In Vitro Leaf Culture and Plant Regeneration of Danxia Primulina minor (Postprint)

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Abstract

Primulina danxiaensis, an endemic species of Danxia landform in the Gesneriaceae family, is characterized by narrow distribution and scarce population, necessitating the use of plant tissue culture technology for propagation and conservation. This study established a rapid tissue culture propagation system for this species using leaf slices as explants, by screening optimal $HgCl_2$ surface sterilization duration, media for adventitious bud induction, bud proliferation, and rooting, as well as transplanting substrates for tissue-cultured seedlings. The results demonstrated: (1) The optimal sterilization protocol for explants consisted of 75% ethanol for 30 s followed by 0.1% $HgCl_2$ for 6 min, achieving a survival rate of 84.95%. (2) The optimal medium for adventitious bud induction was 1/2 MS + 6-BA 2 mg · L^{-1} + NAA 0.1 mg · L^{-1}, yielding a bud induction rate of 100% and an average of 38.35 buds per leaf after 40 days. (3) The optimal bud proliferation medium was 1/2 MS + 6-BA 3 mg · L^{-1} + NAA 0.2 mg · L^{-1}, achieving a bud proliferation coefficient of 7.54 after 50 days. (4) The optimal rooting medium was 1/2 MS + NAA 0.5 mg · L^{-1}, producing a rooting rate of 100% after 30 days with 26.28 roots per plant. (5) Tissue-cultured seedlings transplanted into three substrates—humus soil + perlite + vermiculite (volume ratio 1:1:1) from karst landform, peat soil + perlite + vermiculite (volume ratio 1:1:1), and perlite + vermiculite (volume ratio 1:1)—exhibited 100% survival, vigorous growth, and no significant differences among treatments. This established protocol enables mass propagation of Primulina danxiaensis, facilitating its conservation and utilization.

Full Text

In vitro Culture and Plant Regeneration from Leaves of Primulina danxiaensis

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Abstract

Primulina danxiaensis, an endemic species of the Danxia landform within the Gesneriaceae family, exhibits a narrow distribution range and limited population size, necessitating propagation and conservation via plant tissue culture techniques. This study established a tissue culture and rapid propagation system for *P. danxiaensis* using leaf segments as explants, screening for optimal $HgCl_2$ surface disinfection time, culture media for adventitious bud induction, bud proliferation and rooting, and transplantation substrates for tissue-cultured seedlings. The results were as follows: (1) The optimal disinfection procedure involved a 30-second immersion in 75% alcohol followed by a 6-minute soak in 0.1% $HgCl_2$, achieving an 84.95% survival rate of leaf explants. (2) The most effective bud induction medium was 1/2 MS supplemented with 6-benzyladenine (6-BA) 2 mg·L^{-1} and α-naphthaleneacetic acid (NAA) 0.1 mg·L^{-1}, resulting in a 100% bud induction rate and an average of 38.35 buds per leaf explant after 40 days. (3) The optimal bud proliferation medium was 1/2 MS containing 6-BA 3 mg·L^{-1} and NAA 0.2 mg·L^{-1}, yielding a proliferation coefficient of 7.54 after 50 days. (4) The rooting medium 1/2 MS + NAA 0.5 mg·L^{-1} produced a 100% rooting rate with 26.28 roots per plant after 30 days. (5) When transplanted onto three substrates—leaf mold from Karst landform + perlite + vermiculite (1:1:1, V/V/V), peat soil + perlite + vermiculite (1:1:1, V/V/V), and perlite + vermiculite (1:1, V/V)—the plantlets achieved 100% survival with robust growth and no significant differences among treatments. This study enables large-scale propagation of *P. danxiaensis*, contributing to its conservation and utilization.

Key words: *Primulina danxiaensis*, leaves, in vitro culture, adventitious bud, plant regeneration

Introduction

Primulina danxiaensis, belonging to the Gesneriaceae family, was originally classified under the genus *Chiritopsis* but was later revised and merged into *Primulina* (Wang et al., 2011; Weber et al., 2011). This perennial herb reaches 2–

10 cm in height with rhizomes 5–15 mm long, bearing white flowers from May to June. First reported in 2010, the species has strict environmental requirements, typically growing in rock crevices at 100–250 m altitude near shallow ponds, wet pits, or dark caves, often associated with small plants such as *Boea hygrometrica* (Gesneriaceae), *Pteris ensiformis* (Pteridaceae), *Pellionia radicans* (Urticaceae), *Lindsaea orbiculata* (Lindsaeaceae), and *Lophatherum gracile* (Poaceae) (Shen et al., 2010). Currently, it is only distributed in Danxia landform regions, with 12 populations in Danxiashan, Guangdong, each containing 50–70 individuals including 10–20 seedlings, and three populations in Yongxing, Hunan, and Ningdu and Xingguo, Jiangxi (Zhang and Yu, 2012; Tian et al., 2014), totaling approximately 800 plants. Chen et al. (2021) conducted genetic diversity analysis on 104 *P. danxiaensis* individuals from 12 Danxiashan populations using RAD-seq technology, revealing that *P. danxiaensis* is an extremely small population species with strong genetic structure, necessitating conservation measures for each existing population.

Primulina is the largest genus in Gesneriaceae in China, comprising approximately 230 species globally, with over 210 distributed in China (Ma et al., 2025). These plants hold high potential for horticultural development and possess medicinal value, including anti-inflammatory, analgesic, blood stasis-reducing, swelling-reducing, and snake venom-detoxifying effects (Wang et al., 2023; Luo et al., 2023). However, their narrow distribution and specialized habitats make them vulnerable to environmental destruction, requiring urgent germplasm conservation (Ning, 2017). As a *Primulina* species, *P. danxiaensis* features attractive flowers with exquisite form, offering good ornamental value suitable for indoor potted cultivation or garden bonsai decoration. Researchers from South China National Botanical Garden, including Ning Zulin, successfully hybridized *P. danxiaensis* (as female parent) with *Primulina depressa* from Danxiashan (as male parent) to develop the cultivar 'Zixia', which received international cultivar registration in 2014. Research on artificial propagation of *P. danxiaensis* thus holds important practical significance for conservation and utilization of *Primulina* germplasm resources.

Under natural conditions, *P. danxiaensis* primarily reproduces via seeds, but their small size and collection difficulty limit artificial seed propagation. *Primulina* species can be propagated through cuttings, with studies investigating key factors such as plant growth regulator concentrations, cutting methods, and substrates (Qi et al., 2018; Yan et al., 2019a, 2020). However, cutting propagation suffers from low propagation coefficients, seasonal constraints, and requires numerous leaves as starting material. Plant tissue culture technology overcomes seasonal limitations, enabling mass plant regeneration from minimal tissue, with conservation and propagation results for *Primulina* species being reported successively. Different species exhibit varying regeneration pathways: Ma et al. (2010) and Yang et al. (2012) induced somatic embryogenesis and adventitious buds from *Primulina tabacum* leaves; Fu et al. (2015) obtained regenerated plants from *Chirita ophiopogoides* leaves via adventitious bud induction; Yan et al. (2017) established a rapid propagation system for

Primulina glandaceistriata through both adventitious bud and callus induction; and He et al. (2024) established an in vitro regeneration system for *Primulina shouchengensis* through leaf-induced adventitious buds. These findings demonstrate the need to explore regeneration conditions for each specific species. To date, no studies have reported propagation techniques for *P. danxiaensis*.

This study utilized leaves of wild *P. danxiaensis* as material to compare the effects of different $HgCl_2$ disinfection times on leaf surface sterilization, investigate the effects of different plant growth regulator combinations on adventitious bud induction and proliferation to identify suitable media, and finally observe and statistically analyze the effects of different rooting media and transplant substrates on rooting and acclimatization of tissue-cultured seedlings. The objective was to establish a tissue culture and rapid propagation system for *P. danxiaensis* to achieve germplasm conservation, population expansion, and utilization, while providing technical references for propagation of other *Primulina* species.

Materials and Methods

1.1 Plant Material With approval from the Danxiashan Management Committee, wild *Primulina danxiaensis* plants were collected from their native habitat in Renhua Danxiashan, Shaoguan (95 m altitude, 113°44 09.17 E, 25°01 36.85 N) [Figure 1: see original paper] and subsequently cultivated in the Medicinal Plant Resource Garden at Shaoguan University [Figure 1: see original paper]. After a period of growth, the plants were used for subsequent experiments.

1.2.1 Surface Disinfection of Explants Leaves were excised from *P. danxiaensis* plants in the Medicinal Plant Resource Garden and transported to the laboratory. The leaves were soaked in detergent solution for 10 minutes, then gently washed with clean water before surface disinfection. Disinfection was performed by immersing in 75% alcohol for 30 seconds, rinsing three times with sterile water, then soaking in 0.1% $HgCl_2$ for 6, 8, or 10 minutes, followed by four rinses with sterile water. The leaves were then cut into segments approximately 0.8 cm × 0.8 cm [Figure 1: see original paper] and inoculated onto 1/2 MS + 6-BA 0.5 $mg \cdot L^{-1}$ + NAA 0.1 $mg \cdot L^{-1}$ medium. After 15 days, browning rate, contamination rate, and survival rate were calculated to compare the effects of different $HgCl_2$ disinfection times. Each treatment included at least 30 leaf segments with three replications. Browning rate (%) = (number of browned explants / total inoculated explants) × 100 (mild browning defined as $ $1/3 leaf area), contamination rate (%) = (number of contaminated explants / total inoculated explants) × 100, and survival rate (%) = (number of viable explants / total inoculated explants) × 100.

1.2.2 Adventitious Bud Induction Following disinfection with 75% alcohol for 30 seconds + 0.1% $HgCl_2$ for 6 minutes, leaf segments were used as explants. Based on the method of Ou (2023), 1/2 MS was used as the basal medium (same for all subsequent experiments). Nine adventitious bud induction media were prepared using combinations of 6-BA (0.5, 1, 2 $mg \cdot L^{-1}$) and NAA (0.1, 0.2, 0.5 $mg \cdot L^{-1}$). After 40 days, bud induction rate and average bud number per leaf were recorded. Each treatment included at least 30 leaf segments with three replications. Bud induction rate (%) = (number of explants with buds / number of viable explants) × 100, and average buds per leaf = total number of induced buds / number of leaves with buds.

1.2.3 Adventitious Bud Proliferation Induced adventitious buds (3–5 buds per cluster) were transferred to 12 bud proliferation media containing combinations of 6-BA (0.5, 1, 2, 3 $mg \cdot L^{-1}$) and NAA (0.05, 0.1, 0.2 $mg \cdot L^{-1}$) on 1/2 MS basal medium. Proliferation was assessed after 40 days to calculate the proliferation coefficient. Each treatment included at least 30 bud clusters with three replications. Proliferation coefficient = total number of buds after proliferation / number of buds inoculated.

1.2.4 Rooting Culture Proliferated adventitious buds were separated into individual shoots and transferred to three rooting media containing NAA at 0.1, 0.3, and 0.5 $mg \cdot L^{-1}$ in 1/2 MS. Rooting was observed after 30 days, with rooting rate and average root number recorded. Each treatment included at least 30 shoots with three replications. Seedling survival rate (%) = (number of viable shoots / total inoculated shoots) × 100, rooting rate (%) = (number of rooted shoots / number of viable shoots) × 100, and average root number = total number of roots / number of viable shoots.

1.2.5 Hardening and Transplantation Rooted tissue-cultured seedlings were hardened at room temperature for 7 days, then transplanted onto three different substrates: (1) leaf mold from Karst landform + perlite + vermiculite (1:1:1, V/V/V), (2) peat soil + perlite + vermiculite (1:1:1, V/V/V), and (3) perlite + vermiculite (1:1, V/V). Transplant survival rate and new leaf emergence rate were recorded to compare substrate effects. Transplant survival rate (%) = (number of surviving plants / total transplanted plants) × 100, and new leaf emergence rate (%) = (number of plants with new leaves / number of surviving plants) × 100.

1.2.6 Culture Medium Preparation and Culture Conditions **Culture Medium Preparation:** MS medium stock solutions were prepared using analytical-grade reagents. All media contained 30 $g \cdot L^{-1}$ sucrose and 7 $g \cdot L^{-1}$ agar powder, with pH adjusted to 5.8–6.0, then sterilized at 121 °C for 20 minutes.

Culture Conditions: Adventitious bud induction was performed in darkness for 10 days before transferring to light. Bud proliferation, rooting,

and acclimatization stages were all conducted under light conditions at $(25\$\pm1)°C, with illumination intensity of 1,500–2,000 lx and a 12h \cdot d\hat{}\{-1\}\$$ photoperiod.

1.3 Statistical Analysis Data were organized using Excel software and then analyzed using STST analysis software developed by Wang Shaohua of Nanjing Agricultural University. Single-factor randomized block design was employed for variance analysis (Li et al., 2023).

Results

2.1 Effects of $HgCl_2$ Disinfection Time on Leaf Surface Sterilization After disinfecting leaves with different $HgCl_2$ exposure times and inoculating onto culture medium for 10 days, both mild and severe browning were observed. As shown in , contamination rate, browning rate, and survival rate varied significantly among treatments, with contamination rates ranging from 1.36–7.53%, browning rates from 9.68–34.26%, and survival rates from 68.47–84.95%. Disinfection times of 8–10 minutes reduced contamination but increased browning and decreased survival, indicating that disinfection exceeding 8 minutes caused substantial damage to explants. The 6-minute $HgCl_2$ treatment produced the lowest browning rate (9.68%) and highest survival rate (84.95%), establishing it as the optimal disinfection time for *P. danxiaensis* leaves.

TABLE:1 Effect of different $HgCl_2$ disinfection times on leaf surface sterilization

$HgCl_2$ disinfection time (min)	Pollution rate (%)	Browning rate (%)	Survival rate (%)
6	7.53$\pm1.86a|9.68\pm3.23b|84.95\pm3.72a||8|1.36\pm1.18b|13.68\pm3.00b|68.47\pm4.55b||10|5.		

Note: Different lowercase letters within the same column indicate statistical differences (P<0.05). Mild browning ($ $1/3 leaf area) was included in browning rate calculations, while severe browning (>2/3 leaf area) was considered lethal.

2.2 Effects of 6-BA and NAA Combinations on Adventitious Bud Induction from Leaves After inoculating *P. danxiaensis* leaves onto bud induction medium [Figure 1: see original paper], leaves began to swell, thicken, and curl after 20 days [Figure 1: see original paper], with bud primordia and callus first appearing on leaf margins or surfaces [Figure 2: see original paper]. Differentiated adventitious buds gradually enlarged after 30 days [Figure 2: see original paper]. As shown in , all nine media achieved 100% bud induction rates without significant differences, but the average bud number per leaf varied significantly (21.34–38.35). At constant NAA concentrations, increasing 6-BA from

1–2 mg·L^{-1} tended to increase bud number. The optimal induction occurred at 6-BA 2 mg·L^{-1}, producing 32.23–38.35 buds per leaf—significantly higher than other treatments—with good bud growth and no vitrification. The combination of 6-BA 2 mg·L^{-1} + NAA 0.1 mg·L^{-1} yielded the maximum bud number (38.35), establishing it as the optimal medium for adventitious bud induction.

TABLE:2 Effects of different 6-BA and NAA concentrations on adventitious bud induction

6-BA (mg·L^{-1})	NAA (mg·L^{-1})	Adventitious bud induction rate (%)	Average number of buds per leaf	Growth situation
0.5	0.1	100	21.34$±1.86*d*|*Large, numerousbudswithgoodgrowth*||0.5|0.2|10	Large, numerous buds with good growth

2.3 Effects of 6-BA and NAA Combinations on Adventitious Bud Proliferation After transferring leaf-induced adventitious buds (3–5 buds per cluster) to 12 proliferation media, slow growth was observed within 10 days, followed by accelerated growth. Numerous adventitious buds proliferated around the original buds after 20 days [Figure 2: see original paper]. As shown in , proliferation coefficients varied significantly among the 12 media (1.77–7.54). Low 6-BA concentrations (0.5–1 mg·L^{-1}) combined with low NAA (0.05–0.2 mg·L^{-1}) produced low proliferation coefficients with minimal differences. In contrast, higher 6-BA concentrations (2–3 mg·L^{-1}) with low NAA (0.05–0.2 mg·L^{-1}) significantly increased proliferation coefficients to 3.35–7.54. The highest proliferation coefficient (7.54) was achieved with 6-BA 3 mg·L^{-1} + NAA 0.2 mg·L^{-1}, with good bud growth and no vitrification, establishing this as the optimal proliferation medium. Overall, when NAA ranged from 0.05–0.2 mg·L^{-1}, proliferation coefficients increased with 6-BA concentration from 1–3 mg·L^{-1}.

TABLE:3 Effects of different 6-BA and NAA concentrations on bud proliferation

6-BA (mg·L^{-1})	NAA (mg·L^{-1})	Proliferation coefficient	Bud growth situation
0.5	0.05	2.55$±0.09*fg*|*Slowproliferation, smallbuds*||0.5|0.1|2.66±0.07*f*|*Slowprolifer*	Rapid proliferation, large buds

2.4 Effects of Different Rooting Media and Cultivation Substrates on Rooting and Transplant Survival After transferring proliferated robust

adventitious buds to rooting media, buds grew well and developed adventitious roots at the base after approximately 20 days. As shown in , three rooting media showed no significant differences in seedling survival or rooting rates, both reaching 100%. However, average root number increased significantly with NAA concentration, ranging from 22.41–26.28 roots per plant, with roots appearing slender and 0.5–2 cm long. The medium 1/2 MS + NAA 0.5 $mg \cdot L^{-1}$ was determined to be optimal [Figure 2: see original paper].

Rooted seedlings were hardened and transplanted onto substrates [Figure 2: see original paper]. After 30 days, all three substrates supported 100% transplant survival, with over 97.78% of plants producing new leaves and growing vigorously . No visible differences were observed among seedlings in the three substrates, indicating that leaf mold from Karst landform + perlite + vermiculite (1:1:1, V/V/V), peat soil + perlite + vermiculite (1:1:1, V/V/V), and perlite + vermiculite (1:1, V/V) are all suitable for transplanting *P. danxiaensis* tissue-cultured seedlings.

TABLE:4 Effects of different media on adventitious bud rooting

NAA ($mg \cdot L^{-1}$)	Seedling survival rate (%)	Rooting rate (%)	Average number of roots	Root length (cm)
0.1	100	100	22.41$±2.14*b*|0.5–2||0.5|100|100|25.42±1.68*a*|0.5–2||0.5|100|100	

TABLE:5 Transplantation of rooted tissue-cultured seedlings

Cultivation medium	Transplant survival rate (%)	New leaf emergence rate (%)	Growth situation
Leaf mold from Karst landform + perlite + vermiculite (1:1:1, V/V/V)	100	100$±0.00*a*|*Growingwell*||*Perlite*+ *vermiculite*(1 : 1 : 1, *V*/*V*/*V*)|100|97.78±1.92*a*|*Growingwell*||*Perlite*+ *vermiculite*(1 : 1, *V*/*V*)|100|100±$0.00a	*Growingwell*||*Perlite*+

Discussion

3.1 Explant Treatment and Surface Disinfection Explant surface treatment and disinfection represent the first critical step for successful establishment of a rapid propagation system. Gesneriaceae leaves are typically disinfected using 75% alcohol + 0.1% $HgCl_2$, though specific protocols vary among species. Yang (2020) reported optimal disinfection of *Sinningia speciosa* leaves using 75%

alcohol for 30 seconds + 0.1% $HgCl_2$ for 10 minutes, while Zhang (2018) found 0.1% $HgCl_2$ for 9 minutes optimal for *Hemiboea subacaulis* leaves. In this study, 0.1% $HgCl_2$ treatment for 6–10 minutes on *P. danxiaensis* leaves revealed that 6 minutes provided the optimal balance among contamination rate, browning rate, and survival rate, indicating that *P. danxiaensis* leaves require relatively short disinfection. This aligns with Ou (2023), who achieved high survival rates for *Oreocharis esquirolii* leaves using 75% alcohol for 20 seconds + 0.1% $HgCl_2$ for 5 minutes. However, *O. esquirolii* leaves required 30 minutes of running water rinsing before disinfection, whereas *P. danxiaensis* leaves could not tolerate running water—only gentle washing for about 10 minutes—otherwise they turned white or brown, affecting normal growth. This may be attributed to the tender, thin nature of *P. danxiaensis* leaves, where running water damages mesophyll cells, providing a reference for pre-disinfection washing of Gesneriaceae leaves. This study also observed that contamination rate did not consistently decrease with extended $HgCl_2$ disinfection time; for instance, 10-minute disinfection sometimes resulted in higher contamination than 8 minutes, possibly due to endophytic bacteria and variation among leaf batches.

3.2 Leaf Differentiation and Adventitious Bud Induction Plant growth regulator types and concentrations significantly influence leaf differentiation and plant regeneration in Gesneriaceae. Chen et al. (2016) found that *Chirita swinglei* leaves could directly regenerate adventitious buds on medium containing 6-BA alone, while 6-BA + NAA combinations induced callus formation. Ouyang et al. (2016) reported that *Metabriggsia ovalifolia* leaves produced both adventitious buds and somatic embryos on medium with 6-BA alone (1.13–2.25 $mg \cdot L^{-1}$) or high 6-BA (5.63 $mg \cdot L^{-1}$). In contrast, this study found that *P. danxiaensis* leaves directly regenerated adventitious buds on 6-BA + NAA combination media, similar to Yan et al. (2019b) who observed direct regeneration of *Primulina guigangensis* leaves on 6-BA + IAA medium. Both require appropriate cytokinin-auxin combinations for direct leaf regeneration. These findings demonstrate that different Gesneriaceae species have varying requirements for plant growth regulators, which may control different regeneration pathways. These differences may be related not only to leaf characteristics but also to endogenous hormone content and types (Chen and Zhang, 2022). This study also revealed significant variation in bud number per leaf among different 6-BA (0.5–2 $mg \cdot L^{-1}$) + NAA combinations, with the highest bud number (38.35) achieved at the higher 6-BA concentration of 2 $mg \cdot L^{-1}$ + NAA 0.1 $mg \cdot L^{-1}$. Yan et al. (2018, 2019b) similarly reported maximum bud induction from *Primulina lutea* leaves at 6-BA 4.5 $mg \cdot L^{-1}$ + NAA 0.03 $mg \cdot L^{-1}$, and from *P. guigangensis* leaves at 6-BA 4 $mg \cdot L^{-1}$ + IAA 1.5 $mg \cdot L^{-1}$. These results indicate that higher cytokinin concentrations combined with appropriate auxin levels enhance adventitious bud differentiation capacity in Gesneriaceae leaves.

3.3 Adventitious Bud Proliferation The proliferation capacity of adventitious buds is a key factor determining large-scale propagation and effective

utilization of Gesneriaceae plants. To promote bud proliferation, media are typically supplemented with various cytokinin-auxin combinations. Wang et al. (2018) found that *Oreocharis mileense* adventitious buds proliferated well on low-concentration 6-BA 1 mg·L^{-1} + NAA 0.1 mg·L^{-1} medium. Li et al. (2013) reported that low 6-BA 0.5 mg·L^{-1} alone or combined with NAA 0.1 mg·L^{-1} was suitable for *Lysionotus serratus* bud proliferation. In contrast, this study found that *P. danxiaensis* adventitious buds showed low proliferation coefficients on low 6-BA (0.5–1 mg·L^{-1}) + NAA combinations, while higher 6-BA concentrations (2–3 mg·L^{-1}) with NAA promoted proliferation, with 6-BA 3 mg·L^{-1} + NAA 0.2 mg·L^{-1} being optimal. This finding aligns with Liu and Huang (2010) who reported that higher 6-BA 3 mg·L^{-1} + NAA 0.1 mg·L^{-1} promoted adventitious bud proliferation in *Lysionotus pauciflorus*. These results demonstrate that different Gesneriaceae species require different cytokinin-auxin ratios during the proliferation stage, with variations attributable not only to species and genotype but also to differential sensitivity to plant growth regulators (Lu et al., 2022; Wu et al., 2024).

3.4 Rooting During the rooting stage of Gesneriaceae tissue-cultured seedlings, MS or 1/2 MS basal media are typically used with various concentrations of NAA, IBA, or IAA. Li et al. (2013) found that *L. serratus* adventitious buds rooted best on MS medium containing 0.5 mg·L^{-1} IBA, IAA, or NAA. This study demonstrated that *P. danxiaensis* seedlings could root using NAA alone at low concentrations, with no significant differences in rooting rate (100%) among 0.1–0.5 mg·L^{-1} NAA in 1/2 MS medium, providing a reference for simplifying rooting protocols for Gesneriaceae. However, root number increased significantly with NAA concentration, consistent with findings in *P. lutea* (Yan et al., 2018) and *Saintpaulia ionantha* (Yang, 2020). This suggests that within a certain range, increasing NAA concentration significantly promotes rooting in Gesneriaceae tissue-cultured seedlings, likely by stimulating cell division and elongation in root primordia (Jiang and Zhou, 2000).

3.5 Cultivation Substrate Substrate selection is crucial for tissue-cultured seedling survival. Zhang et al. (2018) reported 100% transplant survival for *Paraisometrum mileense* seedlings when perlite and vermiculite were mixed with humus peat. Li et al. (2016) found that *Chirita eburnea* seedlings achieved 100% survival regardless of whether humus peat was added to perlite and vermiculite. In this study, *P. danxiaensis* seedlings showed robust growth and 100% survival on all three substrates, with or without humus from Karst leaf mold or peat soil, similar to Li et al. (2016). This may be related to the natural habitat of *P. danxiaensis*, which grows in rock crevices with thin soil layers, potentially depending more on soil moisture and aeration than organic matter content. Therefore, any of the three tested substrates can be used for transplanting *P. danxiaensis* seedlings.

In summary, this study used *P. danxiaensis* leaf segments as explants to investigate the effects of $HgCl_2$ disinfection time and plant growth regulators on

adventitious bud induction, proliferation, and rooting, and compared seedling performance across different transplant substrates. The resulting in vitro culture and plant regeneration system provides an effective asexual propagation method for large-scale multiplication of *P. danxiaensis*, offering technical support for its conservation and utilization.

References

CHEN JF, ZHANG JL, 2022. Plant tissue culture: The Third Edition [M]. Beijing: China Agriculture Press: 49-51.

CHEN SF, GUO W, CHEN ZX, et al., 2021. Strong genetic structure observed in *Primulina danxiaensis*, a small herb endemic to Mount Danxia with extremely small populations [J]. Frontiers in Genetics, 12:

CHEN YL, ZHANG YY, CHENG QW, et al., 2016. Plant regeneration via direct and callus-mediated organogenesis from leaf explants of *Chirita swinglei* (Merr.) W. T. Wang [J]. In Vitro Cellular & Developmental Biology-Plant, 52: 521-529.

FU CM, XIAN KH, HE JX, et al., 2015. Rapid propagation technique of *Chirita ophiopogoides* in vitro [J]. Seed, 34(4): 118-122.

HE DP, DUAN DW, XU B, et al., 2024. Adventitious bud induction and plant regeneration of detached leaves from *Primulina shouchengensis* (Z.Yu Li) Z.Yu Li [J/OL]. Molecular Plant Breeding: 1-16 [2024-03-21]. https://link.cnki.net/ur lid/46.1068.s.20240320.1912.017.

JIANG L, ZHOU X, 2000. The effect of auxins and cytokinins on the formation of lateral root primordia and the contents of endogenous hormones in lettuce seedlings [J]. Journal of Nanjing Agricultural University, 23(1): 19-22.

LI CX, JIANG LN, SHAO Y, et al., 2023. Biostatistics[M]. 6th ed. Beijing: Science Press: 237-240.

LI QS, DENG M, ZHANG J, et al., 2013. Shoot organogenesis and plant regeneration from leaf explants of *Lysionotus serratus* D. Don [J]. The Scientific World Journal, 2013: 280384.

LI QS, FAN XL, FANG J, et al., 2016. Experiment on the formula of growth media for *Chirita eburnea* tissue culture plantlet transplant [J]. Journal of Shanghai Institute of Technology (Natural Science), 16(2): 184-188.

LIU W, HUANG Y, 2010. Tissue culture and rapid propagation of *Lysionotus pauciflorus* Maxim [J]. Plant Physiology Communications, 46(2): 159-160.

LU JC, CAO LN, TONG GJ, et al., 2022. Establishment of callus induction and regeneration system of *Anemone silvestris* [J]. Chinese Bulletin of Botany, 57(2), 217-226.

LUO P, DENG ZS, WANG JJ, et al., 2023. Chemical constituents from *Primulina linearifolia* (W. T. Wang) Yin Z. Wang [J]. Guangxi Sciences, 2023, 30(5): 883-890.

MA GH, HE CX, REN H, et al., 2010. Direct somatic embryogenesis and shoot organogenesis from leaf explants of *Primulina tabacum* [J]. Biologia Plantarum, 54 (2): 361-365.

MA HS, WEN SJ, PAN B, et al., 2025. *Primulina gemella*, a newly recorded species of Gesneriaceae in China [J/OL]. Journal of Plant Resources and Environment: 1-5[2025-01-14]. https://link.cnki.net/urlid/32.1339.S.20250113.1439.006.

NING ZL, 2017. Systematic taxonomy and resource conservation on the *Primulina* hance (Gesneriaceae) [D]. Guangzhou: South China Agricultural University: 72-95.

OU MZ, 2023. Research on the endangered reasons and in vitro cultivation techniques of the extremely narrow region endemic plant, *Oreocharis esquirolii* [D]. Guiyang: Guizhou University: 52-53.

OUYANG Y, CHEN YL, LÜ JF, et al., 2016. Somatic embryogenesis and enhanced shoot organogenesis in *Metabriggsia ovalifolia* W. T. Wang [J]. Scientific Reports, 6: 24662.

QI HS, HUANG S, WANG JF, et al., 2018. Screening of leaf cuttage propagation methods for *Chirita heterotricha* [J]. Molecular Plant Breeding, 16(2): 535-540.

SHEN RJ, LIN SS, YU Y, et al., 2010. *Chiritopsis danxiaensis* sp. nov. (Gesneriaceae) from Mount Danxiashan, south China [J]. Nordic Journal of Botany, 28: 728-732.

TIAN J, KONG XL, DU Q, et al., 2014. Three newly recorded species of *Primulina* (Gesneriaceae) from Jiangxi province [J]. Jiangxi Forestry Science and Technology, 42(1): 37-38.

WANG DD, LI XH, CHENG ZY, et al., 2018. In vitro preservation and micropropagation of *Oreocharis mileense* (W. T. Wang) M. Möller & A. Weber (Gesneriaceae) through shoot organogenesis [J]. In Vitro Cellular & Developmental Biology-Plant, 54: 606-611.

WANG N, HU CY, XIAO PG, et al., 2023. Investigation on traditional pharmacology of the Gesneriaceae in China [J]. Journal of Chinese Medicinal Materials, 46(12): 2975-2979.

WANG YZ, MAO RB, LIU Y, et al., 2011. Phylogenetic reconstruction of *Chirita* and allies (Gesneriaceae) with taxonomic treatments [J]. Journal of Systematics and Evolution, 49(1): 50-64.

WEBER A, MIDDLETON DJ, FORREST A, et al., 2011. Molecular systematics and remodelling of *Chirita* and associated genera (Gesneriaceae) [J]. Taxon, 60(3): 767-790.

WU XY, LIAO ML, LI XR, et al., 2024. Establishment of regeneration system of *Chrysanthemum vestitum* with three floret forms [J]. Chinese Bulletin of Botany, 59(2): 245-256.

YAN HX, DENG JL, HUANG CY, et al., 2017. Tissue culture and rapid propagation of *Primulina glandaceistriata* [J]. Guihaia, 37(10): 1270-1278.

YAN HX, GUAN SK, DENG JL, et al., 2018. Adventitious bud induction and plant regeneration of detached leaves from *Primulina lutea* Yan Liu & Y. G. Wei [J]. Molecular Plant Breeding, 16(1): 211-216.

YAN HX, GUAN SK, ZHOU JY, et al., 2019a. Optimization of factors affecting cutting of *Primulina macrorhiza* (D. Fang & D. H. Qin) Mich. Möller & A.Weber based on orthogonal experiment [J]. Journal of Southern Agriculture, 50(11): 2519-2524.

YAN HX, GUAN SK, ZHOU JY, et al., 2020. Effects of different plant growth regulators, leaf positions and substrate components on propagation of three species of *Primulina* [J]. Southwest China Journal of Agricultural Sciences, 33(1): 126-134.

YAN HX, HUANG CY, ZHANG ZB, et al., 2019b. Tissue culture and plant regeneration of leaves of *Primulina guigangensis* [J]. Chinese Journal Tropical Crops, 40(1): 98-106.

YANG CX, 2020. Tissue culture and application of two ornamental plants of Gesneriaceae [D]. Harbin: Northeast Forestry University: 7-26.

YANG XY, LÜ JF, TEIXEIRA DA SILVA JA, et al., 2012. Somatic embryogenesis and shoot organogenesis from leaf explants of *Primulina tabacum* [J]. Plant Cell Tissue Organ Culture, 109:

ZHANG GZ, YU XL, 2012. *Chiritopsis* W. T. WANG: a newly recorded genus of Gesneriaceae from Hunan Province [J]. Journal Central South University of Forestry & Technology, 32(6): 135-137.

ZHANG LN, HE J, ZHANG X, et al., 2018. Root induction and matrix optimization of *Paraisometrum mileense* tissue culture seedling [J]. Journal of West China Forestry Science, 47(4): 69-73.

ZHANG XL, 2018. Study on micropropagation technique of rare and wild plant with extremely small population [D]. Guangzhou: Zhongkai University of Agriculture and Engineering: 57-61.

Note: Figure translations are in progress. See original paper for figures.