Anatomical, morphological, and physiological changes in colchicine-treated protocorm-like bodies of *Catasetum pileatum* Rchb.f. *in vitro*

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Anatomical, morphological, and physiological changes in colchicine-treated protocorm-like bodies of *Catasetum pileatum* Rchb.f. *in vitro*

Masoumeh Kazemi¹ and Behzad Kaviani¹

**Abstract:** *Catasetum pileatum* Rchb.f. is an important potting and cutting plant. This is the first report trying the polyploid induction in *C. pileatum* Rchb.f. Protocorm-like bodies (PLBs) explants of this orchid species were treated *in vitro* with different colchicine concentrations (0.00, 1.00, 2.00, 3.00, 4.00 and 5.00 mg l⁻¹) and exposure time (24, 48 and 72 h) to induce polyploidy. Flow cytometry, chromosome counting (karyotype), and some anatomical, morphological, and physiological parameters were used to detect polyploid induction. Treatment of 4.00 mg l⁻¹ colchicine for 72 h resulted in a mixoploid plantlet. Results showed that none of the treatments induced tetraploidy or other levels of polyploidy, but changes in anatomical, morphological, and physiological parameters were observed. Differences in anatomical, morphological, and physiological parameters between treated plantlets were significant. The chromosome number detected by chromosome counting was 2n = 2x = 54 in diploids. The largest size of stoma guard cells and maximum number of these cells was obtained in leaves of plantlets treated with 4.00 and 3.00 mg l⁻¹ colchicine both for 72 h, respectively. The highest fresh and dry weights of plantlets and chlorophyll index in leaves was obtained in plantlets treated with 4.00 mg l⁻¹ colchicine for 48 h. Average survival rates from treatments were greater than 90%.

**Subjects:** Agriculture & Environmental Sciences; Botany; Plant & Animal Ecology

**Keywords:** antimitotic agents; chromosome doubling; Orchidaceae; polyploidy

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**ABOUT THE AUTHOR**

Behzad Kaviani is currently Associate Professor of Plant Physiology studying and teaching in Department of Horticultural Science at the Faculty of Agricultural Sciences, Rasht Branch, Islamic Azad University, Rasht, Iran. The topics of interest of this researcher are plant tissue culture particularly micropropagation and cryopreservation of ornamental plants especially those in danger of extinction. Miss Masoumeh Kazemi is a M.Sc. of Horticultural Science (Ornamental Plants). This work is part of this researcher’s dissertation supervised by corresponding author (Behzad Kaviani).

**PUBLIC INTEREST STATEMENT**

Behzad Kaviani New ornamental varieties with improved parameters can be produced by polyploidy using colchicine. Polyploid induction plays a significant approach for the development and improvement of vegetative and generative parameters of plants particularly in ornamentals. Orchids are one of the most important families of flowering plants for commercial purposes. *Catasetum pileatum* Rchb.f. is an important pot and cut orchid species. The objective of the present study was to determine the suitable concentration of colchicine for polyploidy induction in *C. pileatum* Rchb.f.
1. Introduction

*Catasetum* (family Orchidaceae) is a genus that includes 166 species, usually lowland epiphytic orchids, that occurs from an area extending from Mexico to Argentina including much of Central and South America. The largest number of species is in Brazil. *C. pileatum* (the felt-capped *Catasetum* or mother of pearl flower) is a rare and near-threatened orchid found from Trinidad to Ecuador. *Catasetum* species is used as pot and cut flowers not only because of their exotic beauty but also for their long shelf life (Chugh et al., 2009).

Biotechnology of ornamental species aims to improve productivity and accelerate breeding programs (Silva et al., 2019). One of the most valuable tools in floriculture is polyploidy induction or chromosome multiplying. Polyploidy plays an important role in the evolution of plant species, plant breeding, genetic and phenotype diversity, and the formation of new species and varieties with useful parameters (Dhooghe et al., 2011). Polyploid plants including orchids showed vigorous growth and better quality (Huy et al., 2019; Miguel & Leonhardt, 2011). Polyploidy may be induced by several antimitotic agents that inhibit mitosis, resulting in chromosome doubling. The most common-used antimitotic agent in plant chromosome set doubling is colchicine. Colchicine is extensively applied for induction of polyploidy in ornamental plants like orchids. The concentration and exposure time duration of colchicine for polyploidy induction is species-dependent (Kim et al., 2003; De Mello Silva et al., 2000). Apical shoot tip, root tip and seed can be used to induce polyploidy. In orchids, the most widely applied plant parts to induce polyploidy are protocorms and protocorm-like bodies (PLBs) (Miguel & Leonhardt, 2011). *Phalaenopsis, Paphiopedilum, Dendrobium, Cymbidium, Vanda* and *Cattleya* are the most important orchid genera that are polyploidized using colchicine (Huy et al., 2019; Hwang et al., 2015). This study is the first to perform the induction of polyploidy in *C. pileatum* Rchb.f. using colchicine. This work aimed to investigate the effect of concentration and time of treatment with colchicine on *in vitro* polyploidy induction and on the anatomical, morphological, and physiological parameters of *C. pileatum* Rchb.f.

2. Materials and methods

2.1. Plant material

*In vitro* fully developed plantlets of *Catasetum pileatum* Rchb.f. containing protocorms were purchased from a tissue culture laboratory, Austria. Protocorm-like bodies (PLBs) were produced from cultivation of these protocorms on Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) enriched with BA and NAA in the Plant Biotechnology Laboratory, Hyrcan Agricultural Sciences and Biotechnology Research Institute, Amol, Iran (data not shown). These PLBs were used as explants.

2.2. Polyploidy induction

PLBs were immersed in vessels containing different concentrations of colchicine (0.00, 1.00, 2.00, 3.00, 4.00, and 5.00 mg l⁻¹) for 24, 48, and 72 h to induce polyploidy. Colchicine was sterilized by a filter because it is heat-sensitive.

2.3. Culture condition

After the end of the treatment period, all treated and untreated (control) PLBs were washed completely with sterilized distilled water. Washed PLBs were then cultured in solid MS medium fortified with 3% sucrose, gelled with 0.8% agar and enriched with 1.00 mg l⁻¹ BA and 0.50 mg l⁻¹ NAA, obtained earlier for shoot multiplication (data not shown). The pH of the media was adjusted to 5.8 ± 0.02 before autoclaving at 121°C for 20 min. All the cultures were kept at 23 ± 2°C under a 16-h photoperiod of 50 μmol m⁻² s⁻¹ photosynthetic photon flux provided by cool-white fluorescent lamps (standard culture conditions).
3. Determination of levels of polyploidy

3.1. Flow cytometry analysis
To determine the ploidy level, the samples were first analyzed with a flow cytometer (BD Science, USA), and subsequently confirmed by chromosome counting. For the flow-cytometry, 0.02 g of fresh leaves were homogenized (chopped with a sharp razor blade) in a 50 mm disposable Petri dish placed on ice containing nuclei extraction buffer, LB01 (Doležel, 1997). Chopping process was performed for 2.5 min and 5 hit per second. The extracts were passed through a 100 μm cell strainer (to remove solid remains of the tissues) and then filtered through a 45 μm nylon mesh filter to prevent clogging of cell analyzer’s nozzle, and collected in a new micro-tube. Prior to injection Ribonuclease A (RNAase A) and Propidium Iodide (PI), both were added to final concentration of 50 μg/ml to obtained nuclei suspension and kept for at least 5 min on ice in a dark place.

3.2. Chromosome number determination
For chromosome number determination, 1.00 cm of root tip of treated and untreated plantlets were cut and kept in a solution containing 0.05 g colchicine/100 ml distilled water for 24 h followed by washing in distilled water. Acetic acid 90% for 30 min was used as fixative solution. After washing, root tips were put in 1 N HCl for 10 min at the temperature of 60°C followed by washing in distilled water. Root tips were stained using Uurile for 15 min at room temperature. Meristem region of root tips were excised and squashed on a microscope slide. Slides were observed and photographed under light microscope at metaphase stage.

3.3. Morphological observations
Morphological, anatomical, and physiological parameters were measured 20 weeks after in vitro cultivation. Length and width of leaves and length of roots were measured by a ruler. Leaf and root length was calculated from tip of these organs to the base of them where they connect to the PLBs. Leaf width was calculated from the widest section of each leaf. The number of organs was counted by naked eye.

3.4. Anatomical observations
A thin layer of epidermis tissues was removed from the abaxial surface of the leaves and stained by iodine solution on slides. The slides were analyzed for stomatal size (guard cells length and width) and number under a light microscope (Standard 4, Zeiss, Germany).

3.5. Physiological observations
Plantlets were weighted immediately after removal from culture medium (fresh weight) and dried in an oven with the temperature of 60°C for 48 h and their dry weight was measured. Chlorophyll index was obtained using a chlorophyll-meter apparatus (SPAD).

3.6. Acclimatization of plantlets
In vitro-grown plantlets were taken out from culture vessels and washed thoroughly under running tap water to remove substrate residual and transplanted to plastic dishes containing perlite. All the dishes were then transferred to the greenhouse with temperature of 24 ± 2°C and 20 ± 2°C (day and night), light intensity of 2000 lux, relative humidity of 80%, and 14 h photoperiod for acclimatization. Survival rate (%) was recorded after 60 days from transfer to greenhouse conditions. Irrigation was done once per 4 days.

3.7. Experimental design and data analysis
The experiment was conducted according to a completely randomized factorial design with three replications per treatment (totally; 162 explants). Colchicine as first factor was used in 6 levels (0.00, 1.00, 2.00, 3.00, 4.00, and 5.00 mg l⁻¹). Exposure time of PLBs to colchicine during 24, 48 and 72 h was selected as second factor. All the statistical analyses were done by using SPSS and MSTATC. The analysis of variance (ANOVA) procedure for the experiments was used to test for significant effect of treatments, followed by contrast and LSD test for comparisons of different means of different treatments.
4. Results

4.1. Effect of colchicine on chromosome change

PLBs of C. pileatum produced from in vitro-grown protocorms were exposed to various concentrations of colchicine (1.00–5.00 mg l\(^{-1}\)) for 24, 48, and 72 h. At these concentrations, colchicine wasn’t effective in polyploidy induction in the treated PLBs. Nevertheless, the growth and development of treated plantlets was proper and survival rate was high (more than 90%). Treatment with colchicine 4.00 mg l\(^{-1}\) for 72 h was efficient to induce mixoploidy in plantlets (Figure 2(b)). Flow cytometry analysis from leaves and chromosome counting from root tip meristems were carried out 5 months after colchicine treatment (Figure 2(c)). In the analysis, the gain value was adjusted so that the peak of nuclei isolated from a control diploid plant was set at channel 50. Mixoploidy was then predicted to display a peak around channel 100. No polyploidy was detected in other treatments and plantlets. Flow cytometry histogram (Figure 2(c)) showed two peaks of nuclear DNA content, which indicates these plantlets were mixoploid. Chromosome counting performed by karyotype analysis confirmed the results obtained by flow cytometry analysis (Figure 2(b)).

4.2. Effect of colchicine on morphological, anatomical and physiological parameters

Significant differences were observed in morphological, anatomical, and physiological parameters measured in plantlets treated with various levels of colchicine at various exposure times (Table 1–4). The leaf length, leaf number, root length, root number, fresh weight, dry weight, chlorophyll index, stomata number, and guard cells size (length and width) were measured. No significant differences were observed in leaf length of plantlets treated with both colchicine concentrations and exposure times individually, leaf and root number, and guard cell width of plantlets treated with colchicine concentrations, as well as root length, dry weight, and chlorophyll index of plantlets treated with different exposure times (Table 1). Plantlets treated with 4.00 mg l\(^{-1}\) colchicine for 24 h did not survive and died in growth stages (Table 4). The induced mixoploidy was characterized with significant larger guard cell length (48 µm) and width (15 µm) than the diploid variants (41 and 10 µm, respectively) (Table 4). Plantlets treated with 4.00 mg l\(^{-1}\) colchicine for 48 h showed significantly higher fresh weight (1.68 g) and dry weight (0.13 g) than control plantlets (0.4 and 0.04 g, respectively (Table 4). These plantlets also had significantly higher chlorophyll index (3.58) than those of untreated plantlets (2.88). Stomatal frequency per square millimeter was calculated. The average of stomata frequency in plantlets treated with 1.00 mg l\(^{-1}\) colchicine for 72 h was 16.26/mm\(^2\), in plantlets treated with
3.00 mg l⁻¹ colchicine for 72 h was 16.03/mm² and in untreated plantlets was 10.33/mm² (Table 4, Figure 1(c)). There was an obvious difference in root number between plantlets treated with 2.00 mg l⁻¹ colchicine for 48 h and control plantlets (Table 4). Based on Table 4, maximum number of root (7.00) was observed in plantlets treated with 2.00 mg l⁻¹ colchicine for 48 h. This root number is two-fold more than that of control. The highest root length (4.16 and 4.12 cm) was induced in plantlets treated with 3.00 mg l⁻¹ colchicine for 72 h and 1.00 mg l⁻¹ colchicine for 48 h, respectively. Leaf length (4.58 cm) was longest in plantlets treated with 3.00 mg l⁻¹ colchicine for 24 h compared to the plantlets treated with 5.00 mg l⁻¹ colchicine for 24 h with shortest length (1.07 cm) (Table 4). The average number of leaf (2.66) was reduced in plantlets treated with 5.00 mg l⁻¹ colchicine for 24 h. Maximum leaf number (6.66) was produced in plantlets treated with 5.00 mg l⁻¹ colchicine for 48 h (Table 4).

5. Discussion
Polyploid induction plays an important role in improvement of ornamental plants. This method provides an appropriate approach in the hybridization and improvement of orchids (Orchidaceae) (Miguel & Leonhardt, 2011). Antimitotic agents have been used to induce polyploidy in some ornamental species and several investigations have reported the success of the use of colchicine to produce polyploid forms in some orchids such as Phalaenopsis, Paphiopedilum, Dendrobium, Cymbidium, Vanda and Cattleya in in vitro condition (Griesbach, 1981; Huy et al., 2019; Hwang et al., 2015). Colchicine as a highly-effective mitotic spindle inhibitor has been used for the improvement of horticultural parameters for a variety of species by polyploidy induction (Dhooghe et al., 2011). The effectiveness of colchicine application and polyploid induction depends on some factors such as type of explant, plant species, colchicine concentration, and duration of treatment (Allum et al., 2007; Hannweg et al., 2013). Colchicine concentration and time duration of treatment are two most important factors. The results of a number of studies revealed that colchicine has different effects on various ornamental plants particularly orchid species and varieties. Current study was not shown the effectiveness of applied colchicine concentrations and exposure time to induce polyploidy in C. pileatum. Explants must be exposed to antimitotic agents at levels and times high enough to saturate plant tissues and induce polyploidy (Allum et al., 2007; Kermani et al., 2003). Optimum concentration and exposure time of colchicine are different for each species and variety, even within the same family of plants (Sarathum et al., 2010). We propose to use higher concentrations of colchicine for polyploidy induction in C. pileatum.

Young tissues containing meristematic cells are preferred to obtain high efficiency in polyploid plant formation through colchicine treatment (Huy et al., 2019). Protocorms and PLBs are suitable explants for polyploid induction in orchids (Huy et al., 2019; Miguel & Leonhardt, 2011). We used PLBs produced from protocorms as explants.

Colchicine has been applied at 0.05–0.20% concentrations and 2–9 days for orchids (L.P. Chen et al., 2006; Huy et al., 2019; Vichiato et al., 2014). In the present study, only a mixoploid plantlet was induced using 4.00 mg l⁻¹ colchicine for 72 h. Study of Atichart (2013) on Dendrobium by in vitro techniques on PLBs showed that the high frequencies of polyploidy were obtained with 0.04% colchicine for a day. Prolonged exposure (4 days) of the protocorms to high concentrations...
Table 1. Analysis of variance of different concentrations of colchicine and exposure time on different morphological, anatomical and physiological parameters of Catasetum pileatum

| Source of variances | df | M.S.                  | Chlorophyll index | Leaf length | Leaf number | Root length | Root number | Fresh weight | Dry weight | Stoma number | Guard cell length | Guard cell width |
|---------------------|----|-----------------------|-------------------|-------------|-------------|-------------|-------------|--------------|------------|---------------|--------------------|-----------------|
|                     | 5  | 6.168**               | 1.638*            | 2.96*       | 4.617**     | 2.818**     | 0.453**     | 0.00282**    | 29.20*     | 229.60**      | 8.16**            |
| Colchicine concentrations (A) | 5  | 2.196*               | 1.820**           | 12.57*      | 0.510**     | 23.120**    | 0.427**     | 0.00089**    | 74.84**    | 598.50**      | 58.16**           |
| Exposure time (B)    | 2  | 1.976*               | 3.970*            | 8.90*       | 3.360*      | 8.596*      | 0.240**     | 0.00153**    | 35.03**    | 393.10**      | 37.96**           |
| A × B                | 10 | 0.888                 | 1.875             | 4.00        | 1.255       | 3.740       | 0.070       | 0.00042      | 9.60       | 57.66         | 8.66               |
| Error               | 36 | 0.888                 | 1.875             | 4.00        | 1.255       | 3.740       | 0.070       | 0.00042      | 9.60       | 57.66         | 8.66               |
| C.V. (%)            | 36 | 70.33                 | 55.50             | 45.37       | 43.79       | 48.12       | 36.26       | 32.49        | 27.77      | 20.52         | 29.27              |

**Non-significant, * and **: Significant at the 0.05 and 0.01 levels, respectively
Table 2. Effect of different concentrations of colchicine on different morphological, anatomical and physiological parameters of Catasetum pileatum

| Colchicine concentrations (mg l⁻¹) | Chlorophyll index | Leaf length (cm) | Leaf number | Root length (cm) | Root number | Fresh weight (g) | Dry weight (g) | Stoma number | Guard cell length (µm) | Guard cell width (µm) |
|-----------------------------------|------------------|-----------------|-------------|-----------------|-------------|-----------------|---------------|--------------|---------------------|----------------------|
| 0.00                              | 2.70             | 2.01            | 4.00        | 1.95⁰           | 3.33⁰       | 0.40⁰           | 0.04          | 10.33⁰       | 41.00⁰              | 10.00⁰               |
| 1.00                              | 0.53 ⁰          | 2.50            | 5.33        | 3.12           | 4.11        | 0.78           | 0.06⁰        | 13.11⁰      | 40.00⁰              | 10.33⁰               |
| 2.00                              | 1.24⁰           | 2.77            | 4.66        | 2.83⁰          | 4.77        | 0.84           | 0.06⁰        | 12.78⁰      | 40.00⁰              | 11.33⁰               |
| 3.00                              | 1.14⁰           | 3.13            | 4.44        | 3.27           | 4.00        | 0.74           | 0.06⁰        | 11.18⁰      | 30.66⁰              | 8.66⁰                |
| 4.00                              | 1.70⁰           | 2.12            | 3.66        | 1.43           | 3.44        | 1.05           | 0.09⁰        | 8.15⁰       | 30.33⁰              | 9.33⁰                |
| 5.00                              | 0.62 ⁰         | 2.25            | 4.33        | 2.73            | 4.44      | 0.57           | 0.05⁰        | 11.38⁰     | 40.00⁰              | 10.66⁰               |

Means with different letters on the same column are significantly different (p < 0.05) based on LSD contrast test.
| Exposure time (h) | Chlorophyll index | Leaf length (cm) | Leaf number | Root length (cm) | Root number | Fresh weight (g) | Dry weight (g) | Stoma number | Guard cell length (µm) | Guard cell width (µm) |
|------------------|-------------------|-----------------|-------------|-----------------|-------------|-----------------|---------------|--------------|----------------------|----------------------|
| 24               | 0.95<sup>b</sup>  | 2.20<sup>a</sup> | 3.44<sup>b</sup> | 2.36<sup>b</sup> | 2.77<sup>b</sup> | 0.57<sup>b</sup> | 0.05<sup>a</sup> | 9.33<sup>b</sup> | 30.50<sup>b</sup> | 8.00<sup>b</sup>     |
| 48               | 1.62<sup>a</sup>  | 2.48<sup>a</sup> | 4.94<sup>a</sup> | 2.66<sup>a</sup> | 5.00<sup>a</sup> | 0.87<sup>a</sup> | 0.07<sup>a</sup> | 11.12<sup>a</sup> | 39.00<sup>a</sup> | 11.33<sup>a</sup>    |
| 72               | 1.44<sup>ab</sup> | 2.71<sup>a</sup> | 4.83<sup>a</sup> | 2.64<sup>a</sup> | 4.27<sup>a</sup> | 0.74<sup>ab</sup> | 0.06<sup>a</sup> | 13.21<sup>a</sup> | 41.50<sup>a</sup> | 10.83<sup>a</sup>    |

Means with different letters on the same column are significantly different (p < 0.05) based on LSD contrast test.
| Concentrations × Time | Chlorophyll index | Leaf length (cm) | Leaf number | Root length (cm) | Root number | Fresh weight (g) | Dry weight (g) | Stoma number | Guard cell length (µm) | Guard cell width (µm) |
|------------------------|-------------------|-----------------|-------------|-----------------|-------------|------------------|---------------|--------------|-----------------------|----------------------|
| 0.00 × 24              | 2.78<sup>abc</sup> | 2.01<sup>bc</sup> | 4.00<sup>abc</sup> | 2.00<sup>cd</sup> | 3.33<sup>bcd</sup> | 0.47<sup>ab</sup> | 0.04<sup>bc</sup> | 10.33<sup>cd</sup> | 41.00<sup>ab</sup> | 10.00<sup>abc</sup> |
| 0.00 × 48              | 2.88<sup>ab</sup> | 2.01<sup>bc</sup> | 4.00<sup>abc</sup> | 1.93<sup>cd</sup> | 3.33<sup>bcd</sup> | 0.40<sup>bc</sup> | 0.04<sup>bc</sup> | 10.33<sup>cd</sup> | 41.00<sup>ab</sup> | 10.00<sup>abc</sup> |
| 0.00 × 72              | 2.92<sup>ab</sup> | 2.01<sup>bc</sup> | 4.00<sup>abc</sup> | 1.93<sup>cd</sup> | 3.33<sup>bcd</sup> | 0.40<sup>bc</sup> | 0.04<sup>bc</sup> | 10.33<sup>cd</sup> | 41.00<sup>ab</sup> | 10.00<sup>abc</sup> |
| 1.00 × 24              | 0.06<sup>cd</sup> | 2.82<sup>bc</sup> | 5.66<sup>abc</sup> | 3.48<sup>bc</sup> | 4.66<sup>bcd</sup> | 0.65<sup>bcd</sup> | 0.04<sup>bc</sup> | 11.93<sup>bc</sup> | 40.00<sup>bc</sup> | 10.00<sup>abc</sup> |
| 1.00 × 48              | 0.51<sup>d</sup> | 2.80<sup>bc</sup> | 6.33<sup>abc</sup> | 4.12<sup>bc</sup> | 4.33<sup>bcd</sup> | 1.03<sup>b</sup> | 0.08<sup>bc</sup> | 11.13<sup>bc</sup> | 41.00<sup>bc</sup> | 11.00<sup>abc</sup> |
| 1.00 × 72              | 0.42<sup>d</sup> | 1.90<sup>bc</sup> | 4.00<sup>abc</sup> | 1.75<sup>bc</sup> | 3.33<sup>bcd</sup> | 0.63<sup>bcd</sup> | 0.06<sup>bc</sup> | 16.26<sup>bc</sup> | 39.00<sup>bc</sup> | 10.00<sup>abc</sup> |
| 2.00 × 24              | 0.76<sup>bc</sup> | 1.88<sup>bc</sup> | 4.66<sup>abc</sup> | 3.37<sup>bc</sup> | 2.66<sup>cde</sup> | 0.80<sup>bc</sup> | 0.06<sup>bc</sup> | 12.30<sup>bc</sup> | 38.00<sup>bc</sup> | 9.00<sup>bc</sup> |
| 2.00 × 48              | 0.80<sup>bc</sup> | 2.76<sup>bc</sup> | 4.00<sup>abc</sup> | 2.29<sup>cde</sup> | 7.00<sup>b</sup> | 0.84<sup>bc</sup> | 0.06<sup>bc</sup> | 13.76<sup>bc</sup> | 41.00<sup>ab</sup> | 13.00<sup>b</sup> |
| 2.00 × 72              | 2.1<sup>bcd</sup> | 3.67<sup>bc</sup> | 5.33<sup>abc</sup> | 2.82<sup>cde</sup> | 4.66<sup>bcd</sup> | 0.88<sup>bc</sup> | 0.06<sup>bc</sup> | 12.30<sup>bc</sup> | 41.00<sup>ab</sup> | 11.00<sup>bc</sup> |
| 3.00 × 24              | 1.11<sup>c</sup> | 4.58<sup>a</sup> | 3.33<sup>abc</sup> | 2.15<sup>cd</sup> | 4.00<sup>bcd</sup> | 0.80<sup>bc</sup> | 0.09<sup>b</sup> | 10.86<sup>cd</sup> | 36.00<sup>bc</sup> | 11.00<sup>bc</sup> |
| 3.00 × 48              | 1.06<sup>c</sup> | 1.56<sup>c</sup> | 3.33<sup>abc</sup> | 3.51<sup>bc</sup> | 2.66<sup>cde</sup> | 0.49<sup>bc</sup> | 0.04<sup>bc</sup> | 6.66<sup>b</sup> | 23.00<sup>bc</sup> | 8.00<sup>c</sup> |
| 3.00 × 72              | 1.27<sup>cd</sup> | 3.25<sup>a</sup> | 6.33<sup>abc</sup> | 4.16<sup>a</sup> | 5.33<sup>bcd</sup> | 0.94<sup>b</sup> | 0.07<sup>bc</sup> | 16.03<sup>bc</sup> | 33.00<sup>bc</sup> | 7.00<sup>c</sup> |
| 4.00 × 24              | 0.00<sup>d</sup> | 0.00<sup>c</sup> | 0.00<sup>c</sup> | 0.00<sup>c</sup> | 0.00<sup>c</sup> | 0.00<sup>c</sup> | 0.00<sup>c</sup> | 0.00<sup>c</sup> | 0.00<sup>c</sup> |
| 4.00 × 48              | 3.58<sup>a</sup> | 3.07<sup>ab</sup> | 5.33<sup>abc</sup> | 2.63<sup>cde</sup> | 6.00<sup>bc</sup> | 1.68<sup>a</sup> | 0.13<sup>a</sup> | 12.13<sup>bc</sup> | 44.00<sup>ab</sup> | 14.00<sup>a</sup> |
| 4.00 × 72              | 1.51<sup>abcd</sup> | 3.30<sup>a</sup> | 5.66<sup>abc</sup> | 1.66<sup>cde</sup> | 4.33<sup>bcd</sup> | 0.87<sup>bc</sup> | 0.07<sup>bc</sup> | 12.33<sup>bc</sup> | 48.00<sup>a</sup> | 15.00<sup>a</sup> |
| 5.00 × 24              | 0.40<sup>d</sup> | 1.07<sup>c</sup> | 2.66<sup>c</sup> | 3.16<sup>cde</sup> | 6.00<sup>bc</sup> | 0.16<sup>a</sup> | 0.02<sup>a</sup> | 9.40<sup>cd</sup> | 28.00<sup>cd</sup> | 8.00<sup>c</sup> |
| 5.00 × 48              | 1.02<sup>c</sup> | 2.72<sup>ab</sup> | 6.66<sup>a</sup> | 1.50<sup>bc</sup> | 5.66<sup>b</sup> | 0.82<sup>bc</sup> | 0.07<sup>bc</sup> | 12.73<sup>bc</sup> | 44.00<sup>ab</sup> | 11.00<sup>bc</sup> |
| 5.00 × 72              | 0.45<sup>d</sup> | 2.12<sup>bc</sup> | 3.66<sup>abc</sup> | 3.52<sup>abc</sup> | 4.66<sup>bcd</sup> | 0.73<sup>bcd</sup> | 0.06<sup>bc</sup> | 12.03<sup>abcd</sup> | 45.00<sup>ab</sup> | 13.00<sup>b</sup> |

Means with different letters on the same column are significantly different (p < 0.05) based on LSD contrast test.
of colchicine (0.10%) reduced survival percentage in *Dendrobium draconis* (Bunnag & Hongthongkham, 2015). Study of Qiang et al. (2009) on *Dendrobium* revealed that 0.06% colchicine for 12 h had the best effect for polyploid induction. Mortality caused by colchicine is different for various species, mainly depending upon its concentration. In many studies, high mortality rate occurred with high concentrations of colchicine (Choopeng et al., 2019; Huy et al., 2019; Vichiato et al., 2014). The inverse relationship between colchicine concentration and plant survival was demonstrated on some orchids (Huy et al., 2019; Petchang, 2010; Saratham et al., 2008). High concentration of colchicine is toxic for plant cell and causes imbalance between physiological and biochemical processes. Most polyploid induction studies were carried out in *in vitro* conditions.

Among all methods for analysis of polyploidy, flow cytometry is the most efficient. Nevertheless, it is better to count chromosome number and apply some morphological, anatomical, physiological, and cytological parameters together with flow cytometry. Polyploidy levels can be precisely identified using a combination of these methods. The chromosome number of diploid *C. pileatum* was 2 n = 2x = 54. Detection of polyploid induction in some orchids was done using chromosome counting (karyotype), morphological, physiological, anatomical, and cytological parameters (Aoyama, 2010; Miguel & Leonhardt, 2011; Vichiato et al., 2014). However, ploidy level determination by morphological or anatomical assays lonely has some limitations (W.H. Chen et al., 2009; Dhooghe et al., 2011).

Comparison of stomatal guard cells size is a simple and effective method to detect polyploids from diploids (Huy et al., 2019). This method was used to identify the genetic characters of *Phalaenopsis*, *Cymbidium*, *Dendrobium*, *Epidendrum*, and *Odontioda* (W.H. Chen et al., 2009; Miguel & Leonhardt, 2011). Assessment of guard cell size needs less time which permits simple and rapid analysis of a large number of species and varieties (W.H. Chen et al., 2009; Dhooghe et al., 2011). However, this method should be applied in combination with other modern methods to obtain clearer results (Huy et al., 2019). Several researchers showed increase in size of guard cell of orchids treated with colchicine in comparison with untreated plants (Atichart & Bunnag, 2007; W.H. Chen et al., 2009; Choopeng et al., 2019; De Mello Silva et al., 2000; Huy et al., 2019; Miguel & Leonhardt, 2011). In our work, guard cells size in mixoploid plantlet was higher than that of other plantlets.

6. Conclusion

(1) Polyploidy plays an important role in improvement of orchids.

(2) In the present study, a mixoploid plantlet was obtained. We think it is necessary to evaluate higher concentrations of colchicine for polyploid induction particularly tetraploid in *C. pileatum* Rchb.f.

(3) The largest size and number of stomatal guard cells were obtained in leaves of plantlets treated with 4.00 and 3.00 mg l⁻¹ colchicine both for 72 h.

(4) Production of polyploid plants without the use of antimitotic agents might be suitable, because some of these materials are poison for plants.

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