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Propagation of orchids using protocorm-like bodies (PLBs): A review

Dr. Manju M George
Department of Botany, Plant Biotechnology Lab, Union Christian College, UCC Post, Aluva, Ernakulam, Kerala, India

Abstract
Protocorm-like bodies (PLBs) represent a critical advancement in orchid micropropagation, enabling mass production of genetically uniform plants for commercial floriculture and conservation. This review synthesizes current knowledge on PLB-based propagation systems across diverse orchid genera, examining explant sources, culture conditions, hormonal requirements, and emerging technologies. PLBs can be induced from various explant types including shoot tips, leaves, protocorms, and roots. Cytokinins, particularly thidiazuron (TDZ) and 6-benzyladenine (BA), are essential for PLB induction, while auxins regulate subsequent development. Advanced systems including thin cell layer culture, temporary immersion bioreactors, and optimized light regimes have significantly enhanced propagation efficiency, with some protocols achieving over 20,000 plantlets annually from minimal starting material.

Keywords: Protocorm-like bodies (PLBs), Orchid micropropagation, *In vitro* regeneration, Plant tissue culture

Introduction

Orchidaceae, comprising over 25,000 species, represents one of the largest and most diverse plant families, with significant economic importance in the global floriculture industry (Cardoso *et al*., 2020) [4]. The commercial value of orchids, particularly genera such as *Phalaenopsis*, *Dendrobium*, *Cymbidium*, and *Cattleya*, has driven extensive research into efficient propagation methods. Traditional propagation through seeds is time-consuming and produces genetically variable offspring, while vegetative division yields limited numbers of plants (Arditti & Ernst, 1993) [1]. These limitations have necessitated the development of *in vitro* micropropagation techniques.

Protocorm-like bodies (PLBs) have emerged as the preferred propagation system for orchids. PLBs are somatic structures that morphologically and developmentally resemble protocorms—the post-germination stage of orchid seeds—but originate from vegetative tissues rather than zygotic embryos (Lee *et al*., 2013) [24]. The PLB-based propagation system enables rapid multiplication, maintains genetic fidelity, and can be applied to a wide range of orchid species (Cardoso *et al*., 2020) [4]. This technology has become indispensable for commercial orchid production, conservation of endangered species, and breeding programs.

Modern approaches incorporate optimized culture media, specific plant growth regulator combinations, advanced culture systems such as temporary immersion bioreactors, and refined environmental controls (Ekmekçigil *et al*., 2019) [7]. This review examines current knowledge on PLB-based orchid propagation, synthesizing findings from recent research to provide a comprehensive overview of methodologies, applications, and future directions.

Biological Nature and Characteristics of PLBs

PLBs are unique morphogenic structures that serve as the foundation for orchid micropropagation. Structurally, PLBs resemble natural protocorms, exhibiting similar morphological features including a globular or ovoid shape, absence of distinct shoot and root poles initially, and the presence of meristematic tissue capable of organogenesis (Lee *et al*., 2013) [24]. However, PLBs differ from true protocorms in their origin, arising from somatic tissues through either direct organogenesis or indirect pathways involving callus formation.

Lee *et al*. (2013) [14] provided compelling evidence that orchid PLBs are functionally equivalent to somatic embryos, demonstrating similar developmental patterns and cellular organization. However, molecular studies have revealed both similarities and differences between PLB formation and classical somatic embryogenesis, suggesting that PLB induction may represent a distinct morphogenic pathway (Cardoso *et al*., 2020) [4].

Histological analyses across multiple species have demonstrated that PLBs typically originate from epidermal or subepidermal cells of explant tissues. In *Epidendrum secundum*, PLBs formed from successive divisions of epidermal cells in both leaf and protocorm explants, with no intermediate callus phase observed (Ferreira *et al*., 2015) [9]. Similarly, in *Coelogyne flaccida*, PLBs developed directly from epidermal cells of young leaves within four weeks of culture initiation (De & Sil, 2015) [6]. This direct organogenic pathway is advantageous for maintaining genetic stability, as it minimizes the risk of somaclonal variation associated with prolonged callus culture.

The capacity for secondary PLB formation represents a key feature that enables exponential multiplication rates. Primary PLBs can produce multiple secondary PLBs through proliferation, which in turn can generate tertiary PLBs, creating a cascading multiplication effect (Ng & Saleh, 2011) [17]. In *Tolumnia Snow Fairy*, PLBs of 1 to ≤2 mm in diameter continued to proliferate, forming secondary PLBs from either whole PLBs or the upper portion of PLBs (Chookoh *et al*., 2019) [5].

An interesting phenomenon observed in some orchid genera is the interconversion between PLBs and other vegetative structures. In *Cymbidium* species, Ogura-Tsujita *et al*. (2007) [18] demonstrated that PLBs could convert to rhizomes under specific culture conditions, and conversely, rhizomes could produce PLBs. This interconversion was regulated by the balance of auxins and cytokinins, with high cytokinin levels promoting PLB formation and auxin favoring rhizome development.

Explant Sources for PLB Induction

The choice of explant tissue significantly influences PLB induction efficiency, with different orchid species showing varying responses to specific explant types. Research has demonstrated successful PLB induction from diverse tissue sources, including shoot tips, leaves, protocorms, roots, and floral tissues.

1. Shoot Tip Explants

Shoot tips represent one of the most commonly used explant sources for PLB induction. In *Phalaenopsis* hybrids, Preetha *et al*. (2017) [20] developed an innovative approach using longitudinally bisected shoot tips from 3-3.5 month old axenic plantlets, which resulted in earlier PLB induction (20-25 days) compared to intact shoot tips (6-8 weeks). The bisected shoot tips produced 13.6-15.2 shoot buds within 8 weeks, representing a nearly three-fold increase over intact shoot tips. This method demonstrated that physical manipulation of explants could enhance morphogenic response, likely by increasing the surface area of exposed meristematic tissue and disrupting apical dominance.

The age and physiological state of shoot tip explants critically affect PLB induction success. Preetha *et al*. (2017) [20] found that axenic plantlets aged 3-3.5 months showed approximately five-fold higher PLB/shoot bud induction percentage (66.7-73.3%) compared to plantlets less than 3 months old (13.3%).

2. Leaf Explants

Leaf tissues have proven to be highly effective explant sources for PLB induction in numerous orchid species. In *Tolumnia Snow Fairy*, Chookoh *et al*. (2019) [5] achieved PLB induction from leaf segments, with inner, expanding leaves cultured on MS medium supplemented with 4 mg·L^{-1} BA and 0.5 mg·L^{-1} NAA yielding an average of 25.5 PLBs per explant. The study demonstrated that leaf position and developmental stage significantly influenced regeneration capacity, with younger, actively growing leaves showing superior response.

In *Epidendrum secundum*, Ferreira *et al*. (2015) [9] found that 2-month-old leaves (0.3 cm length) were most responsive, achieving 58% PLB induction, while older 6-month-old leaves showed reduced regeneration capacity. Histological studies have revealed that PLBs from leaf explants typically originate from epidermal cells. In *Coelogyne flaccida*, PLBs formed directly from epidermal cells of young leaves without an intervening callus phase, with the highest number of plantlets (35-36 per explant) regenerating through PLBs after 15 weeks when cultured with NAA (2 mg/l) and kinetin (2 mg/l) (De & Sil, 2015) [6].

3. Protocorm and Other Explants

Protocorms derived from seed germination serve as excellent explant sources for PLB induction. In *Epidendrum secundum*, protocorm explants achieved 100% PLB induction, independent of light conditions, demonstrating their superior morphogenic capacity compared to leaf explants utilized protocorms from *Phalaenopsis circus* seeds as explants, achieving the highest number of PLBs (75.0) on medium enriched with 1.0 mg l^{-1} 2,4-D. (Ferreira *et al*., 2015) [9].

In *Cypripedium macranthos var. rebunense*, Shimura and Koda (2004) [22] successfully induced PLBs from mature seeds following a 3-month cold stratification period. The resulting PLBs could be subcultured eight times over 2 years without losing regeneration ability, with one PLB aggregate producing about 10 plants within a year.

4. Thin Cell Layer (TCL) Explants

Thin cell layer (TCL) technology represents an advanced explant preparation method that has significantly enhanced PLB induction efficiency. TCLs consist of explants sectioned to 2-3 cell layers, maximizing the exposure of competent cells to culture medium (Teixeira da Silva, 2013) [24]. In *Phalaenopsis circus*, transversal TCLs with 2-3 cell layers cultured on medium supplemented with 0.5 mg l^{-1} IBA combined with 1.0 mg l^{-1} TDZ produced the highest number of plantlets.

The TCL approach proved particularly effective when combined with temporary immersion bioreactor systems. Ekmekçigil *et al*. (2019) [7] demonstrated that transverse thin cell layers of PLBs (tTCL-PLB) cultured in RITA® bioreactors produced 111.9 PLBs per explant—21 times higher than semi-solid culture—and 199.9 shoots per explant, representing a 95-fold increase over conventional methods.

Culture Media and Hormonal Requirements

The composition of culture media and the specific combinations of plant growth regulators (PGRs) are critical determinants of PLB induction, proliferation, and subsequent plantlet development.

1. Basal Media Formulations

Murashige and Skoog (MS) medium serves as the foundation for most orchid propagation protocols, though optimal nutrient concentrations vary by species and culture stage. Kishi *et al*. (1997) [13] found that one-fourth strength MS medium containing 30 g/l sucrose was optimal for PLB induction in monopodial orchids, while one-fourth strength MS with 10 g/l sucrose and modified iron source proved best for PLB micropropagation.

Half-strength MS medium has shown particular effectiveness for certain genera. In *Paphiopedilum rothschildianum*, Ng and Saleh (2011) [17] achieved optimal secondary PLB formation (4.1 per explant) using half-strength MS medium with 4.0 µM kinetin. Alternative basal media have been explored for specific applications. New Dogashima Medium (NDM) proved highly effective for continuous PLB multiplication in *Phalaenopsis*, with Preetha *et al*. (2017) [20] maintaining PLB and shoot bud proliferation through bimonthly subcultivation in hormone-free NDM.

2. Cytokinins for PLB Induction

Cytokinins play a central role in PLB induction, with thidiazuron (TDZ) and 6-benzyladenine (BA) emerging as the most effective compounds across diverse orchid genera. TDZ, a phenylurea-type cytokinin with both cytokinin and auxin-like activities, has demonstrated exceptional potency in promoting PLB formation. In *Phalaenopsis* hybrids, TDZ at 4.54 µM combined with 10% coconut water produced 15.2 shoot buds per shoot tip within 8 weeks (Preetha *et al*., 2017) [20].

BA has proven effective across numerous species. In *Ipsea malabarica*, MS medium with 13.3 µM BA and 2% sugar yielded a mean of 33.1 PLBs within 50 days, with subsequent subculture producing 47.5 PLBs within 50 days (Martin & Madassery, 2005) [15]. For *Tolumnia Snow Fairy*, 4 mg·L^{-1} BA combined with 0.5 mg·L^{-1} NAA resulted in optimal PLB induction from inner leaf explants (Chookoh *et al*., 2019) [5].

Kinetin has shown species-specific effectiveness. In *Paphiopedilum rothschildianum*, 4.0 µM kinetin in half-strength MS medium produced the highest number of secondary PLBs (4.1 per explant) after 8 weeks (Ng & Saleh, 2011) [17]. Meta-topolin (mT), a naturally occurring aromatic cytokinin, has emerged as a promising alternative. In *Cymbidium chloranthum*, a combination of 0.5 mg/L mT and 0.5 mg/L BA promoted 2.97±0.53 shoots and 3.83±1.07 roots, while also yielding an average of 2.24±0.55 PLBs for protocorm multiplication.

3. Auxins and Their Interactions

While cytokinins are essential for PLB induction, auxins play critical roles in regulating PLB development, proliferation, and subsequent organogenesis. The synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) has proven particularly effective for PLB induction in several species. In *Eulophia nuda*, MS medium supplemented with 2 mg/l 2,4-D was optimal for PLB development within 6-8 weeks (Shroti & Upadhyay, 2014) [23]. For *Phalaenopsis circus*, 1.0 mg l^{-1} 2,4-D produced the highest number of PLBs (75.0), while combinations of 0.5 mg l^{-1} 2,4-D with 2.0 mg l^{-1} NAA yielded maximum somatic embryos (12.3/explant) Naphthaleneacetic acid (NAA) is frequently combined with cytokinins to optimize PLB induction and development. In *Dendrobium alba Ascanda dongtarm*, 1.0 mg/l NAA produced the highest number of PLBs (18.58/explant) after 90 days (Islam *et al*., 2014) [23]. Indole-3-butyric acid (IBA) is commonly employed for root induction and plantlet development stages. In *Phalaenopsis amabilis var. Jawa*, a combination of 1.00 mg l^{-1} kinetin with 1.00 mg l^{-1} IBA was suitable for maximum PLB regeneration (30.40/plantlet), leaf number (5.93/plantlet), and root number (8.36/plantlet) (Asa & Kaviani, 2020) [2].

The ratio of cytokinin to auxin is a critical determinant of morphogenic outcome. High cytokinin-to-auxin ratios generally favor shoot and PLB formation, while high auxin-to-cytokinin ratios promote root development or callus formation.

4. Organic Supplements and Carbohydrates

Various organic supplements have been shown to enhance PLB induction and development. Coconut water (CW), a complex mixture of amino acids, vitamins, sugars, and growth factors, has demonstrated consistent benefits across multiple species. In *Paphiopedilum rothschildianum*, 20% coconut water in half-strength MS medium yielded the best plantlet regeneration (67.9%) from tertiary PLBs after 8 weeks (Ng & Saleh, 2011) [17].

Banana homogenate has proven effective for PLB proliferation. In *Paphiopedilum rothschildianum*, tertiary PLBs subcultured onto half-strength plant growth regulator-free MS medium with 60 g/L banana homogenate proliferated to form 9.5-12.1 new PLBs (Ng & Saleh, 2011) [17]. Protein hydrolysates, including peptone, tryptone, and casein hydrolysate, have been incorporated into culture media to enhance PLB formation and growth. In *Vanilla planifolia*, MS medium containing BA 1 mg/l, IBA 0.5 mg/l, and tryptone/peptone 2 g/l successfully induced PLBs from axillary bud explants (Jose & Babu, 2018) [11].

Sucrose concentration significantly influences PLB induction, proliferation, and subsequent development. Most protocols employ sucrose concentrations ranging from 20-30 g/l analyzed endogenous carbohydrate levels during PLB induction and development in *Cattleya tigrina*, finding that the presence of sucrose, fructose, and glucose was related to cell signaling functions. Increased indole-acetic acid (IAA) levels and reduced sugar

levels were strongly linked to morphogenetic response, including PLB formation and leaf primordia development.

Advanced Propagation Technologies

Recent advances in culture systems and environmental control have significantly enhanced the efficiency and scalability of PLB-based orchid propagation.

1. Temporary Immersion Bioreactor Systems

Temporary immersion bioreactors (TIBs) represent a major advancement in orchid micropropagation. The RITA® system, a widely adopted TIB design, periodically immerses explants in liquid medium, providing enhanced nutrient and oxygen availability while preventing hyperhydricity associated with continuous liquid culture. Ekmekçigil *et al*. (2019) [7] demonstrated the exceptional effectiveness of combining thin cell layer technology with RITA® bioreactors for *Cattleya forbesii*. Transverse thin cell layers of PLBs cultured in RITA® bioreactors with optimized conditions (1 min/4 h immersion, 250 mL medium, 20 explants) produced 111.9 PLBs per explant—21 times higher than semi-solid culture. For shoot regeneration, optimal conditions yielded 199.9 shoots per explant, representing a 95-fold increase over conventional methods. This dramatic improvement in multiplication rates has enabled commercial-scale adoption of the technology.

Winarto and Rachmawati (2013) [26] successfully applied liquid culture with orbital shaking for PLB proliferation in *Dendrobium 'Gradita 31'*, using liquid half-strength MS medium supplemented with 0.3 mg/l TDZ and 0.1 mg/l NAA. Periodic subcultures (4-5 times) of 15 days each in this system facilitated rapid PLB multiplication.

2. Light Quality and Photoperiod Manipulation

Light quality, intensity, and photoperiod significantly influence PLB induction, proliferation, and development. Mehbub *et al*. (2022) [16] demonstrated that green LED illumination increased PLB number by 81.1% and fresh weight by 80.8% compared to white fluorescent light in *Dendrobium okinawense*. This substantial improvement highlights the importance of light spectrum in regulating morphogenic processes.

Photoperiod manipulation has proven effective for controlling PLB development. Enoki and Takahara (2014) [8] developed an innovative system for *Phalaenopsis* using elongated PLBs (ePLBs) induced by skotomorphogenesis under dark conditions. Culturing PLBs in darkness suppressed shoot formation and produced ePLBs approximately twice as long as normal PLBs. After acclimation under low light conditions, ePLBs transferred to high light with partial incision yielded 6 times more secondary PLBs than normal PLBs with partial incisions, and 8 times more than normal PLBs without incisions. This method enabled production of over 20,000 plantlets from 5 PLBs within a year.

3. Novel Chemical Additives

Novel chemical additives have emerged as tools for enhancing PLB regeneration. Mehbub *et al*. (2022) [16] investigated the effects of p-chlorophenoxyisobutyric acid (PCIB), an anti-auxin compound, and hexaconazole-mancozeb-iprodione (HMI), an anti-fungal agent, on PLB regeneration in *Dendrobium okinawense*. Low concentrations of PCIB (0.01 mg/L) increased PLB number by 35.9% and fresh weight by 98.3% compared to control cultures. The combination of green LED with 0.01 mg/L PCIB produced synergistic effects, significantly increasing PLB number by 69.0% over white fluorescent light without PCIB.

Hyaluronic acid (HA), a naturally occurring glycosaminoglycan, has shown promise as a novel supplement. Sharmin *et al*. (2020) [21] investigated the effects of two molecular weight forms of HA on *Oncidium Aloha 'Iwanaga'* organogenesis. The highest number of PLBs was obtained at 0.1 mg/l of HA12 (20.7/explant) and HA20 (23.1/explant).

4. Encapsulation and Synthetic Seed Technology

Encapsulation of PLBs in sodium alginate to create synthetic seeds represents an innovative approach for short-term storage, transportation, and direct field planting. Bustam *et al*. (2013) [3] developed protocols for *Dendrobium Shavin White*, finding that PLBs of 3-5 mm with shoot showed significantly higher conversion percentages (85% and 90%) to plantlets. Encapsulated PLBs regenerated best in semi-solid half-strength MS basal medium, with 96% germination and 76% conversion to plantlets. PLBs stored at 25 ± 2 °C survived longer, retaining 80-92% survival up to 75 days and 52% survival up to 135 days.

Species-Specific Protocols and Applications

While general principles of PLB propagation apply across orchid genera, optimal protocols vary considerably among species.

1. Phalaenopsis Species and Hybrids

Phalaenopsis, commonly known as moth orchids, represents the most economically important orchid genus in commercial floriculture. Preetha *et al*. (2017) [20] developed a protocol using longitudinally bisected shoot tips that can produce approximately 50,000 hardened plantlets from a single flower stalk with 10 dormant buds within 2 years, ensuring genetically stable plants without detectable somaclonal variation.

For *Phalaenopsis circus*, established protocols using both protocorms and thin cell layers, achieving 75.0 PLBs per explant with 1.0 mg l^{-1} 2,4-D and 100% survival after acclimatization. Enoki and Takahara (2014) [8]

developed a highly efficient system using elongated PLBs induced under dark conditions, achieving production of over 20,000 plantlets from 5 PLBs within a year.

2. Dendrobium Species

Dendrobium, one of the largest orchid genera with over 1,500 species, includes numerous commercially important ornamental and medicinal species. Winarto and Rachmawati (2013) [26] established a comprehensive protocol for *Dendrobium 'Gradita 31'* using varied Growmore media formulations. Half-strength MS medium containing 1 mg/l TDZ and 0.5 mg/l BA proved effective for high PLB formation from shoot explants, while GM-6 medium was optimal for growth, proliferation, and regeneration, stimulating growth up to 309 mg per month (17 PLBs/month) and inducing 76% PLB regeneration. Plantlets were successfully acclimatized with 100% survivability.

For *Dendrobium okinawense*, Mehbub *et al*. (2022) [16] demonstrated that green LED illumination combined with low concentrations of anti-auxin (PCIB) significantly enhanced PLB regeneration, increasing PLB number by 69.0% over conventional white fluorescent light without PCIB.

3. Other Genera

Cymbidium orchids present unique challenges and opportunities for PLB-based propagation. Mehbub *et al*. (2022) [16] developed an efficient protocol for *Cymbidium chloranthum*, finding that a combination of 0.5 mg/L meta-topolin and 0.5 mg/L BA promoted optimal shoot and root formation, while yielding 2.24±0.55 PLBs for protocorm multiplication. The 66% survival rate during acclimatization enabled successful reintroduction of regenerated plants into their natural habitat.

Cattleya species and hybrids have been successfully propagated using advanced PLB technologies. Ekmekçigil *et al*. (2019) [7] achieved exceptional multiplication rates in *Cattleya forbesii* by combining thin cell layer technology with RITA® temporary immersion bioreactors, producing 111.9 PLBs per explant and 199.9 shoots per explant under optimized conditions.

Paphiopedilum (slipper orchids) are notoriously slow-growing and challenging to propagate. Ng and Saleh (2011) [17] achieved propagation of *P. rothschildianum* via secondary PLB formation from stem-derived callus, with half-strength MS medium containing 4.0 µM kinetin producing the highest number of secondary PLBs (4.1 per explant) after 8 weeks.

4. Conservation Applications

PLB-based propagation has proven particularly valuable for conservation of endangered terrestrial orchids. Martin and Madassery (2005) [15] developed a rapid propagation protocol for the threatened endemic orchid *Ipsea malabarica*, achieving conversion of axillary buds to PLBs with MS medium containing 13.3 µM BA and 2% sugar yielding 33.1 PLBs within 50 days. Reintroduction of PLB-derived plantlets in the natural habitat was attempted to assist in in situ conservation.

For *Eulophia nuda*, another endangered terrestrial orchid, Shroti and Upadhyay (2014) [23] established a micropropagation protocol using 4 mm axillary bud segments, with PLBs developing within 6-8 weeks on MS medium supplemented with 2 mg/l 2,4-D. Thuy *et al*. (2015) developed a rapid micropropagation protocol for the endangered medicinal orchid *Anoectochilus setaceus* through PLBs, achieving 29.66 plantlets per PLB with 86.25% explant regeneration efficiency in a 120-day process.

Molecular and Biochemical Aspects

Understanding the molecular and biochemical mechanisms underlying PLB formation and development is essential for rational optimization of propagation protocols.

1. Genomic and Molecular Studies

The sequencing of the *Phalaenopsis equestris* genome has enabled more detailed molecular studies of PLB formation and development (Cardoso *et al*., 2020) [4]. This genomic resource has facilitated identification of genes involved in morphogenesis, hormone signaling, and stress responses that may regulate PLB induction. Cardoso *et al*. (2020) [4] noted that molecular characterization of plantlets obtained from PLB propagation allows for comprehensive evaluation of the technique's real applications and main limitations, particularly regarding somaclonal variation.

Despite morphological similarities to somatic embryogenesis, molecular differences suggest that PLB induction is not yet fully characterized as a type of somatic embryogenesis, indicating that PLBs may represent a unique developmental pathway specific to orchids (Cardoso *et al*., 2020) [4]. PLB induction has been used as a tool to identify genes of interest and in breeding techniques, including obtaining transgenic plants.

2. Hormonal and Metabolic Regulation

The role of plant hormones in regulating PLB formation has been extensively studied. Many analyzed endogenous indole-acetic acid (IAA) and carbohydrate levels during PLB induction and development in *Cattleya tigrina*, finding that increased IAA and reduced sugar levels were strongly linked to morphogenetic response, including PLB formation and leaf primordia development. The study demonstrated that hormonal signals and carbohydrates alter metabolism, initiating and developing PLBs.

The balance between auxins and cytokinins is a key determinant of developmental fate in orchid tissues. In *Cymbidium* species, high cytokinin levels promote PLB formation while auxin favors rhizome development, demonstrating that the ratio of these hormones can direct tissues toward alternative developmental pathways (Ogura-Tsujita *et al*., 2007) [18].

3. Genetic Stability

A critical concern in PLB-based propagation is the potential for somaclonal variation—genetic or epigenetic changes that occur during tissue culture. While PLBs generally maintain genetic stability better than callus-based systems, particularly when formed through direct organogenesis, some degree of variation can occur. Cardoso *et al*. (2020) [4] emphasized that molecular characterization of plantlets obtained from PLB propagation allows for comprehensive evaluation of somaclonal variation as a main limitation for mass propagation.

The risk of somaclonal variation appears to be influenced by several factors, including the explant source, culture duration, plant growth regulator concentrations, and the developmental pathway (direct vs. indirect organogenesis). Direct PLB formation from epidermal cells, as observed in *Epidendrum secundum* (Ferreira *et al*., 2015) [9] and *Coelogyne flaccida* (De & Sil, 2015) [6], likely minimizes variation by avoiding prolonged callus phases where genetic instability is more likely to occur.

De and Sil (2015) [6] verified that regenerated plants of *Coelogyne flaccida* maintained the same chromosome number (2n = 40) as the mother plant, demonstrating chromosomal stability through the PLB propagation process.

Challenges and Future Directions

Despite significant advances in PLB-based orchid propagation, several challenges and limitations remain.

1. Current Challenges

One of the most significant challenges in orchid micropropagation is the high degree of genotype-specific variation in response to culture conditions. Paek *et al*. (2011) [19] noted that not all genotypes of *Phalaenopsis* respond to the same protocol under the same culture conditions and often result in the development of undesirable characteristics. This genotypic variation necessitates protocol optimization for each new cultivar or species, increasing the time and resources required for commercial implementation.

While some protocols achieve exceptional multiplication rates, many orchid species, particularly terrestrial and temperate taxa, exhibit slow growth and relatively low PLB proliferation rates. Shimura and Koda (2004) [22] noted that PLB growth in *Cypripedium macranthos var. rebunense* was slow, though plantlets could be easily regenerated. The slow growth characteristic of many orchid species reflects their natural ecology and physiology but poses challenges for commercial propagation.

The transition from *in vitro* culture to ex *vitro* conditions represents a critical bottleneck in orchid propagation, with survival rates during acclimatization varying widely among species and protocols. Plantlets produced *in vitro* often have poorly developed cuticles, non-functional stomata, and limited root systems, making them vulnerable to desiccation and pathogen attack when transferred to ambient conditions.

2. Future Directions

The increasing availability of genomic resources for orchids, particularly *Phalaenopsis* species, provides opportunities for molecular approaches to understanding and optimizing PLB formation. Transcriptomic and proteomic analyses comparing PLB-forming and non-forming tissues could identify key regulatory genes and pathways that control morphogenic competence. CRISPR-Cas9 and other genome editing technologies offer potential for modifying genes involved in PLB formation, hormone signaling, or stress responses to create genotypes with enhanced propagation characteristics.

Further refinement of temporary immersion bioreactor systems and development of novel culture platforms could enhance propagation efficiency and reduce costs. The finding that green LED illumination significantly enhances PLB regeneration in *Dendrobium okinawense* (Mehbub *et al*., 2022) [16] suggests that systematic investigation of light quality effects across diverse orchid genera could reveal species-specific optimal light spectra.

The discovery that low concentrations of anti-auxin compounds and anti-fungal agents can enhance PLB regeneration (Mehbub *et al*., 2022) [16] suggests that systematic screening of diverse chemical compounds could identify additional substances with beneficial effects. While encapsulation technology enables short-term storage of PLBs (Bustam *et al*., 2013) [3], development of effective cryopreservation protocols would enable long-term germplasm storage for conservation and breeding purposes.

Development of standardized protocols for major orchid genera would facilitate technology transfer and commercial adoption. While genotype-specific optimization will always be necessary to some degree, establishing baseline protocols that work reasonably well across multiple species within a genus would reduce the time and resources required for protocol development.

PLB-based propagation technology has enormous potential for conservation of endangered orchid species, but realizing this potential requires integration with broader conservation strategies. Protocols must be developed not only for propagation but also for successful reintroduction and establishment in natural habitats. The success of Martin and Madassery (2005) [15] in reintroducing PLB-derived *Ipsea malabarica* plantlets into natural habitat

demonstrates the feasibility of this approach, though long-term monitoring is needed to assess establishment and reproductive success.

Conclusion

Protocorm-like body (PLB) technology has revolutionized orchid propagation, enabling mass production of genetically uniform plants for commercial floriculture, conservation of endangered species, and support of breeding programs. This review has synthesized current knowledge on PLB-based propagation systems, examining explant sources, culture media and hormonal requirements, advanced technologies, species-specific protocols, and molecular aspects of PLB formation and development.

Key findings from the literature demonstrate that PLBs can be successfully induced from diverse explant types including shoot tips, leaves, protocorms, and roots, with optimal explant choice varying by species. Cytokinins, particularly TDZ and BA, are essential for PLB induction, while auxins regulate subsequent development and organogenesis. The balance between these hormones, along with carbohydrate sources and organic supplements, critically influences propagation outcomes. Advanced technologies including thin cell layer culture, temporary immersion bioreactors, optimized light regimes, and novel chemical additives have dramatically enhanced propagation efficiency, with some protocols achieving over 20,000 plantlets annually from minimal starting material.

Despite these advances, significant challenges remain. Genotype-specific variation in culture response necessitates protocol optimization for each new species or cultivar. Slow growth rates in some species, particularly terrestrial and temperate taxa, limit commercial viability. Somaclonal variation, though generally lower in PLB systems than callus-based approaches, remains a concern for maintaining genetic uniformity. Acclimatization and ex *vitro* establishment represent critical bottlenecks where survival rates vary widely.

Future research directions include integration of molecular tools to understand and manipulate PLB formation pathways, optimization of culture systems and environmental controls, discovery of novel chemical additives, development of cryopreservation protocols, and standardization of propagation methods. Applications in conservation and restoration require integration of propagation technology with broader ecological knowledge to ensure successful reintroduction and establishment of endangered species.

The continued evolution of PLB-based propagation technology, driven by advances in plant biology, engineering, and molecular genetics, promises to further enhance the efficiency, reliability, and applicability of this essential tool for orchid cultivation and conservation. As our understanding of the molecular and biochemical mechanisms underlying PLB formation deepens, opportunities will emerge for rational design of optimized propagation systems tailored to specific species and applications. The integration of traditional tissue culture approaches with modern molecular and computational tools will enable the next generation of orchid propagation technologies, ensuring the continued availability of these remarkable plants for future generations.

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