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Thin Cell Layer-Mediated Somatic Embryogenesis in *Phalaenopsis gigantea*: Effect of 2,4-D Concentration

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Abstract: *Phalaenopsis gigantea* is an endemic orchid species with high ornamental value but limited propagation efficiency using conventional methods. Somatic embryogenesis offers a promising approach for large-scale propagation; however, successful SE induction depends on appropriate plant growth regulator concentrations. This study aimed to evaluate the effect of different concentrations of 2,4-D on somatic embryogenesis induction in *P. gigantea* using the thin cell layer technique. The experiment was arranged in a completely randomized design with six concentrations of 2,4-D (0, 1, 2, 3, 4, and 5 mg L^{-1}), each replicated five times. Leaf-derived TCL explants were cultured on half-strength Murashige and Skoog (½ MS) medium and maintained for eight weeks. The observed parameters included percentage of somatic embryogenesis and embryogenic developmental stage. The results showed that somatic embryogenesis was induced only in explants cultured on medium supplemented with 5 mg L^{-1} 2,4-D, with a somatic embryogenesis frequency of 60%. Lower concentrations of 2,4-D failed to induce embryogenic structures. Somatic embryos first appeared after eight weeks of culture and were morphologically identified as the globular stage, with no further development observed during the culture period. These findings demonstrate that 5 mg L^{-1} 2,4-D was the only concentration within the tested range that induced somatic embryogenic structures in P. gigantea through the TCL technique.

Keywords: Conservation; Globular embryo; Somatic embryogenesis; Thin cell layer; Orchid.

Introduction

Orchids represent one of the most economically valuable ornamental plant groups, with a continuously increasing global market demand. Among them, *Phalaenopsis gigantea* is a particularly valuable species due to its distinctive giant foliage, attractive floral characteristics, and unique ornamental appeal (Aziz *et al*., 2015). However, natural populations of *P. gigantea* have declined considerably because of habitat degradation and excessive collection from the wild, resulting in its limited distribution in natural ecosystems. Consequently, this species has been listed under Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), indicating that international trade must be strictly regulated to prevent further population decline (CITES, 2025).

Natural and conventional propagation of *P. gigantea* is constrained by several biological limitations. This species exhibits an exceptionally slow growth rate, requiring approximately 5–7 years to develop from seed germination to the flowering stage under natural conditions (Hinsley *et al*., 2017). Furthermore, seed germination in *P. gigantea* is inherently restricted because orchid seeds are microscopic and lack endosperm, rendering them devoid of sufficient nutrient reserves to support autonomous embryo development. Consequently, successful

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germination depends entirely on a highly specific symbiotic association with mycorrhizal fungi, which considerably limits seedling establishment under natural conditions (Rasmussen *et al*., 2015; Bedő *et al*., 2023). Therefore, in vitro propagation has become an essential conservation strategy to overcome these biological constraints and facilitate the large-scale production of clonally propagated planting materials.

Among the available regeneration approaches, somatic embryogenesis (SE) enables bipolar embryo development directly from somatic cells without gametic fusion (Zulkarnain, 2009). This process relies on plant cell totipotency, enabling somatic cells to regain embryogenic competence and develop into complete plants under suitable culture conditions (Lestari, 2011). In orchids, somatic embryogenesis is initiated by cellular dedifferentiation, followed by the sequential formation of the globular, heart, torpedo, and cotyledonary embryo stages (Daud *et al*., 2018). The induction of somatic embryogenesis is primarily regulated by controlled exogenous stimuli, particularly the application of plant growth regulators (PGRs), which trigger cellular reprogramming and activate the molecular pathways required for embryogenic development (Mohammadi *et al*., 2023).

Synthetic auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) is commonly used as a PGRs to induce SE because of its strong ability to trigger embryogenic competence in differentiated somatic cells. During the induction phase, 2,4-D promotes cellular dedifferentiation and reprogramming by modulating the expression of genes associated with cell proliferation and developmental regulation while simultaneously eliciting controlled physiological stress that promotes the conversion of somatic cells into an embryogenic state (Raghavan, 2004). At an optimal concentration, 2,4-D has also been reported to enhance antioxidant defense systems, thereby maintaining cellular redox homeostasis during embryogenic induction, while activating key regulatory genes associated with somatic embryogenesis (Gao *et al*., 2022; Singh *et al*., 2022). The effectiveness 2,4-D to induce somatic embryogenesis has been reported in various orchid species, such as Noli *et al*. (2025) reported that half-strength Murashige and Skoog (½ MS) + 2 mg L^{-1} 2,4-D significantly enhanced somatic embryogenesis induction in *Dendrobium discolor*. Similarly, Astuti *et al*. (2019) found that culturing *Vanda sumatrana* on ½ MS + 3 mg L^{-1} 2,4-D resulted in a somatic embryogenesis frequency of 75%, highlighting the species-specific response of orchids to exogenous auxin during embryogenic induction.

Thin Cell Layer (TCL) is a micropropagation technique that utilizes thin explant sections, typically ranging from 0.5 to 2.0 mm in thickness, to enhance morphogenetic responses (Karami *et al*., 2018). The reduced tissue thickness increases the surface-area-to-volume ratio, thereby facilitating the diffusion of plant growth regulators, oxygen, and nutrients into the explant. More importantly, the extensive wound response generated during TCL excision stimulates cellular dedifferentiation and enhances embryogenic competence by promoting direct exposure of competent cells to exogenous signaling molecules and culture medium components (Tan *et al*., 2023). Campos-Boza *et al*. (2022) reported that transverse TCL explants excised from lateral shoots of *Bactris gasipaes* achieved a 60% somatic embryogenesis frequency, substantially exceeding that obtained using conventional explant preparation methods. Therefore, this study aimed to assess the effects of 2,4-D concentrations in combination with the TCL technique on ES induction in *P. gigantea*.

Materials and Methods

Plant Material and Culture Medium

Young leaves excised from *P. gigantea* plantlets were used as explants. The explants were cultured on ½ MS medium supplemented with 30 g L^{-1} sucrose, 7 g L^{-1} agar, 1 g L^{-1} activated charcoal, and 2,4-D (0, 1, 2, 3, 4, and 5 mg L^{-1}), according to the experimental treatments. The medium pH was adjusted to 5.8–6.0 prior to autoclaving at 121°C for 15 min.

Experimental Design and Treatments

The experiment was arranged in a Completely Randomized Design (CRD) with a single factor, namely the concentration of 2,4-D. Six concentrations of 2,4-D (0, 1, 2, 3, 4, and 5 mg L^{-1}) were evaluated. Each treatment was replicated five times, resulting in a total of 30 experimental units.

Explant Culture

Leaves excised from *P. gigantea* plantlets were transversely sectioned into TCL explants approximately 1 mm in thickness and inoculated onto the respective culture media according to the experimental treatments. The cultures were maintained at 24–26°C for eight weeks and monitored periodically for morphological responses and somatic embryogenesis development.

Observation Parameters

The observed parameters included SE induction frequency (%) and the developmental stage of somatic embryos. Embryogenic structures were identified based on their morphological characteristics under a stereomicroscope according to the criteria described by Sasmita *et al*. (2022).

Results and Discussion

Somatic Embryogenesis Frequency (%)

As shown in Table 1, SE was induced exclusively in TCL explants grown on a medium containing 5 mg L^{-1} 2,4-D, indicating that embryogenic induction in *P. gigantea* showed a response only at the highest nominal auxin concentration tested. The enhanced embryogenic response observed in TCL explants may be attributed to the extremely thin tissue sections, which increase the proportion of cells directly exposed to the culture medium, PGRs, and environmental cues, thereby facilitating cellular reprogramming and embryogenic induction (Bravo-Ruiz *et al*., 2022).

Somatic embryogenic structures first became visible after 8 weeks of culture and developed predominantly along the wounded margins of the explants. The relatively long induction period suggests that differentiated somatic cells underwent a sequential process of dedifferentiation, cellular reprogramming, and acquisition of embryogenic competence before entering the embryogenic pathway, consistent with previous reports in orchid species (Guan *et al*., 2016). The preferential formation of somatic embryos at wound sites further supports the role of wound-induced signaling in embryogenic induction. Tissue injury is known to activate a cascade of molecular and hormonal responses, including auxin redistribution and the expression of regeneration-associated genes, thereby creating a favorable microenvironment for somatic embryo initiation. Similar observations were reported by Kuo *et al*. (2005), cells adjacent to wound sites possess a greater embryogenic potential than cells located in intact tissues.

Table 1. Somatic embryogenesis frequencyof*P. gigantea* TCL explants at different 2,4-D concentrations

2,4-D concentrations (mg L^{-1})	Somatic embryogenesis frequency (%)
0	0
1	0
2	0
3	0
4	0
5	60

The successful somatic embryogenesis induction in TCL explants was likely associated with the wound response triggered during explant excision. Wounding initiates a complex network of physiological and molecular processes, including the accumulation of ROS, redistribution, and the activation of dedifferentiation-associated regulators (Iwase *et al*., 2011; Ikeuchi *et al*., 2019). However, these wound-induced signals resulted in somatic embryogenesis only in explants grown on medium containing 5 mg L^{-1} 2,4-D, suggesting that *P. gigantea* requires a relatively high auxin concentration to initiate the embryogenic developmental program. As a synthetic auxin, 2,4-D promotes cellular reprogramming through extensive transcriptional and epigenetic reorganization, leading to the activation of key embryogenesis-associated regulators (Nic-Can & Loyola-Vargas, 2016; Elhiti *et al*., 2022).

Morphological Characterization of Somatic Embryos

Based on morphological observations, SE was first detected in *P. gigantea* TCL explants

grown on 5 mg L^{-1} 2,4-D-supplemented medium after eight weeks of culture (Figure 1). The embryogenic structures exhibited morphological characteristics typical of the globular stage, including a spherical and compact appearance, a creamy white coloration, and preferential development along the wounded margins of the explants (Figure 2), consistent with the morphological criteria described by Von Arnold *et al*. (2002). The globular stage represents the earliest recognizable phase of SE development, marking the successful transition of differentiated somatic cells into embryogenic cells through intensive mitotic activity and the establishment of a bipolar developmental program (Fehér, 2019).

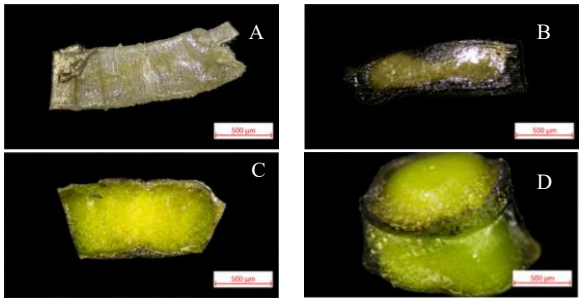


Figure 1. Morphological characteristics of *P. gigantea* TCL explants cultured on medium containing 0–5 mg L^{-1} 2,4-D: (A) norespon (B) browning (C) yellow-green and (D) ES

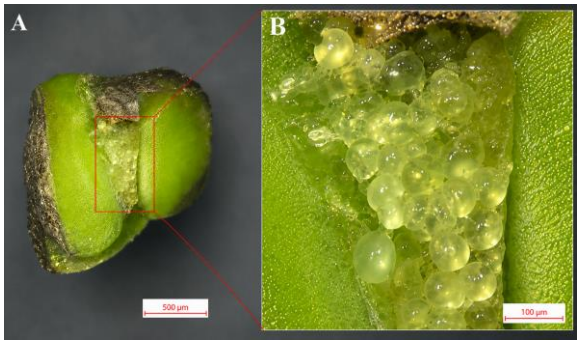


Figure 2. Globular-stage somatic embryos of *P. gigantea* after 8 weeks of culture. Scale bars = 500 µm (A) and 100 µm (B).

The preferential formation of somatic embryos at wound sites further demonstrates the importance of the TCL technique in enhancing embryogenic competence through wound-induced signaling. Tissue injury activates a complex network of physiological and molecular responses that initiate cellular dedifferentiation, whereas 2,4-D promotes cellular reprogramming and directs competent cells toward the embryogenic developmental pathway (Ikeuchi *et al*., 2019; Nic-Can & Loyola-Vargas, 2016). Nevertheless, somatic embryo development was arrested at the globular stage throughout the 8-week culture period, indicating that the culture conditions were sufficient to induce embryogenic competence but inadequate to support subsequent embryo maturation and progression to later developmental stages.

These findings are consistent with those of Tokuhara and Mii (2001) and Chen *et al*. (2006), who identified the globular stage as the earliest morphological stage of SE in *Phalaenopsis*. Similar developmental arrest at the globular stage has also been reported in several orchid species when the culture medium or PGR balance failed to provide the physiological conditions required for embryo maturation (Tan *et al*., 2023).

Conclusion

The addition of 5 mg L^{-1} 2,4-D was the only tested concentration that induced somatic embryogenic structures in *P. gigantea* TCL explants, with a reported induction frequency of 60%. Nevertheless, embryo development remained at the globular stage after eight weeks of culture, suggesting that further optimization of the culture system is necessary to facilitate embryo maturation and subsequent developmental progression.

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