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# *In vitro* PROPAGATION OF *Liparis nervosa* (Thunb.) Lindl., AN ENDANGERED MEDICINAL ORCHID

Yan Ren[1], Jin-Rong Gao[1], Shou-Meng Cai[1], Hong-Mei Yan[2*], Heng-Yu Huang[✉1]

[1] Yunnan Breeding and Cultivation Research and Development Center of Endangered and Daodi Chinese Medicinal Materials, Yunnan University of Chinese Medicine, 1076, Yuhua Road, Chenggong District, Kunming 650500, China

[2] Institute of Quality Standard and Testing Technology, Yunnan Academy of Agriculture Science, Kunming 650205, China

## ABSTRACT

*In vitro* regeneration was studied to protect the rare Chinese medicinal orchid *Liparis nervosa* (Thunb.) Lindl. The mixtures of protocorm and seeding and the stem tip were used as explants. The results revealed that the best essential medium for *L. nervosa* growth was 1/3 MS medium with 25 g · $L^{-1}$ sucrose, 50 g · $L^{-1}$ banana puree, 40 g · $L^{-1}$ mashed potato, and 1.0 g · $L^{-1}$ AC (MS1); MS1 medium with 0.5 mg · $L^{-1}$ BA, 0.05 mg · $L^{-1}$ 2,4-D, and 1.5 mg · $L^{-1}$ NAA was optimal for proliferation. When stem tips were cultured in a proliferation medium, four types of proliferation occurred: basal stem cluster bud (occurring at the basal node), tiller bud (occurring at the root), protocorm-like body (occurring at the plant's base incision), and high-position bud (occurring on plant stem nodes other than the basal nodes). Four methods produced 10.12 proliferation coefficients. In the MS1 medium with 0.5 mg · $L^{-1}$ NAA, the plantlets rooted 100%, and the rooted plantlets survived 100% after domestication and transplantation.

**Key words:** *in vitro* regeneration, proliferation method, basal stem cluster bud, tiller bud, protocorm-like body, *Liparis nervosa* (Thunb.) Lindl.

## INTRODUCTION

China is one of the countries with the richest orchid resources in the world, with 171 genera and 1,247 species. Most are terrestrial orchids whose roots grow in organic-rich soil and frequently indicate fungal symbiosis, such as *Cymbidium goeringii* (Rchb. f.) Rchb. f., *C. sinense* (Jackson ex. Andr.) Willd., *C. kanran* Makino, *C. ensifolium* (L.) Sw., and *Liparis nervosa* (Thunb.) Lindl. [Pant and Shrestha 2011, Liu et al. 2014].

*Liparis nervosa* is a terrestrial medicinal orchid belonging to the family *Orchidaceae* that is predominantly found in tropical and subtropical regions of China, such as Jiangxi, Hunan, and Guizhou. This species grows under trees in grassy, shady places and has a well-developed rhizome [Flora of China Editorial Committee and Chinese Academy of Sciences 1999]. Miao people consider the entire *L. nervosa* plant to be an 'effective medicine of hemostasis' for its use in treating pyretic pulmonary syndrome, arthralgia owing to wind dampness, and infantile convulsion [Zhang et al. 2020]. Researchers have analyzed the chemical composition of *L. nervosa* and extracted nervosine from it [Chen et al. 2018]. In addition, it was discovered that the alkaloids found in *L. nervosa*, such as nervine, protoveratrine, and pyrrolizidine, have a positive anti-inflammatory and hemostasis effect [Chen et al. 2019].

Seed germination and nutritional reproduction are two methods of natural reproduction for orchids. The

✉ hhyhllhbb@163.com

seeds of orchids are tiny, lack endosperm, and need symbiosis with suitable fungi to germinate, and the germination rate is extremely low; the nutritional reproduction is not only small in number but also slow in reproduction [Castillo-Pérez et al. 2021]. According to the survey, with the accelerated exploitation and use of orchid resources, the natural resources of many species are decreasing at an alarming rate [Pant and Shrestha 2011]. Plant tissue culture technology has been an essential method for medicinal orchid propagation, and the seeds of some orchids can achieve non-symbiotic germination and *in vitro* regeneration from organ or embryo pathways by culturing explants [Pant and Shrestha 2011, Xi et al. 2021]. *Phaius tancarvilleae* (L'Her.) used stem tips as explants to induce clumped buds for fast reproduction [Pant and Shrestha 2011]. In *Dendrobium fimbriatum* Lindl. var. oculatum Hk. f., the callus was produced from the stem tip, and protocorm-like bodies were subsequently induced from the callus [Roy and Banerjee 2003]. Seed germination of *Rhynchostylis retusa* (L.) Bl produced protocorms were used as material to induce protocorm-like bodies for *in vitro* fast reproduction [Xi et al. 2021]. Due to *L. nervosa*'s low sexual reproduction efficiency, extended asexual reproduction cycle, and low genetic diversity, it is difficult to locate its traces in its natural habitat. There are few reports on the artificial breeding of *L. nervosa*, while *in vitro* regeneration is not reported at all. Therefore, there is an urgent need to establish a rapid *in vitro* propagation system to improve the artificial mass propagation of *L. nervosa*. This study aimed to establish the entire technical system for *L. nervosa* plant regeneration and lay the technical groundwork for protecting natural resources and advancing artificial cultivation. Meanwhile, this protocol also guided the rapid *in vitro* propagation of other *Orchidaceae* plants.

## MATERIALS AND METHODS

**Materials.** The capsules of *L. nervosa* were provided by Jishou University in Hunan Province, China, and were picked from mature *L. nervosa* plants by Hua Long, a professor at Jishou University. The 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (Kin), N-(phenylmethyl)-9H purin-6-amine (BA), zeatin (Zt), sucrose, activated charcoal (AC), and agar were purchased from Beijing Dingguo Changsheng Biotech Co., Ltd. (Beijing, China), and the main component content of all reagents is greater than or equal to 99.7%.

**Sterilization and aseptic germination of capsules.** The mature capsules were selected and underwent a 30 s immersion in 75% (*v/v*) ethyl alcohol, 30 min of surface sterilization with 0.1% (*w/v*) $HgCl_2$, and three lengthy rinses with sterile distilled water. The sterile capsules were then placed on sterile blotting paper to dry the surface for 3 min, and the seeds were removed and deposited on an MS medium with 0.5 g · $L^{-1}$ AC and 4.0 g · $L^{-1}$ agar for seed germination. Except for the germination experiment, all other media were supplemented with 1.0 g · $L^{-1}$ AC and 0.47% agar. All media were adjusted to a pH range of 5.4–5.8 and then autoclaved at 122°C for 20 min. The hormone concentrations in this study are all mass (*w/v*).

**Screening of natural additives and initial culture.** Table 1 shows the experimental design for $L_{16}(4^5)$ orthogonal experiments in which the varieties of medium (MS, 1/2 MS, 1/3 MS, and 1/4 MS), concentrations of sucrose (10, 15, 20, and 25 g · $L^{-1}$), banana

**Table 1.** Selection of basal medium by $L_{16}(4^5)$ orthogonal experiment

| Level | Factor | | | |
|---|---|---|---|---|
| | A<br>MS | B<br>sugar concentration (g · $L^{-1}$) | C<br>banana puree (g · $L^{-1}$) | D<br>mashed potatoes (g · $L^{-1}$) |
| 1 | 1 | 10 | 30 | 30 |
| 2 | 1/2 | 15 | 40 | 40 |
| 3 | 1/3 | 20 | 50 | 50 |
| 4 | 1/4 | 25 | 60 | 60 |

puree (30, 40, 50, and 60 g · $L^{-1}$), and mashed potatoes (30, 40, 50, and 60 g · $L^{-1}$) were used as factors. The protocorm and seedling mixtures were trimmed to 1.0 × 1.0 cm (false root hairs were removed) and then inoculated into the media mentioned above. The essential medium types, carbon source, and natural addition concentrations suitable for the growth of *L. nervosa* were determined based on the growth conditions of each group for the following 90 days, called MS1 medium. The protocorm and seedings obtained from seed germination were consistently cultured in the MS1 medium to obtain more plantlets.

Proliferation coefficient = the number of available buds produced/the number of materials for initial inoculations.

**Screening experiment on types and concentrations of PGR.** The stem tip and segment (the stem tip and segment contained two leaves and one node) were chosen, and these two materials were inoculated into the MS1 medium with different kinds and concentrations of plant growth regulators (PGRs). Zt, Kin, and NAA concentrations were set at 0.1, 0.5, 1.0, and 1.5 mg · $L^{-1}$; 2,4-D concentrations were set at 0.05, 0.1, 0.5, 1.0, and 1.5 mg · $L^{-1}$; and the concentrations of BA were set at 0.1, 0.5, 1.0, and 2.0 mg · $L^{-1}$. Each experimental group's proliferative and growth conditions were evaluated to determine which PGR types and concentration ranges were most effective against *L. nervosa* for the following 90 days.

**Experimentation in proliferation.** A (BA), B (2,4-D), and C (NAA) were used as factors to perform the $L_9(3^4)$ orthogonal experiment (Tab. 2) based on the preliminary experiment results of the screening experiment on the types and concentrations of PGR. The stem tip with leaves (2.0 to 3.0 cm) served as the experimental material for the present research. The proliferation pathway and proliferation coefficient were recorded after 90 days of culture.

Proliferation coefficient = the number of available buds produced/the number of materials for initial inoculations.

Basal stem cluster bud incidence (%) = the number of materials with two or more adventitious buds/the number of materials initially inoculated × 100%.

Incidence of high-position bud (%) = the number of materials with two or more stem node buds/number of total materials inoculated × 100%.

Incidence of protocorm-like bodies (%) = the number of materials with protocorm-like bodies/the number of total materials inoculated × 100%.

Incidence of tiller bud (%) = the number of materials with tiller bud from succulent root/the number of total materials inoculated × 100%.

**Rooting culture and plantlet transplantation.** Based on the results of the screening experiment on the types and concentrations of PGR, regeneration buds with a length of 3 to 4 cm were used as materials and then cultured in MS1 medium supplemented with NAA (0.1, 0.5, and 1.0 mg · $L^{-1}$). The rooting rate and root growth condition were observed and recorded once every 15 days.

Rooting rate (%) = the number of plantlets with new roots/the total number of plantlets inoculations × 100%.

The culture bottle's plantlets were exposed to natural light for seven days after they reached a height of 5 to 6 cm. They were then removed from the sealing film of the culture vessels and exposed to light for two days. Then, we removed the plantlets, washed the residual agar solid from the root, and submerged them

Table 2. $L_9(3^4)$ orthogonal design of the stem tip proliferation

| Level | Factor | | |
|---|---|---|---|
| | A<br>6-BA concentration/(mg · $L^{-1}$) | B<br>2,4-D concentration/(mg · $L^{-1}$) | C<br>NAA concentration/(mg · $L^{-1}$) |
| 1 | 0.5 | 0.05 | 0.5 |
| 2 | 1 | 0.1 | 1.0 |
| 3 | 2 | 0.5 | 1.5 |

for 5 min in 0.1% chlorothalonil. Finally, we transplanted them into humus soil sterilized with 0.2% (*w/v*) potassium permanganate (humidity maintained between 50 and 60% and temperature between 23 and 25 degrees Celsius). The survival rate and growth were recorded after 90 days.

The survival rate of transplanting (%) = the total number of survival plants/the total number of transplanting plants × 100%.

**Culture conditions and inoculation methods.** The temperature in the culture room was maintained at 20 ±2°C, and the illumination was 10 h · $d^{-1}$ with a light intensity of 1500–2000 lx.

Natural additive screening required the mixture of protocorm and seedings to be cut to 1.0 × 1.0 cm in size, with ten bottles in each group and nine materials per bottle. The PGR screening, proliferation, and rooting experiments were inoculated according to growth direction, except that the rooting experiment was to inoculate ten materials per bottle. Every other medium was inoculated with 14 materials, with ten bottles in each experiment group. Except for the germination experiment, each experiment was repeated three times, and if the plants died or were polluted, they were replaced in time.

**Statistical analysis.** The results of each treatment group were quantified as the mean plus or minus the standard error. The analysis of variance (ANOVA) and Duncan's test was carried out by SPSS 26.0 (IBM Corp., Armonk, USA) software, and the significant difference between each treatment method was determined with a significance level of 5% ($P \leq 0.05$).

## RESULTS

**Sterile germination of seeds.** After 60 days of sowing, some yellow and white seeds turned green (Fig. 1A). After 100 days, the seeds continued germinating. The number of green protocorms grew (Fig. 1B). A portion of the protocorms grew into plant-

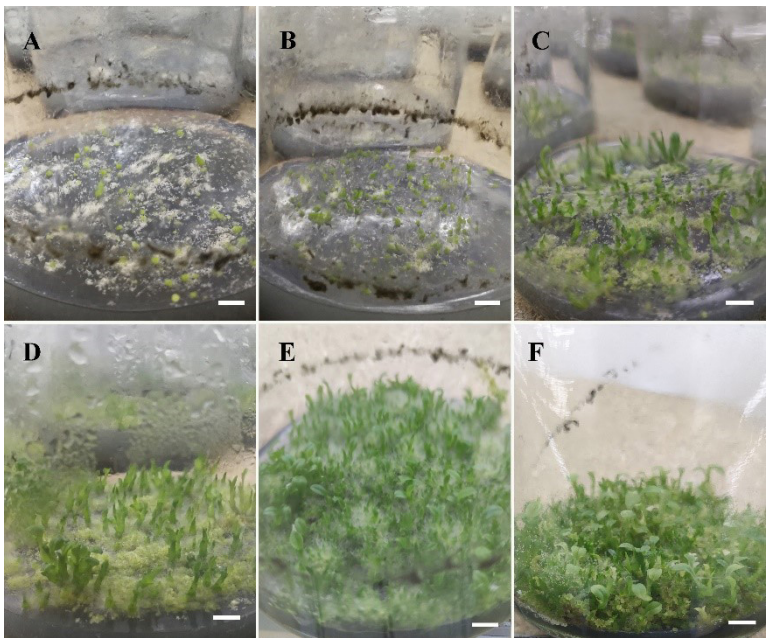


**Fig. 1.** Sterile germination of seeds of *L. nervosa*. (A) The seeds began to germinate after 60 days; (B) seed germination increased after 100 days; (C) after 140 days of culture, some protocorm developed into seedings; (D) after 180 days, the seeds germinated basically, and the false roots appeared; (E) after 220 days of culture, the leaves of the partial seedlings were open; (F) after 260 days, the medium was covered by the mixtures of protocorm, seedlings, and fake root hair (Bar: 1.5 cm)

lets after 140 days (Fig. 1C). Protocorms and plantlets continued to expand, and false root hairs were observed on the medium's surface after 180 days (Fig. 1D). After 220 days, the surface of the medium was covered with false root hair, protocorms, and seedings; inferior seeds were covered in false root hairs (Fig. 1E). The surface of the medium was covered with composites of protocorms and seedings; the growth of some seedings was inhibited, and the leaves were yellowed for the following 260 days (Fig. 1F).

**Selection of basal medium.** In germination mediums, protocorms and seedlings grew slowly, with small plants and yellow leaves; thus, it was essential to identify the primary medium suitable for seedlings' average growth. The type of medium had the most significant impact on *L. nervosa* growth and development, followed by banana puree, mashed potatoes, and sucrose from $R_A > R_C > R_D > R_B > R_{Error}$ (Tab. 3). Meanwhile, the range of four factors was greater than that of the blank column (0.59), indicating that the four factors had a more significant impact on *L. nervosa* expansion. According to the analysis of variance (Tab. 4), the type of medium had a significant effect on the proliferation coefficient ($P < 0.05$), whereas sucrose, banana puree, and mashed potatoes did not ($P > 0.05$). Table 5 demonstrates that Duncan's test on the type of medium showed that level 3 (1/3 MS) had the best response, which was significantly different from level 2 (1/2 MS), level 1 (MS), and level 4 (1/4 MS). The known mean value analysis determined that the most suitable basal medium for *L. nervosa* growth was 1/3 MS medium with 25 g · L$^{-1}$ sucrose, 50 g · L$^{-1}$ banana puree, and 40 g · L$^{-1}$ mashed potatoes. It was referred to as MS1 medium, and the maximal proliferation coefficient of MS1 medium was 5.57.

After 160 days of seed sowing, the mixture of protocorm and seedings where false root hairs were removed was transferred into MS1 culture medium

**Table 3.** The $L_{16}(4^5)$ orthogonal experiment results for the base medium

| No. | Medium A | Sugar (g · L$^{-1}$) B | Natural additives (g · L$^{-1}$) | | Error | Proliferation coefficient |
|---|---|---|---|---|---|---|
| | | | C (banana puree) | D (mashed potatoes) | | |
| C01 | 1 | 1 | 1 | 1 | 1 | 2.62 ± 0.56 |
| C02 | 1 | 2 | 2 | 2 | 2 | 3.21 ± 0.13 |
| C03 | 1 | 3 | 3 | 3 | 3 | 2.55 ± 0.38 |
| C04 | 1 | 4 | 4 | 4 | 4 | 2.43 ± 0.43 |
| C05 | 2 | 1 | 2 | 3 | 4 | 4.11 ± 0.66 |
| C06 | 2 | 2 | 1 | 4 | 3 | 3.48 ± 0.42 |
| C07 | 2 | 3 | 4 | 1 | 2 | 3.43 ± 0.18 |
| C08 | 2 | 4 | 3 | 2 | 1 | 5.57 ± 0.38 |
| C09 | 3 | 1 | 3 | 4 | 2 | 4.96 ± 0.89 |
| C10 | 3 | 2 | 4 | 3 | 1 | 5.00 ± 1.22 |
| C11 | 3 | 3 | 1 | 2 | 4 | 5.50 ± 0.74 |
| C12 | 3 | 4 | 2 | 1 | 3 | 5.05 ± 0.33 |
| C13 | 4 | 1 | 4 | 2 | 3 | 2.07 ± 0.18 |
| C14 | 4 | 2 | 3 | 1 | 4 | 3.07 ± 0.52 |
| C15 | 4 | 3 | 2 | 4 | 1 | 2.31 ± 0.39 |
| C16 | 4 | 4 | 1 | 3 | 2 | 3.14 ± 0.85 |
| Multiplication coefficient | $K_1$ | 2.70 | 3.44 | 3.69 | 3.54 | 3.88 | – |
| | $K_2$ | 4.15 | 3.69 | 3.67 | 4.09 | 3.69 | – |
| | $K_3$ | 5.13 | 3.45 | 4.04 | 3.70 | 3.29 | – |
| | $K_4$ | 2.65 | 4.05 | 3.23 | 3.30 | 3.78 | – |
| | $R$ | 2.48 | 0.61 | 0.81 | 0.79 | 0.59 | – |

No. – The group number of the experiment; proliferation coefficient was average ± standard error; $K$ – mean; $R$ – range ($R = K_{max} - K_{min}$)

**Table 4.** Variance analysis results for the base medium

| Factors | Source | Type III sum of squares | DOF | Mean square | *F* | Significant |
|---|---|---|---|---|---|---|
| Multiplication coefficient | A | 17.332 | 3 | 5.777 | 15.740 | $P < 0.05$ |
| | B | 0.978 | 3 | 0.326 | 0.188 | $P > 0.05$ |
| | C | 1.304 | 3 | 0.435 | 0.255 | $P > 0.05$ |
| | D | 1.325 | 3 | 0.442 | 0.260 | $P > 0.05$ |
| | Error | 0.797 | 3 | 0.266 | – | – |

DOF – degree of freedom

**Table 5.** Duncan's test of three levels of basic medium

| Factors | Levels | Mean | Significant |
|---|---|---|---|
| Basic medium | 3 | 5.128 | a |
| | 2 | 4.148 | b |
| | 1 | 2.703 | c |
| | 4 | 2.648 | c |

Different lowercase letters in the same column indicated significant differences at 0.05 level

(Fig. 2A). The protocorm expanded, and leaves of plantlets appeared, but false root hairs were hard to find after 20 days of culture (Fig. 2B). Most protocorms developed into plantlets after 50 days (Fig. 2C). Then the plantlet developed luxuriant stems and leaves, and the protocorm was difficult to locate for the following 80 days (Fig. 2D). At this time, the result was calculated by cluster (a cluster of 5–8 seedlings), the proliferation coefficient could reach 5.50, and a large number of stem tips and stem segments were obtained.

**Experiment results for screening PGR species and concentrations.** The effects of NAA, 2,4-D, Kin, Zt, and BA on the stem tip and segment were distinct; there were significant differences in growth and bud differentiation between the two materials. Stem segments grew slowly in all groups, and only a few materials in the BA treatment group (1.0 mg · $L^{-1}$) developed 1 to 2 buds (Fig. 3A). A significant number of basal stem cluster buds were found in the basal portion of the stem tip in BA concentrations ranging from 0.5–2.0 mg · $L^{-1}$, but the cluster buds' leaves were relatively small, and the stem tips grew slowly (Fig. 3B). Basal stem cluster buds were occasionally observed at the base of the stem tip, and the leaf area of the plant was relatively large at the Zt concentration range of 0.5–1.5 mg · $L^{-1}$ (Fig. 3C). Rare regenerated buds were found only in Kin concentrations of 0.1–1.0 mg · $L^{-1}$, and in the later period of culture, the leaves turned yellow and the plantlets were in a sub-health state (Fig. 3D). In the NAA treatment group, the stem tips grew faster, and the adventitious roots were induced by NAA at 1.5 mg · $L^{-1}$ (Fig. 3E). The plantlets grew well and produced more leaves, but there were hardly any buds at the stem node within the range of 0.05 to 1.5 mg · $L^{-1}$ 2,4-D (Fig. 3F). In terms of plantlet elongation, auxin performed better than cytokinin in the experiment. The cytokinin group was more effective than the auxin group at inducing bud regeneration. To summarize, stem tips were more appropriate than the stem segment for rapid *in vitro* propagation of *L. nervosa*. Meanwhile, Zt was abandoned because its biological activity was comparable to that of BA and was expensive. Consequently, BA, NAA, and 2,4-D were chosen for the next experiment.

**The result of the proliferation experiment.** Table 6 ($R_{BA} > R_{2,4\text{-}D} > R_{NAA} > R_{Error}$) revealed that three PGRs

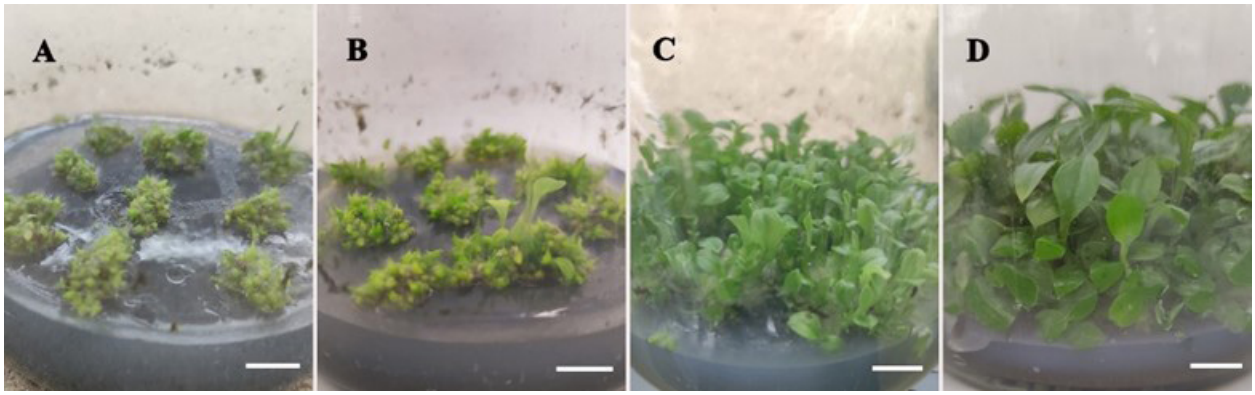


**Fig. 2.** The protocorm growth of *L. nervosa*. (A) Protocorm and seedling mixture; (B) 20 days after culture; (C) 50 days after culture; (D) 80 days after culture (Bar: 1.5 cm)

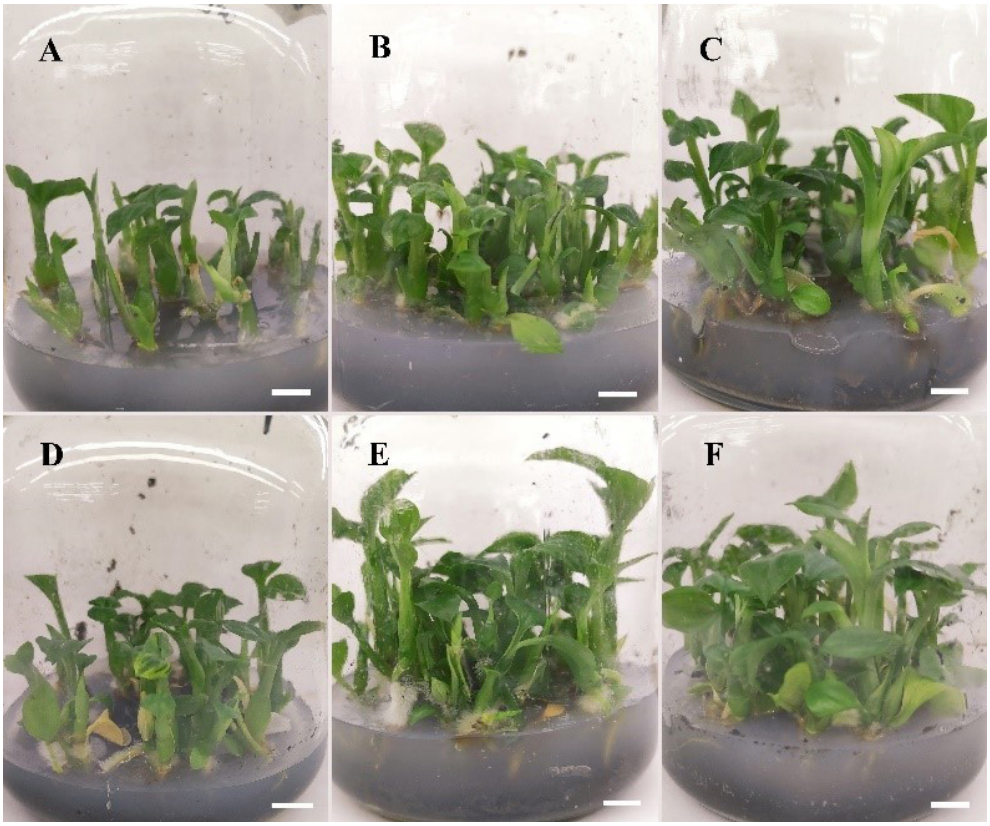


**Fig. 3.** Experiment results for screening PGR species and concentrations. (A) Stem segments were cultured in MS1 with 1.0 mg · $L^{-1}$ BA for 60 days; (B) the stem tips were cultured in MS1 with 1.0 mg · $L^{-1}$ BA for 60 days; (C) the stem tips were cultured in MS1 with 0.5 mg · $L^{-1}$ Zt for 60 days; (D) the stem tips were cultured in MS1 with 0.5 mg · $L^{-1}$ Kin for 60 days; (E) the stem tips were cultured in MS1 with 1.5 mg · $L^{-1}$ NAA for 60 days; (F) the stem tips were cultured in MS1 with 0.1mg · $L^{-1}$ 2,4-D for 60 days (Bar: 1.5 cm)

were effective at inducing basal stem cluster buds at the stem tip. Among them, BA had the best response for basal stem cluster bud occurrence, followed by 2,4-D and NAA. According to the variance analysis results (Tab. 7), BA was statistically significant ($P < 0.05$) for the proliferation coefficient, whereas 2,4-D and NAA were not ($P > 0.05$). The results of Duncan's test to induce basal stem cluster bud at three levels of BA (Tab. 8) indicated that level 1 (0.5 mg · $L^{-1}$) produced the best response, which was significantly different from levels 2 (1.0 mg · $L^{-1}$) and 3 (1.5 mg · $L^{-1}$). MS1 medium with 0.5 mg · $L^{-1}$ BA, 0.05 mg · $L^{-1}$ 2,4-D, and 1.5 mg · $L^{-1}$ NAA was the optimal PGR combination for *L. nervosa* cluster bud formation.

In repeated experiments, the green buds appeared on the nodes where the base of the material contacted the medium after 30 days of culture (Figs 4A, B). The basal stem buds gradually increased for 50 days (Figs 4C, D). Then, the material grew progressively after 70 days of incubation, and the regenerated buds grew slower than the main stem. The color of yellow and white roots was found at the base of the material. (Figs 4E, F). The leaves of the material were green, and basal stem cluster buds grew luxuriantly after 90 days (Figs 4G, H). At this time, basal stem cluster bud incidence was 100%, and the proliferation coefficient was up to 8.84. Aside from basal stem cluster buds, it was discovered that there were three bud regeneration methods: tiller bud (occurring at the root) (Fig. 5), protocorm-like body (occurring at the plant's base incision), and high-position bud (occurring on plant stem nodes other than the plant's basal nodes) (Fig. 6). The sum of the proliferative coefficients of basal stem cluster buds, tiller buds, protocorm-like bodies, and high-position buds was 10.12.

The tiller buds were found at the roots (Fig. 5A). Figure 5B depicts the transformation of tiller seedlings into plantlets 60 days later. The tiller buds transform into robust plantlets with a low regeneration rate of 8.33% 90 days later (Fig. 5C).

The protocorm-like body appeared at the base of the stem tip incision (Fig. 6A). The protocorm-like bodies gradually developed into plantlets along the culture period (Figs 6B–D), and the occurrence rate was about 17.14%. However, the space was congested and inhibited by the main plant. So, the regeneration process was slow, and the regenerated plantlets were weak; this circumstance needed to be rejuvenated before transplanting. As the main plantlet grew and elongated, the stem nodes at the upper end wrapped by the leaf sheath grew small buds (Figs 6E, F). The buds in the high position grew and developed into conventional plantlets for the following 120 days (Figs 6G, H). The incidence rate of this method of regeneration accounted for 48.87%.

**Results of experimental rooting and transplantation.** In the rooting experiment (Tab. 9), the rooting rate of the blank control was only 31.76%, which was significantly lower than that of other experimental groups, and the adventitious roots were short, thin, and lacked root hair. The regeneration buds' rooting rate was significantly higher in the MS1 medium containing 0.1 mg · $L^{-1}$ of NAA, and the growth conditions were favorable. However, the rooting rate decreased when the NAA concentration exceeded 1.0 mg · $L^{-1}$. In other words, elevated auxin concentrations inhibited the development of adventitious roots in *L. nervosa*. The optimal medium for adventitious root induction was MS1 medium containing NAA at a concentration of 0.5 mg · $L^{-1}$ with a 100% rooting rate. The plantlets

**Table 7.** Variance analysis results of the proliferation coefficient in *L. nervosa*

| Factors | Source | Type III sum of squares | DOF | Mean square | *F* | Significant |
|---|---|---|---|---|---|---|
| Proliferation coefficient | A | 10.040 | 2 | 5.020 | 5.174 | $P < 0.05$ |
| | B | 4.722 | 2 | 2.361 | 1.272 | $P > 0.05$ |
| | C | 0.747 | 2 | 0.374 | 0.148 | $P > 0.05$ |
| | Error | 0.351 | 2 | 0.176 | – | – |

DOF – degree of freedom

**Table 8.** Duncan's test of three levels of 6-BA

| Factors | Levels | Mean | Significant ($P < 0.05$) |
|---|---|---|---|
| 6-BA | 1 | 8.053 | a |
| | 2 | 6.717 | ab |
| | 3 | 5.467 | b |

Different lowercase letters in the same column indicated significant differences at 0.05 level

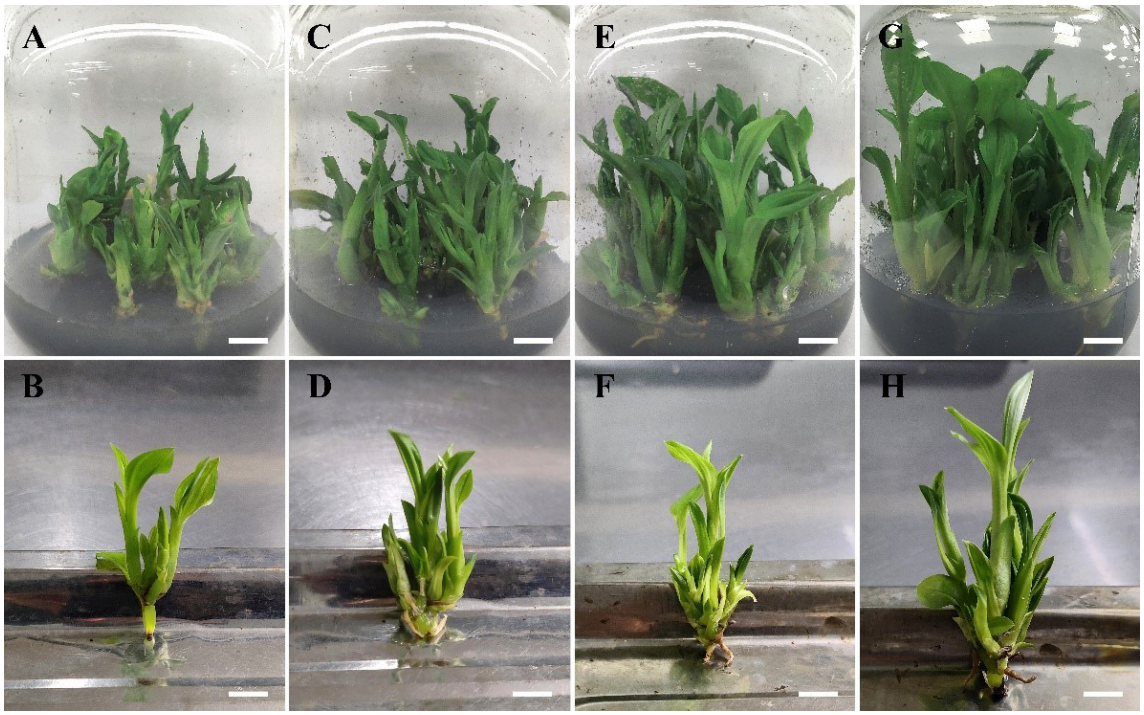


**Fig. 4.** Stem tip propagation culture of *L. nervosa*. (A, B) Buds appeared at the base of the material after 30 days of culture; (C, D) the buds began to grow after 50 days of culture; (E, F) buds continuously appeared at the base with the growth of plantlets after 70 days of culture; (G, H) bud clusters on the basal stem after 90 days of culture (Bar: 2.0 cm)

grew significantly in this medium, and roots appeared at the base of the plantlets for the following 30 days (Fig. 7A). After 60 days of culture, the plantlets had green leaves but grew slowly, with numerous yellow and white roots visible through the bottom of the container (Figs 7B, C). The 60-day-old rooted plantlets were transferred into the foam boxes containing substrates for a sealed, insulated, and moisturized incubation (Fig. 7D). The plant's root system developed after 30 days of soil cultivation (Fig. 7E). The rooted plantlets were transplanted into the flower pot at this time. The plants had successfully adapted to their natural environment after 30 days in the flower pot (Fig. 7F). The plantlets were transplanted successfully.

## DISCUSSION

**Method for the *in vitro* proliferation of *L. nervosa*.** There were four varieties of regeneration methods for *in vitro* rapid reproduction of orchids: protocorm

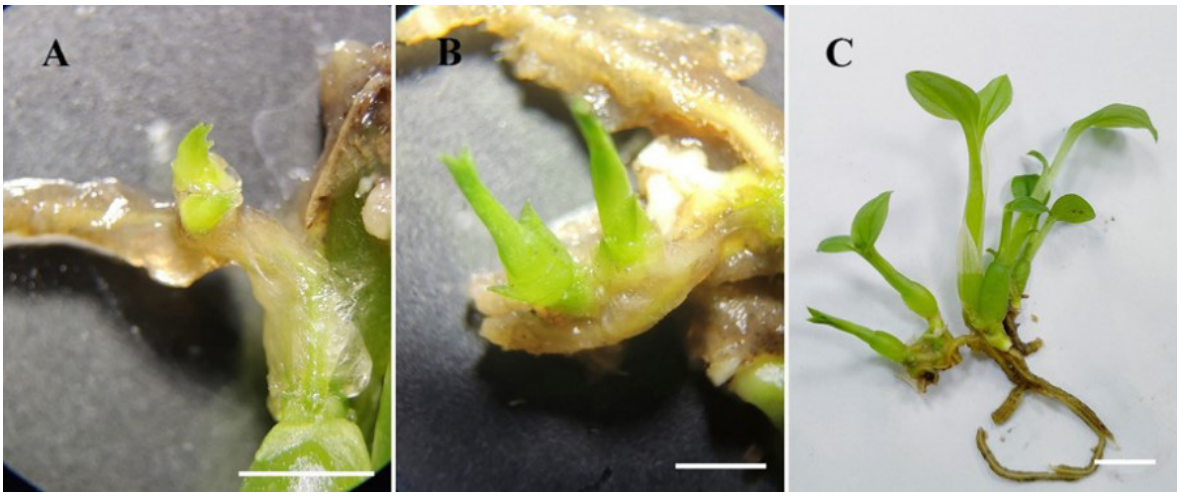


**Fig. 5.** The incidence of *L. nervosa* in tiller buds. (A) Juvenile buds of tiller buds; (B) tiller buds after 60 days; (C) plantlets grew from tiller buds after 90 days (Bar: 2.0 cm)

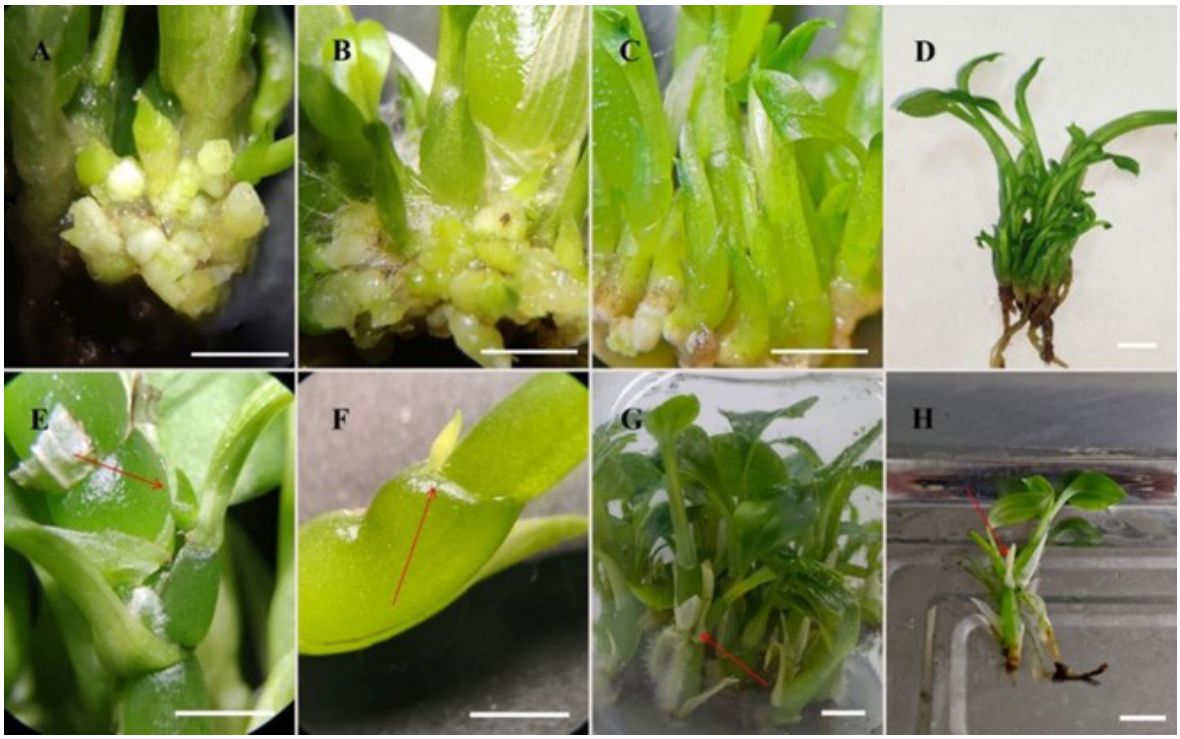


**Fig. 6.** Protocorm-like body and high-position bud regeneration of *L. nervosa*. (A) After 30 days, protocorm-like bodies appeared at the base of stem tip; (B) after 60 days, protocorm-like bodies proliferated and fluff appeared; (C) protocorm-like bodies grew into bud clusters after 90 days; (D) protocorm-like bodies developed into seedlings after 120 days; (E, F) buds appearing on nodes of the stem; (G, H) plantlet regeneration from high-position bud after 120 days (the red arrow points to the high-position bud, bar: 2.0 cm)

formation, induction of callus-adventitious cluster bud formation, protocorm-like body formation, and stem node bud occurrence [Roy and Banerjee 2003, Pant and Shrestha 2011, Xi et al. 2021, Aung et al. 2022]. Except for the induction of callus-adventitious cluster buds, *L. nervosa* in our study appeared to regenerate in the three methods described above. In the seed germination stage, the protocorm was formed directly from the seed rupture without forming a callus. Based on the medium and certain species characteristics, protocorms obtained from seeds proliferated and gradually formed plantlets. When the mixtures of protocorm and seeding were subcultured, the maximum multiplication coefficient was 5.57. After the protocorms developed into plantlets, it was speculated that the proliferation of protocorms was significantly inhibited due to the restriction of growth space and the endogenous auxin produced by the stem tip of the plantlets. Thus, as plantlets grew more extensive, the capacity of protocorms to proliferate diminished, resulting in fewer protocorms. This phenomenon has also been observed in the *Cymbidium Golden* Elf 'Sundust' [Xi and Yang 2022]. In the proliferation culture of the stem tip of *L. nervosa*, the incidence of protocorm-like bodies at the base wound was approximately 17.14%, but there was no callus. The protocorm-like body could be derived from epidermal, vascular, subepidermal, and single cells in the callus [Lee et al. 2013]. The protocorm-like body in this study came from the wound, and the plantlets developed by protocorm-like bodies under the microscope were not connected with the main stem by vascular bundles. Therefore, the protocorm-like body in this study should come from cortical cells, and some cells with meristematic ability in the epidermis and under the epidermis divided many times to form a mass of meristem and then formed protocorm-like bodies. In *Anthurium andreanum* cv. CanCan, it has also been reported that protocorm-like bodies originated from the stem tip incision, where the incision was first swollen and gradually formed small nodules, which produced protoplasts in the subsequent culture [Gantait et al. 2012].

In orchids reported, stem tips usually induce protocorm-like bodies or calluses [Roy and Banerjee 2003, Jainol et al. 2016]. In contrast to previous reports, the cluster buds of *L. nervosa* were directly formed on the nodes near the medium at the base of the stem tip, and no callus or protocorm-like bodies occurred. It was its primary propagation method, with a 100% incidence rate. Basal stem cluster buds in Orchidaceae were uncommon, having only been reported in *Paphiopedilum rothschildianum* Pfitzer [Chyuam-Yih et al. 2010] and *Vanilla fragrans* (Salisb.) Ames. [Kalimuthu et al. 2006], whereas it was common in other non-orchids [Li et al. 2020, Xi and Huang 2021]. The stem nodes contained meristematic tissues, and the meristematic tissues on the nodes near the medium produced many cluster buds under the stimulation of exogenous hormones. Due to the severe inhibition of apical dominance, only a small number of high-position stem nodes (nodes near the top) could differentiate new buds. The roots

**Table 9.** Effect of different NAA concentrations on rooting of plantlets in *L. nervosa*

| No. | NAA concentration ($mg \cdot L^{-1}$) | Rooting rate (%) | Growth state |
|---|---|---|---|
| 1 | 0 | 31.76 ±0.81d | the root was short and thin, without adventitious bud differentiation at the base |
| 2 | 0.1 | 60.26 ±0.67c | the root was long and strong, with a few adventitious buds at the base |
| 3 | 0.5 | 100.00 ±0.13a | the root was long and shaped, with many adventitious buds at the base |
| 4 | 1.0 | 75.68 ±0.55b | the root was long and strong, with some adventitious bud differentiation at the base |

The rooting rate was average ± standard error; different lowercase letters in the same column indicated a significant difference at 0.05 level

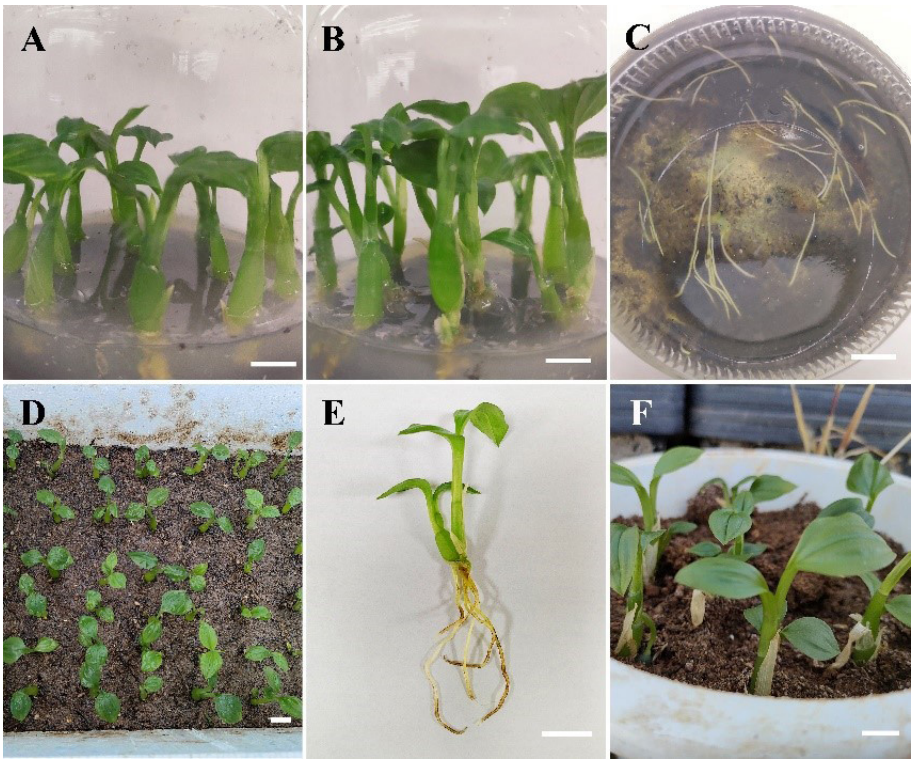


**Fig. 7.** Rooting and transplanting of *L. nervosa*. (A) After 30 days of culture; (B) after 60 days of culture; (C) the adventitious roots cultured for 60 days; (D) transplant plantlet to a foam box for culture; (E) root system in foam box after 30 days; (F) plants grown in pots for 30 days (Bar: 2.0 cm)

of some orchids have strong meristematic ability, and in *Cyrtopodium paranaense* Schltr., the root tips were cultured to eventually produce protocorm-like bodies from the root vascular bundles [Guo et al. 2010]. Tiller buds were produced in the roots of the blood green, indicating that the tissues in the roots of the blood green also could differentiate.

**Effects of PGRs on the rapid propagation *in vitro* of *L. nervosa*.** Due to embryos and natural products, after the seeds germinated aseptically, the protocorms proliferated and developed very well in the MS1 medium without hormones, with a proliferation coefficient of approximately 5.57. Therefore, it prompted our research team to incorrectly conclude that efficient propagation was possible without PGRs. However, when the stem tips were cultured in MS1 medium, they grew slowly and eventually died. In addition, the stem tips were cultured in MS1 medium supplemented with PGR, and their growth conditions were superior to those in MS1 medium without PGR, indicating that PGRs were required for the *in vitro* propagation of *L. nervosa*. From the results of PGR screening, stem tips of *L. nervosa* were able to have bud formation in all growth regulators. However, cytokinin was able to form more buds with lower plant height than the auxin treatment group, indicating that cytokinin stimulated bud formation while also inhibiting bud elongation, similar to the results of *Catasetum fimbriatum* (Morren) Lindl. [Suzuki et al. 2006]. In plant tissue culture, cytokinin and auxin must be added during the proliferation stage. In *Doritis pulcherrima* Lindl., a combination of exogenous auxin and cytokinin was shown to suppress apical dominance, leading to the formation of axillary buds at the nodes [Mondal et al. 2013]. In this study, BA significantly affected the buds' induction. In combina-

tion with 2,4-D and NAA, it caused the formation of cluster buds on the nodes at the base of the stem tip, which increased the proliferation efficiency of *L. nervosa*, indicating that the synergistic effect of multiple hormones had a significant effect on the proliferation of *L. nervosa*. In addition, under the stimulation of the three exogenous hormones, some stem tips produced protocorm-like bodies at the incision. Some studies have suggested that the induction of protocorm-like bodies requires high concentrations of BA [Nayak et al. 2002, Kumar et al. 2022]. There was no protocorm-like body in the medium supplemented with BA only, and the frequency of protocorm-like body was low under the effect of the three hormones, further proving that the synergistic effect of hormones was beneficial to the protocorm-like body. However, the concentration of BA used was probably too low for the protocorm-like body to be fully stimulated.

**Effect of natural additives on the rapid propagation of *L. nervosa in vitro*.** In the *in vitro* rapid propagation of many orchids, natural organic additives such as coconut juice, mashed bananas, mashed potatoes, and apple juice are essential in stimulating cell differentiation and development [Momtaj et al. 2021]. However, natural additive products affect different orchids differently; in *Cymbidium pendulum* (Roxb.) Sw., coconut water promoted proliferative protocorm, and banana puree promoted root development and plant growth [Kaur and Bhutani 2012]. Banana puree in *Dendrobium* could promote the growth and development of protocorm-like bodies [Islam et al. 2015]. In *Bulbophyllum nipondhii* Seidenf., potato mash promoted the induction of pseudobulbs into protocorms [Pakum et al. 2016]. We tried to complete the rapid propagation without adding natural organic additives to *L. nervosa*, and although the stem tips were still able to produce basal stem cluster buds without adding natural organic additives, the plant leaves were vanishingly yellow, and the growth rate was slow. The protocorm-like body was also found to only appear in the medium with natural organic additives. Natural organic additives had an essential influence on the growth and development of *L. nervosa* and the formation of the protocorm-like body. Some studies showed that the total nitrogen content in orchids was roughly twice that of legumes, indicating that the growth and development of orchids require more organic material than other plants [Stöckel et al. 2014]. Banana puree and mashed potatoes contained some substances needed for the growth of *L. nervosa*, which could ensure its healthy growth during *in vitro* propagation.

## DISCUSSION

This is the first study reporting *in vitro* rapid propagation of *L. nervosa*, providing artificial breeding technology for further research and development of this precious orchid medicinal plant. The results indicated that the suitable essential medium for *L. nervosa* growth was 1/3 MS medium supplemented with 25 g · $L^{-1}$ sucrose, 50 g · $L^{-1}$ banana puree, 40 g · $L^{-1}$ mashed potato, and 1.0 g · $L^{-1}$ AC. Then, the stem tips were cultured on MS1 medium containing 0.5 mg · $L^{-1}$ BA, 0.05 mg · $L^{-1}$ 2,4-D, and 1.5 mg · $L^{-1}$ NAA, with a proliferation coefficient 10.12. In this medium, the stem tip proliferated in four distinct ways: basal stem cluster bud, tiller bud of roots, high-position bud, and wound protocorm-like body. All plantlets developed roots in MS1 medium and 0.5 mg · $L^{-1}$ NAA. The survival rate was 100% after domestication and transplantation.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest to report regarding the present study.

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