

Endopolyploidy and ploidy stability during protocorm-like body morphogenesis and regeneration in *Cattleya tigrina*

A. Richard

Rafaela Duarte de LIZ¹, Gabriela Ferreira NOGUEIRA^{2,*}, Paulo César Poeta FERMINO Jr.³, Jonny Everson SCHERWINSKI-PEREIRA^{2,*}, José Marcello Salabert de CAMPOS⁴, Inaê Mariê de Araújo SILVA-CARDOSO², Lírio Luiz DAL VESCO¹, and Rosete PESCADOR^{1,*}

¹ Graduate Program in Plant Genetic Resources, Federal University of Santa Catarina, Florianópolis, SC 88034-001, Brazil

² Embrapa Genetic Resources and Biotechnology, Laboratory of Tissue Culture II, Brasília, DF 70770-917, Brazil

³ Department of Natural and Social Science, Federal University of Santa Catarina, Curitibanos, SC 89520-000, Brazil

⁴ Department of Biology, Laboratory of Genetic and Biotechnology, Federal University of Juiz de Fora, Juiz de Fora, MG 36036-900, Brazil

*Corresponding authors: E-mails: jonny.pereira@embrapa.br, rosete.pescador@ufsc.br

Abstract

Background: Protocorm-like bodies (PLBs) represent a major morphogenic pathway in orchids and are characterized by cellular reprogramming, tissue differentiation, and developmental reorganization. Although PLB formation is widely explored for orchid micropropagation, endopolyploidy dynamics during PLB morphogenesis remain poorly understood.

Aims: This study aimed to characterize endopolyploidy patterns and evaluate nuclear DNA content maintenance during PLB morphogenesis and plant regeneration in *Cattleya tigrina*.

Methods: Basal leaf explants were cultured under PLB-inducing conditions *in vitro*. Morphological and histological analyses were performed throughout PLB development, while nuclear DNA content was estimated by flow cytometry in leaf regions, PLBs at successive developmental stages, and regenerated plants.

Results: The leaf base was the most responsive region for PLB induction, showing intense cell proliferation and meristematic activity. Flow cytometric profiles consistently revealed 2C, 4C, and 8C nuclear DNA peaks, indicating endopolyploidy in somatic tissues. PLBs exhibited transient shifts in ploidy distribution during active proliferation phases, particularly at intermediate developmental stages, when 4C nuclei became predominant. Regenerated plants maintained nuclear DNA contents comparable to the original explants.

Conclusions: Endopolyploidy is an intrinsic and developmentally regulated feature of PLB morphogenesis in *Cattleya tigrina*. PLB-mediated regeneration maintained overall nuclear DNA content under the evaluated *in vitro* conditions.

Keywords: endopolyploidy, flow cytometry, morphogenesis, Orchidaceae, ploidy stability, protocorm-like bodies.

Introduction

Orchidaceae is the second largest family of flowering plants (Dressler, 2005), comprising 736 genera and approximately 28 000 species (Christenhusz and Byng,

2016), and ranks among the top 10 angiosperm families in terms of species richness (BFG, 2015). However, because of their ornamental and landscape value, orchids are frequently removed from their natural habitats, which has driven some species to extinction and makes

Received 10 January 2026, last revision 22 April 2026, accepted 21 May 2026.

Abbreviations: FC – flow cytometry; LM – light microscope; MS – Murashige and Skoog; PLB – protocorm-like bodies; PGR – plant growth regulators; SM – stereoscope microscope; TDZ – thidiazuron.

Acknowledgements: This work was supported by the National Council for Scientific and Technological Development (CNPq, Brazil), the Coordination for the Improvement of Higher Education Personnel (CAPES, Brazil), and the State of Santa Catarina Research Foundation (FAPESC). The authors acknowledge the staff of the EMBRAPA Genetic Resources and Biotechnology (Cenargen, Brasília, Federal District) and the Physiology of Plant Development and Genetics Laboratory (LFDGV) of the Federal University of Santa Catarina, Brazil.

Conflict of interest: The authors declare that they have no conflict of interest.

the maintenance of their biodiversity increasingly difficult (Swarts and Dixon, 2009; Hinsley *et al.*, 2018). More than 50% of orchid species assessed in the IUCN Global Red List are threatened with extinction (Gale *et al.*, 2018). Vitt *et al.* (2023) identified 278 orchid species that represent unique evolutionary lineages and/or display unique morphological features requiring urgent conservation actions, and more than 70% of these are not yet protected through *ex situ* approaches.

Cattleya tigrina is an orchid species endemic to the Atlantic Forest, occurring in different regions of Brazil, including the South (Rio Grande do Sul), Southeast (São Paulo), and Northeast (southern portions of Bahia and Pernambuco) (Barros *et al.*, 2015; POWO, 2024). As in other orchids, its *in vitro* morphogenetic responses share features with classical regeneration systems (Yam and Arditti, 2017). Tissue culture has been successfully used for the propagation of various terrestrial and epiphytic orchid species (Santos Díaz and Carranza Álvarez, 2009; Nongdam *et al.*, 2023), many of which are considered vulnerable to environmental change, including climate and land-cover change (Yudaputra *et al.*, 2024). This approach allows rapid multiplication and can therefore facilitate the preservation and propagation of endangered species. Furthermore, tissue culture serves as a model system for studying metabolism and differentiation, providing information on the physiological and biochemical events that underlie modulation during *in vitro* morphogenesis (Chugh *et al.*, 2009).

During *in vitro* establishment, a morphogenic response pathway involving the formation of protocorm-like bodies (PLBs) has been described for several orchid species (Zhao *et al.*, 2008). This pathway has a high potential to replace conventional micropropagation techniques due to its broad application in clonal propagation, conservation, phytosanitary cleaning, and breeding (Cardoso *et al.*, 2020). PLBs are globular structures so named because of their similarity to protocorms of zygotic origin (Yam and Arditti, 2017). The induction of these structures has been reported from leaf tips of *Dendrobium malones* (Anjum *et al.*, 2006), flower-stalk buds of *Phalaenopsis* (Chen *et al.*, 2009), and leaf segments of *Aerides crispum* (Sheelavanthmath *et al.*, 2005) and *C. tigrina* (De Conti *et al.*, 2018; 2020; Fritsche *et al.*, 2022a). This morphogenic pathway has been investigated in *C. tigrina* through morpho-histodifferentiation and biochemical analyses (Liz, 2013; De Conti *et al.*, 2018), which revealed signaling events that promote and regulate PLB formation. PLBs are composed of differentiated tissues (Sujjarithurakarn and Kanchanapoom, 2011) and can develop into plantlets when grown on culture media supplemented with plant growth regulators (Ng and Saleh, 2011). They can proliferate rapidly and regenerate whole plants, and thus represent suitable targets for genetic transformation studies in orchids (Sreeramanan *et al.*, 2008), as well as for the production of polyploids *via* PLBs, as reported for *Dendrobium officinale* (Zhang and Gao, 2020).

In orchids, estimating nuclear DNA content is a useful tool for phylogenetic analyses in generic classification (Burke *et al.*, 2008) and for understanding variation in genome size (Leitch *et al.*, 2009). It is also valuable

for identifying and mapping DNA replication levels (“C-levels”) in tissues and inferring ploidy level in orchids (Kron, 2015; Grosso *et al.*, 2018), as well as for supporting species identification and characterization as an auxiliary tool (Rewers *et al.*, 2021), particularly when interpreted in conjunction with other sources of taxonomic information. In addition, it enables studies of endoreduplication, a process responsible for generating somatic polyploidy in some differentiated cells owing to the absence of mitosis after nuclear DNA amplification (Sliwinska, 2022). Therefore, it is essential to identify and standardize reliable methods for estimating the C-value in orchids. Despite the availability of flow cytometry protocols for Orchidaceae, their application often requires species-specific optimization to account for differences in tissue characteristics and to ensure reliable estimates of nuclear DNA content. The DNA content of a haploid or gametic nucleus of an organism is referred to as the DNA C-value (Bennett *et al.*, 2008), whereas a nucleus in the G1 phase of the cell cycle containing two copies of the non-replicated genome has a DNA 2C value following the conceptual standardization proposed by Greilhuber *et al.* (2005).

Flow cytometry (FC) has been widely used to analyze genetic variation in micropropagated plants and can detect, early in morphogenesis, changes in nuclear DNA content in cells, tissues, or organs grown *in vitro*, including whole plants (Orbović *et al.*, 2008; Escobedo-Gracia Medrano *et al.*, 2014). As a complementary technique, FC provides information that supports plant breeding programs and related research (Sevilleno *et al.*, 2023). This technique stands out for its high accuracy, high throughput, relatively simple sample preparation (Trávníček *et al.*, 2015), rapid analysis, and relatively low cost (Sliwinska, 2022).

In this study, we used FC to estimate nuclear DNA C-values in different types of *C. tigrina* explants grown *in vitro* and integrated these data with morphological and histological analyses of PLB morphogenesis. Specifically, we characterized endopolyploidy patterns during PLB development and assessed whether PLB-mediated regeneration affects nuclear DNA content and ploidy stability in regenerated plants.

Materials and methods

Plant material and *in vitro* culture: Seedlings of *Cattleya tigrina* A. Rich. were obtained by asymbiotic seed germination on half-strength Murashige and Skoog (1962) medium without plant growth regulators (PGRs), supplemented with Morel vitamins (Morel and Wetmore, 1951), sucrose (30 g L⁻¹), activated charcoal (1.5 g L⁻¹) and agar (7 g L⁻¹) as a gelling agent. Cultures were incubated at 25 ± 2°C under a 16-h photoperiod and an irradiance of 50–60 μmol m⁻² s⁻¹. Plantlets were subcultured every 60 d.

Leaves (≈ 1 cm) excised from *in vitro*-grown plantlets of *C. tigrina* were inoculated onto half-strength MS medium supplemented with 9 μM thidiazuron (TDZ), Morel vitamins, soy peptone (1 g L⁻¹), sodium dihydrogen phosphate (NaH₂PO₄; 170 mg L⁻¹), sucrose (20 g L⁻¹) and Phytigel (2 g L⁻¹), following the protocol adapted

by Fritsche (2012) for PLB induction. Cultures were maintained in a growth chamber (PERCIVAL I-35LLVL, Percival Scientific Inc., Boone, IA, USA) at $25 \pm 2^\circ\text{C}$ under a 16-h photoperiod and an irradiance of $50\text{--}60 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Morphological and histological analyses: Representative samples of cultures containing protocorm-like bodies (PLBs) were observed under a Leica EZ4 HD stereomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with an image capture system and LAS EZ 2.0 software (Leica Microsystems, Wetzlar, Germany), in order to record key morphological events at 0 (control), 7, 30, 60, 120, and 180 d of cultivation.

Samples collected at 7, 30, 60, 120, and 180 d were fixed in 2.5% (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) and dehydrated in an ethanol series. For light microscopy, samples were infiltrated with hydroxyethyl methacrylate (Leica Historesin, Heidelberg, Germany), sectioned with a SLEE Cut 4055 rotary microtome (Slee Technik GmbH, Mainz, Germany), and stained with toluidine blue (O'Brien et al., 1964). Sections were examined using an Olympus BX40 light microscope (Olympus Optical Co., Tokyo, Japan), and relevant structures and events were recorded with a DP71 digital camera (Olympus Optical Co., Tokyo, Japan) and Image Q Capture Pro 5.1 software (QImaging Corporation, Austin, TX, USA).

Fresh mass increase and tissue oxidation: Fresh mass increase of PLBs and the percentage of oxidized explants were recorded at 7, 30, 60, 120, and 180 d of cultivation. Fresh mass increase was calculated as fresh mass increase = final fresh mass of PLBs – initial fresh mass of PLBs, and the percentage of oxidized explants as percentage of oxidized explants = (number of oxidized explants $\times$ 100)/total number of cultured explants.

Estimation of nuclear DNA content by flow cytometry: Leaf explants (entire leaves, leaf tips, and leaf bases) were analyzed at 0 and 30 d of cultivation. PLBs were analyzed at 0, 7, 30, 60, 120, and 180 d of cultivation. Leaves and root apices of *C. tigrina* plantlets maintained *in vitro* without PGRs were also analyzed to estimate nuclear DNA content.

Approximately 100–150 mg of leaf explants, PLBs, leaves and roots of *C. tigrina*, and leaves of the external reference standard (*Pisum sativum* L.) were used for sample preparation. To obtain nuclear suspensions, tissues were chopped in Petri dishes containing 1 mL of cold Marie extraction buffer (Marie and Brown, 1993). The resulting suspensions were aspirated with a Pasteur pipette and filtered through a 42- μm mesh (Millipore Corporation, Billerica, MA, USA). Nuclei suspensions were stained with 25 μL of a 1 mg L^{-1} propidium iodide solution.

Histograms were acquired using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with BD Accuri CFlow Plus software (BD Biosciences, San Jose, CA, USA). Nuclear DNA

contents (pg) were calculated according to Doležel et al. (2007) using the formula: DNA content (pg) = (position of the G1 peak of the sample/position of the G1 peak of the reference) $\times$ DNA content of the reference.

For each sample type, 12 replicates were analyzed. Data were processed using the SISVAR statistical software (Ferreira, 2011), and means were compared by Tukey's test at the 5% probability level. All FC analyses were performed at the Tissue Culture Laboratory II (LCTII), Embrapa Genetic Resources and Biotechnology, Brasília, Federal District, Brazil.

Results

Morpho-histological analyses and fresh mass increase:

Morphological and histological observations showed that the leaf base region is the most responsive site for PLB formation in *C. tigrina* (Fig. 1). Evidence of PLB development was detected after 7 d of culture (Fig. 1B). In leaf explants at 60 and 120 d of culture, PLB proliferation was evident at the leaf base and on previously formed PLBs (Fig. 1D,E), although progressive tissue oxidation was also observed. At 180 d of cultivation, leaf primordia were clearly distinguishable (Fig. 1F). Increases in fresh mass were associated with PLB proliferation at the leaf base (Figs. 1, 2). PLBs showed a gradual increase in fresh mass with increasing *in vitro* culture time (Fig. 2).

During histodifferentiation, epidermal cells undergoing both periclinal and anticlinal divisions were observed (Fig. 1H). At 30 d, cells with meristematic features, characterized by conspicuous nuclei, dense cytoplasm, and few intercellular spaces, were present in the leaf mesophyll (Fig. 1I). At 120 d, PLBs exhibited an organized shoot apical meristem (Fig. 1K). At 180 d, PLBs showed apices with well-developed leaf primordia and the formation of provascular tissues (Fig. 1L). Explants in culture exhibited progressive oxidation over time (Fig. 1C–E), which affected the quality of the peaks obtained by FC.

Estimation of DNA content in different leaf regions:

Entire leaves, leaf tips, and leaf bases of *C. tigrina* did not differ in DNA content when compared within each cultivation time (0 or 30 d; Table 1). However, DNA contents differed significantly ($P < 0.05$) between 0 and 30 d for all leaf regions. On average, an increase of approximately 17.3% in 2C DNA content was observed in the tissues between 0 and 30 d of *in vitro* culture.

Estimation of DNA content during PLBs development:

Flow cytometric analyses generated DNA content histograms with distinct peaks for leaf explants and PLBs of *C. tigrina* (Fig. 3). The 2C DNA value estimated for the leaf base of *C. tigrina* was 5.06 pg, and three peaks corresponding to 2C, 4C, and 8C nuclei were detected in the somatic tissues of this species. The estimated DNA content values associated with the 2C, 4C, and 8C peaks varied during PLB development (Table 2). For the 2C class, peak positions at 7, 30, 60 and 120 d were significantly higher ($P < 0.05$) than those observed for the control and

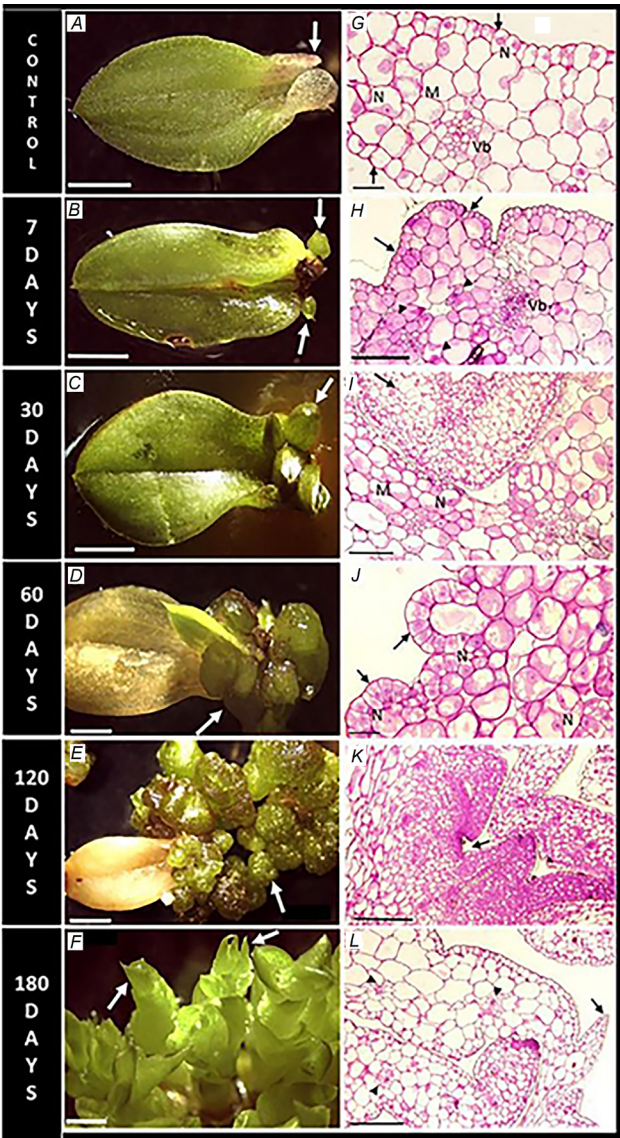


Fig. 1. Protocorm-like bodies (PLBs) formed between 7 and 180 d after inoculation in *Cattleya tigrina*. A – Control leaf, showing the absence of PLBs at the leaf base (arrow). B – Leaf explant at 7 d with initial PLB formation (arrows). C, D – PLBs (arrows) at the leaf base at 30 and 60 d of cultivation, respectively. E – At 120 d, increased numbers of PLBs at the leaf base (arrow). F – At 180 d, PLBs in the regeneration phase. G – Cross-section of a control leaf showing the epidermis (arrows), mesophyll (M), nuclei (N), and a vascular bundle (Vb). H – Cross-section of a leaf explant at 7 d of culture, showing cells with meristematic activity (arrows and arrowheads). I – PLB at 30 d of cultivation, with a peripheral layer of meristematic cells (arrow) and mesophyll nuclei (N). J – PLB with meristematic cells in the epidermis (arrows). K – At 120 d, PLB clusters, some showing a shoot apex (arrow). L – Longitudinal section of PLBs with leaf primordia (arrow) and provascular tissues (arrowheads). Bars: A–F = 2 mm; H, I, K, L = 200 µm; G, J = 50 µm.

180 d samples. In contrast, the histogram at 120 d (Fig. 3E) suggests a shift in the relative distribution of nuclei, with a greater contribution of the 4C class compared with the other time points. Thus, Fig. 3E illustrates changes in

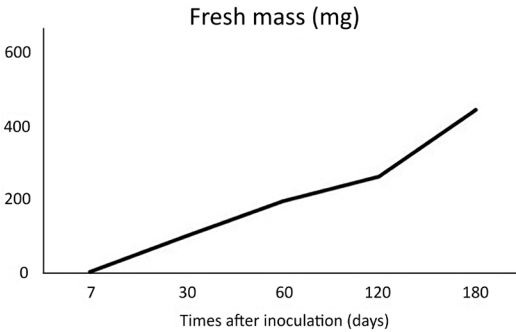


Fig. 2. Fresh mass increase (mg) of protocorm-like bodies (PLBs) of *Cattleya tigrina* at different *in vitro* culture times after inoculation ($n = 10$).

Table 1. Relative DNA content (pg) of leaf explants (entire leaves, leaf tips, and leaf bases) of *Cattleya tigrina* at 0 (control) and 30 days of *in vitro* cultivation. $n = 20$. Means followed by the same lowercase letter in the column do not differ significantly according to Tukey's test ($P < 0.05$). Superscript numbers indicate cultivation times (¹ = 0 d, control; ² = 30 d). Values were calculated from the mean G0/G1 peak position of *Cattleya tigrina* relative to the external reference standard *Pisum sativum*. CV = coefficient of variation.

Parameters	Time	2C value (pg)	CV (%)
Leaves	0 (control)	4.99 ^{a1}	3.73
Leaf tips	0 (control)	5.04 ^{a1}	3.86
Leaf bases	0 (control)	5.06 ^{a1}	4.13
Leaves	30 d	5.84 ^{a2}	6.11
Leaf tips	30 d	5.72 ^{a2}	3.89
Leaf bases	30 d	6.14 ^{a2}	5.82

the relative abundance of nuclei among ploidy classes, while Table 2 presents the estimated DNA content corresponding to each peak position.

Estimation of DNA content in leaves and root tips from plantlets: Flow cytometric analysis of leaves and root tips of *C. tigrina* plantlets maintained *in vitro* without PGRs revealed three peaks related to DNA content (Fig. 4), corresponding to 2C, 4C, and 8C nuclei. This pattern was similar to that observed for most PLBs analyzed, which also showed three peaks (2C, 4C, and 8C) in somatic tissues.

Discussion

Leaf explants of *C. tigrina* used for PLB development showed that the leaf base is the region with the greatest morphogenic activity for PLB differentiation, in agreement with Fritsche et al. (2022a) for this species. Leaf bases can be more responsive to embryogenesis and/or organogenesis than median and apical regions. In monocotyledons, this region, being closer to the axillary meristem, may contain meristematic zones. These tissues are young, metabolically active, and associated with the intercalary meristem, and

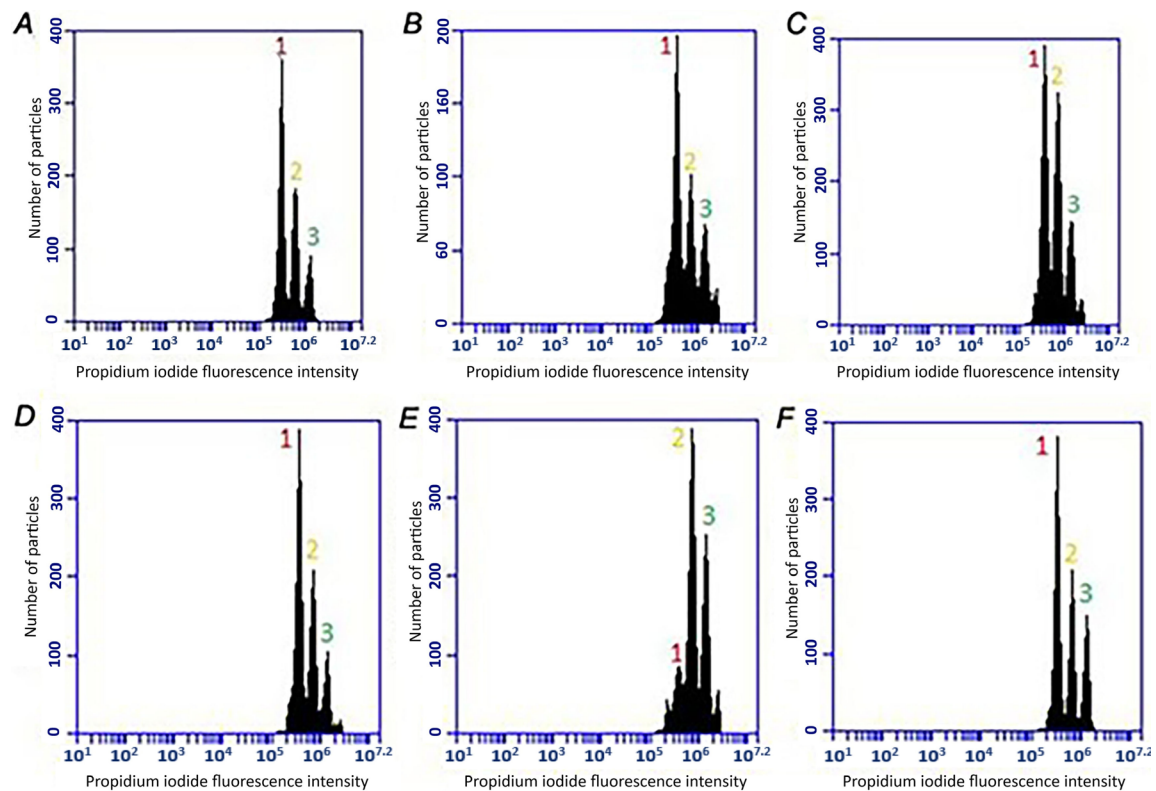


Fig. 3. Histograms of relative fluorescence intensity (linear scale) obtained from propidium iodide-stained nuclei isolated from *Cattleya tigrina* leaf explants and PLBs at different developmental stages. A – Leaf base used as control. B – Leaf base with PLBs at 7 d of cultivation. C – PLBs at 30 d of cultivation. D – PLBs at 60 d of cultivation. E – PLBs at 120 d of cultivation. F – Leaves regenerated from PLBs at 180 d of cultivation. Peak 1 corresponds to 2C nuclei, peak 2 to 4C nuclei, and peak 3 to 8C nuclei.

Table 2. Relative DNA content (pg) and fresh mass (mg) increase in protocorm-like bodies (PLBs) of *Cattleya tigrina* at different *in vitro* culture times. $n = 20$. Means followed by the same letter in the column do not differ significantly according to Tukey's test ($P < 0.05$). Values were calculated from the mean G0/G1 peak position of *Cattleya tigrina* relative to the external reference standard *Pisum sativum*. CV = coefficient of variation.

Development of PLBs (days)	Quantity of DNA (pg)			CV (%)
	2C	4C	8C	
Control (0)	5.06 ^b	10.09 ^c	20.35 ^c	3.53
7	5.56 ^a	11.04 ^{ab}	22.05 ^a	5.05
30	5.49 ^a	10.80 ^b	21.29 ^{ab}	4.51
60	5.49 ^a	10.73 ^b	21.17 ^{ac}	4.73
120	5.68 ^a	11.31 ^a	22.04 ^a	4.93
180	5.11 ^b	10.28 ^c	20.49 ^{bc}	4.10

they often include large vacuolated cells (Corredor-Prado et al., 2015). In the present study, an increase in fresh mass and intense cell proliferation were observed in the leaf base region, resulting in PLB regeneration. The onset of cell differentiation and PLB tissue formation in *C. tigrina* occurred after 7 d of culture. Histodifferentiation of the leaf bases, forming protrusions, began from epidermal cells, as also reported by Chookoh et al. (2019) in *Tolumnia*

orchids, who highlighted the recurrent observation that cells from the leaf base show high responsiveness and are largely responsible for PLB formation in different orchid species, which is consistent with the findings for *C. tigrina*.

The increase in PLB volume was associated with the proliferation of corpus cells, and secondary PLBs were formed from tunica cells of the initial PLB (Liz, 2013). De Conti et al. (2018) also reported, in this same species, large amounts of starch grains and proteinaceous material, followed by elongation of epidermal cells and PLB formation. Most PLBs can proliferate to produce secondary PLBs originating from cells either inside or outside the embryogenic callus (Zhao et al., 2008). Similarly, De Conti et al. (2020) reported the formation of PLBs from pre-existing PLBs in *C. tigrina* after 30 d of culture, and Fritsche et al. (2022b) observed the formation of secondary PLBs after 60 d of culture in *Epidendrum fulgens*. Taken together, these studies and the present results reinforce the idea that PLB multiplication in orchids frequently involves secondary PLB formation from pre-existing structures.

Methodological issues, such as the interference of secondary metabolites, including phenolic compounds, can cause variation in flow cytometry peaks (Greilhuber et al., 2007). However, these effects are primarily associated with intrinsic properties of the plant tissue rather than being exclusively methodological artifacts. In this study, explants at 30 d of culture exhibited oxidation and had

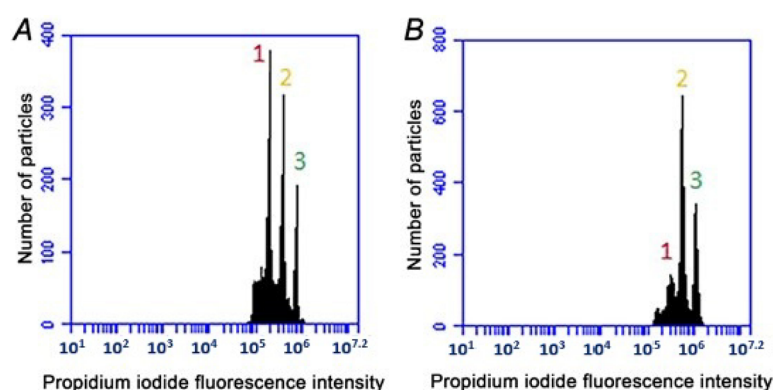


Fig. 4. Histograms of relative fluorescence intensity (linear scale) obtained from propidium iodide-stained nuclei isolated from leaves (A) and root tips (B) of *Cattleya tigrina* plantlets cultivated *in vitro* without plant growth regulators. Peak 1 corresponds to 2C nuclei, peak 2 to 4C nuclei, and peak 3 to 8C nuclei.

coefficients of variation exceeding 5%, likely due to phenolic compounds released during excision, which generated additional events and debris that affected peak quality. The presence of multiple peaks, as observed here, made flow cytometry analysis challenging, particularly in selecting an appropriate reference standard to avoid overlap between standard and sample peaks. The interpretation of DNA content histograms can be particularly difficult in orchids. Three peaks were observed for all somatic tissues of *C. tigrina*, indicating endopolyploidy. The lack of data on nuclear DNA content for this large plant family – genome sizes are known for only about 1.5% of orchid species so far (Rewers *et al.*, 2021) – may be partly due to methodological problems (Lee and Lin, 2005) and to difficulties in interpreting DNA content histograms (Trávníček *et al.*, 2015).

In general, estimating genome size in orchids using flow cytometry presents several challenges: (i) several peaks corresponding to different ploidy levels almost always appear in DNA content histograms; and (ii) fluorescence peaks of nuclei at the G1 phase with a 2C DNA content may be small or even absent in some somatic tissues, leading to overestimation of genome size. These limitations indicate that FC does not allow the identification of individual cells or the direct determination of chromosome number (Doležel and Bartoš, 2005; Trávníček *et al.*, 2015; Sliwinska, 2022). Therefore, complementary cytogenetic approaches, such as chromosome counting, are important to support the interpretation of ploidy variation and endopolyploidy patterns. Flow cytometric analysis in orchids may also hinder the selection of an appropriate reference standard that does not overlap with sample peaks (Trávníček *et al.*, 2015). In this context, and corroborating Trávníček *et al.* (2015), Brown *et al.* (2017) argued that many estimates of genome size in orchids are erroneous because, in preparations from leaf tissue, the 2C nuclear population has often been overlooked; they emphasize the importance of using plant tissue in which a clear 2C population is expressed, to facilitate nuclear handling and improve histogram quality. Few studies have used flow cytometry to investigate the *in vitro* development and regeneration of orchid PLBs. Therefore, the present study

is important because it elucidates the developmental pattern of *C. tigrina*. Estimation of ploidy level in leaf explants, PLBs, and leaves and roots from plantlets cultivated *in vitro* was effective in *C. tigrina*, and different levels of 2C, 4C, and 8C DNA content were observed at different culture times, in the presence or absence of PGRs. Among the 105 described species of *Cattleya* (Barros *et al.*, 2015), nuclear DNA content has been quantified in only six species (Leitch *et al.*, 2019), and this work contributes to filling that gap.

Regeneration of *C. tigrina* via PLB development, as assessed by flow cytometry, showed that during morphogenesis and PLB regeneration, the DNA content of regenerated leaves remained stable when compared with the C-value of leaves used as explants. During *in vitro* maintenance of *C. tigrina* genotypes over extended culture periods and successive subcultures, DNA content estimates indicated maintenance of DNA content, as nuclear DNA contents were maintained in induced and regenerated leaves. In *Dendrobium crumenatum*, DNA content also does not change regardless of whether the material originates from greenhouse-grown or *in vitro*-grown plants (Meesawat *et al.*, 2008). In a study estimating DNA content in *Dendrobium*, regenerants obtained after the first culture maintained the same ploidy level as the parent plant, whereas in subsequent cultures a large number of polyploids and chimeras were produced (Meesawat *et al.*, 2008). Likewise, in a study on *C. tigrina* regenerants, all were found to possess twice the ploidy level of the tissue of the plants used as explant sources, and histograms displayed three fluorescence peaks corresponding to 2C, 4C, and 8C nuclei (Fritsche *et al.*, 2022a). In the present study, all types of *C. tigrina* explants analyzed also exhibited three fluorescence peaks. For *C. tigrina*, estimating the C-value for different tissues of the same species and avoiding misinterpretation of DNA content histograms were crucial. By analyzing six different types of somatic tissue, the occurrence of endopolyploid cells was documented, and an attempt was made to understand the endoreduplication pattern.

Endopolyploidy is the condition in which certain cells of an otherwise diploid organism become polyploid as

a result of endoreduplication, a process involving DNA replication without mitosis, leading to the formation of cells with different ploidy levels within the same tissue (polysomaty) (Barow, 2006; Inzé and De Veylder, 2006; Fukai et al., 2002; Kołodziejczyk et al., 2023). It generally occurs in differentiated cells (Masonbrink et al., 2013). Flow cytometric analysis of *C. tigrina* showed that PLBs at 120 d of culture exhibited a shift in ploidy distribution. At earlier stages, PLBs were predominantly diploid (2C), a condition typically associated with active cell proliferation (Scholes and Paige, 2015). At 120 d, however, there was a greater relative contribution of 4C nuclei, coinciding with a period of intense PLB proliferation. Endopolyploidy has been associated with plant growth processes, particularly cell expansion and metabolic activity (Kołodziejczyk et al., 2023), which is especially relevant for *in vitro* growth.

In endoreduplicating tissues, nuclei with 2C DNA content may represent only a small fraction, and their peaks can be underestimated in flow cytometric profiles (Trávníček et al., 2015), which may explain the pattern observed for *C. tigrina* at 120 d of culture. At 180 d, the diploid predominance returned to the pattern observed at other stages. Endopolyploid cells are typically associated with differentiated states, contributing to cell expansion and metabolic activity, whereas actively proliferating tissues are generally enriched in cells with lower DNA content (2C) (Trávníček et al., 2015; Kołodziejczyk et al., 2023). Thus, the observed increase in diploid nuclei at later stages may reflect a shift in the relative contribution of proliferative and differentiated cell populations during plant regeneration. Endoreduplication has been reported in several orchid species, including *Epidendrum* spp. (Assis et al., 2013; Fritsche et al., 2022b), *Vanilla* spp. (Brown et al., 2017) and *Phalaenopsis equestris*, in which leaves contain higher ploidy levels than other organs (Lin et al., 2001), consistent with the pattern observed in *C. tigrina*. In protocorms and PLBs of *Phalaenopsis aphrodite*, variation in ploidy levels and the presence of endopolyploid cells were also detected (Chen et al., 2009). Polysomaty was similarly observed in PLBs of *Cymbidium*, with peaks corresponding to 2C, 4C, 8C, and 16C (Fukai et al., 2002). High levels of endopolyploidy have also been reported during orchid seed development (Jean et al., 2011), and genomic amplification of up to 64-fold has been described in some species (Brown et al., 2017). In the present study, PLBs of *C. tigrina* exhibited endopolyploidy at all six cultivation times, with a maximum peak of 8C and only minor variation between the control and cultured tissues.

During protocorm development in *Phalaenopsis*, endopolyploidy appears after germination, and different DNA content levels have been reported in protocorms after 30 d of culture (Chen et al., 2009). These observations indicate the existence of different levels of endopolyploid cells in protocorms, as also observed in the different tissues of *C. tigrina*. Diploid plants maintain diploidy in meristematic cells, whereas endopolyploidy arises during differentiation (Teixeira da Silva et al., 2014). Under various biotic and abiotic stress conditions, endopolyploidy can function as an adaptive mechanism

that maintains organismal homeostasis by supporting tissue growth and regeneration (Lee et al., 2009; Sliwinski et al., 2022; Kołodziejczyk et al., 2023). This may explain its frequent occurrence in *in vitro* cultures, as observed in *C. tigrina*. Endopolyploidy is common in some plant families, and its degree usually differs among organs and tissues (Loureiro et al., 2006; Barow and Jovtchev, 2007; Torres-Silva et al., 2020). It may also vary within the same organ, as observed in the leaves of *Spathoglottis plicata*, where endopolyploidy decreases from the base to the apex (Yang and Loh, 2004). Endopolyploid cells grow mainly by cell expansion that is mediated or accompanied by endoreduplication (Kocová and Mártonfi, 2011), and endoreduplication is well documented for endosperm and cotyledons during seed development (Lee et al., 2009).

A constant feature of *C. tigrina* was the presence of endopolyploid tissues, with 2C, 4C, and 8C peaks. This phenomenon has been reported in other Orchidaceae genera, such as *Cymbidium* (Fukai et al., 2002), *Polystachya* (Rupp et al., 2010), *Spathoglottis* (Yang and Loh, 2004), and *Vanilla* (Lepers-Andrzejewski et al., 2011). It results from the production of polyploid tissues and may lead to the generation of polyploid descendants (Lepers-Andrzejewski et al., 2011). Some authors relate this phenomenon to increased expression of specific genes and to control of leaf growth (Bourdon et al., 2011; Massonnet et al., 2011). However, many aspects of the specific role of endoreduplication in vegetative development remain unknown. Polysomaty, indicating the presence of cells with different ploidy levels within the same tissue, such as leaves, roots and PLBs of *C. tigrina*, may be a pre-existing feature of the parent plant (genotype), of the explant type (tissue nature, including replication cycles not followed by division), and/or of the culture conditions (medium composition, PGRs, stress, light).

Conclusions

Knowledge of endopolyploidy is important for understanding the regulation of gene expression in differentiated tissues and the nature of explants used for plant regeneration and transformation. Such understanding can help to reduce the occurrence of somaclonal variation and undesired polyploidy in *in vitro* cultures. In *C. tigrina*, estimation of nuclear DNA content in different tissues and culture conditions, in the presence or absence of PGRs, clearly demonstrated genomic stability during *in vitro* maintenance, including throughout PLB morphogenesis and regeneration.

This study also presents an efficient approach to estimate ploidy levels in plants grown *in vitro* and during PLB development in tissue cultures derived from long-term subcultures in the presence of PGRs. Nevertheless, cytogenetic analyses employing chromosome counts are still needed to validate and refine the interpretation of polysomaty and endopolyploidy patterns observed by flow cytometry. Future studies comparing *in vitro*-derived plants with field-grown individuals will be important to clarify whether polyploidy in *C. tigrina* is predominantly

genetically determined or can also be induced by *in vitro* cultivation.

References

- Anjum, S., Zia, M. & Chaudhary, M.F. (2006) Investigations of different strategies for high frequency regeneration of *Dendrobium malones* 'Victory'. *African Journal of Biotechnology*, 5, 1738-1743.
- Assis, F.N.M., Souza, B.C.Q., Medeiros-Neto, E., Pinheiro, F., Silva, A.E.B. & Felix, L.P. (2013) Karyology of the genus *Epidendrum* (Orchidaceae: Laeliinae) with emphasis on subgenus *Amphiglottium* and chromosome number variability in *Epidendrum secundum*. *Botanical Journal of the Linnean Society*, 172, 329-344.
- Barow, M. (2006) Endopolyploidy in seed plants. *BioEssays*, 28, 271-281.
- Barow, M. & Jovtchev, G. (2007) Endopolyploidy in plants and its analysis by flow cytometry. In: Doležel, J., Greilhuber, J. & Suda, J. (Eds.) *Flow Cytometry with Plant Cells: Analysis of Genes, Chromosomes and Genomes*. Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA, pp. 349-372.
- Barros, F., Vinhos, F., Rodrigues, V.T. et al. (2015) Orchidaceae in Lista de Espécies da Flora do Brasil. Rio de Janeiro: Jardim Botânico do Rio de Janeiro. Available at: <https://floradobrasil.jbrj.gov.br/>. [In Portuguese]
- Bennett, M.D., Price, H.J. & Johnston, J.S. (2008) Anthocyanin inhibits propidium iodide DNA fluorescence in *Euphorbia pulcherrima*: implications for genome size variation and flow cytometry. *Annals of Botany*, 101, 777-790.
- BFG (2015) Growing knowledge: an overview of seed plant diversity in Brazil. *Rodriguésia*, 66, 1085-1113.
- Bourdon, M., Coriton, O., Pirrello, J. et al. (2011) *In planta* quantification of endoreduplication using fluorescent *in situ* hybridization (FISH). *The Plant Journal*, 66, 1089-1099.
- Brown, S.C., Bourge, M., Maunoury, N. et al. (2017) DNA remodeling by strict partial endoreplication in orchids, an original process in the plant kingdom. *Genome Biology and Evolution*, 9, 1051-1071.
- Burke, J.M., Bayly, M.J., Adams, P.B. & Ladiges, P.Y. (2008) Molecular phylogenetic analysis of *Dendrobium* (Orchidaceae), with emphasis on the Australian section *Dendrocoryne*, and implications for generic classification. *Australian Systematic Botany*, 21, 1-14.
- Cardoso, J.C., Zanello, C.A. & Chen, J.-T. (2020) An overview of orchid protocorm-like bodies: mass propagation, biotechnology, molecular aspects, and breeding. *International Journal of Molecular Sciences*, 21, 985.
- Corredor-Prado, J.P., Schmidt, E.C., Guerra, M.P., Bouzon, Z.L., Dal Vesco, L.L. & Pescador, R. (2015) Histodifferentiation and ultrastructure of nodular cultures from seeds of *Vriesea friburgensis* Mez var. *paludosa* (L.B. Smith) L.B. Smith and leaf explants of *Vriesea reitzii* Leme & A. Costa (Bromeliaceae). *Journal of Microscopy and Ultrastructure*, 3, 200-209.
- De Conti, D., Corredor-Prado, J.P., Bueno, J.C.F. et al. (2020) Differentially expressed proteins in the development of protocorm-like bodies of *Cattleya tigrina* A. Richard. *Acta Physiologiae Plantarum*, 42, 181.
- De Conti, D., Corredor-Prado, J.P., Roecker Jr., D., Suzuki, R.M., Guerra, M.P. & Pescador, R. (2018) Determination of endogenous IAA and carbohydrates during the induction and development of protocorm-like bodies of *Cattleya tigrina* A. Richard. *Acta Scientiarum: Biological Sciences*, 40, 37874.
- Doležel, J. & Bartoš, J. (2005) Plant DNA flow cytometry and estimation of nuclear genome size. *Annals of Botany*, 95, 99-110.
- Doležel, J., Greilhuber, J. & Suda, J. (2007) Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols*, 2, 2233-2244.
- Dressler, R.L. (2005) How many orchid species? *Selbyana*, 26, 155-158.
- Escobedo-Gracia Medrano, R.M., Maldonado-Borges, J.I., Burgos-Tan, M.J., Valadez-González, N. & Ku-Cauich, J.R. (2014) Using flow cytometry and cytological analyses to assess the genetic stability of somatic embryo-derived plantlets from embryogenic *Musa acuminata* Colla (AA) ssp. *malaccensis* cell suspension cultures. *Plant Cell, Tissue and Organ Culture*, 116, 175-185.
- Ferreira, D.F. (2011) Sisvar: a computer statistical analysis system. *Ciência e Agrotecnologia*, 35, 1039-1042.
- Fritsche, Y. (2012) [Regeneration of protocorm-like bodies and flow cytometry applied to genetic enhancement and to the study of the nuclear genome of orchids.] Master's thesis. Florianópolis: Federal University of Santa Catarina, pp. 138. [In Portuguese]
- Fritsche, Y., Deola, F., da Silva, D.A., Holderbaum, D.F. & Guerra, M.P. (2022a) *Cattleya tigrina* (Orchidaceae) *in vitro* regeneration: main factors for optimal protocorm-like body induction and multiplication, plantlet regeneration, and cytogenetic stability. *South African Journal of Botany*, 149, 96-108.
- Fritsche, Y., Ornellas, T.S., Stefenon, V.M. & Guerra, M.P. (2022b) Ploidy mosaics: does endopolyploidy in explants affect the cytogenetic stability of orchids regenerated from PLBs? *Plant Cell, Tissue and Organ Culture*, 149, 697-713.
- Fukai, S., Hasegawa, A. & Goi, M. (2002) Polysomaty in *Cymbidium*. *HortScience*, 37, 1088-1091.
- Gale, S.W., Fischer, G.A., Cribb, P.J. & Fay, M.F. (2018) Orchid conservation: bridging the gap between science and practice. *Botanical Journal of the Linnean Society*, 186, 425-434.
- Greilhuber, J., Doležel, J., Lysák, M.A. & Bennett, M.D. (2005) The origin, evolution and proposed stabilization of the terms 'genome size' and 'C-value' to describe nuclear DNA contents. *Annals of Botany*, 95, 255-260.
- Greilhuber, J., Temsch, E.M. & Loureiro, J.C.M. (2007) Nuclear DNA content measurement. In: Doležel, J., Greilhuber, J. & Suda, J. (Eds.) *Flow Cytometry with Plant Cells: Analysis of Genes, Chromosomes and Genomes*. Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA, pp. 67-101.
- Grosso, V., Farina, A., Giorgi, D., Nardi, L., Diretto, G. & Lucretti, S. (2018) A high-throughput flow cytometry system for early screening of *in vitro* made polyploids in *Dendrobium* hybrids. *Plant Cell, Tissue and Organ Culture*, 132, 57-70.
- Hinsley, A.M.Y., de Boer, H.J., Fay, M.F. et al. (2018) A review of the trade in orchids and its implications for conservation. *Botanical Journal of the Linnean Society*, 186, 435-455.
- Chen, W.H., Tang, C.Y. & Kao, Y.L. (2009) Ploidy doubling by *in vitro* culture of excised protocorms or protocorm-like bodies in *Phalaenopsis* species. *Plant Cell, Tissue and Organ Culture*, 98, 229-238.
- Chookoh, N., Chiu, Y.-T., Chang, C., Hu, W.-H. & Dai, T.-E. (2019) Micropropagation of *Tolumnia* orchids through induction of protocorm-like bodies from leaf segments. *HortScience*, 54, 1230-1236.
- Christenhusz, M.J.M. & Byng, J.W. (2016) The number of known plant species in the world and its annual increase. *Phytotaxa*, 261, 201-217.
- Chugh, S., Guha, S. & Rao, I.U. (2009) Micropropagation of orchids: a review on the potential of different explants. *Scientia Horticulturae*, 122, 507-520.

- Inzé, D. & De Veylder, L. (2006) Cell cycle regulation in plant development. *Annual Review of Genetics*, 40, 77-105.
- Jean, G.-T., Kao, Y.-L., Tang, C.-Y. & Chen, W.-H. (2011) Distribution of nuclei of different ploidy levels during ovule, seed and protocorm development in *Phalaenopsis aphrodite* subsp. *formosana* (Orchidaceae). *American Journal of Plant Sciences*, 2, 325-333.
- Kocová, V. & Mártonfi, P. (2011) Endopolyploidy in *Trifolium pratense* L. *Caryologia*, 64, 419-426.
- Kołodziejczyk, I., Tomczyk, P. & Kaźmierczak, A. (2023) Endoreplication – Why are we not using its full application potential? *International Journal of Molecular Sciences*, 24, 11859.
- Kron, P. (2015) Endopolyploidy, genome size, and flow cytometry. *Cytometry Part A*, 87, 887-889.
- Lee, H.-C. & Lin, T.-Y. (2005) Isolation of plant nuclei suitable for flow cytometry from recalcitrant tissue by use of a filtration column. *Plant Molecular Biology Reporter*, 23, 53-58.
- Lee, H.O., Davidson, J.M. & Duronio, R.J. (2009) Endoreplication: polyploidy with purpose. *Genes & Development*, 23, 2461-2477.
- Leitch, I.J., Johnston, E., Pellicer, J., Hidalgo, O. & Bennett, M.D. (2019) Plant DNA C-values Database (Release 7.1, April 2019). Available at: <https://cvalues.science.kew.org/>.
- Leitch, I.J., Kahandawala, I., Suda, J. et al. (2009) Genome size diversity in orchids: consequences and evolution. *Annals of Botany*, 104, 469-481.
- Lepers-Andrzejewski, S., Siljak-Yakovlev, S., Brown, S.C., Wong, M. & Dron, M. (2011) Diversity and dynamics of plant genome size: an example of polysomaty from a cytogenetic study of Tahitian vanilla (*Vanilla × tahitensis*, Orchidaceae). *American Journal of Botany*, 98, 986-997.
- Lin, S., Lee, H.-C., Chen, W.-H. et al. (2001) Nuclear DNA contents of *Phalaenopsis* sp. and *Doritis pulcherrima*. *Journal of the American Society for Horticultural Science*, 126, 195-199.
- Liz, R.D. (2013) [Elucidation of morpho-histodifferentiation of the development of protocorm-like bodies (PLBs) of micropropagated *Cattleya* Lindl. species.] Master's thesis. Florianópolis: Federal University of Santa Catarina, pp. 119. [In Portuguese]
- Loureiro, J., Rodriguez, E., Doležel, J. & Santos, C. (2006) Comparison of four nuclear isolation buffers for plant DNA flow cytometry. *Annals of Botany*, 98, 679-689.
- Marie, D. & Brown, S.C. (1993) A cytometric exercise in plant DNA histograms, with 2C values for 70 species. *Biology of the Cell*, 78, 41-51.
- Masonbrink, R.E., Fu, S., Han, F. & Birchler, J.A. (2013) Heritable loss of replication control of a minichromosome derived from the B chromosome of maize. *Genetics*, 193, 77-84.
- Massonnet, C., Tisné, S., Radziejewski, A. et al. (2011) New insights into the control of endoreduplication: endoreduplication could be driven by organ growth in *Arabidopsis* leaves. *Plant Physiology*, 157, 2044-2055.
- Meesawat, U., Srisawat, T., Eksomtramage, L. & Kanchanapoom, K. (2008) Nuclear DNA content of the pigeon orchid (*Dendrobium crumenatum* Sw.) with the analysis of flow cytometry. *Songklanakarin Journal of Science and Technology*, 30, 277-280.
- Morel, G. & Wetmore, R.H. (1951) Fern callus tissue culture. *American Journal of Botany*, 38, 141-143.
- Murashige, T. & Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, 15, 473-479.
- Ng, C.-Y. & Saleh, N.M. (2011) In vitro propagation of *Paphiopedilum* orchid through formation of protocorm-like bodies. *Plant Cell, Tissue and Organ Culture*, 105, 193-202.
- Nongdam, P., Beleski, D.G., Tikendra, L. et al. (2023) Orchid micropropagation using conventional semi-solid and temporary immersion systems: a review. *Plants*, 12, 1136.
- O'Brien, T.P., Feder, N. & McCully, M.E. (1964) Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma*, 59, 368-373.
- Orbović, V., Čalović, M., Vilorio, Z. et al. (2008) Analysis of genetic variability in various tissue culture-derived lemon plant populations using RAPD and flow cytometry. *Euphytica*, 161, 329-335.
- POWO – Plants of the World Online (2024) *Cattleya tigrina* A. Rich. Royal Botanic Gardens, Kew. Available at: <https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:622256-1>.
- Rewers, M., Jedrzejczyk, I., Rewicz, A. & Jakubska-Busse, A. (2021) Genome size diversity in rare, endangered, and protected orchids in Poland. *Genes*, 12, 563.
- Rupp, B., Samuel, R., Russell, A., Temsch, E.M., Chase, M.W. & Leitch, I.J. (2010) Genome size in *Polystachya* (Orchidaceae) and its relationships to epidermal characters. *Botanical Journal of the Linnean Society*, 163, 223-233.
- Santos Díaz, M.d.S. & Carranza Álvarez, C. (2009) Plant regeneration through direct shoot formation from leaf cultures and from protocorm-like bodies derived from callus of *Encyclia mariae* (Orchidaceae), a threatened Mexican orchid. *In Vitro Cellular and Developmental Biology – Plant*, 45, 162-170.
- Sevillano, S.S., Cabahug-Braza, R.A., An, H.R., Lim, K.B. & Hwang, Y.J. (2023) The role of cytogenetic tools in orchid breeding. *Korean Journal of Agricultural Science*, 50, 235-248.
- Sheelavanthmath, S.S., Murthy, H.N., Hema, B.P., Hahn, E.J. & Paek, K.Y. (2005) High frequency of protocorm-like bodies (PLBs) induction and plant regeneration from protocorm and leaf sections of *Aerides crispum*. *Scientia Horticulturae*, 106, 395-401.
- Scholes, D.R. & Paige, K.N. (2015) Plasticity in ploidy: a generalized response to stress. *Trends in Plant Science*, 20, 165-175.
- Sliwinska, E. (2022) Investigations on nuclear DNA content and DNA synthesis in plants and fungi using flow cytometry and fluorescence microscopy. *Genes*, 13, 417.
- Sreeramanan, S., Vinod, B., Sashi, S. & Xavier, R. (2008) Optimization of the transient *gusA* gene transfer of *Phalaenopsis violacea* orchid via *Agrobacterium tumefaciens*: an assessment of factors influencing the efficiency of gene transfer mechanisms. *Advances in Natural and Applied Sciences*, 2, 77-88.
- Sujjarittharakarn, P. & Kanchanapoom, K. (2011) Efficient direct protocorm-like bodies induction of dwarf *Dendrobium* using thidiazuron. *Notulae Scientia Biologicae*, 3, 88-92.
- Swarts, N.D. & Dixon, K.W. (2009) Terrestrial orchid conservation in the age of extinction. *Annals of Botany*, 104, 543-556.
- Teixeira da Silva, J.A., Giang, D.T.T., Dobránszki, J., Zeng, S. & Tanaka, M. (2014) Ploidy analysis of *Cymbidium*, *Phalaenopsis*, *Dendrobium* and *Paphiopedilum* (Orchidaceae), and *Spathiphyllum* and *Syngonium* (Araceae). *Biologia*, 69, 750-755.
- Torres-Silva, G., Matos, E.M., Correia, L.F. et al. (2020) Anatomy, flow cytometry, and X-ray tomography reveal tissue organization and ploidy distribution in long-term *in vitro* cultures of *Melocactus* species. *Frontiers in Plant Science*, 11, 1314.
- Trávníček, P., Ponert, J., Urfus, T. et al. (2015) Challenges of

- flow-cytometric estimation of nuclear genome size in orchids, a plant group with both whole-genome and progressively partial endoreplication. *Cytometry Part A*, 87, 958-966.
- Vitt, P., Taylor, A., Rakosy, D. et al. (2023) Global conservation prioritization for the Orchidaceae. *Scientific Reports*, 13, 6718.
- Yam, T.W. & Arditti, J. (2017) Micropropagation of Orchids. Chichester: John Wiley & Sons, pp. 2368.
- Yang, M. & Loh, C.S. (2004) Systemic endopolyploidy in *Spathoglottis plicata* (Orchidaceae) development. *BMC Cell Biology*, 5, 33.
- Yudaputra, A., Munawaroh, E., Usmadi, D. et al. (2024) Vulnerability of lowland and upland orchids in their spatially response to climate change and land cover change. *Ecological Informatics*, 80, 102534.
- Zhang, X. & Gao, J. (2020) In vitro tetraploid induction from multigenotype protocorms and tetraploid regeneration in *Dendrobium officinale*. *Plant Cell, Tissue and Organ Culture*, 141, 289-298.
- Zhao, P., Wu, F., Feng, F.S. et al. (2008) Protocorm-like body (PLB) formation and plant regeneration from the callus culture of *Dendrobium candidum* Wall ex Lindl. *In Vitro Cellular and Developmental Biology – Plant*, 44, 178-185.