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A complete and efficient in vitro micropropagation system for the endangered wild Lilium nepalense using scale explants

Linyan Qi
Southwest Forestry University
Xiaodong Wu
Southwest Forestry University
Shengjie Sun
Southwest Forestry University
Rong Wang
Southwest Forestry University
Yinlong Zhang
Southwest Forestry University
Xiahong He
Southwest Forestry University
Dan Li
lidan@swfu.edu.cn

Southwest Forestry University

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Abstract

Lilium nepalense is an endangered wild lily with high ornamental and horticultural value, yet its natural propagation is slow and wild populations are declining. Using scale segments as explants, we developed an efficient *in vitro* micropropagation system for Chinese *L. nepalense*. The optimal disinfection protocol (75% ethanol for 30 s followed by 2.5% sodium hypochlorite for 7 min) achieved a 94% explant survival rate. The highest adventitious bud induction rate (88%) was obtained from basal scale segments on MS medium with 0.5 mg/L NAA and 1.0 mg/L 6-BA. The optimal multiplication medium (MS with 0.7 mg/L NAA and 1.0 mg/L 6-BA) yielded a proliferation coefficient of 11.03 ± 0.6. Half-strength MS medium with 0.1 mg/L NAA and 0.5 mg/L IBA resulted in 100% rooting and an average of 10.8 roots per plantlet, without abnormal callus formation. For acclimatization, a 1:1 (v/v) mixture of peat and river sand was most effective. Notably, we introduced a microbial priming strategy using native soil microbial filtrate during acclimatization to enhance plantlet adaptability. This optimized system supports germplasm conservation, artificial propagation, and horticultural use of this endangered species.

1 Introduction

Liliaceae are perennial herbaceous plants characterized by their distinctive underground bulbs and elegant flower forms [1, 2]. The genus *Lilium* comprises approximately 100 species worldwide, mainly distributed in temperate zones of the Northern Hemisphere [3, 4]. As one of the key centers of origin and diversity of the genus, China harbors rich germplasm resources [5, 6]. The species are valued not only for their ornamental appeal and prominent position in global horticulture, attributed to their elegant flower morphology, diverse coloration, and intense fragrance [7–9], but also for their potential medicinal, cosmetic, and edible applications. Research indicates that the lilyosides present in these plants can modulate the nervous system, thereby improving sleep and reducing anxiety [10, 11]. The mucilaginous substances and vitamins in these plants may stimulate skin metabolism, thereby increasing skin elasticity and radiance [12, 13]. In addition, the bulbs are rich in starch, protein, dietary fiber, and various bioactive substances, suggesting promising potential for culinary and industrial processing [14, 15].

Wild *Lilium nepalense* is a perennial bulbous plant belonging to the section *Martagon* of the genus *Lilium* within the family Liliaceae. It is native to Yunnan and Tibet in China and is usually found in mixed forest scrublands at altitudes ranging from 1,400 to 2,900 meters [16, 17]. Its flowers have a unique shape, resembling a trumpet or a lantern with recurved petals, while their color ranges from light yellow to greenish-yellow, with distinct purple spots on the throat. With a fragrance and high ornamental value, it is an excellent material for cut flower production and for use in landscape gardening [18–20]. In addition, *L. nepalense* exhibits rapid growth and strong adaptability. It possesses desirable traits such as stress tolerance, which are often rare in cultivated lilies. These traits make it highly valuable for lily germplasm innovation and new variety development [21, 22]. However, due to factors such as unsustainable harvesting and habitat destruction, the natural populations of *L. nepalense* are declining, characterized by sharply decreasing population sizes and a slow natural reproduction rate. It is now considered

threatened, making it imperative to employ artificial propagation techniques to achieve both germplasm conservation and large-scale utilization.

Tissue culture technology is an effective method for plant asexual propagation. It offers advantages such as rapid multiplication, a high propagation coefficient, and season-independent operation. This technique can effectively circumvent common problems associated with traditional propagation methods, such as the spread of pests and diseases, and germplasm degeneration. Consequently, it is well suited for large-scale industrial seedling production [23, 24]. Currently, preliminary progress has been made in tissue culture research on *L. nepalense*. Using bulb scales as explants, Li Biao et al. (2001) established a three-step culture system encompassing “induction, proliferation, and rooting” and identified the appropriate basic culture medium formulations for each distinct stage [25]. Wawrosch et al. (2001) developed a protocol for *L. nepalense* using zeatin as the cytokinin source, achieving efficient shoot multiplication from twin-scale explants [26].Subsequently, Malla and Malla (2004) further investigated the *in vitro* propagation of this Himalayan herb, contributing to the conservation efforts in Nepal [27].Wang Hongxia et al. (2008) optimized the culture protocol, thereby enhancing propagation efficiency [28]. Meanwhile, Wu Yingying et al. (2013), focusing specifically on *L. nepalense*, systematically screened hormone combinations for callus induction, bud proliferation, and rooting, ultimately establishing an *in vitro* regeneration system [29]. However, conventional tissue culture protocols primarily focus on optimizing nutrient media and plant growth regulator combinations, often overlooking the potential role of beneficial rhizosphere microorganisms in enhancing plantlet resilience during the critical transition from *in vitro* to ex vitro conditions. A common yet often overlooked issue in lily micropropagation is the formation of dysfunctional "pseudoroots". Such pseudoroots lack structural integrity, are prone to shedding during handling, and consequently reduce the survival rate of transplanting.Recent studies have highlighted the importance of microbial priming—a phenomenon in which prior exposure to beneficial microbes primes the plant's defense and growth pathways, leading to improved stress tolerance and performance [30, 31]. To address these gaps, this study conducted a comprehensive trial encompassing the entire process for the Chinese provenance of *L. nepalense*, from explant disinfection, induction, and proliferation to rooting culture, and achieved systematic optimization of tissue culture technology tailored to the physiological characteristics of this Chinese endemic germplasm. Notably, we introduced an innovative microbial priming strategy during the acclimatization phase, applying a native soil microbial filtrate to simulate natural rhizosphere interactions, prime plantlet defense and growth pathways, and enhance stress tolerance and adaptability, thereby overcoming the limitations of conventional media- and hormone-centric protocols and improving post-transplant establishment. This study aims to establish an efficient tissue culture and micropropagation system for *L. nepalense*, providing comprehensive technical support for the germplasm conservation, large-scale artificial cultivation, and horticultural development of this threatened species.

2 Materials and methods

2.1Plant materials, reagents, and equipment

For this study, wild bulbs of *L. nepalense* were collected from Langhe village, Chenghai Town, Yongsheng County, Lijiang City. Healthy, plump bulbs with dense scales, no visible damage, and a diameter of 10–13 cm were selected. After collection, bulbs were stored at 4℃ for no more than two weeks before use. The outer scales were removed, and the inner healthy scales were used as explants. Agar, Naphthaleneacetic acid (NAA); 6-Benzylaminopurine (6-BA); Indole-3-butyric acid (IBA); 75% ethanol; Carbendazim; Sucrose;NaOH solution; HCl solution.Peat soil, river sand, perlite, and vermiculite were purchased from the Baihanmei brand. Pipette; scalpel; forceps; culture flask; sterilizer; inoculation tray; pH test paper.

2.2 Explant preparation

Healthy bulbs of *L. nepalense* were selected. Surface impurities were removed with a soft brush and then rinsed with running tap water. The scales were then immersed in a mild detergent solution for 5 min. After this immersion, the scales were gently washed with sterile cotton pads, washed again with running water for 30 min, and finally transferred to the laminar flow chamber for further use.

2.3 Explant sterilization

After transfer to the laminar flow chamber, the lily scales were first sterilized by immersion in 75% ethanol for 30 s, followed by 2 to 3 rinses with sterile distilled water. They were then transferred to sterile containers filled with sodium hypochlorite solutions of different concentrations (2.0%, 2.5%, and 3.0%) and agitated for 6, 7, and 8 minutes, respectively, for sterilization. After sterilization, the explants were washed with 3 to 5 rinses of sterile distilled water, transferred to sterile containers, and set aside for further use. Finally, they were inoculated on Murashige and Skoog medium [32, 33], each treatment consisting of 30 explants with three replicates. The inoculation time was recorded, and the survival rate of the explants was recorded at 10 days post-inoculation.

Table 1
Experimental design of different sterilization treatments

Treatment	75% Ethanol (s)	Sodium Hypochlorite (%)	Total Explants (n)	Sterilization Time (s)
A1	30	2.0	30	6
A2	30	2.0	30	7
A3	30	2.0	30	8
A4	30	2.5	30	6
A5	30	2.5	30	7
A6	30	2.5	30	8
A7	30	3.0	30	6
A8	30	3.0	30	7
A9	30	3.0	30	8

2.4 Adventitious bud induction experiment

MS medium was used as the basal medium and was supplemented with different concentrations of NAA (0.1, 0.5, and 1.0 mg/L) and 6-BA (0.1, 0.5, and 1.0 mg/L). The medium contained sucrose at 30 g/L and agar at 7 g/L. The pH of all media was adjusted to 5.8 using NaOH or HCl solution, followed by autoclaving at 121°C for 20 minutes. Culture conditions were maintained at a temperature of 25 ± 1°C, with a photoperiod of 16 h light / 8 h dark and a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Each 250 mL culture flask was filled with 50 mL of medium. At inoculation, the lily scales were divided equally into upper, middle, and lower sections (Fig. 1B), resulting in a total of nine treatment groups. Each group was inoculated with 30 explants in triplicate, and adventitious bud induction was recorded after 30 days.

Table 2
Experimental design for induction culture

Serial number	Hormone combinations for culture media
B1	MS + 6-BA 0.1mg/L + NAA 0.1mg/L
B2	MS + 6-BA 0.1mg/L + NAA 0.5mg/L
B3	MS + 6-BA 0.1mg/L + NAA 1.0mg/L
B4	MS + 6-BA0.5mg/L + NAA 0.1mg/L
B5	MS + 6-BA 0.5mg/L + NAA 0.5mg/L
B6	MS + 6-BA 0.5mg/L + NAA 1.0mg/L
B7	MS + 6-BA 1.0mg/L + NAA 0.1mg/L
B8	MS + 6-BA 1.0mg/L + NAA 0.5mg/L
B9	MS + 6-BA 1.0mg/L + NAA 1.0mg/L

2.5 Experimental design for proliferation

The tissue-cultured plantlets were transferred to a proliferation medium containing different concentrations of NAA (0.1, 0.3, 0.5, 0.7, 1.0 mg/L) and a constant concentration of 6-BA (1.0 mg/L) [34, 35]. The medium also contained 30 g/L sucrose and 7 g/L agar. These treatment groups were designated as C1 to C5 (Table 3). Each treatment consisted of three replicates with a total of 90 explants per treatment. Proliferation results were recorded after 30 days of culture.

Table 3
Experimental design for proliferation culture

Serial number	Hormone combinations for culture media
C1	MS + 6-BA 1.0 mg/L + NAA 0.1mg/L
C2	MS + 6-BA 1.0 mg/L + NAA 0.3mg/L
C3	MS + 6-BA 1.0 mg/L + NAA 0.5mg/L
C4	MS + 6-BA 1.0 mg/L + NAA 0.7mg/L
C5	MS + 6-BA 1.0 mg/L + NAA 1.0mg/L

2.6 Experimental design for rooting

The lily plantlets, after proliferation when they reached a height of 2–3 cm, were transferred to half-strength Murashige and Skoog (1/2 MS) medium [36, 37] containing 30 g/L sucrose and 7 g/L agar, supplemented with different concentrations of NAA (0.1, 0.5, 1.0 mg/L) and IBA (0.1, 0.3, 0.5 mg/L). These treatment groups were designated as D1 to D9 (Table 4), aimed at promoting root system development. Each treatment, comprising three replicates, included a total of 90 explants. Rooting results were recorded after 30 days of culture.

Table 4
Experimental design for rooting culture

Serial number	Hormone combinations for culture media
D1	1/2MS + NAA 0.1mg/L + IBA 0.1 mg/L
D2	1/2MS + NAA 0.1mg/L + IBA 0.3 mg/L
D3	1/2MS + NAA 0.1mg/L + IBA 0.5 mg/L
D4	1/2MS + NAA 0.5mg/L + IBA 0.1 mg/L
D5	1/2MS + NAA 0.5mg/L + IBA 0.3 mg/L
D6	1/2MS + NAA 0.5mg/L + IBA 0.5 mg/L
D7	1/2MS + NAA 1.0mg/L + IBA 0.1 mg/L
D8	1/2MS + NAA 1.0mg/L + IBA 0.3 mg/L
D9	1/2MS + NAA 1.0mg/L + IBA 0.5 mg/L

2.7 Experimental design for acclimatization and transplantation

Acclimatization was performed 7–10 days before transplantation. Initially, the caps of culture vessels are loosened to create a small opening, which is followed by full removal. This process allowed the plantlets to acclimatize to the external environmental conditions.

To simulate the natural rhizosphere microbial environment and enhance the stress tolerance of plantlets during the transition from *in vitro* to ex vitro conditions, a native soil microbial filtrate was introduced during the acclimatization process.Rhizosphere soil collected from the native habitat of *L. nepalense* was mixed with sterile water (100 g soil per 200 mL water), shaken for 30 min, allowed to settle for 24 h, and then sequentially filtered through qualitative filter paper and a 0.45 μm membrane filter. The resulting filtrate was stored at 4°C and allowed to return to room temperature before use.Tarting from the first day of acclimatization, immediately after loosening the vessel caps, 5 mL of the microbial filtrate was uniformly sprayed onto the plantlets and the surface of the culture medium. As the vessel caps were gradually opened, the same volume of filtrate was applied daily. One day before transplantation, a final 5 mL of filtrate was administered. This step was integrated as a routine procedure into the acclimatization protocol for all plantlets prior to transplantation.

After acclimatization, the plantlets were carefully removed using forceps. The roots were rinsed with running tap water to remove the adhesion medium and then immersed in 500 mg/L of carbendazim solution for 5–10 minutes to allow for disinfection. After disinfection, the plantlets were transplanted into various substrates that had been autoclaved. Five substrate formulations were prepared, designated as E1 to E5 (Table 5). For each treatment, 10 pots of plantlets were transplanted, and three replicates were maintained. Plantlets were regularly monitored for growth, and root length, root number, and plant height were recorded after 30 days.

Table 5
Experimental design for acclimatization and transplantation

Serial number	Peat soil	River sand	Perlite	Vermiculite
E1	1			
E2	1	1		
E3	1		1	
E4	1			1
E5	1	1	1	1

2.8 Statistical analysis

All experiments were conducted using a completely randomized design with three replicates per treatment. Data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS software (IBM Corp., Armonk, NY, USA).One-way analysis of variance (ANOVA) was used to compare means among treatments, followed by multiple comparisons. A significance level of $p < 0.05$ was considered statistically significant.

3 Results

3.1 Efficiency of the *L. nepalense* explant disinfection procedures

The effectiveness of the sterilization protocol depends on both disinfectant concentration and exposure time. Excessively high or low concentrations of sodium hypochlorite, as well as inappropriate exposure times, can lead to higher contamination rates and reduced survival rates of the explants. An analysis of all sterilization combinations revealed that the sodium hypochlorite concentration and the sterilization time had a highly significant effect on the contamination and survival rates of the explants (Fig. 1). Treatment A2 exhibited the lowest survival rate (57%) under the sterilization condition of 75% ethanol for 30 s followed by 2.0% sodium hypochlorite for 7 min. Treatment A5 exhibited the highest survival rate (94%) under the sterilization condition of 75% ethanol for 30 s followed by 2.5% sodium hypochlorite for 7 min.The results of this study indicate that Treatment A5 is the optimal sterilization method for *L. nepalense* scales.

3.2 Effects of different plant growth regulators (PGRS) on the induction of various parts of L. nepalense scales

Different plant growth regulator combinations had a significant effect on the bud induction rate, with particularly notable differences observed among various parts of the scales (Fig. 2). Overall, explants from the lower section exhibited the highest average bud emergence rate (88%), followed by those from the middle section (81%), with the upper section showing the lowest rate (44%).Among the nine plant growth regulator combinations, B3 (NAA 0.1 mg/L + 6-BA 1.0 mg/L), B6 (NAA 0.5 mg/L + 6-BA 1.0 mg/L), and B9 (NAA 1.0 mg/L + 6-BA 1.0 mg/L) all exhibited relatively high bud emergence rates from the lower section (≥ 93%). Furthermore, as the concentration of 6-BA increased, the bud emergence rates from the middle and lower sections exhibited an upward trend, while those from the upper section showed no significant change. An increase in NAA concentration inhibited bud emergence from the upper section, while the middle and lower sections maintained a relatively high budding capacity. Overall, regarding the promotive effect of plant growth regulator combinations on budding, the combination of a low concentration of NAA and a high concentration of 6-BA applied to explants from the lower section yielded the optimal results.

3.3 Effects of different plant growth regulator (PGRS) combinations on the proliferation of *L. nepalense*

In the proliferation experiment conducted with tissue-cultured plantlets of L. nepalense, different plant growth regulator combinations were found to influence their proliferation efficacy, with C4 (0.7 mg/L NAA + 1.0 mg/L 6-BA) exhibiting the best proliferation performance, while the other treatments showed relatively inferior results (Fig. 3). Comparative analysis revealed that increasing the NAA concentration from 0.1 mg/L to 0.7 mg/L enhanced the proliferation efficiency of the tissue-cultured plantlets. The number of proliferated buds initially decreased and then increased with rising NAA concentration,

reaching an optimum at 0.7 mg/L and the lowest value at 0.1 mg/L. The results of this study indicate that Treatment C4 is the optimal medium for the proliferation of *L. nepalense*.

Figure 3. A:Effects of different plant growth regulator (PGRS) combinations on the proliferation of *L. nepalense*.Different lowercase letters above the bars indicate significant differences at $p < 0.05$.B:Proliferation of *in vitro* Plantlets of *L. nepalense*.

3.4 Influence of various plant growth regulators (PGRS) and their concentrations on the rooting of L. nepalense tissue-cultured plantlets

Different concentrations of indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA) exerted distinct effects on the rooting of L. nepalense (Fig. 4). These results indicated that the concentration of NAA plays a critical role in the rooting process.Compared to the treatment groups with 0.5 mg/L and 1 mg/L NAA, the 0.1 mg/L NAA treatment resulted in superior rooting. In contrast, 1 mg/L NAA inhibited rooting and substantially reduced the rooting rate. Regarding IBA concentration, the 0.5 mg/L and 0.1 mg/L IBA treatments showed comparable rooting effects. The D7 treatment (0.1 mg/L NAA + 0.5 mg/L IBA in 1/2MS medium) exhibited the best rooting performance, with an average of 10.8 roots per plantlet and a rooting rate as high as 100%, which was significantly superior to that of the other treatments. In contrast, the B6 treatment (1 mg/L NAA + 0.3 mg/L IBA in 1/2MS medium) showed the poorest rooting performance, with an average root number of only 3.87. This may be attributed to a synergistic inhibitory effect between the high concentration of NAA and the moderate concentration of IBA, which hindered the normal growth and development of the root system in the *in vitro* plantlets. Therefore, the D7 medium was identified as the optimal medium for the rooting culture of *L. nepalense*.

Figure 4. A:Influence of various plant growth regulators (PGRS) and their concentrations on the rooting of *L. nepalense* tissue-cultured seedlings.Different lowercase letters above the bars indicate significant differences at $p < 0.05$.B:Rooting response of *L. nepalense* to different treatments.

3.5 Effects of different substrates on the acclimatization and transplantation of *L. nepalense*

In the acclimatization and transplantation experiment of *L. nepalense*, different substrates and their formulations affected plant growth, among which Treatment E2 (peat soil : river sand = 1:1) demonstrated the best growth performance (Fig. 5). In this substrate, the transplanted plants exhibited vigorous growth and clearly superior root development, with an average of 11.93 roots and an average root length of 7.04 cm, significantly outperforming all other treatment groups.Due to its poor aeration, Treatment E1 (peat soil) caused hypoxia in the root system, which severely compromised rooting efficacy. Consequently, both the average root number and root length were significantly lower than those in the other groups.The experimental results indicate that Treatment E2 (peat soil : river sand = 1:1) ensures both good water retention and suitable aeration, providing an ideal environment for root growth of *L. nepalense* tissue-cultured plantlets. Therefore, it is the optimal substrate choice for acclimatization and transplantation.

Figure 5. A: Effects of different substrates on the acclimatization and transplantation of *L. nepalense*. B: Growth performance of plantlets under different substrate treatments. C: Transplantation performance of plantlets under different substrate treatments.

4 Discussion

Wild *Lilium nepalense*, a species belonging to the spotted group of the genus Lilium, is both rare and of high ornamental value. Owing to habitat destruction and its intrinsically slow propagation rate, this species is currently at risk of endangerment. Tissue culture technology represents a key approach for its germplasm conservation and large-scale propagation. In this study, using scale segments of *L. nepalense* collected from China as explants, we successfully established a complete *in vitro* micropropagation system covering the entire process from explant disinfection to the transplantation of acclimatized plantlets. This system was optimized according to the physiological characteristics of the Chinese provenance, thus providing reliable technical support for the conservation and utilization of this endangered species.

Explant disinfection is a prerequisite for establishing an aseptic tissue culture system. The compatibility of disinfectant type, concentration, and treatment duration directly determines the contamination rate and explant viability [38]. Compared with previous studies [29], the disinfection protocol in this study was optimized by treating explants with 75% ethanol for 30 s followed by 2.5% sodium hypochlorite for 7 min, which reduced the contamination rate to 6% and increased the survival rate to 94%. This significant improvement can be primarily attributed to the compatibility between the mode of action of the disinfectants and the characteristics of the explants: although mercuric chloride exhibits strong disinfection efficacy, its high toxicity readily induces explant browning; in contrast, sodium hypochlorite exerts its bactericidal effect through oxidation, causing less tissue damage and reducing the risk of browning, albeit with relatively weaker disinfection efficacy.

Regarding the induction of adventitious buds from scale segments, the results of this study demonstrated that the basal scale segments exhibited the highest induction efficacy, with an average induction rate of 88%, which was significantly higher than that of the middle and apical segments. This trend is consistent with the findings of Song et al. (2025) in the Oriental lily cultivar 'Pacific', namely that the basal part of scale segments possesses higher regenerative potential [39]. This phenomenon may be attributed to the higher endogenous cytokinin activity, denser vascular bundle distribution, and more efficient transport of nutrients and hormones in the basal tissue [40, 41]. Furthermore, the combination of 0.5 mg/L NAA and 1.0 mg/L 6-BA used in this study demonstrated a clear synergistic effect during the induction phase, indicating that an appropriate ratio of exogenous plant growth regulators can significantly enhance the adventitious bud induction rate.

In contrast to studies that commonly employ 6-BA as the primary cytokinin during the proliferation stage [25, 28, 29], Wawrosch et al. (2001) adopted a different strategy for propagating *L. nepalense* by incorporating 20 μM zeatin as the sole cytokinin in MS medium, which induced an average of more than

seven adventitious buds per explant [26]. Although this zeatin-based system exhibited a certain level of effectiveness, its efficiency was considerably lower than the 11.03 adventitious buds per explant achieved in the present study using a combination of 1.0 mg/L 6-BA and 0.7 mg/L NAA. This study found that the combination of MS medium supplemented with 1.0 mg/L 6-BA and 0.7 mg/L NAA achieved a proliferation coefficient of 11.03 ± 0.6, which was significantly higher than those reported in the four aforementioned studies. The key to this improvement lies in the appropriate increase of NAA concentration, which optimized the cytokinin-to-auxin ratio. This balance promoted the formation of shoot clusters while preventing the development of slender, weak plantlets. This discrepancy indicates that the proliferation of *L. nepalense* responds to auxin concentration within a narrower optimum range. In the three aforementioned studies, the employed NAA concentrations were relatively low, thus failing to fully exploit the proliferation potential of the explants.

The main differences during the rooting stage lie in the basal medium and the plant growth regulator formulations. This study found that the combination of half-strength MS medium with 0.1 mg/L NAA and 0.5 mg/L IBA not only achieved a 100% rooting rate but also produced an average of 10.8 robust roots per plantlet without abnormal callus formation. This result is superior to those obtained with the single plant growth regulator treatment and the low-concentration IBA treatment used in previous studies [28, 29]. Half-strength MS medium reduced the salt concentration, thereby alleviating osmotic stress on the developing root system. Furthermore, the synergistic interaction between NAA and IBA promoted both rooting and root quality. In contrast, the two aforementioned studies did not address such hormonal synergy, which may account for the insufficient root number or vigor observed in their treatments.

In addition to optimizing the culture medium composition and hormone ratios, this study introduced an ecologically inspired step during the acclimatization phase: as the culture vessel caps were gradually opened, a native soil microbial filtrate was sprayed onto the plantlets. This procedure simulates the natural contact between plants and rhizosphere microorganisms during the transition from a sterile *in vitro* environment to the soil environment, representing an application of a microbial priming strategy during the late stage of tissue culture. Pandey et al. (2000) first demonstrated that inoculating rhizosphere bacteria during acclimatization could significantly improve the transplantation survival rate of micropropagated tea plants, a process they termed "biotization." Mechanistically, plant growth-promoting rhizobacteria can enhance plant stress tolerance by inducing systemic resistance and modulating hormonal balance [42, 43]. These effects are underpinned by the phenomenon of "priming"—whereby prior exposure to a stimulus enables plants to activate defense responses more rapidly and robustly upon subsequent stress encounters[30]. In this study, the application of native microbial filtrate during the gradual opening of culture vessel caps harnesses these microorganisms as "priming signals," allowing the plantlets to establish early contact with microbial communities from their native habitat prior to transplantation, thereby activating relevant physiological pathways and preparing them for post-*in vitro* establishment.

The growth substrate significantly influences the transplantation survival rate of plantlets. In this study, a 1:1 (v/v) mixture of peat and river sand was found to be the most effective substrate during the

acclimatization and transplantation process. Plantlets cultivated in this substrate exhibited the most vigorous growth, with well-developed root systems, and showed significantly greater average root length and root number compared to those in other treatments. This advantage can be attributed to the favorable balance achieved by this mixture among water retention, aeration, and structural stability. This finding is corroborated by the work of Liu et al. (2021) on the nurse culture of *Lilium regale* bulblets, in which an optimized substrate formulation of imported peat, vermiculite, and river sand (1:1:1, v/v/v) also achieved the maximum fresh weight, circumference, and yield of bulblets under fertilizer-free conditions [44]. Both cases demonstrate that an appropriate substrate composition can optimize the root growth environment, promote the efficient utilization of nutrients and water, and thereby enhance transplantation survival rates and plant growth vigor.

5 Conclusions

In this study, scales of *L. nepalense* were used as explants to systematically optimize key technical links in its *in vitro* regeneration process, including explant sterilization, adventitious bud induction, shoot proliferation, rooting culture, and acclimatization and transplantation. A complete and efficient tissue culture and rapid propagation system was successfully established, which not only addresses the technical bottlenecks of low explant survival rate and inefficient proliferation but also integrates a microbial priming step during acclimatization—using native soil filtrate—to enhance plantlet adaptability upon transplantation. Building upon previous research, the optimized system enables efficient clonal propagation of *L. nepalense* and provides robust technical support for its germplasm conservation, large-scale artificial cultivation, and germplasm innovation. Compared with earlier protocols, this system achieves significant improvements in both proliferation efficiency and transplantation survival, effectively overcoming the limitations imposed by slow natural propagation and habitat destruction. By tailoring a comprehensive technical system to the physiological characteristics of the Chinese provenance of *L. nepalense*, this study lays a foundation for the sustainable utilization of this species and enriches the tissue culture technology system for spotted-group lilies. Future research should focus on genotypic differences among geographic populations, optimization of environmental factors during acclimatization, assessment of the genetic stability of long-term subcultured plantlets, and elucidation of the microbial priming mechanisms, in order to further enhance the potential for industrial application of this propagation system.

Figure 6. The protocol of *L. nepalense* micropropagation and plant regeneration.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare no competing interests.

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Author Contributions

Conceptualization: Linyan Qi, Xiahong He, Dan Li; Data curation: Linyan Qi, Xiaodong Wu, Rong Wang, Yinlong Zhang; Formal analysis: Linyan Qi, Xiaodong Wu, Shengjie Sun, Dan Li, Rong Wang; Investigation: Linyan Qi, Xiahong He, Dan Li; Methodology: Linyan Qi, Xiaodong Wu, Xiahong He, Dan Li;Software: Linyan Qi, Shengjie Sun, Rong Wang, Yinlong Zhang; Writing—original draft: Linyan Qi, Xiaodong Wu, Xiahong He, Dan Li; Funding acquisition: Xiahong He, Dan Li.

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Figures

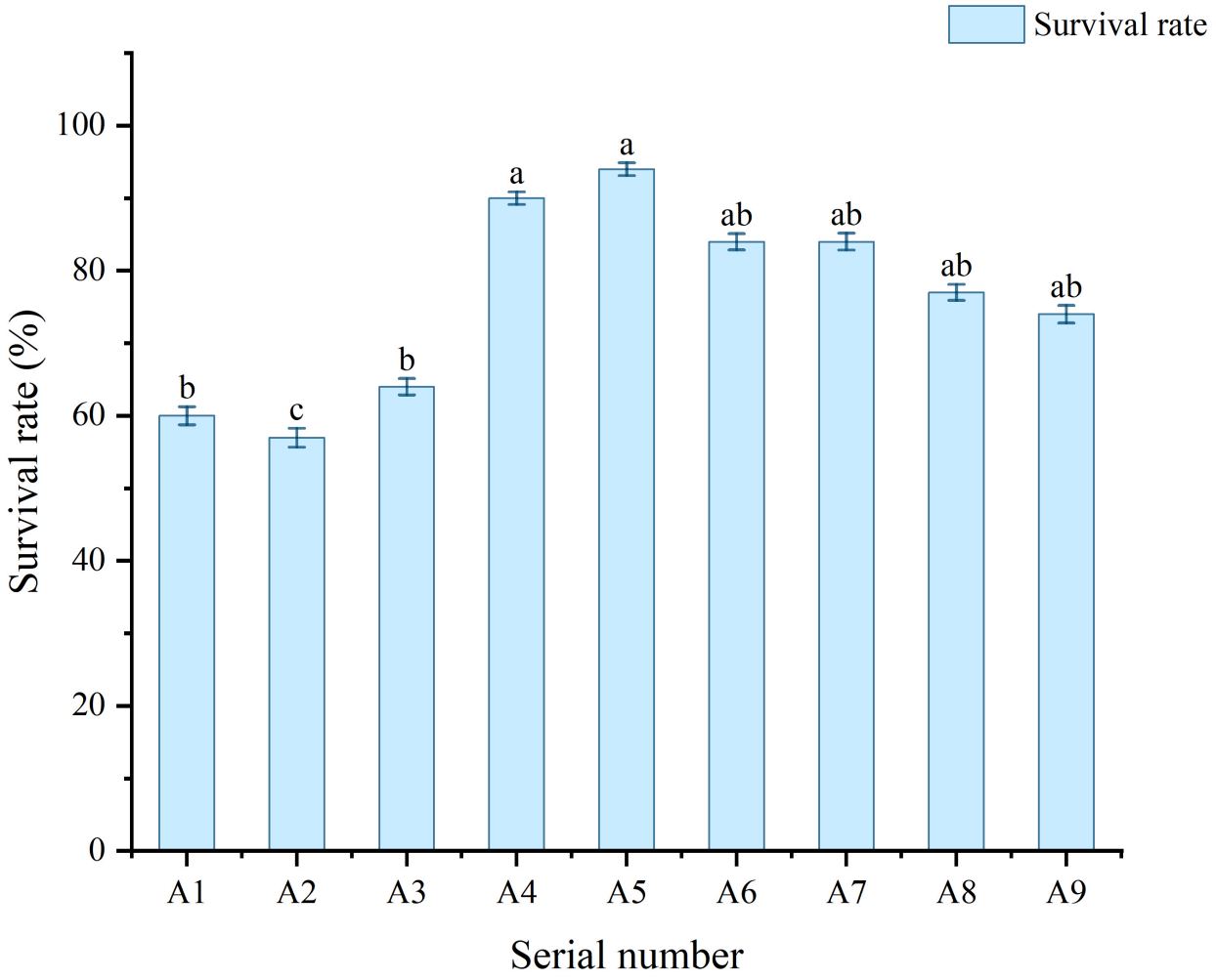


Figure 1

Effect of different sterilization protocols on the survival rate of *L. nepalense* explants. Different lowercase letters above the bars indicate significant differences at $p < 0.05$.

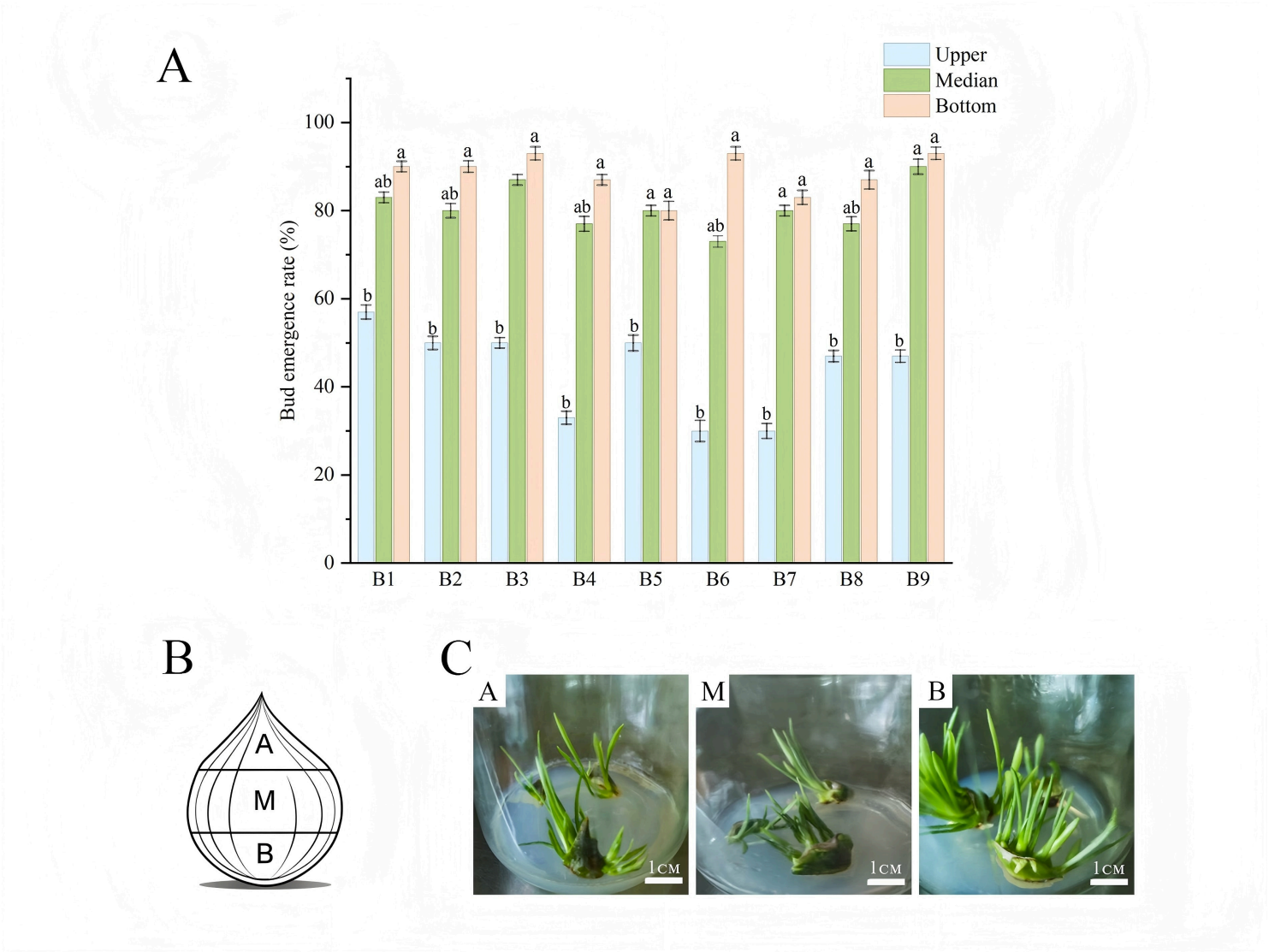


Figure 2

A:Effects of different plant growth regulators (PGRS) on the induction of various parts of *L. nepalense* scales.Different lowercase letters above the bars indicate significant differences at $p<0.05$.B:Schematic diagram of scale cutting and preparation for micropropagation of *L. nepalense.* C:Adventitious bud induction from explants of different parts of *L. nepalense.*

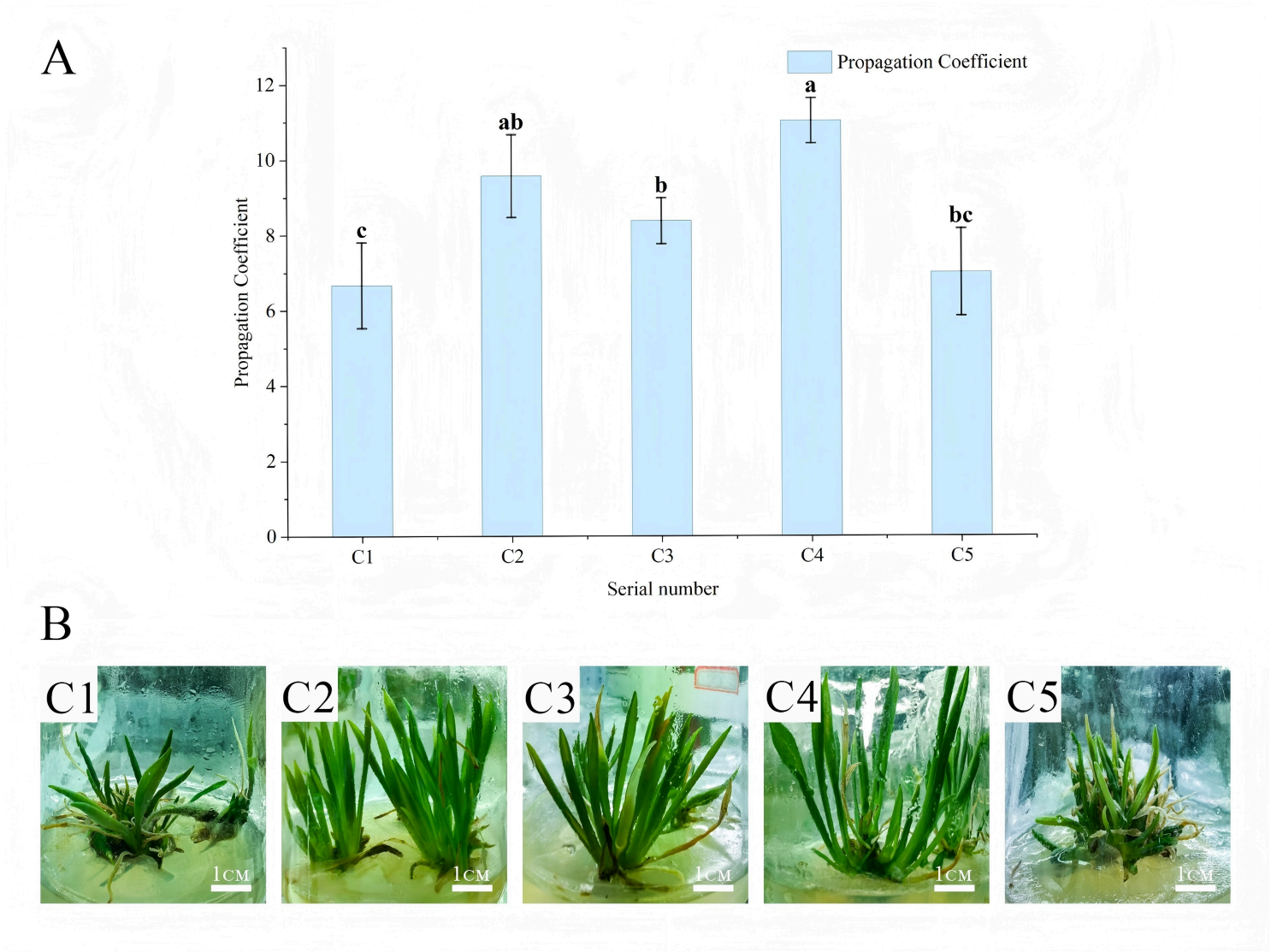


Figure 3

A:Effects of different plant growth regulator (PGRS) combinations on the proliferation of *L. nepalense*.Different lowercase letters above the bars indicate significant differences at p<0.05.B:Proliferation of *in vitro* Plantlets of *L. nepalense*.

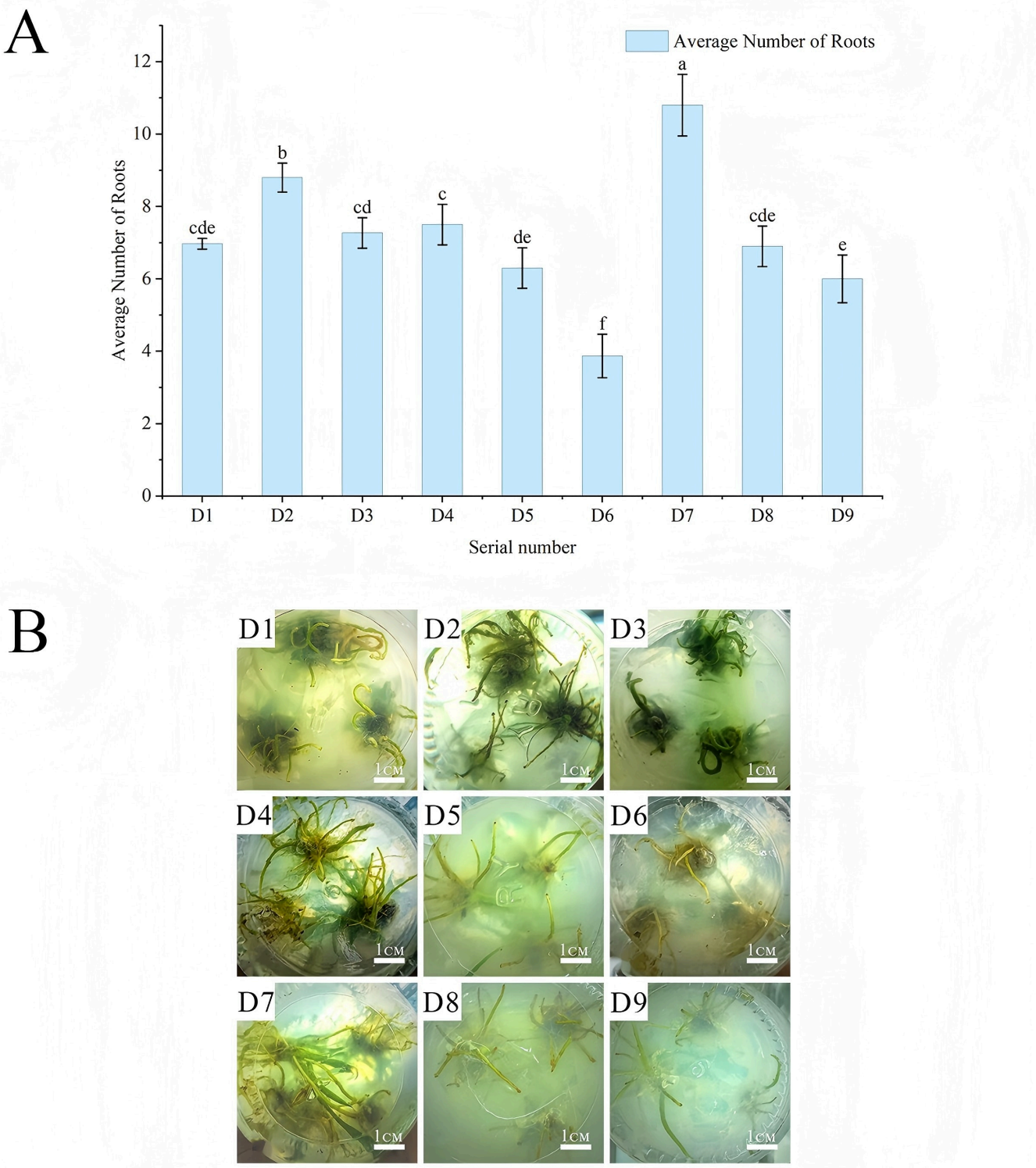


Figure 4

A:Influence of various plant growth regulators (PGRS) and their concentrations on the rooting of *L. nepalense* tissue-cultured seedlings.Different lowercase letters above the bars indicate significant differences at $p<0.05$.B:Rooting response of *L. nepalense* to different treatments.

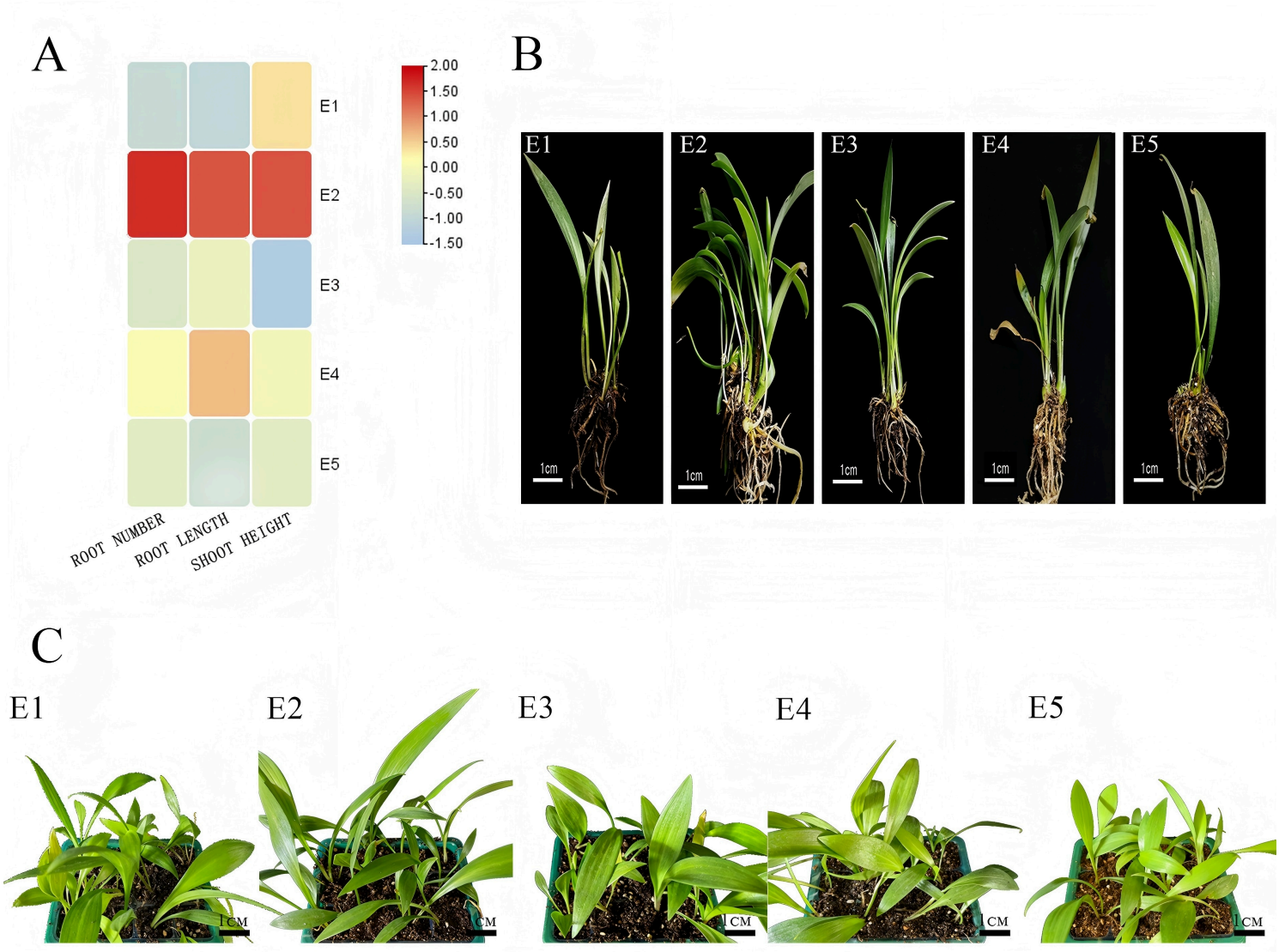


Figure 5

A: Effects of different substrates on the acclimatization and transplantation of *L. nepalense*. B: Growth performance of plantlets under different substrate treatments. C: Transplantation performance of plantlets under different substrate treatments.

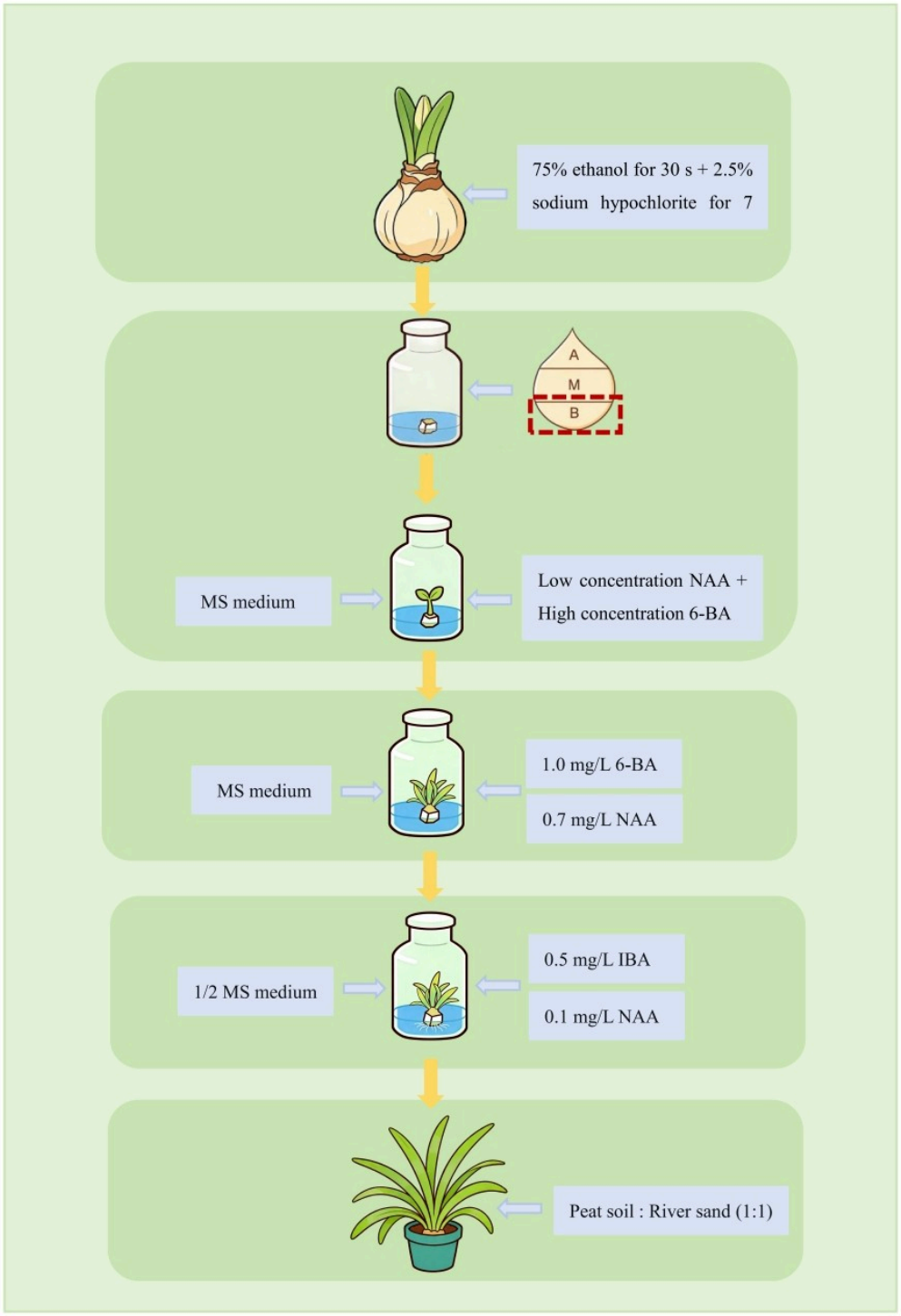


Figure 6

The protocol of *L. nepalense* micropropagation and plant regeneration.