Postprint of Research on Efficient Plant Regeneration System for Cymbidium tracyanum

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Abstract

Cymbidium tracyanum is a perennial herbaceous plant in the family Orchidaceae and genus Cymbidium, and is a wild flower of great ornamental value. Currently, research reports on tissue culture rapid propagation of Cymbidium tracyanum are scarce. This study used wild Cymbidium tracyanum seeds as explants, and by analyzing the effects of different basal media and plant hormone combinations on protocorm induction, proliferation, and differentiation, as well as the effects of photoperiod and culture temperature on in vitro seedling growth, screened for suitable conditions for efficient plant regeneration of Cymbidium tracyanum. The results showed that: the suitable basal medium for Cymbidium tracyanum growth was 1/2 MS; the optimal medium for seed germination and protocorm induction was 1/2 MS + 1.0 mg · L-1 6-BA + 0.5 mg · L-1 NAA, after 50 days of culture, 95.00% of seeds developed into protocorms; the optimal medium for protocorm proliferation was 1/2 MS + 2.0 mg · L-1 NAA, after 30 days of culture, the proliferation coefficient was 4.25; the optimal differentiation medium for protocorms was 1/2 MS + 2.0 mg · L-1 NAA + 60 g · L-1 potato homogenate + 0.5 g · L-1 activated charcoal, after 10 days of culture, the adventitious bud induction rate was 98.33%, and when cultured to 40 days, the seedling rooting rate was 94.67%; when in vitro seedlings were cultured under conditions of 20°C temperature, 12 h · d-1 photoperiod, and 2 000 lx light intensity, seedlings exhibited vigorous growth, and the incidence of physiological leaf tip necrosis was only 3.33%; using humus soil as the cultivation substrate, the transplant survival rate of in vitro seedlings was 97.78%. These results provide a scientific basis and technical support for the conservation of wild Cymbidium tracyanum resources and industrialized seedling production.

Full Text

Preamble

Research on an Efficient Plant Regeneration System for *Cymbidium tracyanum*

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Abstract: *Cymbidium tracyanum* is a perennial herbaceous plant in the Orchidaceae family that possesses exceptional ornamental value as a wild flower. However, research on its tissue culture and rapid propagation remains scarce. This study utilized wild *C. tracyanum* seeds as explants to investigate the effects of different basal media and plant hormone combinations on protocorm induction, proliferation, and differentiation, as well as the influence of photoperiod and culture temperature on plantlet growth, in order to identify optimal conditions for efficient plant regeneration.

The results demonstrated that 1/2 MS was the most suitable basal medium for *C. tracyanum* growth. The optimal medium for seed germination and protocorm induction was 1/2 MS supplemented with 1.0 mg · L^{-1} 6-BA and 0.5 mg · L^{-1} NAA, achieving a 95.00% protocorm formation rate after 50 days of culture. For protocorm proliferation, the best medium was 1/2 MS + 2.0 mg · L^{-1} NAA, yielding a proliferation factor of 4.25 after 30 days. The optimal differentiation medium was 1/2 MS + 2.0 mg · L^{-1} NAA + 60 g · L^{-1} mashed potato + 0.5 g · L^{-1} activated carbon, which produced an adventitious bud induction rate of 98.33% after 10 days and a rooting rate of 94.67% after 40 days. When plantlets were cultured at 20°C under a 12 h · d^{-1} photoperiod with 2,000 lx light intensity, they exhibited vigorous growth with only 3.33% physiological leaf tip scorch. Using humus as the cultivation substrate, the transplanting survival rate reached 97.78%. These findings provide a scientific basis and technical support for the conservation of wild *C. tracyanum* resources and its commercial-scale micropropagation.

Keywords: *Cymbidium tracyanum*, seed, protocorm, tissue culture, plant regeneration

Introduction

Cymbidium tracyanum is an epiphytic, perennial herbaceous orchid species native to southwestern Guizhou, southwestern to southeastern Yunnan, and southeastern Tibet, China. Characterized by its long, green leaves and bold, magnificent floral displays with delicate fragrance, it is also known as "Chenxiang Huto Lan" and holds significant ornamental and economic value as a potted or cut flower. Under natural conditions, *C. tracyanum* seeds exhibit extremely low germination rates, and the species primarily reproduces through division, resulting in slow resource regeneration. Consequently, it is protected under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and listed as a Category I protected plant in both the *China Species Red List* (Flora Section) and the *National Key Protected Wild Plants List* (Second Batch Discussion Draft).

Despite its conservation status and commercial potential, few studies have focused on *C. tracyanum*. Existing research primarily addresses environmental stress response mechanisms and tissue culture techniques. While domestic scholars have conducted some micropropagation studies using *C. tracyanum* seeds as explants, these efforts have been hampered by low protocorm induction and differentiation rates, weak seedlings, and inconsistent culture cycles. To address these limitations, this study systematically investigated seed-induced protocorm formation and the conditions for proliferation and regeneration, aiming to establish an efficient rapid propagation system that would support both wild resource conservation and commercial seedling production.

Materials and Methods

Plant Material

Undehisced mature capsules of wild *C. tracyanum* [FIGURE:2] were collected from Medog County, Nyingchi City, Tibet Autonomous Region.

Seed Sterilization and Inoculation

Capsules were first washed with detergent and surface debris was gently scraped away with a scalpel before rinsing under running tap water for one hour. Under aseptic conditions, capsules were surface-sterilized in 75% ethanol for 5 minutes, rinsed three times with sterile water, then treated with 0.1% $HgCl_2$ solution for 15 minutes with gentle agitation to ensure complete contact, followed by three additional sterile water rinses and a 30-minute soak in sterile water. Sterilized capsules were blotted dry on sterile filter paper, longitudinally split with a scalpel to release the seeds, and the seeds were gently shaken onto protocorm induction medium using forceps.

Protocorm Induction

Fourteen different induction media were prepared using either 1/2 MS or MS basal medium supplemented with various ratios of 6-BA and NAA . Six culture vessels were inoculated per treatment, with 3 mL of liquid medium of the same formulation added every two weeks to maintain moisture and nutrient levels. Seed germination, protocorm induction, and growth were monitored to identify the optimal basal medium and induction conditions.

Protocorm Proliferation

Based on the optimal basal medium identified above, proliferation media were prepared using 1/2 MS supplemented with different NAA concentrations, with the best induction medium serving as control . Healthy protocorms were inoculated into test media (six vessels per treatment, 50 protocorms per vessel). After 30 days, proliferation effects were evaluated and multiplication rates were calculated using the formula: Proliferation factor = (Number of protocorms after proliferation - Number of inoculated protocorms) / Number of inoculated protocorms.

Protocorm Differentiation

Differentiation media were prepared by adding 60 $g \cdot L^{-1}$ mashed potato and/or 0.5 $g \cdot L^{-1}$ activated carbon to the optimal proliferation medium, with the proliferation medium alone as control . Healthy protocorms cultured for 30 days on proliferation medium were inoculated into test media (six vessels per treatment, 50 protocorms per vessel). Adventitious bud induction rate was recorded on day 10, and rooting rate was assessed on day 40, with seedling growth status documented throughout.

The formulas used were:
Adventitious bud induction rate (%) = (Number of protocorms producing buds $ $0.5 cm / Total inoculated protocorms) × 100
Rooting rate (%) = (Number of seedlings with roots and shoots $ $2 cm / Total inoculated protocorms) × 100

All media were solidified with 6.5 $g \cdot L^{-1}$ agar and contained 25 $g \cdot L^{-1}$ sucrose at pH 5.8. Culture conditions were maintained at (25 ± 2)°C with a 16 $h \cdot d^{-1}$ photoperiod at 2,000 lx light intensity.

Screening of Environmental Factors for Plantlet Growth

Healthy plantlets approximately 1 cm tall without physiological leaf tip scorch were selected and inoculated onto the optimal differentiation medium. They were cultured at (25 ± 2)°C and 2,000 lx light intensity under photoperiods of 8, 12, 16, and 20 $h \cdot d^{-1}$. Based on the optimal photoperiod identified, plantlets were then cultured at temperatures of 15, 20, 25, and 30°C . Each photoperiod and temperature treatment consisted of 20 plantlets (one vessel with 50 mL

medium) in three replicates. Physiological leaf tip scorch rate was calculated as: (Number of scorched plantlets / Total inoculated plantlets) × 100.

Acclimatization and Transplanting

Robust plantlets with well-developed root systems and approximately 10 cm height were selected for acclimatization. Culture vessels were opened and exposed to natural light for 3 days before seedlings were carefully removed, washed free of residual medium, and transplanted into one of five substrates: (I) farmland soil, (II) humus, (III) farmland soil:humus (1:1), (IV) humus:vermiculite:perlite (8:1:1), or (V) mixed soil:vermiculite:perlite (8:1:1). Substrates were thoroughly watered before and after transplanting. Each treatment comprised 30 plantlets in three replicates. Transplanted seedlings were maintained in a greenhouse at (20 ± 2)°C with 70-80% humidity for 20 days, after which growth status and survival rates were recorded. Transplanting survival rate was calculated as: (Number of wilted/dead plantlets / Total plantlets) × 100.

Results

Seed-Induced Protocorm Formation

Among the 14 induction media prepared with different basal media and hormone combinations, significant variations in protocorm induction were observed . The 1/2 MS basal medium supported faster protocorm induction and growth compared to MS medium. The A5 medium (1/2 MS + 1.0 mg·L^{-1} 6-BA + 0.5 mg·L^{-1} NAA) produced the best results, with seeds beginning to swell and turn light yellow after (36.83 ± 0.90) days, and 95.00% developing into yellow-green or dark green, granular protocorms within approximately 50 days [FIGURE:2]. In contrast, MS-based media resulted in slower germination and lower induction rates. Both 6-BA and NAA positively influenced seed germination and protocorm induction: 0.5 mg·L^{-1} NAA promoted germination and reduced initiation time, while 1.0 mg·L^{-1} 6-BA enhanced protocorm formation and growth. However, 6-BA at 2.0 mg·L^{-1} inhibited growth and caused severe browning. The combination of 1.0 mg·L^{-1} 6-BA and 0.5 mg·L^{-1} NAA significantly shortened germination time and increased induction rates, producing vigorous protocorms. In hormone-free 1/2 MS and MS media, germination required (66.17 ± 0.55) and (86.17 ± 0.69) days respectively, with only 10.00% induction and slow growth after 3-4 months. Therefore, the optimal induction medium was identified as A5: 1/2 MS + 1.0 mg·L^{-1} 6-BA + 0.5 mg·L^{-1} NAA.

Protocorm Proliferation

Healthy green protocorms from the A5 medium were transferred to proliferation media containing different NAA concentrations, with the A5 medium as control

. Protocorm proliferation and vigor increased with NAA concentration up to 2.0 mg · L^{-1}, reaching a maximum proliferation factor of (4.25 ± 0.05) at this concentration. These protocorms appeared yellow-green or dark green, mostly spherical or conical with protrusions at one or both ends, often clustering together in mulberry-like aggregates [FIGURE:2]. NAA concentrations exceeding 2.0 mg · L^{-1} inhibited proliferation. The control A5 medium yielded a proliferation factor of only (2.03 ± 0.10) with fewer protrusions. Thus, the optimal proliferation medium was B4: 1/2 MS + 2.0 mg · L^{-1} NAA.

Protocorm Differentiation

Protocorms with protrusions, cultured for 30 days on proliferation medium, were transferred to differentiation media supplemented with mashed potato and activated carbon . The addition of 60.0 g · L^{-1} mashed potato significantly enhanced adventitious bud induction and rooting rates while promoting vigorous seedling growth. Incorporating 0.5 g · L^{-1} activated carbon markedly reduced basal browning and further stimulated bud induction and root development. On the C2 medium, buds and roots emerged within approximately 10 days, with an adventitious bud induction rate of (98.33 ± 1.37)%. New dark green protocorms formed simultaneously, clustering tightly with white, hair-like rhizoids [FIGURE:2]. After 40 days, the rooting rate reached (94.67 ± 2.21)%, with most seedlings exceeding 2 cm in height and developing robust root systems [FIGURE:2]. Therefore, the optimal differentiation medium was C2: 1/2 MS + 2.0 mg · L^{-1} NAA + 60 g · L^{-1} mashed potato + 0.5 g · L^{-1} activated carbon.

Effects of Photoperiod and Temperature on Plantlet Growth

During subculture, *C. tracyanum* plantlets frequently exhibited physiological leaf tip scorch. To address this, healthy seedlings were cultured on the optimal differentiation medium under various photoperiods and temperatures [TABLE:4, TABLE:5]. Photoperiod significantly affected both growth vigor and scorch incidence: longer photoperiods increased scorch severity, while shorter periods resulted in slow, weak growth. A 12 h · d^{-1} photoperiod produced robust, fast-growing seedlings despite a (28.33 ± 2.36)% scorch rate, making it the optimal choice. When this photoperiod was combined with different temperatures, 20°C proved ideal, producing vigorous growth with minimal leaf tip scorch (3.33%) [FIGURE:2]. Temperatures of 25-30°C exacerbated scorch, while 15°C produced weak growth without scorch. Consequently, the optimal conditions for subculture were identified as 20°C with a 12 h · d^{-1} photoperiod.

Plantlet Transplanting

When *C. tracyanum* plantlets with roots reached approximately 10 cm in height [FIGURE:2], they were acclimatized and transplanted. Substrate composition significantly affected survival rates

. Humus-based substrates outperformed farmland soil, with pure humus achiev-

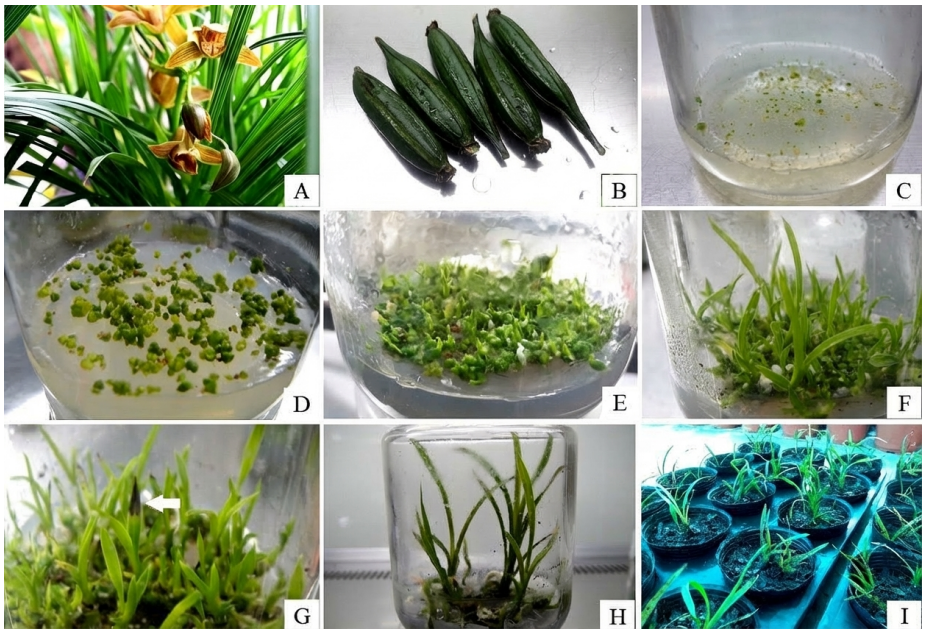

Figure 1: Figure 1

ing the highest transplanting survival rate of (97.78 ± 1.57)% and producing robust, vigorously growing plants [FIGURE:2]. Therefore, humus (Treatment II) was identified as the optimal transplanting substrate.

Discussion and Conclusion

Cymbidium tracyanum is a valuable orchid species with high ornamental and economic potential. Previous tissue culture studies have reported varying results. Li et al. (2005) used 1/2 MS with 6-BA and NAA for seed germination, KT and NAA for protocorm induction, and 6-BA with NAA for differentiation. Wang et al. (2009) reported over 90% germination on both 1/2 MS and MS media supplemented with potato extract, though MS was considered superior for germination and protocorm proliferation, and noted that high 6-BA concentrations weakened plantlets. Lan et al. (2010) achieved 90-98% germination using Hyponex medium with banana and apple extracts, with optimal differentiation on MS + 1.0 mg $\cdot$ L^{-1} NAA + banana extract (89% bud differentiation), and rooting on 1/2 MS + 1.0 mg $\cdot$ L^{-1} NAA + banana extract. Chen et al. (2015) found that MS with 0.5 mg $\cdot$ L^{-1} NAA, potato extract, and activated carbon was suitable for germination and seedling proliferation, while MS with 1.0 mg $\cdot$ L^{-1} NAA, banana extract, and activated carbon promoted rooting. These studies provided fragmented data without comprehensive technical protocols.

This research systematically investigated the complete micropropagation process from seed germination through protocorm induction, proliferation, differentiation, environmental optimization, and transplanting, establishing a highly efficient regeneration system for *C. tracyanum*. Our results demonstrate that 1/2 MS is superior to MS for all stages of protocorm development. The combination of 1.0 $mg \cdot L^{-1}$ 6-BA + 0.5 $mg \cdot L^{-1}$ NAA significantly improved induction efficiency and reduced germination time compared to hormone-free or single-hormone treatments. Protocorms achieved a 4.25-fold proliferation on 1/2 MS + 2.0 $mg \cdot L^{-1}$ NAA within 30 days, and the addition of 60 $g \cdot L^{-1}$ mashed potato + 0.5 $g \cdot L^{-1}$ activated carbon effectively promoted differentiation. The complete process from seed to 2 cm rooted plantlet required only 4-5 months, with both induction and differentiation rates exceeding 95%. Subsequent subculture for two months at 20°C under 12 $h \cdot d^{-1}$ photoperiod produced robust 10 cm plantlets. Compared with previous studies, our protocol simplifies medium formulations and procedures while achieving higher efficiency and shorter culture cycles, reducing production costs.

Physiological leaf tip scorch, which sometimes affected over half of the leaves in culture vessels, was identified as a critical issue affecting plantlet quality. This phenomenon is typically caused by low/high humidity, excessive light, or high temperature. Standard culture conditions of (25 ± 2)°C, 16 $h \cdot d^{-1}$ photoperiod, and 2,000 lx are unsuitable for *C. tracyanum* subculture. As an epiphytic orchid that can tolerate brief direct sunlight, *C. tracyanum* requires moderate shading. Our optimization revealed that 12 $h \cdot d^{-1}$ photoperiod at 20°C minimized scorch incidence to 3.33%.

This efficient regeneration system provides a solid foundation for *C. tracyanum* propagation and offers valuable technical guidance for wild resource conservation and sustainable utilization.

References

China Flora Editorial Board, 1999. *China of Flora* [M]. Beijing: Science Press, 18: 201.

CHEN HM, LÜ FB, LI Z, et al, 2015. The tissue culture and rapid propagation of *Cymbidium tracyanum* L. Castler. [D]// Chinese Society for Horticultural Science. *Advances in Ornamental Horticulture of China*. Beijing: China Forestry Publishing House: 253-255.

Institute of Botany, the Chinese Academy of Sciences, 2013. List of rare and endangered plants in China [DB/OL]. http://rep.iplant.cn/protlist.

KUANG ML, ZHANG BS, 2015. Physiological response to high light in *Cymbidium tracyanum* and *C. sinense* [J]. *Plant Diversity and Resources*, 37(1): 55-62.

LAN YT, WU TG, LIU SY, et al, 2010. Study on tissue culture of wild *Cymbidium tracyanum* L. Castler [J]. *Journal of Fujian Forestry Science and Technology*, 37(1): 77-79.

LI ZL, YU CX, WANG YY, et al, 2005. Research on aseptic budding and fast propagation of the *Cymbidium tracyanum* seed [J]. *Chinese Agricultural Science Bulletin*, 21(8): 269-270, 281.

LI HM, 2014. Advances in studies of tissue culture and rapid multiplication of Orchids [J]. *Agricultural Research and Application*, (1): 53-56.

LIU SS, CHEN J, GUO SX, 2015. Review on germination of Orchid seeds [J]. *Seed*, 34(6): 43-49.

LI JW, Zhang SB, 2016. Differences in the responses of photosystems I and II in *Cymbidium sinense* and *C. tracyanum* to long-term chilling stress [J]. *Frontiers in Plant Science*, doi: 10.3389/fpls.2015.01097.

LI JW, Chen XD, Hu XY, et al, 2018. Comparative physiological and proteomic analyses reveal different adaptive strategies by *Cymbidium sinense* and *C. tracyanum* to drought [J]. *Planta*, 247(1): 69-97.

QIU Y, GONG N, ZHANG KY, 2010. Seed germination and the protocorm multiplication of *Anoectochilus roxburghii* [J]. *Guihaia*, 30(4): 555-559.

WANG LH, JIANG YL, YU JY, et al, 2009. Tissue culture and rapid propagation of *Cymbidium tracyanum* L. Castler [J]. *Plant Physiology Communications*, 45(1): 51-52.

WANG XL, 2018. Evaluation of endangered grade of wild Orchidaceae in Tibet [J]. *Tibet Science and Technology*, (1): 65-67.

WANG ZH, 2003. Diagnosis and treatment of physiological scorch of leaf tip of orchid [J]. *Flower Plant & Penjing*, (6): 32.

WU YG, 2003. Diagnosis and treatment of bacterial scorch of leaf tip of orchid [J]. *Flower Plant & Penjing*, (6): 32.

YAO SC, LING ZZ, LAN ZZ, et al, 2012. Study on seed germination and the protocorm multiplication of *Phaius tankervillese* [J]. *Acta Botanica Boreali-Occidentalia Sinica*, 32(7): 1474-1479.