

Alteration of *Spathoglottis eburnea* Gagnep. Ploidy Level after Colchicine Treatments

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Abstract

Improvement of *Spathoglottis eburnea* Gagnep (*Orchidaceae*) with alterative morphology was performed and observed by polyploidy induction. Two millimeters long shoot tips were immersed and cultured in ½ MS liquid medium supplemented with 0, 0.25, 0.5, 0.75, and 1 % (w/v) of sterilized colchicine solutions for 1 d. After 3 months of culture, the highest polyploidy induction was obtained with 1 % (w/v) colchicine for 1 d, which gave a shoot survival rate of about 45 % and which regenerated into 40 % polyploid plantlets. Five polyploid plantlets were further maintained by subculturing every 2 months on ½ MS agar medium supplemented with 8 g L⁻¹ activated charcoal. After the third subculture, all of them were morphological and cytological chimeras, which had alterations in leaf morphology. The variation of ploidy level was determined again by flow cytometry and chromosome counting. A flow cytometry showed that tetraploid plantlets changed to mixoploid plants, where phenotype instability was further observed, in correlation with chromosome instability. The density of stomata and the stoma index were observed and showed no significant differences between mixoploids and diploids. However, the guard cell size of mixoploids was larger than those of diploids. In the light of our findings, we can consider polyploidy induction to be an effective technique for plant improvement; however, there is a necessity to study genetic stability between the clones after micropropagation.

Keywords: Mixoploid, flow cytometry, chromosome count, stomata analysis

Introduction

Spathoglottis eburnea Gagnep, a medicinal orchid, is a native and endangered species in Southeast Asia [1]. In Thailand, it is found in the North and Northeast area, where traditional doctors use fresh tubers as poultices for wounds and boiling bulbs for antipyretics. This genus contains 40 species of ornamental and medicinal plants [2] and which has a high potential to be a commercial orchid in the future. According to plant breeding, polyploid induction is used in agriculture for novelty ornamental plants [3], high productivity of secondary metabolites [4], and fertility restoring [5]. Polyploidy is known as a major mechanism for plant adaptation and speciation occurring in nature. Induction of polyploidy can be induced via 2 mechanisms, mitotic and meiotic polyploidization, from somatic cells or tissues [6] and gametes [7]. The basic method for chromosome doubling is achieved by using antimetabolic reagents, such as colchicine (C₂₂H₂₅O₆N). At the meiosis or mitosis stage, this compound binds to tubulin dimers that cause disruption of microtubule formation, in which polyploidy plants can be formed.

In orchids, Griesbach [8] applied 50 mg L⁻¹ colchicine to *Phalaenopsis* protocorms, which produced about 50 % tetraploids. Also, in *Cattleya intermedia* Lindl., Silva *et al.* [3] studied the effect of colchicine concentration and suggested that 0.05 and 0.1 % (w/v) of colchicine treatment were suitable for

polyploidy induction. Likewise, many studies based on orchid polyploidy induction were performed using *Dendrobium chrysotoxum* [9], *Dendrobium secundum* [10], *Dendrobium scabrilingue* [11], and *Rhynchostylis gigantea* var. *rubrum* Sagrik [12].

The ploidy stability of micropropagated plants has great importance for breeding programs. Verifications are rarely reported, and some of the indicators used make identification of mixoploid and polyploid plants difficult. Mostly, morphology and anatomy are used as indicators for polyploidy screening, because most polyploid plants show larger or higher amounts of plant forms [3,7,10,12-14]. However, this criterion is not suitable to identify mixoploid plants. The staining of nuclear DNA content by flow cytometry is used to compare fluorescence intensities between tested samples and the DNA standard [12-15]. This method is quick and reliable in distinguishing ploidy level. However, there was no report on the autotetraploid induction and chromosome estimation in *Spathoglottis* orchids. Therefore, this study develops an *in vivo* protocol of polyploid induction and determination by colchicine in *Spathoglottis eburnea* Gagnep.

Materials and methods

Plant materials and culture conditions

Self-pollination of *Spathoglottis eburnea* Gagnep was pollinated by hand and fruits were collected after 25 d. The fruits were surface sterilized through 70 % (v/v) ethanol for 1 to 2 min and then transferred into a laminar air-flow cabinet. After that, the fruits were further soaked in 95 % (v/v) ethanol and flamed immediately with an alcohol burner before sowing on half-strength Murashige and Skoog ($\frac{1}{2}$ MS) [16]. The germinated protocorms were cultured in $\frac{1}{2}$ MS liquid medium supplemented with 15 g L⁻¹ sucrose, 0.5 mg L⁻¹ α -naphthaleneacetic acid (NAA), 1 mg L⁻¹ 6-benzylaminopurine (BAP) and pH 5.8 [17] for 3 months. All explants were cultured on a rotary shaker at 110 rpm and incubated at 25 \pm 2 °C under illumination of approximately 36 μ mol m⁻² s⁻¹ provided by cool white fluorescent light (Philips) for 16 h per d. Then, protocorm-like bodies were transferred to culture on $\frac{1}{2}$ MS agar medium supplemented with 0.8 g L⁻¹ activated charcoal for plantlet induction. After 3 months of culture, apical shoots from 4 cm high *S. eburnea* plantlets were used as plant materials.

Colchicine treatments

Two millimeters long shoot tips were immersed and cultured in $\frac{1}{2}$ MS liquid medium supplemented with 0, 0.25, 0.5, 0.75 and 1 % (w/v) of sterilized colchicine solutions for 1 d on Petri dishes (9 cm in diameter). Consequently, the treated shoots were washed 3 times with sterile distilled water and transferred to $\frac{1}{2}$ MS agar medium supplemented with 15 g L⁻¹ sucrose and pH 5.8 (**Figure 1**). After 1 month of culture, a total number of ten shoots per treatment were observed as the percentage of survival shoots and the number of proliferated shoots (shoots per explant). These plantlets were transferred to $\frac{1}{2}$ MS agar medium supplemented with 0.8 g L⁻¹ activated charcoal.

Ploidy analysis

The different parts of *in vitro* plantlets (without colchicine treatment)- root tips (1 cm segment from root tips), mature root segments (1 cm from the root tips), young leaves (1 cm segments in length), and mature leaves (1 - 3 cm from leaf apices)- were used for flow cytometry analysis (**Figure 1**). Samples were cut with razor blades into strips of less than 1 mm and suspended in 1 mL Otto I solution [15]. The suspensions were filtered through 20 μ m nylon mesh to eliminate large debris and cell fragments. The filtrate were centrifuged at 3,500 rpm for 10 min and the pellets collected, before resuspension in 1 mL Otto I solution. After 10 min of incubation, staining solution, which contained 400 μ L of Otto II solution plus 50 μ g RNase A (Sigma, USA), 50 μ g propidium iodide (Sigma, USA), and 2 μ L β -mercaptoethanol, was added. The relative nuclei content of each sample was analyzed by using a flow cytometer (BD FACSCanto™, USA). In the colchicine treatment, 0.2 \times 2.0 cm of young leaf tissue were used as plant materials and extracted the same as the previous method. The ratio between average channels of tissue was analyzed and the 2C peak of the standard was determined along with samples for ploidy evaluation.

For chromosome counting, the active growing root tips of plantlets (approximately 1 - 2 cm long) were excised and pretreated in 2 mM 8-hydroxyquinoline at 18 °C for 24 h. Treated roots were removed to a fixative solution which contained acetic acid, chloroform, and ethanol (1:1:2). After 24 h, the fixed root tips were stored in 75 % ethanol at 4 °C before hydrolysis in 1 N hydrochloric acid (HCl) at 60 °C for 13 min. The macerated root tips were stained with aceto-orcein and a coverslip was applied over the slides. Finally, the slide of each sample was observed under a light microscope at 100× magnification.

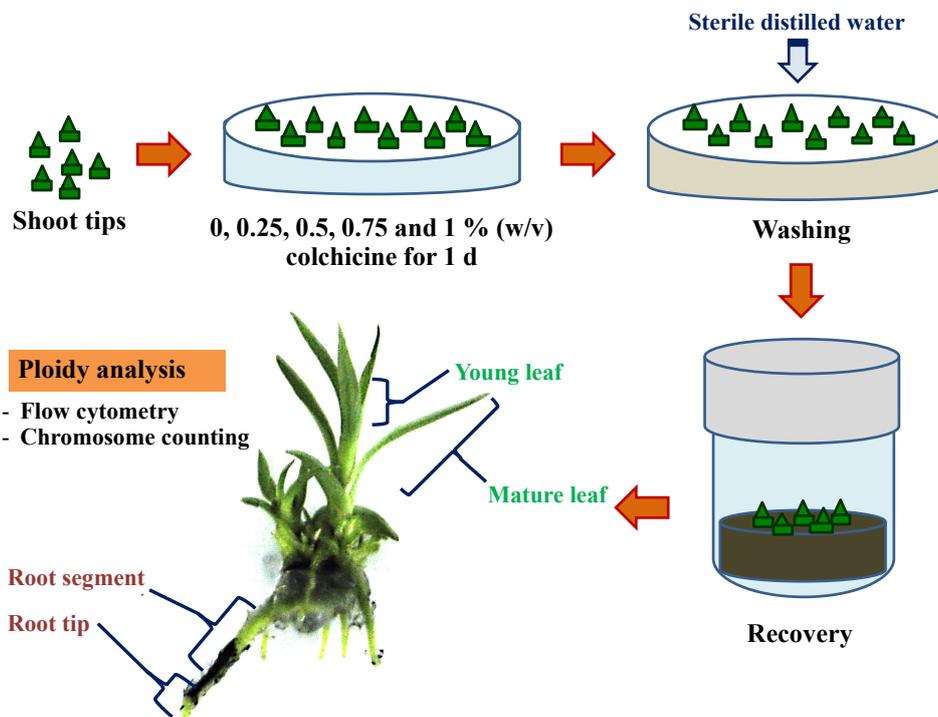


Figure 1 Polyplody induction via shoot tips of *S. eburnea* via colchicine solution and ploidy analysis using difference parts of plant. Schematic drawing of protocol started from soaking the shoot tips in colchicine for 1 d then plantlets were recovered and analyzed.

Stomata measurement

Fully expanded leaves of plantlets were prepared and used for leaf prints. An abaxial, or lower, surface was painted with clear nail varnish for 60 min. The dried layer was peeled off and mounted on a slide by using transparent tape. The images of leaf sections, which were obtained by a BX50 microscope (Olympus) using an Olympus DP50 digital camera with a Viewfinder Lite version 1.0 program (Pixeria, Los Gatos, CA, USA) mounted on a light-phase microscope, were observed at 4× and 10× magnification. Then, stomatal density (SD), stomata index (SI), and sizes were measured. The calculation of SD and SI was achieved via the total number of stomata and epidermal cells in 1 mm² unit. SD and epidermal cell densities (ED) were identified and used for the stomata index (SI) calculation as per the following equation;

$$SI (\%) = \frac{SD \times 100}{SD + ED} \quad (1)$$

For the guard cell size, closing stomata were used to avoid any variation from natural opening or closing stomata. The estimation was taken randomly from untreated and colchicine treated plants.

Data analysis

The experiment was organized in a completely randomized design, with 3 replications and ten subsamples per replication. All treatments were organized using completely randomized design. Data were subjected to analysis of variance. The means of the shoot survival rates (%) and the average number of proliferated shoots were compared using the Duncan Multiple Range Tests (DMRT) and the Pair Sample T-test, at $P \leq 0.5$.

Results and discussion

Survival and number of shoots

Different concentrations of colchicine solution were applied to shoot tips of *S. eburnea*. After recovery on 1/2 MS agar medium for 1 month, the survival rates and number of proliferated shoots were observed (Table 1). The highest survival percentage was found in the treatment without colchicine, and the lowest in 1 % (w/v) colchicine. According to DMRT analysis, the percentage of shoot survival rates showed that all treatments were significantly different at $P < 0.05$. The verification was evaluated together with the shoot survival rates, via the number of proliferated shoots which gradually regenerated into the whole plantlet after 3 months of culture. The most and least numbers of proliferated shoots were 11.9 and 1.1 shoots/explant, when cultured in the treatment without colchicine and with 0.5 % (w/v) colchicine solution, respectively. At 0.5 % (w/v) colchicine solution, the regenerated shoots were decreased and stopped about at 10 % of the control (Table 1) and there was significant difference between treatments at $P < 0.05$. The highest polyploid induction was achieved when shoot tips were treated in 1 % (w/v) colchicine. In contrast, 0.25 % (w/v) colchicine gave the lowest polyploid induction, which produced only one mixoploid plant.

Table 1 The effects of colchicine concentration on shoot survival rates, number of proliferated shoots, and percentage of ploidy induction of *S. eburnea* after exposure in colchicine solution for 1 d.

Colchicine concentration (% w/v)	Shoot survival rates (%)	Number of proliferated shoots (shoots/explant)	Ploidy induction (%)	
			Diploid (2n)	Mixoploid (2n + 4n)
0	100.0 ± 0.0 ^a	11.9 ± 2.2 ^a	100	0
0.25	65.2 ± 2.9 ^b	3.7 ± 0.9 ^b	90	10
0.50	55.6 ± 2.9 ^{bc}	1.1 ± 0.1 ^b	80	20
0.75	48.1 ± 6.1 ^c	2.4 ± 0.4 ^b	80	20
1.00	45.0 ± 2.9 ^c	1.5 ± 0.2 ^b	60	40

Data are presented as means ± Standard error.

Means followed by different letters in each column are significantly different according to DMRT.

Colchicine treatments are used as antimetabolic agents to generate polyploids in plant breeding [5-8]. In this study, the range from 10 to 40 % of polyploid induction depended on the concentrations used; for example, culturing in 1 % (w/v) colchicine for 1 d indicated the highest polyploidy induction of about 4 chimeras (40 %). Increasing colchicine concentration tended to decrease shoot survival rates, as shown in Table 1. The highest tetraploid induction was obtained when the shoot tips were immersed in 1/2 MS supplemented with 1 % (w/v) colchicine for 1 d, which gave about a 45 % shoot survival rate and regenerated 40 % polyploidy plantlets. For polyploidy screening in the first culture, chromosome counting was prepared and further maintained by subculturing every 2 months on basal agar medium

supplemented with 8 g L⁻¹ activated charcoal. After the third subculture, the determination of ploidy level of polyploid plantlets was determined again by flow cytometry and chromosome counting. Similar to our results, a high frequency of polyploidy induction was done in a high colchicine concentration substance, where the survival rate was decreased [4,8,11]. Comparison between colchicine concentration and duration time for polyploidy induction was conducted in protocorms of *Dendrobium* [10] and the results gave high percentages of tetraploid plantlets in mid-level concentrations and short periods of time.

Ploidy determination

The various tissues, root tips, root segments, young leaves, and mature leaves were extracted and analyzed by using a flow cytometer in order to assess ploidy level (Figure 2). The 2C peak was represented as a standard for ploidy level evaluation. The flow cytometric histograms of untreated control plants showed the nuclear DNA content released from the root tips and mature root segments, which are demonstrated in 3 peaks at 40, 80, and 160 of the relative nuclear DNA content as 2c, 4c, and 8c, individually (Figures 2a and 2b). However, a relative nuclear DNA content of 50 (Figures 2c and 2d) of young and mature leaves were exposed only to the 2c peak. Therefore, the young leaf tissue was further used as plant material for normal and variegated structures of untreated and treated plants.

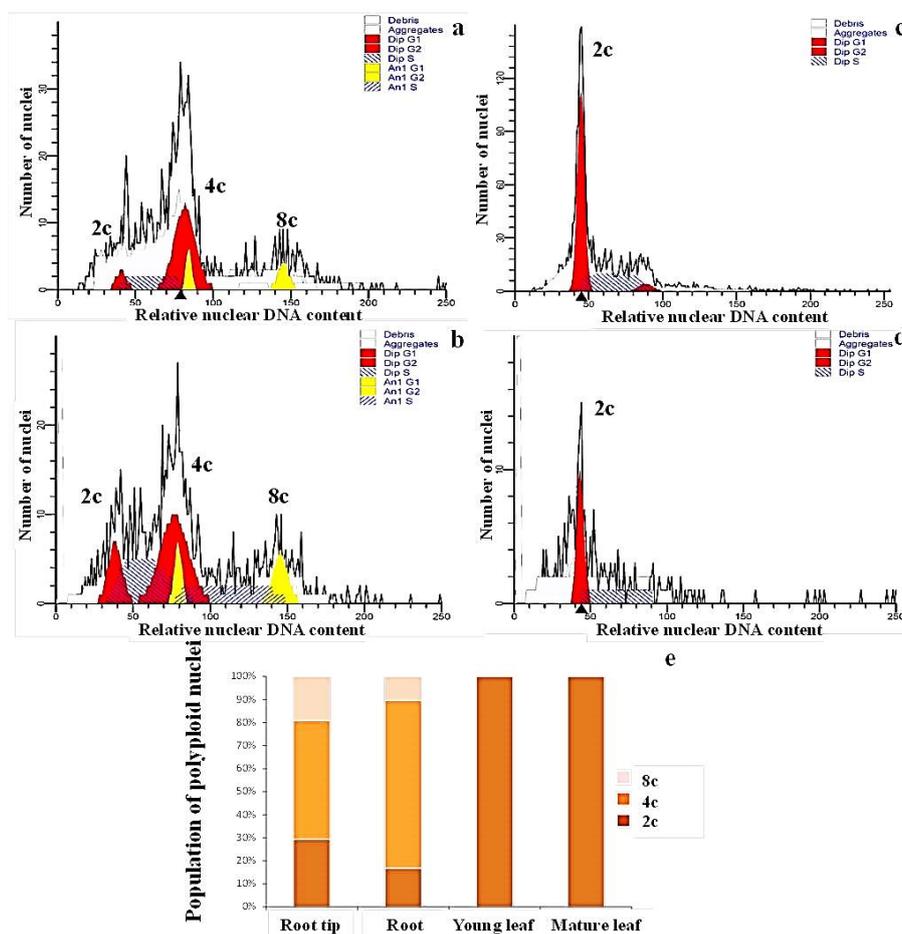


Figure 2 Relative nuclear DNA content in different tissues of *S. eburnea* by flow cytometry: root tips (a), root segments (b), young leaves (c), mature leaves (d), and population of polyploidy nuclei of control (e).

In each experiment, the diploid plant was analyzed parallel to the samples as an external control (Figure 3a). When shoot tips of *S. eburnea* were treated in colchicine solution for 1 d, 2 types of ploidy level were found (Figure 3). After identification, the regenerated plantlets were additionally characterized in root tip cells by the chromosome counting method. From Figure 4, the cytological clarifications revealed that diploid chromosome numbers were $2n = 2 \times = 40$, while chimera or mixoploid established in a different combination of both diploid and tetraploid cells as $2n = 2 \times = 40$ and $2n = 4 \times = 80$, respectively.

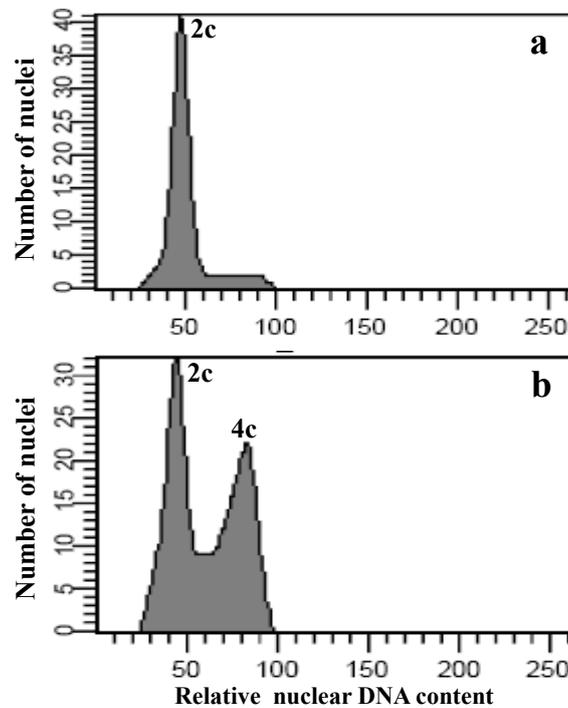


Figure 3 Flow cytometric histogram of nuclei isolated from leaves of diploid (a) and mixoploid (b) plants regenerated from untreated and treated with colchicine.

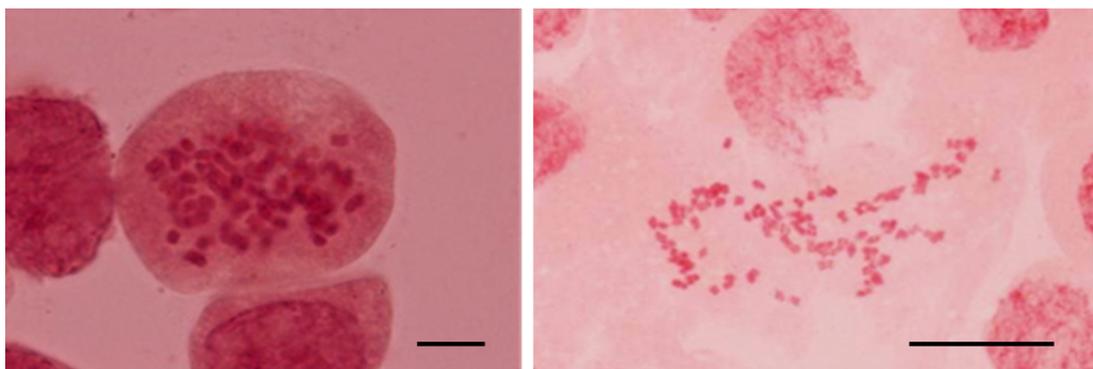


Figure 4 Chromosomes of root tips in diploid ($2n = 40$) (left) and tetraploid ($2n = 80$) (right) cells of *S. eburnea* under a light microscope (100× magnification). Bar = 10 µm.

Due to this ploidy analysis, a flow cytometry was used in this work, because it can analyze a lot of samples very quickly and reliably. Our study produced 17 diploids and 5 mixoploids which were further observed by chromosome counting (Table 1 and Figure 4). This chromosome counting was analyzed and showed 2 types of chromosome numbers. In this study, an alteration of ploidy level was found. According to first culture, 5 tetraploids were established from regenerated plantlets of *S. eburnea* after colchicine treatment (data not shown). Then, subculturing was done every 2 months for micropropagation; however, morphological and cytological chimeras were exhibited (Figure 5). Therefore, the stability of ploidy level was determined, and the results showed that all tetraploid plantlets were reversed to mixoploid (Figure 4). The polyploidization was affected due to the stage of cells which caused normal and tetraploid types in the same shoot tip. These results are called chimeras, or mixoploid, and have been found in many researches [7,12,14]. The results from polyploidy induction, chromosome counting, flow cytometry, and an assessment of morphological or anatomical parameters were used to determine ploidy levels in plants.

Comparison of morphological characteristics between diploid and mixoploid plants

After 6 months of culturing, the control plants produced proliferated shoots and regenerated into whole plantlets. There were more green leaves and faster growth than in colchicine treated plantlets (Figure 5). The morphology of diploid and mixoploid orchids was observed (Table 2). Some of them exhibited a chimera phenotype as variegated leaves with yellow or white color instead of green color. A normal phenotype was found in control treatment, which regenerated the highest shoot multiplication (Figure 5a). However, mixing phenotypes, such as a white color of young shoots and a variegated structure with green color at the tip of a leaf, were exhibited in mixoploids (Figure 5d). Normal plantlets regenerated into the mature stage as corm-structure (Figure 5a), whereas variegated phenotypes were approximately 2 cm long without corm-structure (Figures 5c and 5d). Moreover, white or yellow shoots and lack of corm formation developed a normal phenotype after being subcultured 3 times. The averaged leaf sizes were 0.56/5.52 (width/length) and 0.33/2.34 in diploid and mixoploid plantlets, respectively (Table 2).

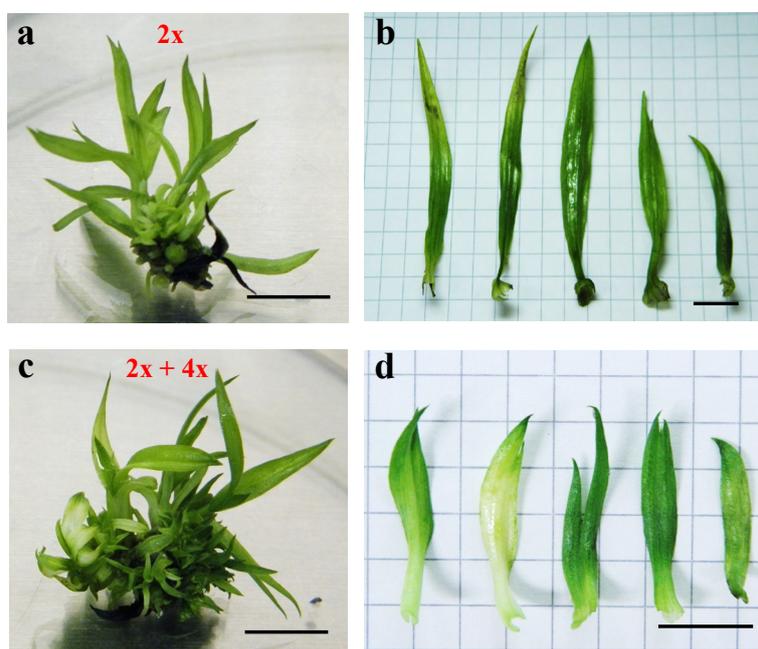


Figure 5 Plantlets regenerated from untreated control (a and b) and 0.25 % (w/v) colchicine treated shoot (c and d) after culture for 1 year. Bar = 1 cm.

Furthermore, the fully expanded leaves of diploid and mixoploid plantlets were prepared as leaf prints for the stomata density (SD), stomata index (SI), and stomata size. From **Table 2**, a number of stomata cells were measured and presented as SD. The distribution ranged from 11 - 48 cells/mm², which had an average of about 32.1 cells/mm² in normal (2×), and the chimera (2× + 4×) was 20 - 46 cells/mm² and 31.1 cell/mm², respectively. The numbers of guard cells were not only standard for SD but also SI. The SI of these 2 variants is very similar (8.4 - 8.3) since there is no significant difference in SD and SI between both plantlets (**Table 2**). Furthermore, stomata size was considered (**Table 2** and **Figure 6**). The width and length of diploid were 45.3 and 57.3 μm and mixoploid were 50.1 and 61.7 μm, respectively. In contrast, significant differences were found in both the width and length of guard cells at *P* = 0.05.

Table 2 Comparison of leaf and stomata morphology between diploid and mixoploid of *S. eburnea*.

Ploidy	Leaf size (cm)		Stomata density (cells/mm ²)	SI (%)	Size of guard cell (μm)	
	Width	Length			Width	Length
Diploid (2×)	0.56 ± 0.0 ^b	5.52 ± 0.4 ^b	32.1 ± 1.2 ^a	8.4 ± 0.3 ^a	45.3 ± 0.8 ^a	57.3 ± 1.0 ^a
Mixoploid (2× + 4×)	0.33 ± 0.0 ^a	2.34 ± 0.1 ^a	31.1 ± 1.0 ^a	8.3 ± 0.3 ^a	50.1 ± 1.2 ^b	61.7 ± 1.5 ^b

Data are represented as means ± standard error of 2 ploidy levels. Means followed by different letters in each column are significantly different according to DMRT at *P* = 0.05.

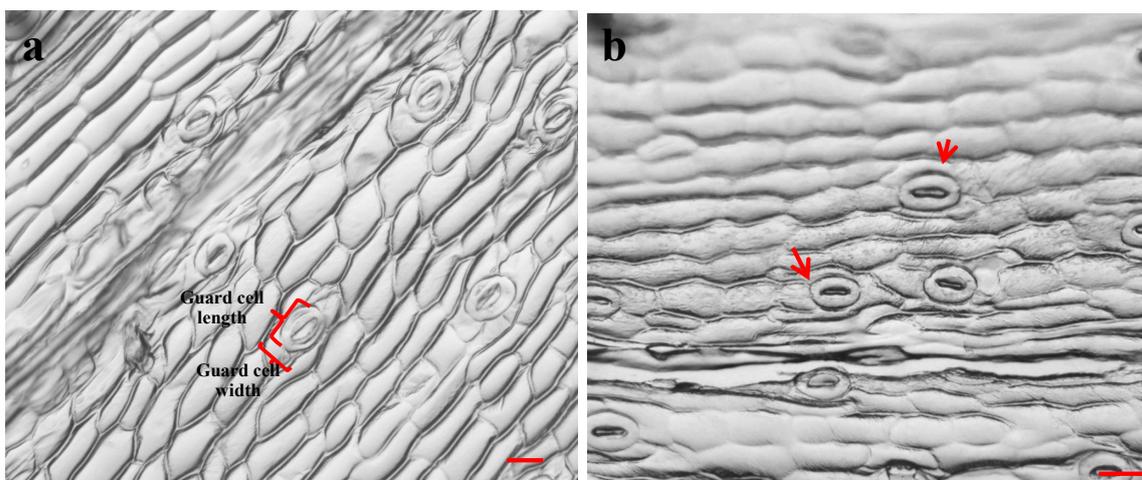


Figure 6 Leaf prints of diploid (a) and mixoploid (b) plantlets grown from *in vitro* plantlets. Bar = 100 μm.

As a result of polyploidy induction, chromosome counting, flow cytometry, and an assessment of morphological or anatomical parameters were used to determine ploidy levels in plants. Previously, flow cytometry was performed in this work, because it analyzes a lot of samples very quickly and reliably. Moreover, chromosome counting was analyzed and compared to the ploidy level, which showed 2 types of chromosome number (**Figure 4**). However, these 2 techniques have some limitation, as the flow cytometry needs specific equipment and proper sample preparation. Chromosome counting is time

consuming. Therefore, the stomata characters (SD and/or sizes) and leaf size were used for preliminary screening of polyploid plants in *Alocasia* 'Green Velvet' [14], *Aframomum corrorima* [4], *Cattleya intermedia* [3], and *Dendrobium secundum* [10], which showed significant difference among diploid and polyploidy plants.

Conclusions

Shoot tips treated with 1 % (w/v) colchicine for 1 d gave about a shoot survival rate of 45 % and regenerated the highest polyploidy induction (40 %). The results showed that morphology was not a reliable indicator for the identification of polyploidy plants, whereas flow cytometry, guard cell, and leaf size were verified to be effective protocols. There was significant difference in the size of leaves and guard cells between diploid and polyploidy plantlets; nevertheless, confirmation of mixoploids by flow cytometry was required. Therefore, leaf and guard cell size are suitable for the primary screening of polyploids in breeding programs, and flow cytometry is recommended in order to confirm analysis of ploidy levels in *Spathoglottis eburnea*.

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