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Chromosome doubling in *Cattleya tigrina* A. Rich

Duplicação cromossômica em *Cattleya tigrina* A. Rich

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Chromosome doubling induction in orchids may benefit their production for resulting in flowers of higher commercial value, larger size and higher content of substances that intensify the color and fragrance when compared with diploid orchids. This work aimed to induce and confirm artificial polyploidization, using flow cytometry and stomatal analysis. Explants were treated with colchicine at concentrations of 0, 2.5, 7.5, and 12.5 mM, for 24 and 48 hours and with oryzalin, at concentrations of 0, 10, 30, and 50 μM, for three and six days. For the flow cytometric analysis, a sample of leaf tissue was removed from each plant, crushed to release the nuclei and stained with propidium iodide. In addition to flow cytometry, the ploidy of the antimitotic treated plants was evaluated by stomata analysis. Young leaves were used where the density, functionality and stomatal index were evaluated. Colchicine provided induction of satisfactory polyploidy in *C. tigrina* at all concentrations and times of exposure, obtaining a greater number of polyploid individuals in the concentration of 12.5 mM for 48 hours. Oryzalin did not induce chromosome duplication at the tested concentrations.
Keywords: Orchidaceae, polyploidy, flow cytometry.

A duplicação cromossômica induzida em orquídeas pode beneficiar sua produção por possuírem tamanho e teor de substâncias que intensificam a cor e a fragrância quando comparadas com orquídeas diplóides. Este trabalho teve como objetivo induzir e confirmar a poliploidização artificial, utilizando para isso a citometria de fluxo e análise estomática. Os explantes foram tratados com colchicina nas concentrações de 0; 2,5; 7,5 e 12,5 mM, por 24 e 48 horas e com orizalina, nas concentrações de 0, 10, 30 e 50 μM, por três e seis dias. Para as análises de citometria de fluxo, uma amostra de tecido foliar foi retirada de cada planta, triturada para liberação dos núcleos e corada com iodeto de propídio. Além da citometria de fluxo a ploidia das plantas tratadas com antimitóticos foi avaliada por meio da análise dos estômatos. Para isso utilizou-se folhas jovens onde foram avaliadas a densidade, funcionalidade e índice estomático. A colchicina proporcionou indução de poliploidia satisfatória em *C. tigrina* em todas as concentrações e tempos de exposição, obtendo maior número de indivíduos poliploides na concentração de 12,5 mM em 48 horas. Orizalina não induziu a duplicação cromossômica nas concentrações testadas.
Palavras-chave: Orchidaceae, Poliploidia, Citometria de fluxo.

1. INTRODUCTION

Species of the family Orchidaceae are ornamental plants of botanical, economic, food, medicinal, and cosmetic interest [13]. The genus *Cattleya* Lindley is known as the "queen of orchids" owing to its beauty [32]. For being highly appreciated by the market, some species of this genus are endangered, such as *C. tigrina* [4].

The devastation of the Atlantic Forest, which occurs from the State of Pernambuco to Rio Grande do Sul, and the predatory collection results in the loss of genetic material and interferes with genetic variability of species of that habitat. In this way, the study of chromosome duplication becomes relevant, in order to reduce the focus of native species threatened with extinction, since it can provide an increase in vegetative structures of the species, allowing the development of a new cultivar.

Moreover, polyploidy can increase the genetic base by recovering the fertility of interspecific hybrids, reaching higher reproduction rate in a short period [16, 27, 34]. Polyploid plants may

occur naturally due to some cytological procedures, such as the production of unreduced gametes [3]. Nevertheless, they can be synthetically generated by the chromosome doubling induction of the somatic cellusing antimitotic agents [20]. Many antimitotic agents bind to the proteins that form the fibers of the spindle (tubulins), preventing their polymerization and suppressing fibers formation, which makes it impossible the chromosomes separation at the anaphase, forming cells with a duplicated chromosomal complement [8].

Colchicine, the most commonly used antimitotic agent to induce chromosome doubling, is an alkaloid extracted from the plant *Colchicum autumnale*. However, besides being highly toxic to both plants and humans, this substance acts efficiently only on dividing cells. Since the substance does not reach all cells of the treated material, mixoploids may occur [5].

Therefore, colchicine needs to be applied at higher amounts, which is highly toxic. Oryzalin is another antimitotic that has been successfully used for the polyploidization of ornamental plants, despite being little explored in orchids [26]. Polyploids induction with colchicine and oryzalin has been used in several plant species, such as *Cattleya intermedia* [1, 31], *Dendrobium nobile* [37, 38], *Oncidium flexuosum* [35], *Dendrobium scabrilingue* [30], *Dendrobium chrysotoxum* [2] and *Dendrobium, Cymbidium, Epidendrum, Odontioda, Phalaenopsis* [26]. However, no studies have reported the application of these antimitotic agents for *in vitro* polyploids induction in *C. tigrina*.

The polyploidy level changes several anatomical traits, such as leaf thickness and length, stomata size, flower size and texture, and flowering period [40]. Chromosome doubling can be proven by cytogenetic, flow cytometry, which quantifies the nuclei DNA content [9, 42] and by stomatal analysis, which studies the size and density of stomata.

Due to the relevance of *C. tigrina*, this study aimed to analyze the production of polyploid plants, to confirm polyploid or mixoploid plants, and to evaluate investigate the effects of artificial polyploidization through flow cytometry and stomatal analysis.

2. MATERIAL AND METHODS

2.1 In vitro polyploidy induction

Two hundred and forty *in vitro C. tigrina* plants, were subject to treatments with colchicine ($C_{22}H_{25}NO_6$) and oryzalin (3,5-dinitro-N4, N4-dipropylsulfanilamide). Colchicine and oryzalin were tested at concentrations of 0, 2.5, 7.5, and 12.5 mM, 0, 10, 30, and 50 µM, respectively [28]. Inoculation was performed in 125 ml Erlenmeyer flask with 30 ml of MS liquid medium under agitation (60 rpm), for 24 and 48 hours for colchicine and three and six days for oryzalin, using 1000 ml Erlenmeyer flasks with 200 ml of MS liquid medium, with constant air bubbling by domestic aquarium air pumps.

The experiments consisted of a completely randomized design, in a 4x2 factorial scheme, with four concentrations of an antimitotic agent (colchicine or oryzalin), at two exposure times (24 and 48 h for colchicine and three and six days for oryzalin), with six replications composed of five tubes with one plant each.

After the treatments, plants were subject to a triple wash with distilled water and transferred to the MS medium supplemented with 30 g l^{-1} of sucrose and 7 g l^{-1} of agar for 90 days. Plants were kept in a growth room, with a photosynthetic photon flux density of 40 µmol m^{-2} s^{-1}, 12-hour photoperiod, at 25 ± 2°C.

At the end of the 90 days, the plants induced new shoots, which were individualized and cultivated in MS medium supplemented with 30 g l^{-1} sucrose and 7 g l^{-1} agar for 60 days. At the end of the 60 days after the cultivation of the individualized shoots, the ploidy level was evaluated by flow cytometry analysis and stomatal analysis.

2.2 Flow cytometric analysis of plants treated with antimitotic

Young leaves of *Cattleya tigrina* and "Sunki Maravilha" (*Citrus sunki* Hort ex Tan.) were used to determine the DNA content. *Citrus sunki* presented 2C = 0.745 pg of DNA and was used as an internal reference standard. Samples the middle part of *C. tigrina* leaves treated with antimitotic

and of the reference standard were macerated together in 1ml of ice-cold Galbraith buffer (1983) [12] for the release of the nuclei. The nuclei suspension was filtered in a CellTrics® 30 µm (Partec), stained with 25 µl of a 1 mg/1ml solution of propidium iodide, and stored in a container with crushed ice, in the darkness for two minutes. Samples were analyzed using the Attune™ NxT Acoustic Focusing Cytometer, and histograms were obtained with the software Attune cytometer. The fluorescence data were acquired from at least 10,000 nuclei of each sample.

The coefficients of variation were obtained in the analysis software, and the nuclear DNA content (pg) of the plants was estimated using the formula:

$$\text{Sample 2C (pg)} = \frac{\text{sample G1 peak mean}}{\textit{Citrus sunki}\ \text{G1 peak mean}} x\ \text{2C DNA content}$$

Anatomical analyses of plants subject to chromosome doubling induction

Samples were collected from the middle of the first fully expanded leaf of *in vitro* plants.

Leaf paradermic sections were clarified in sodium hypochlorite (1% active chlorine) and washed in distilled water. The sections were stained with Astra Blue and Safranin (1:1 v/v) and then mounted on semipermanent slides with glycerinated water [36].

For the analyses of density (number of stomata/area), functionality (polar diameter of the stomata/equatorial diameter of the stomata), and stomatal index [(number of stomata/number of stomata + number of epidermal cells) x100], 30 paradermal sections were used, which were photographed with an optical microscope equipped with a LEICA DM500 camera, and visualized on the computer with the LAS EZ® software.

All measurements were taken by the image analysis software UTHSCSA Image Tool (University of Texas, San Antonio, USA), using calibrations with microscopic rulers, photographed at the same photomicrograph magnification.

Stomatal analysis data were subject to analysis of variance, and the means were compared by the Tukey's test at 5% probability, using the SISVAR software [10], when necessary.

3. RESULTS AND DISCUSSION

In the treatments with colchicine, 1,141 plantlets were obtained from 240, with 557 plants with duplicate chromosomes and 584 plants with non-duplicated chromosomes. In the treatments with oryzalin, 916 plantlets were obtained from 240, but no duplicate plant was observed. *C. tigrina* presented a mean DNA content of 3.21 pg in diploid plants and 6.69 pg in autotetraploid plants. The survival rate was 100% for plants subject to treatments with colchicine and oryzalin, regardless of the concentrations or exposure time.

The initial growth of the plants at 45 days, in relation to the diploid control, was slow for plants with duplicated chromosomes. It can be inferred that, due to the endoreduplication process existing in this species, which allows its slow growth, and with a new chromosome duplication, the decrease of growth occurred in relation to the diploid control.

Colchicine at concentrations of 2.5 (128 plants), 7.5 (197 plants), and 12.0 (232 plants) mM induced plants with duplicated chromosomes when applied for 24 and 48 hours, as confirmed by flow cytometry (Table 1; Figure 1) and stomatal analysis (Figure 2).

Considering the results analyzed, the seedlings submitted to treatments with colchicine, regardless of concentrations and time of exposure were duplicated. The 12.0 concentration at 24 and 48 hours showed a higher number of duplicate plants (Table 1).

Plants with duplicated chromosomes presented thick leaves, with a more intense color when compared with those of the diploid plants. This result suggests that thick leaves are related to the increase of the enzymes and cellular pigments, accentuating the color of the leaves. Therefore, this phenomenon can be the primary factor for a more intense color verified in the leaves of the tetraploid plant [14, 21]. These parameters were also reported in other species, with artificial polyploidization, such as *Musa* spp. [28], rangpur lime (*Citrus limonia*) [1], *Brassica campestris* [18] and citrus [19].

Table 1. Flow cytometric analysis of C. tigrina subject to treatment with colchicine.

Colchicine (mM)	**Total number of plants (n)**	**Plants with duplicated chromosomes (%)**		**Plants with non-duplicated chromosomes**	
		Number	**Average**	**Number**	**Average**
0	89	0 (0.00%)	0.00 a	89	2.96 a
2.5 for 24 h	177	55 (31.07%)	1.83 a	122	4.06 a
7.5 for 24 h	167	97 (58.08%)	3.23 a	70	2.33 a
12 for 24 h	166	100 (60.24%)	3.33 b	66	2.20 a
0	85	0 (0.00%)	0.00 a	85	2.83 a
2.5 for 48 h	130	73 (56.15%)	2.43 a	57	1.90 b
7.5 for 48 h	145	100 (68.96%)	3.33 a	45	1.50 b
12 for 48 h	182	132 (72.52%)	4.40 a	50	1.66 b
CV (%)			27.83		12.37

* Means followed by same letter, between 24 and 48 h, do not differ by Tukey's test ($p \leq 0.05$).

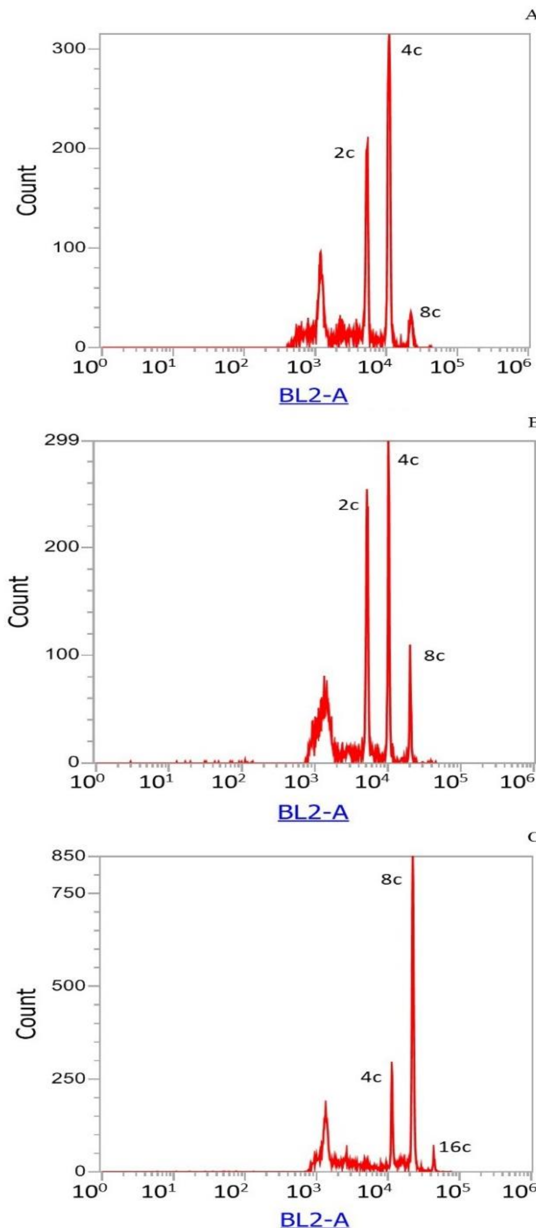

Figure 1. Flow cytometry histograms of Cattleya tigrina plants treated with colchicine using Citrus sunki as an internal reference standard. A) Control; B) Treatment with oryzalin; C) Treatment with 2.5 mM colchicine for 24 hours

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Cattleya tigrina presents endopolyploidy, with cells of three different ploidies in the same tissue in their natural environment. Thus, the control plant had more diploid and tetraploid cells (Figure 1A). The chromosome doubling induction resulted in plants with more tetraploid and octoploid cells (Figure 1C).

There was a significant difference between the concentrations and the estimated amount of DNA. All the histograms obtained indicated 3 distinct peaks and in all of them the result was similar. The control seedlings, that is, without passing through antimitotic substances showed lower DNA indexes. Seedlings grown in the other treatments on antimitotic substances had higher DNA contents (Table 2). In all the histograms it was possible to observe that the three peaks showed values that are multiples of each other (Table 2), thus demonstrating that endoreduplication occurred, which leads us to infer that ploidies are 2C in the first peak, 4C, in the second and 8C on the third. Figure 1 shows the histograms of the analyzed treatments and in all of them we can observe three peaks of *C. tigrina* and one peak of the internal reference standard (represented by the first peak), which was used to calculate the DNA indexes.

Table 2. DNA index (ID) of C. tigrina submitted to treatment with colchicine and analyzed on a flow cytometer.

Colchicine (mM)	**ID Peak 1**	**ID Peak 2**	**ID Peak 3**
0	3.23 b	6.40 b	12.68 b
2,5 for 24 h	6.11 a	12.18 a	24.32 a
7,5 for 24 h	6.21 a	12.34 a	24.52 a
12,5 for 24 h	6.32 a	12.37 a	24.55 a
0	3.23 b	6.40 b	12.68 b
2,5 for 48 h	6.20 a	12.29 a	24.43 a
7,5 for 48 h	6.20 a	12.40 a	24.44 a
12,5 for 48 h	6.25 a	12.46 a	24.49 a
CV(%)	8.43	7.36	7.24

* Means followed by the same letter, in the columns, do not differ by the Tukey test ($p \leq 0.05$).

The endoreduplication process was possibly indicated during evolution for the benefit of plants and organ development. According to the diverse situations identified in several species, in relation to the plant, organ or cellular physiology, many functional roles were described to seek to clarify the importance of endoreduplication [7]. The endoreduplication occurs during differentiation of cells that are highly specialized in their morphology, such as succulent leaves of *C. tigrina* that stores large amount of water internally. The survival rate was 100% for *C. tigrina* plants subject to oryzalin treatments, regardless of the concentrations and exposure time. Thus, oryzalin did not impair plants survival.

Moreover, oryzalin induces chromosome doubling at concentrations lower than those used for colchicine owing to the high-affinity binding of dinitroanilines to tubulin and the stability of the oryzalin-tubulin complex [11, 28]. In the present study, the concentrations of the antimitotic agent tested appear to have been very low and ineffective for chromosomes doubling (Table 3). Oryzalin did not provide duplicate plants at the times and treatments tested (Figure 1B), therefore, in the future, new studies are needed for this species, testing higher concentrations and varying the immersion times.

Cattleya tigrina responded differently to the antimitotic agents tested since the chromosomal doubling capacity of the substances (colchicine and oryzalin) was different. However, colchicine concentrations favored doubling. Results confirm that chromosome doubling varies according to the type, species, genotype, exposure time, age, and application technique [8, 39]. This fact was also verified in *Dendrobium nobile* [37, 38], *Oncidium flexuosum* [35], and *Dendrobium scabrilingue* [30].

Plant ploidy can be safely determined in the flow cytometric analysis since the DNA content is not influenced by external factors, such as water content in the plant tissue, leaf blade development, and light intensity [28]. Another methodology used to identify chromosome number counts is cytogenetics as seen in *Dendranthema nankingense* [23] and *Lilium leichtlinii* [15].

Table 3. Flow cytometric analysis of C. tigrina subject to treatment with oryzalin.

Oryzalin (µM)	**Total number of plants**	**Number of plants with duplicated chromosomes (%)**	**Number of plants with non-duplicated chromosomes**
0	62	0 (0.00%)	62
10 for 3 days	115	0 (0.00%)	115
30 for 3 days	161	0 (0.00%)	161
50 for 3 days	166	0 (0.00%)	166
0	68	0 (0.00%)	68
10 for 6 days	136	0 (0.00%)	136
30 for 6 days	102	0 (0.00%)	102
50 for 6 days	106	0 (0.00%)	106

During the development of some plants the normal cell cycle can be modified by a different cell cycle, in which it does not perform mitosis [7]. This altered cycle called the endoreduplication cycle is based on one or several cycles of DNA synthesis in the absence of mitosis, enabling the process of cell expansion, and collaborating for the growth of plant organs [25]. This event can occur in several cells of the plant, especially those submitted to aging or differentiation [29].

Endopolyploidy in plants has been seen in several different tissues [24]. In higher plants is a common characteristic, being analyzed in tissues and organs of the species [29]. In the family Orchidaceae the occurrence of endopolyploidy in the genus *Cattleya* was verified, with 40 chromosomes, in the species *C. trianae*, *C. grandis, C. guttata, C. labiata, C. cernua, C. tenius, C. elongata, C. crispata, C. rupestres, C. aclandiae, C. amethystoglossa, C. pfisterii, C. rupestris, C. sincorana, C. loddigesii,* and *C. granulosa*, resulting in the presence of 2C, 4C, and 8C peaks [33]. It was identified that this species also has this characteristic, confirmed by flow cytometry.

Stomatal analysis is a method that allows the identification of polyploid and diploid plants via counting and comparative verification of stomata, since the length of the stomata increases with the number of chromosomes [6, 28]. The increase in the ploidy level is directly proportional to the size and number of the stomata. Several authors have reported larger stomata and with a lower density of tetraploid plants on diploid plants in different plant species [40, 41]. This technique is considered one of the indicators to estimate the rate of polyploidy in several species such as *Cattleya intermedia* [31], *Vanda* [22], *Cymbidium* [17], *Dendrobium nobile* [36] and *Dendrobium chrysotoxum* [2].

In the present study, the stomatal analysis was efficient to determine the occurrence or not of chromosome doubling, corroborating the results obtained by flow cytometry. Most of the plants classified as having duplicated chromosomes by flow cytometry were also identified in the stomatal analysis. Thus, due to its lower cost, this analysis can be used to pre-select possible polyploids, reducing the number of samples for the flow cytometry analysis.

The function of stomata in plants with duplicated chromosomes (1.25) was significantly higher, and stomatal density (3.30 mm^2) was lower when compared with plants with non-duplicated chromosomes (1.09) (Table 4). These data corroborate with the results with studies of other species, such as *Dendrobium nobile* and *Musa* spp., which stated that polyploid plants have a lower density of stomata than the control [28, 36]. The stomata are important because they have the function to perform gas exchanges, however in doubling the stomata do not change the physiology of the plant. In ornamental plants the stomata has no influence on commercial characteristics, while duplication favors the increase of floral structures, with a more rounded conformation and greater content of substances that intensify the color and fragrance, when compared with the diploid orchids.

Table 4. Density, functionality, and stomatal index in leaves of C. tigrina plants without and with duplicated chromosomes, treated with oryzalin.

Ploidy level	**Density (mm^2)**	**Functionality (polar diameter/equatorial diameter)**	**Stomatal index**
Non-duplicated chromosomes	7.61 a	1.09 b	6.96 a
Duplicated chromosomes	3.30 b	1.25 a	4.28 b
CV (%)	3.48	7.79	3.85

* Means followed by the same letter, in the columns, do not differ by the Tukey test ($p \leq 0.05$).

The stomatal index in diploid plants (6.96) was higher in relation to plants with duplicated chromosomes. Thus, the increase in the stomatal index (frequency of stomata) in the diploid leaves of *C. tigrina* may be related to the smaller size of the stomata and the smaller size of the epidermal cells.

The non-duplicated plants showed smaller stomata and in major quantity (Figures 2A and 2B), while the duplicated plants had larger stomata and in minor quantity (Figures 2C and 2D). Flow cytometry confirmed the occurrence of duplication of chromosomes.

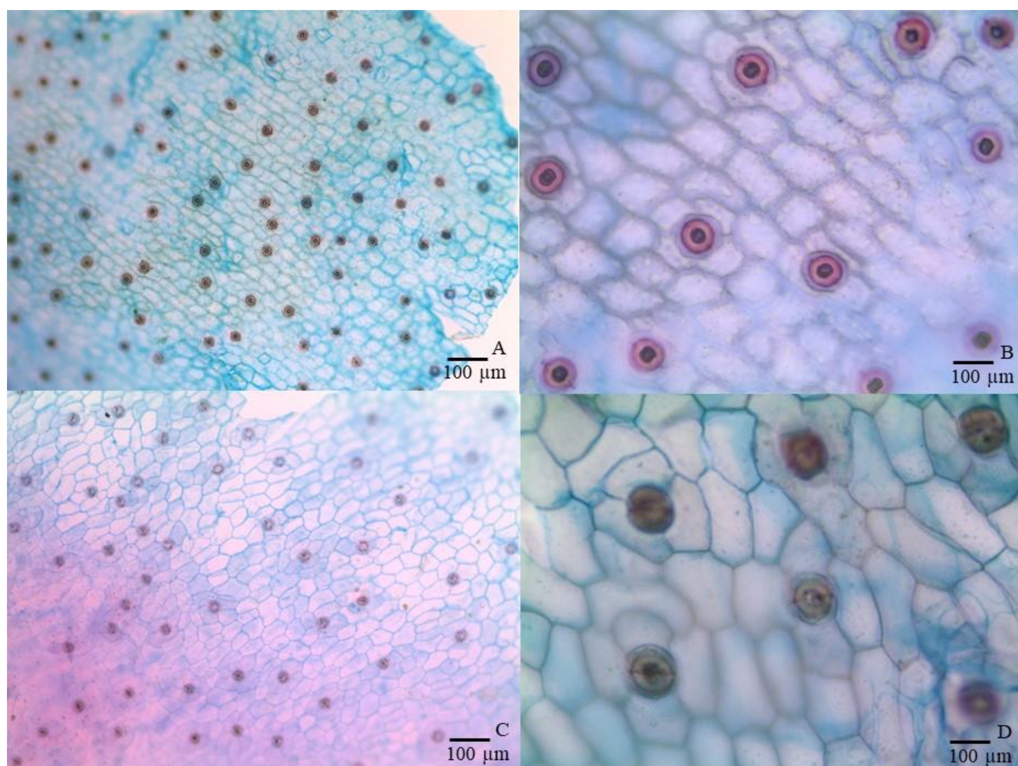

Figure 2. Stomatal analysis of a control plant (A and B) and a plant with duplicated chromosomes (C and D) of Cattleya tigrina. Fonte: Os autores

4. CONCLUSION

Colchicine provided induction of satisfactory polyploidy in *C. tigrina* at all concentrations and times of exposure, obtaining a greater number of polyploid individuals in the concentration of 12.5 mM for 48 hours. Oryzalin did not induce chromosome duplication at the tested concentrations.

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