Induction and identification of polyploidy by colchicine treatment in balloon flower (*Platycodon grandiflorus*)

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Abstract
Balloon flower (Platycodon grandiflorus) is a valuable medicinal plant in traditional medicine. Experiments on shoot formation and rapid multiplication have been conducted to create a source of mutation-inducing material. The Murashige and Skoog (MS) medium supplemented with 1.0 mg/l BAP; 0.4 mg/l BAP and 0.1 mg/l NAA showed good responses in shoot formation and rapid multiplication of shoots. The shoot clusters were immersed in different concentrations of colchicine solutions (0%; 0.01%; 0.02%; 0.03%; 0.04%; w/v) for 24, 48 and 72h.

The results indicated that treatment at 0.02% colchicine for 48h had the highest polyploid shoot formation induction. The polyploid shoots exhibited lager stomatal size and denser distribution. The ploidy levels of polyploid shoots were assessed by counting number chromosomes and by flow cytometry.

Keywords: Balloon flower, *Platycodon grandiflorus*, *in vitro*, BAP, NAA, colchicine, ploidy, stomata, chromosome, flow cytometry.

Introduction

Balloon flower (*Platycodon grandiflorus*) is a herbaceous plant belonging to the Campanulaceae family and its primary chemical component[5] is a group of saponins[16,21]. Notably, platycodin D_2 constitutes 8.62% of its chemical composition[21], it is recognized as a strong adjuvant, increasing immune response fluid against the antigen that causes hepatitis B, treating cancer by inhibiting growth generate cell types such as lung cancer, ovarian cancer, colon cancer and malignant tumors[14,26].

The pharmacological action of *Platycodon grandiflorus* is mainly due to saponins, flavonoids, phenolic and other compounds. It has been shown to be beneficial in relieving cough and asthma and to have anti-tumor, anti-inflammatory, anti-diabetic activities (successfully studied on mice)[15] and it was used in numerous traditional herbal formulations in Vietnam, China, South Korea and India[6,7,26]. Besides, balloon flower is also used as a food, preserved fruits and health drinks; it makes high ornamental value for the landscape[13] and also contributes to the beauty and cosmetics industry[17].

Balloon flower (*P. grandiflorus*) is currently in high demand in the global market. In China, annual production typically reaches 1,000 tons, with exports accounting for half of this volume. Meanwhile, Japan requires 150 tons of balloon flowers annually[13]. In Vietnam, since 2019, *P. grandiflorus* has ranked 18th among the 100 medicinal plant species with high health and economic value for focused development. Looking ahead to 2030, *P. grandiflorus* is one of the 16 domestically cultivated medicinal plant varieties being actively promoted. In Bac Ha (Lao Cai), the cultivation area for *P. grandiflorus* covers nearly 100 hectares, with a yield ranging from 5 to 6 tons per hectare (as of 2020).

Polyploidy induction research on plants aims to enhance varieties and achieve high economic efficiency, particularly for medicinal plants. The common method for inducing polyploidy involves treating explants with colchicine. In general, polyploid plants exhibit favorable characteristics such as increased secondary compound production[22], larger plant size, higher leaf yield, robust stems and resilience[3,8,23]. The purpose of this study is to establish an *in vitro* propagation process for balloon flowers and isolate a stable and uniform polyploid line for consistent growth *in vitro*, this will provide primary material for selecting high - yield balloon flower varieties in the future.

Material and Methods

Plant materials: The seeds of Balloon flower (*Platycodon grandiflorus*) were purchased from the natural source in Lao Cai province, Vietnam. Seeds were washed with tap water (10 minutes), shaken in a 10% (v/v) soap solution (10 minutes) and rinsed again with tap water. Next, the seeds were shaken in 70% ethanol solution (90 seconds) and then soaked in a 10% (w/v) Javel solution (10 minutes). After sterilization, the seeds were cultured on MS medium supplemented with 0.7% agar (w/v) and 2% sucrose (w/v). Cultivation was carried out at a temperature of 25 ± 2°C, light intensity of 2800 - 3000 lux [4,11] and a photoperiod of 14 hours/day. The germinated seedlings were used in further experiments.

Shoot induction and multiplication: Nodal explants at the size of 3 - 5 mm were excised from 4-week-old *in vitro* seedlings and cultured on MS medium supplemented with BAP at different concentrations (0 - 2.5 mg/l). Monitor shoot cluster quantity and shoot height after 4 weeks of cultivation. Then, isolate shoot clusters containing 3 - 4 shoots, each measuring 3 - 5 mm in height and culture them on MS medium supplemented with 0.1 mg/l NAA and BAP at different concentrations (0 - 0.5 mg/l). Monitor shoot quantity, shoot height and shoot morphology after 2 weeks of cultivation.

Induction of polyploidy[2]: Isolate shoot clusters containing 3 to 4 shoots, each measuring from 3 to 5 mm in height; these shoot clusters are the starting materials for the experiment. The shoot clusters were immersed in different concentrations of colchicine solutions (0 - 0.04%; w/v) and at treatment durations of 24, 48, 72 hours. After the treatment, the shoot clusters were washed in sterile distilled water, with 3 replications then cultured on MS medium supplemented with 0.4 mg/l BAP + 0.1 mg/l NAA for rapid shoot multiplication for 4 weeks; observe the survival rate of the explants of 4 weeks; note whether any shoots exhibit morphological variations due to induced polyploidy.

Identification of the polyploid shoots

Analysis of morphological characteristics: After treating with colchicine and culturing for 4 weeks, separate shoots or shoot clusters that exhibit morphological variations. These variations may include enlarged stems, thicker leaves and darker coloration. Transfer these selected shoots to an optimal shoot multiplication medium. Perform subculturing every 2 weeks for a total of 2 months. The monitored parameters include stem diameter, leaf color, shoot size, leaf size and internodal distance.

Stomata observations: Cut the third leaf from the top of 4-week-old polyploid shoots; a peeled section of the polyploid leaves was separated from the lower surface of the leaf. Place the leaf peel on the slide. Take a cover slip and place it under an optical microscope and observe at 400x magnification. Capture the resulting image. Do the same for the control.

Chromosomes counting [12]: Polyploid shoot apices with a length of approximately 2 - 3 mm were pretreated in a 0.05% colchicine solution for 2 hours and subsequently fixed in Carnoy's solution for 24 hours. The staining and observation of nuclear structures were conducted following the procedure described by Huynh et al[12] and this staining method has also been performed on a few subjects such as the vertex growth of onion (*Allium*)[24] and callus cells of tobacco (*Nicotiana tabacum*)[25]. Photos were taken under magnifying power of 1000x and the photos were further processed by Adobe Photoshop software. A similar process was carried out for the control explants.

Flow cytometric analysis of ploidy level: To assess DNA content, thin sections of young leaf tissue from polyploid plants were prepared in a solution containing 20 mM Tris base, 4 mM $MgCl_2$ and pH 7.2 to obtain isolated nuclei. The entire cell suspension was then passed through a cell strainer (70 mM, Cell Strainer, BD) to collect the isolated nuclei. A 0.5% (v/v) Triton X - 100 solution was added to the cell suspension for cell permeabilization. After 2 minutes incubation at room temperature, the cell suspension was divided into two groups: group I and group II.

Group I was supplemented with 0.04 µg/µL Propidium Iodide (Sigma). Subsequently, the suspension was further incubated in the dark for 15 minutes at room temperature. Finally, DNA content in each cell was determined using a Flow Cytometry device (BD Accuri C6 Plus), which included analyzing cell size (FSC) and fluorescence signal from propidium iodide (PerCP) bound to DNA. The maximum flow rate was set at 400 cells/second and a total of 50,000 cells were analyzed per explant.

Statistical analysis: The data in the result tables were statistically processed using IBM SPSS Statistics version 20.0 for Windows. Analysis of variance (ANOVA) was performed and pairwise comparisons of mean values were conducted using the LSD or Duncan test at a significance level of 5%.

Results and Discussion

Shoot induction and multiplication: In an *in vitro* shoot regeneration study, sterilized seeds were cultured on MS medium for 2 weeks. The results showed a clean culture rate of 82% and a germination rate of 92%, with uniform seedling growth. After 5 weeks of culture, the resulting seedlings exhibited stable development, healthy green coloration and had 3 - 4 pairs of leaves. Monitor the formation from stem segments (0.3 - 0.5 cm) of the seedling (5-week-old). After 4 weeks of culture in MS medium supplement with BAP at a concentration of 0 - 2.5 mg/L.

In the control treatment (C0) where MS medium was not supplemented with cytokinins, the explants exhibited normal shoot development. The green shoots had 3 - 4 pairs of leaves and reached a height of 1.7 cm, but no new shoot clusters were formed from the leaf axils. For treatments supplemented with varying concentrations of BAP ranging from 0.5 to 2.5 mg/L, the formation of new shoot clusters was observed.

Table 1
Effect of BAP on shoot proliferation from nodal shoot explants after 4 weeks of culture

Treatment	BAP (mg/l)	No. of shoot/ explant	Shoot lengths (cm)
C0	0	1.33 ± 0.33 c	1.70 ± 0.12 a
C1	0.5	3.38 ± 0.07 b	1.30 ± 0.06 b
C2	**1**	**5.40 ± 0.38 a**	**0.97 ± 0.12 cd**
C3	1.5	3.17 ± 0.35 b	1.23 ± 0.12 bc
C4	2	2.17 ± 0.26 c	0.83 ± 0.09 d
C5	2,5	1.93 ± 0.13 c	0.70 ± 0.13 d

The highest cluster formation occurred in treatment C2 (1.0 mg/l BAP), with an average of 5.4 shoots per cluster and these shoots displayed uniform sizes. However, in treatment C3 (1.5 mg/l BAP), the number of shoots decreased to 3.17 shoots per cluster, with a height of 1.23 cm.

Treatment C5 (2.5 mg/l) had the lowest shoot count at 1.93 shoots per explant, with a height of 0.7 cm. These shoots exhibited uneven growth and both stems and leaves showed abnormal swelling. Based on these results, we conclude that the medium in treatment C2 (1 mg/l BAP) facilitated shoot cluster formation, with an average of 5.4 shoots per explant. These shoots were healthy and well-developed, with an average height of 0.97 cm.

Rapidly propagate shoot clusters: In the control treatment (CX0), shoot growth increased in height and limited shoot regeneration occurred (1 - 2 shoots per explant) after 1 week of culture. In treatments supplemented with varying concentrations of benzyl adenine (BAP) ranging from 0.1 to 0.4 mg/l, the number of shoots increased progressively. Treatment CX4 (0.4 mg/l BAP and 0.1 mg/L NAA) exhibited the highest shoot count (13.77 shoots per explant), with an average height of 0.71 cm. These shoots displayed uniform shape, robust development and balanced leaf morphology. However, in treatment CX5 (0.5 mg/l BAP and 0.1 mg/l NAA), as BAP concentration increased, the shoot count decreased to 10.93 shoots per explant, with a height of 0.97 cm. These shoots had smaller leaves, abnormal stem swelling, uneven growth and a paler color.

Overall, the medium containing 0.4 mg/L BAP and 0.1 mg/L NAA (CX4) was the optimal environment for efficient shoot cluster proliferation. The multiplication factor for shoots over 1 year was calculated as $K = (3.44)^{48}$, considering a real-time duration of 1 year (48 weeks) with weekly subculturing and an initial shoot count of 4, resulting in 13.77 shoots per cluster after one subculture.

Induction of polyploid shoots: The young shoots were cultured in MS medium supplemented with colchicine at different concentrations and treatment durations. After 4 weeks of cultivation on the optimized shoot multiplication medium CX4 (containing 0.4 mg/l BAP and 0.1 mg/l NAA), the results were recorded as shown in table 3.

Table 2
Effect of BAP and NAA on rapidly propagate shoot clusters after 1 weeks of culture

Treatment	BAP (mg/l)	NAA (mg/l)	After 1 weeks	
			No. of shoot/ explant	Shoot lengths in cm
CX0	0	0	5.00 ± 0.58 e	1.60 ± 0.04 a
CX1	0.10	0.1	7.67 ± 0.41 d	1.07 ± 0.09 b
CX2	0.20	0.1	8.73 ± 0.15 cd	0.97 ± 0.07 b
CX3	0.30	0.1	10.33 ± 0.43 bc	0.87 ± 0.12 bc
CX4	**0.40**	**0.1**	**13.77 ± 1.25 a**	**0.71 ± 0.04 cd**
CX5	0.50	0.1	10.93 ± 0.64 b	0.63 ± 0.03 d

Table 3
Effect of colchicine concentration and duration on *in vitro* shoot polyploid induction

Treatment	Duration (hours)	Colchicine concentration (%)	Explant regeneration rate (%)	Polyploid explant rate (%)	No. of regenerated shoots have different morphologies
L0	24	0	100.00 ± 0.00 a	0.00 ± 0.00 d	0.00 ± 0.00 f
L1		0.01	89.00 ± 0,03 ab	7.33 ± 0,04 c	1.60 ± 0.10 c
L2		0.02	86.00 ± 0.03 b	12.33 ± 0.07 c	1.93 ± 0.09 b
L3		0.03	77.33 ± 0.02 bc	14.00 ± 0.00 c	1.33 ± 0.09 d
L4		0.04	52.67 ± 0.03 de	20.00 ± 0.12 bc	1.10 ± 0.10 e
L01	48	0	100.00 ± 0.00 a	0.00 ± 0.04 d	0.00 ± 0.00 f
L5		0.01	71.67 ± 0.06 c	32.67 ± 0.05 ab	2.00 ± 0.12 b
L6		**0.02**	**58.33 ± 0.08 d**	**40.00 ± 0.12 a**	**2.33 ± 0.09 a**
L7		0.03	44.33 ± 0.06 e	3.33 ± 0.05 ab	1.57 ± 0.07 c
L8		0.04	16.33 ± 0.10 f	0 ± 0.00 d	0 ± 0.00 f
L02	72	0	100.00 ± 0.00 a	0.00 ± 0.00 d	0.00 ± 0.00 f
L9		0.01	16.67 ± 0.05 f	0.00 ± 0.00 d	0.00 ± 0.00 f
L10		0.02	0.00 ± 0.00 g	0.00 ± 0.00 d	0.00 ± 0.00 f
L11		0.03	0.00 ± 0.00 g	0.00 ± 0.00 d	0.00 ± 0.00 f
L12		0.04	0.00 ± 0.00 g	0.00 ± 0.00 d	0.00 ± 0.00 f

From the obtained results, it is observed that as the colchicine concentration increases from 0.01% to 0.04%, the rate of shoot regeneration gradually decreases. Within 24 hours, treatment L1 (0.01%; 24 hours) exhibited the highest shoot regeneration rate at 89%, decreasing to 52.67% in treatment L4 (0.04%; 24 hours). When explants were treated for 48 hours, the trend of decreasing shoot regeneration was like observed at 24 hours as colchicine concentration increased. Meanwhile, extending the treatment time from 24 to 48 hours resulted in an increasing trend in shoot induction rate, with the highest rate observed in treatment L6 (0.02%; 48 hours) at 40%, yielding morphologically distinct shoots at 2.33 shoots per explant. In treatment L7 (0.03%; 48 hours), the shoot count decreased to 1.57 shoots per explant.

In general, the colchicine concentration significantly influences shoot induction. As the concentration increases, the explant's regenerative capacity decreases, while the colchicine induction rate rises, resulting in morphologically distinct shoots. However, excessively high colchicine concentrations beyond the cell's induction capacity can lead to reverse inhibition, disrupting cell division processes and causing imbalances in physiological function. Treatment L6 (0.02%; 48 hours) exhibited a 58.33% reduction in explant regeneration rate and a 40% increase in polyploid induction rate. The recorded number of shoots with polyploid induction was 2.33 shoots per explant.

Morphological differences between control shoots and polyploid shoots: The shoot clusters obtained after treatment with colchicine were cultured in the optimized shoot multiplication medium (MS + 0.4 mg/l BAP + 0.1 mg/l NAA). After every 3 weeks of cultivation, newly regenerated shoots were separated, exhibiting morphological characteristics similar to the expected polyploid plants such as thick stems, large shoot size, dark-colored leaves and increased leaf thickness. Following 2 weeks of cultivation, the results for polyploid mutant shoots and control shoots on the MS medium were recorded as shown in table 4.

In some polyploid species, similar results were also recorded: the number of leaves, number of branches, plant height and stem length increased such as the white orchid (*Bauhinia acuminate*)[3] and the tobacco (*Nicotiana alata*) [8]. On the other hand, there is a change in leaf color for some polyploid plants such as marigold (*Tagates erecta*) [23], chaste tree (*Vitex agnus-castus*)[1].

Size and density of stomata: It was observed that control shoots had a lower stomatal count per observation area (14.17 stomata), sparse density and smaller stomatal size (7.8 µm). In contrast, polyploid mutant shoots exhibited a higher stomatal count (26.83 stomata), denser distribution and larger stomatal size (12.5 µm) as recorded in table 5. This result was similar with some polyploid plants because stomatal cells and pollen cells exhibit an increase in size, stomatal cells are larger[20]. However, their frequency per unit area is lower compared to diploid plants. This phenomenon has been observed in some typical polyploid plants such as feverfew (*Tanacetum parthenium*) [18] and petunia (*Petunia hybrida*)[10].

The preliminary assessment method based on stomatal characteristics is considered effective in determining polyploidy. However, there is still a limitation. Besides the large sized stomatal cells, there are still a few smaller sized stomatal cells similar to the stomatal cells in control plants. These may be the mosaic polyploidy.

Table 4
Morphology between control shoots and polyploid shoots

Parts of plants	Characteristics	Control plants	Polyploid plants
Roots	No. of Root	2.00 ± 0.57	5.33 ± 0.88
	Length of roots (cm)	0.80 ± 0.05	1.05 ± 0.05
	Describe	Thin roots	Roots are long, thick and have secondary roots
Leaf	Describe	Light green leaves, few leaves in each stem node, leaves grow symmetrically	Dark green leaves, thick leaf blades, many leaves and alternate leaves
Stem	Distance between segments (cm)	0.43 ± 0.04	1.03 ± 0.29
	Stem diameter (cm)	< 0.1	0.1 - 0.15
	Describe	Dark green stem	Light green stem

Table 5
Effect of colchicine on stomata number and size per unit area of control and polyploid plant in 400X magnification.

	Stomata number per unit area	Average size of stomata per unit area (µm)
Control	14.17 ± 0.73	7.8 ± 0.24
Polyploid	26.83 ± 2.17	12.5 ± 0.45

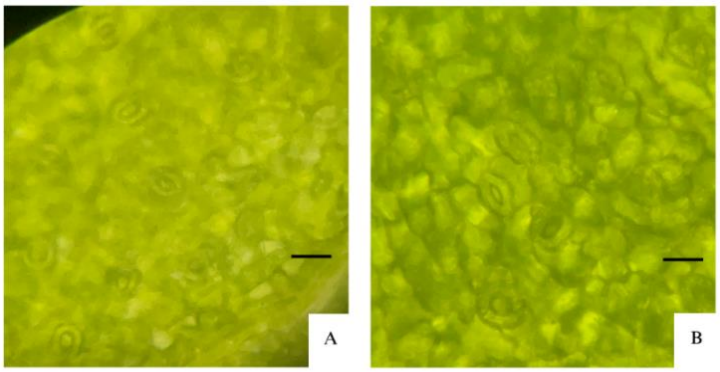


Figure 1: Stomata of leaves diploid (A) and polyploid (B) plants (400X)
Note: The length of the bar is 10 µm.

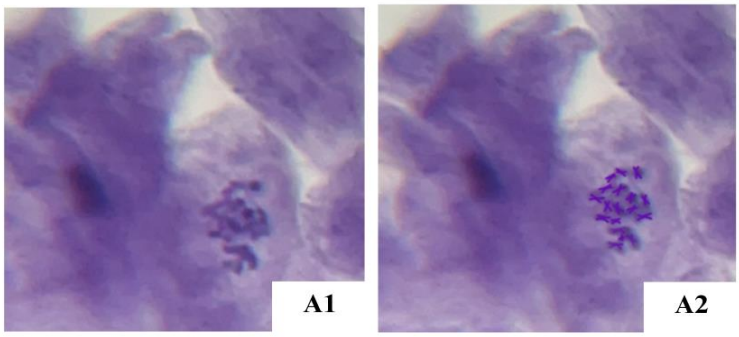


Figure 2: Chromosome number in diploid plant (2n = 18); (A1) Image before processing by photoshop; (A2) Image after processing by photoshop.

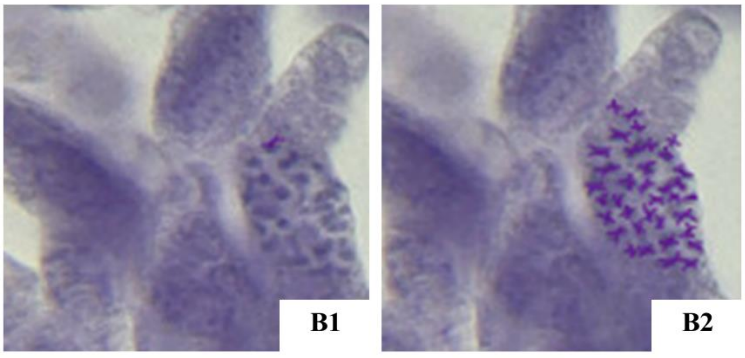


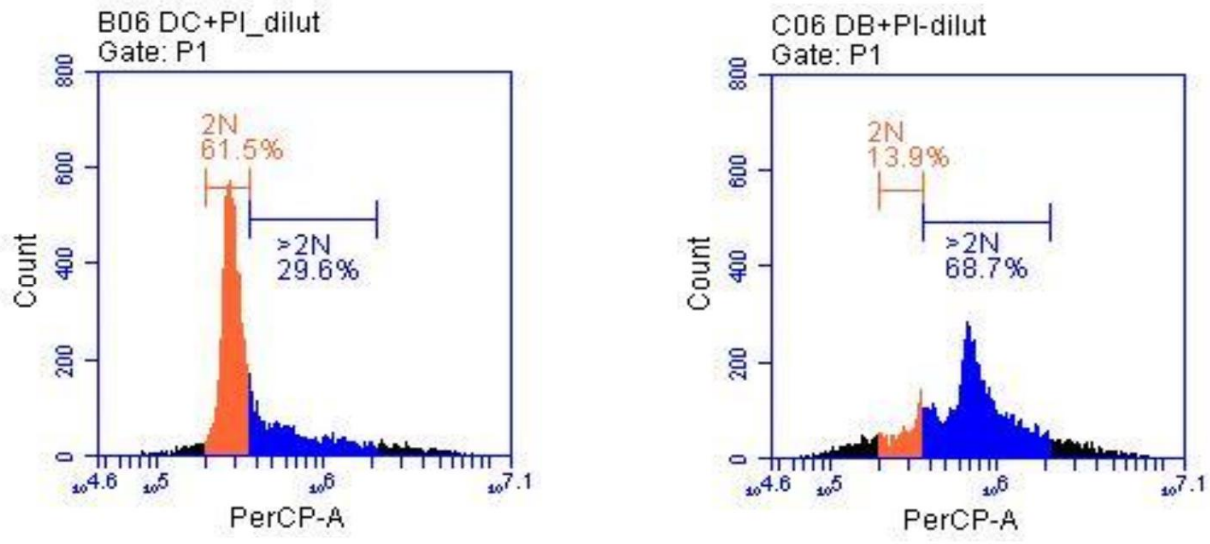
Figure 3: Chromosome number in tetraploid plant (4n = 36); (B1) Image before processing by photoshop; (B2) Image after processing by photoshop.

Chromosome number determination: To determine whether there are changes within the chromosomes of polyploid shoots, we collected apical shoots from both polyploid mutant and control shoots to count the number of chromosomes per cell. The results, as recorded in figure 2 and figure 3, polyploid plants (4n = 36) have more chromosomes than diploid plants (2n = 18). The mutant cells exhibited larger, more intensely stained nuclei under the influence of colchicine. Colchicine acts as an inhibitor of spindle formation during cell division, preventing chromosomes from segregating into the two poles of the cell, resulting in cells with an increased number of chromosomes double or more than normal cells. In some research, similar results have been found: chromosomes exist in the same cell and double the number of chromosomes [9,19] and had similar results with induction of polyploidy in Periwinkle (*Catharanthus roseus* (L.) G. Don)[12].

Table 6
Percentage of diploid (2n) and polyploid (> 2n) cells in the analyzed sample population

	Diploid (2n)	Polyploid (> 2n)
2n	61.46%	13.91%
> 2n	29.61%	68.74%

Flow Cytometric Analysis of Ploidy Level: To accurately determine the polyploid level of the polyploid shoot lines, we collected the shoots (after 3 weeks of culture) for flow cytometry analysis. The results are recorded in table 6 and figure 4.



(A) **(B)**

(A): Control shoot (B): Polyploid shoot

Figure 4: Flow cytometric analysis of *Platycodon grandiflorus* (A) 2n and (B) 4n.

From figure 4 and table 6, the control shoot (2n) has a "peak" at the 500,000 line as compared to the polyploid shoot, which has a "peak" at the 1,000,000 line B corresponding to the ploidy level 4n (B). This result shows that the polyploid shoot has a large morphology, the chromosome (4n=36) has doubled the DNA content compared to the control. However, in polyploid shoot, there were 68.74% cells > 2n cells and 13.91% of 2n cells. With this data, we noted that the polyploid shoot samples were still mixed with a few 2n cells, not completely isolated.

Conclusion

The suitable medium for shoot cluster formation from nodal segments of *Platycodon grandiflorus* is MS + 1 mg/l BAP. The optimal medium for multiple shoot induction consists of MS + 0.4 mg/l BAP + 0.1 mg/l NAA. *In vitro* polyploid induction in *Platycodon grandiflorus* with colchicine at a concentration of 0.02% for 48 hours resulted in the formation of shoots with the most optimal polyploid. Compared to the control shoots, polyploid shoots exhibit the following characteristics: increased stomatal density per unit area and larger stomatal cell size, a chromosome number greater than 18, with each cell containing 36 chromosomes and cells with doubled DNA content.

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