following the development of the embryo into the ellipsoidal stage was similarly related to the progressive accumulation of lignin-like substances in the seed coat, which reduced its water permeability[38]. In this study, the decline in germination rates observed in *P. purpuratum* after 240 DAP was likely attributable to the similar progressive deposition of lignin-like substances

Table 7. Effects of different cultivation substrates on the growth of *P. purpuratum*.

Substrate	Survival rate (%)	Crown width (cm)	Soluble sugar content (mg/g)	Chlorophyll a content (mg/g.FW)	Chlorophyll b content (mg/g.FW)	Total chlorophyll content (mg/g.FW)
Bark: limestone	36.11 ± 2.41c	2.72 ± 0.69a	69.90 ± 11.33a	0.10 ± 0.02a	0.21 ± 0.03a	0.31 ± 0.05a
In-situ substrate: limestone	75.93 ± 1.60a	2.86 ± 0.42a	68.57 ± 8.63a	0.19 ± 0.04a	0.39 ± 0.07a	0.58 ± 0.11a
Bark: volcanic rock	63.89 ± 2.41b	3.04 ± 0.40a	45.57 ± 6.20a	0.10 ± 0.02a	0.20 ± 0.04a	0.30 ± 0.06a

The data are mean ± standard deviation, and different letters after the same line of numbers indicate significant differences between different treatments ($p < 0.05$).

Fig. 3 Effects of different cultivation substrates on transplantation of *P. purpuratum* (scale bars = 2 cm), (a) bark: limestone (1:1), (b) *in-situ* substrate: limestone (1:1), (c) bark: volcanic rock (1:1).

within the seed coat. This physical dormancy induced by seed coat thickening serves as a protective mechanism against adverse environmental conditions in natural settings. However, in *in-vitro* cultivation, it becomes a primary barrier that must be overcome. To address this obstacle in artificial conservation and propagation, the germination rate of seeds could be significantly enhanced through sodium hypochlorite treatment[39].

Interspecific variations and the implications for conservation practices

Although a common germination pattern exists, the optimal germination period varies among species within the *Paphiopedilum* genus, reflecting potential adaptive responses to their respective native habitats. For instance, *P. delenatii* reached its peak germination rate of 79.3% at 210 DAP[36]. Germination in *P. villosum* commenced at 150 DAP, with the highest germination rate of 31.33% observed at 200 DAP[40]. For *P. wardii*, studies have reported that the optimal germination period occurs at 180 DAP[22]. In this study, the optimal germination period for *P. purpuratum* was determined to be 230 DAP. Such variations highlight the necessity of identifying and selectively targeting the specific seed age when developing *in-vitro* propagation protocols for the endangered *Paphiopedilum* species.

Effects of light on the physiological responses of *P. purpuratum*

Adaptation to low-light conditions and chlorophyll stability

Paphiopedilum purpuratum is naturally distributed in southeast-facing forest understories with heavy canopy shade, where its growth requirements are met by moderate diffuse light conditions[28]. Although studies have reported low light intensity in the natural habitat of *P. purpuratum*, the effects of varying light intensities on its growth remain poorly understood. Studies have demonstrated that *P. emersonii* can autonomously regulate leaf chlorophyll content to maintain consistent levels within a certain light intensity range, thereby optimizing the capture and utilization of diffuse light[41]. The present study revealed that chlorophyll content in *P. purpuratum* exhibited no statistically significant variation during vigorous seedling cultivation under two distinct PPFD conditions of 4.67 and 66.13 $\mu M \cdot M^{-2} \cdot S^{-1}$. This finding aligns with the previously mentioned research results and suggests that the *Paphiopedilum* genus may possess the ability to autonomously regulate chlorophyll metabolism to stabilize pigment content, thereby effectively capturing and utilizing light energy in varying light environments.

Carbon metabolism balance under different light intensities and research limitations

Paphiopedilum purpuratum exhibited marginally higher soluble sugar content under high light intensity compared to low light conditions, though the difference was not statistically significant. This suggests the species may possess an efficient carbon metabolism system capable of maintaining metabolic homeostasis across varying light regimes. Under low light conditions, *P. purpuratum* likely maintains soluble sugar content by reducing respiratory rate or enhancing photoassimilate accumulation, whereas under high light intensity, it may stabilize sugar levels through increased respiratory rate or promoted translocation and utilization of photoassimilates to dissipate excess photosynthetic products. This carbon metabolic regulation pattern may be closely related to the photosynthetic characteristics of variegated leaves in *P. purpuratum*. The dark-colored regions possess higher chlorophyll content and serve as the primary sites for light energy absorption, whereas the light-colored regions, although lower in chlorophyll content, exhibit higher energy capture efficiency of the photosystem II (PSII) reaction center, enabling better utilization of low light for photosynthesis[42]. This study established two distinct light intensity gradients, which preliminarily revealed the stability of physiological indicators in *P. purpuratum*. However, this experimental design still exhibits evident limitations.

Effect of cultivation substrate on the growth of *P. purpuratum*

Influence of substrate physical structure on transplant survival

Substrate type is one of the key factors determining the transplant survival of slipper orchids. Commonly used substrates for slipper orchids include volcanic rock, pine bark, peat soil, and their mixtures[43]. Volcanic rock exhibits high porosity, combining superior aeration and water retention properties, thus serving as an ideal substrate for orchid cultivation. Limestone, characterized by its porous structure, excellent drainage properties, and moderate hardness, is a commonly used component in mixed cultivation substrates for terrestrial orchids. *P. hirsutissimum* exhibited the highest transplant survival rate of 97% in shredded bark cultivation substrate, demonstrating uniform root development and vigorous growth[44]. In contrast, *P. emersonii* achieved a 75.93% survival rate in a mixed substrate composed of bark: volcanic rock: humus soil (1:1:1)[45]. For *P. villosum*, a substrate mixture of charcoal, brick fragments, and soil at a 1:1:1 ratio, topped with a layer of moss, proved most suitable for seedlings, demonstrating satisfactory survival rates up to 63.1%[46].

In this transplantation experiment of *P. purpuratum*, a 1:1 (v/v) mixture of *in-situ* substrate and limestone fragments was identified as the optimal growing medium, achieving a high survival rate of 75%. The *in-situ* substrate was collected from the *P. purpuratum* population in Zhongshan, primarily composed of humus and leaf litter. This substrate is rich in rhizosphere fungi and their metabolites specific to *P. purpuratum*, with high soil fertility that maintains excellent nutrient availability. When combined with limestone fragments (1:1 ratio), the mixed medium provides optimal water retention without waterlogging, effectively preventing root rot and consequently achieving the highest survival rate.

Influence of substrate pH and mineral nutrients on survival rate

Both volcanic rock and limestone exhibit large interparticle voids with loose, aerated structures, making them common orchid cultivation substrates when combined with bark or humus. However, in this experiment, *P. purpuratum* showed markedly different survival rates: merely 36.11% in bark: limestone (1:1) substrate vs 63.89% in bark: volcanic rock (1:1). This significant difference may be related to the contrasting pH-regulating capacities of the two substrates. Measured data show that the irrigation water used in the experiment had a pH of 7.5, while the leachate from the bark: volcanic rock substrate had a pH of 6.8, which was lower than the pH of 7.1 from the bark: limestone substrate. This indicates that volcanic rock likely possesses a greater buffering or acidifying capacity, enabling it to modulate the substrate's pH to some extent and better align with orchids' requirements for their growth environment. In addition to the pH effect, volcanic rock is rich in iron, calcium, potassium, magnesium, manganese, phosphorus, and other trace elements. These nutrients can be gradually absorbed by roots through irrigation, thereby enhancing the growth and development of *P. purpuratum*. This study did not investigate the synergistic effects of combining native habitat substrate with volcanic rock on *P. purpuratum* growth. Future research could explore the potential optimization benefits of such mixed substrates.

Conclusions

This study successfully developed a methodology for asymbiotic seed germination and acclimatized transplantation of *P. purpuratum*. Key findings include: (1) 230 DAP capsules showed the highest germination rate, with all protocorms developing into normal seedlings; (2) organic additives demonstrated specific effects—banana promoted shoot expansion and root growth, coconut water increased root diameter, while potato enhanced soluble sugar accumulation in leaves; (3) light intensity differentially influenced growth—high light favored shoot expansion, whereas low light promoted root elongation; (4) a 1:1 mixture of *in-situ* substrate and limestone proved optimal for transplanting tissue-cultured seedlings. These results provide a comprehensive technical framework for large-scale propagation, supporting both *ex-situ* conservation and wild population restoration of this endangered orchid species.

Author contributions

The authors confirm their contributions to the paper as follows: study conception and design: Wang M; data collection: Wang K, Chen P, Wang Y; analysis and interpretation of results: Xiu X, Chen J, Zhang S; draft manuscript preparation: Wang K, Xiu X. manuscript revision: Wang M, Li J, All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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References

[1] Moonlight PW, Baldaszti L, Cardoso D, Elliott A, Särkinen T, et al. 2024. Twenty years of big plant genera. *Proceedings of the Royal Society B: Biological Sciences* 291:20240702

[2] Pérez-Escobar OA, Bogarín D, Przelomska NAS, Ackerman JD, Balbuena JA, et al. 2024. The origin and speciation of orchids. *New Phytologist* 242:700–716

[3] POWO. 2025. *Plants of the World Online*. https://powo.science.kew.org/

[4] Zhang J, Fang L, Zeng J, Li L, Wu K, et al. 2024. Species and geographical distribution of *Paphiopedilum*. *Chinese Journal of Tropical Crops* 45:1572–1584

[5] IPNI. 2025. *International Plant Names Index*. http://www.ipni.org

[6] CoL China. 2025. *Catalogue of Life China: 2025 Annual Checklist*. http://www.sp2000.org.cn

[7] Luo YB, Jia JS, Wang CL. 2003. Conservation strategy and potential advantages of the Chinese *Paphiopedilum*. *Biodiversity Science* 11:491–498

[8] Fang L, Xu X, Li J, Zheng F, Li M, et al. 2020. Transcriptome analysis provides insights into the non-methylated lignin synthesis in *Paphiopedilum armeniacum* seed. *BMC Genomics* 21:524

[9] Wu RH, Zhang X. 2013. Research advance on reproductive biology of *Paphiopedilum*. *Journal of Henan Agricultural Science* 42:6–10

[10] Chen X, He M, Wang W, Li X, Zheng Y, et al. 2020. Effects of high temperature stress on physiological and biochemical indexes of two species of *Paphiopedilum*. *Chinese Agricultural Science Bulletin* 36:72–77

[11] UNEP-WCMC. 2015. *The checklist of CITES species website*. http://checklist.cites.org

[12] Nhut DT, Trang PTT, Vu NH, Thuy DTT, Khiem DV, et al. 2005. A Wounding method and liquid culture in *Paphiopedilum delenatii* propagation. *Propagation of Ornamental Plants* 5:158–163

[13] Zhang YY, Wu KL, Zhang JX, Deng RF, Duan J, et al. 2015. Embryo development in association with asymbiotic seed germination *in vitro* of *Paphiopedilum armeniacum* S. C. Chen et F. Y. Liu. *Scientific Reports* 5:16356

[14] Zeng S, Huang W, Wu K, Zhang J, Teixeira da Silva JA, et al. 2016. *In vitro* propagation of *Paphiopedilum* orchids. *Critical Reviews in Biotechnology* 36:521–534

[15] Diengdoh RV, Kumaria S, Tandon P, Das MC. 2017. Asymbiotic germination and seed storage of *Paphiopedilum insigne*, an endangered lady's slipper orchid. *South African Journal of Botany* 112:215–224

[16] Jiang N, Zhang JX, Teixeira Da Silva JA, Duan J, Liu HT, et al. 2016. Stimulatory effects of sodium hypochlorite and ultrasonic treatments on tetrazolium staining and seed germination *in vitro* of *Paphiopedilum* SCBG Red Jewel. *Seed Science and Technology* 44:77–90

[17] Zeng S, Guo B, Kong X, Fang L, Li L, et al. 2020. Advances in propagation *in vitro* of *Paphiopedilum*. *Chinese Journal of Tropical Crops* 41:2080–2089

[18] Lee YI. 2007. The asymbiotic seed germination of six *Paphiopedilum* species in relation to the time of seed collection and seed pretreatment. *Acta Horticulturae* 755:381−386

[19] Lan VT, Uyen NTM. 2024. The influence of fruit maturity and basic media on seed germination of *Paphiopedilum emersonii* Koop & P.J. Cribb. *TNU Journal of Science and Technology* 229:120−127

[20] Li XL, Hang CY, Song Q, Zhou JY, Wang XG, et al. 2016. *In vitro* germination and propagation of *Paphiopedilum concolor* (Lindl.) Pfitz. *Plant Science Journal* 34:127−134

[21] Chen ZL, Ye XL, Liang CY, Duan J. 2004. Seed germination *in vitro* of *Paphiopedilum armeniacun* and *P. micranthum*. *Acta Horticulturae Sinica* 31:540−542

[22] Zeng S, Wu K, Teixeira da Silva JA, Zhang J, Chen Z, et al. 2012. Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh., an endangered terrestrial orchid. *Scientia Horticulturae* 138:198−209

[23] Yang Y, Huang J, Hu H, Zhang S. 2021. Progress on conservation and utilization of *Paphiopedilum* species in China. *Journal of West China Forestry Science* 50:108−112+119 (in Chinese)

[24] Liu Z, Chen X, Chen L. 2009. *The Genus Paphiopedilum in China*. Beijing: Science Press.

[25] National Forestry and Grassland Administration. 2021. National Key Protected Wild Plants of China. National Forestry and Grasslan Administration, Beijing. www.gov.cn/zhengce/zhengceku/2021-09/09/content_5636409.htm

[26] Liu ZJ, Zhang JY, Ru ZZ, Lei SP, Chen LJ. 2004. Conservation biology of *Paphiopedilum purpuratum* (Orchidaceae). *Biodiversity Science* 12:509−516

[27] Chen XD, Peng DH, Lan SR, Chen J, Fu WQ. 2019. The complete chloroplast genome sequence of *Paphiopedilum purpuratum*(Orchidaceae). *Mitochondrial DNA Part B* 4:3910−3911

[28] Du XJ, Yi QF, Ye QL, Zhong MZ, Wang YQ, et al. 2017. Study on the community characteristics and conservation of endangered species *Paphiopedilum purpuratum* (Lindl.) Stein. *Plant Science Journal* 35:844−850

[29] Zhu Y, Huang J, Tao K, Wang Y, Zhang J, et al. 2024. Floral morphology and anther development in diandrous orchid *Paphiopedilum purpuratum* with their taxonomic implications. *Guihaia* 44:102−112

[30] Stewart SL, Zettler LW. 2002. Symbiotic germination of three semi-aquatic rein orchids (*Habenaria repens, H. quinquiseta, H. macroceratitis*) from Florida. *Aquatic Botany* 72:25−35

[31] Hu QM, Li YY, Huang YF, Hu RC, Wu KL. 2016. Aseptic seeding and rapid propagation of *Paphiopedilum helenae*. *Plant Physiology Journal* 52:1443−1448 (in Chinese)

[32] Meng W, Jia Y, Zhang Y, Hu J, Ding A, et al. 2024. Effect of element deficiency and deficiency recovery on tissue culture seedling of *Dendrobium officinale*. *Journal of Plant Resources and Environment* 33:39−49

[33] Liu HY, Wang HH, Cui CH, Wang M, Guo JJ, et al. 2013. Experiment improvement of the soluble sugar content determination by enthrone colorimetric method. *Laboratory Science* 16:19−20

[34] Zhu X, Liang M, Ma Y. 2020. A review report on the experiments for the determination of root activity by TTC method. *Guangdong Chemical Industry* 47:211−212

[35] Chen WA, Hsu ST, Lee YI. 2024. Embryology of a lady's slipper orchid, *Paphiopedilum spicerianum* and cytokinin requirements for seed germination and protocorm development. *HortScience* 59:1158−1164

[36] Liao H, Li X, Fan J, Bu Z, Ning Y. 2025. Aymbiotic germination and tissue culture rapid propagation technology of *Paphiopedilum delenatii*. *Molecular Plant Breeding* 23:7169−7177 (in Chinese)

[37] You J, Zhang Y, Liu Y, Chen J, Pan H, et al. 2014. Seed development and asymbiotic germination of *Paphiopedilum barbigerum* Tang et Wang. *Plant Physiology Journal* 50:275−282

[38] Zhang J, Yan N, Hu H. 2013. The seed development of three *Paphiopedilum* species in relation to asymbiotic germination. *Plant Diversity and Resources* 35:33−40

[39] Chen Y, Goodale UM, Fan XL, Gao JY. 2015. Asymbiotic seed germination and in vitro seedling development of *Paphiopedilum spicerianum*: an orchid with an extremely small population in China. *Global Ecology and Conservation* 3:367−378

[40] Long B, Niemiera AX, Cheng ZY, Long CL. 2010. *In vitro* propagation of four threatened *Paphiopedilum* species (Orchidaceae). *Plant Cell, Tissue and Organ Culture (PCTOC)* 101:151−162

[41] Qin H, Zou R, Wei X, Chai S, Tang F. 2023. Effect of light intensity on photochemistry and dry weight accumulation of *Paphiopedilum emersoniii*. *Guangxi Sciences* 30:1171−1179

[42] Wang Y, Wen Z, Cai J, Tan Z, Wang Y, et al. 2011. Study on the photosynthesis characteristics of four species of *Paphiopedilum*. *Modern Agricultural Science and Technology* 20:200−02+04

[43] Poniewozik M, Parzymies M, Szot P, Rubinowska K. 2021. *Paphiopedilum insigne* morphological and physiological features during *in vitro* rooting and *ex vitro* acclimatization depending on the types of auxin and substrate. *Plants* 10:582

[44] Tian F, Yan FX, Jiang YL, Wang LP. 2017. Research on asymbiotic seed germination and tube seedling formation techniques of *Paphiopedilum hirsutissimum*. *Jiangsu Agricultural Sciences* 45:30−33

[45] Zhang M, Hu J, Zhou Y, Li JY, Feng YH, et al. 2019. Tissue culture and rapid propagation of *Paphiopedilum emersonii*. *Seed* 38:45−49

[46] Diengdoh RV, Das MC, Nongsiang A, Kumaria S. 2023. Efficient utilization of phytohormones for the *in vitro* proliferation of *Paphiopedilum* villosum Lindl. Stein - a Lady's Slipper orchid. *South African Journal of Botany* 154:387−393