



## Article

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## MICROPROPAGATION AND CELLULAR BEHAVIOUR CHANGES DURING *in vitro* FLOWERING OF *Impatiens balsamina*

*Micropropagação e Mudanças de Comportamento das Células Durante o Florescimento in vitro de Impatiens balsamina*

**ABSTRACT** - In this study, the micropropagation of *Impatiens balsamina* was established from stem and shoot explants. The effects of GA<sub>3</sub> and glutathione on the morphogenesis of this species were also investigated, in order to induce *in vitro* flowering. It was found that the optimum *in vitro* plant regeneration was achieved on MS medium supplemented with 1.0 mg L<sup>-1</sup> GA<sub>3</sub> and *in vitro* flowering was also obtained from the same medium after 4 weeks of culture. To understand cellular behavior during *in vitro* flowering, Mitotic Index (MI), chromosome counts, measurement of mean cell and nuclear areas, DNA measurements and ploidy levels were analyzed from *in vivo* plants, *in vitro* grown plants and plantlets that flowered *in vitro*. The chromosome count was the same for all, 2x=2n=14 or n=7. However, it was observed that *in vitro* flowering plants of *Impatiens balsamina* had the highest percentage of polyploid cells (30.7%), based on a histogram plotted by the AxioVision 4.7 software. It was found that plant growth regulators, especially GA<sub>3</sub>, increased the polyploidy level of the meristematic root cells.

**Keywords:** regeneration, gibberellic acid, glutathione, flowering response, cellular behavior and acclimatization.

**RESUMO** - Neste estudo, a micropropagação de *Impatiens balsamina* foi estabelecida a partir de explantes de caule e parte aérea. Os efeitos do GA<sub>3</sub> e da glutatona na morfogênese dessa espécie também foram investigados, de modo a induzir o florescimento *in vitro*. Verificou-se que a melhor regeneração *in vitro* de plantas foi obtida em meio MS adicionado com 1,0 mg L<sup>-1</sup> de GA<sub>3</sub>, e o florescimento *in vitro* também foi obtido a partir do mesmo meio após quatro semanas de cultura. Para entender o comportamento das células durante o florescimento *in vitro*, o Índice Mitótico (MI), a contagem de cromossomos, a medição da área média de célula e núcleo, as medições de DNA e os níveis de ploidia foram analisados a partir de plantas *in vivo*, plantas cultivadas *in vitro* e plântulas que floresceram *in vitro*. A contagem cromossômica foi a mesma para todos: 2x=2n=14 ou n=7. No entanto, observou-se que as plantas de *Impatiens balsamina* com florescimento *in vitro* apresentaram a maior porcentagem de células poliploides (30,7%), com base em um histograma executado pelo software AxioVision 4.7. Verificou-se que os reguladores de crescimento das plantas, especialmente o GA<sub>3</sub>, aumentaram o nível de poliploidia das células de raiz meristemáticas.

**Palavras-chave:** regeneração, ácido giberélico, glutatona, resposta de florescimento, comportamento das células e aclimação.

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## INTRODUCTION

*Impatiens balsamina* L. belongs to the Balsaminaceae family and is a member of the *Impatiens* genus. *Impatiens* comprises more than 1000 species and is one of the largest genera of flowering plants (Janssens et al., 2006). *Impatiens balsamina*, known as 'garden balsam', is one of Malaysia's traditional medicinal plants (Ong et al., 2011). It has also been cultivated as an ornamental plant in many parts of the world. The flower of *Impatiens balsamina* is an ideal model for analyzing the flowering process as it has an absolute requirement for short day conditions in order to flower, and flower bud reversion can be obtained in a predictable way after changing to long day conditions (Pouteau et al., 1998).

Flowering is a complex process regulated by both internal plant factors and environmental signals. A mature plant only flowers when there is a genetic response to the required environmental conditions, including photoperiod (Tisserat and Galetta, 1995). These conditions can often be altered, so that the plant can be induced to undergo an early reproductive phase. *In vitro* flowering serves as an important tool for studying flower induction, initiation and the floral developmental process by using plant growth regulators such as cytokinins, gibberellins and auxins (Ziv and Naor, 2006).

*Impatiens balsamina* was cultured *in vitro* to study *in vitro* morphogenesis and the effect of various hormones on plant regeneration, in an attempt to achieve the efficient mass propagation of this species. Cellular behavior studies, such as the measurement of mean cell and nuclear areas, Mitotic Index (MI) and ploidy level, can also be very informative, for example to differentiate embryogenic and non embryogenic calli (Moghaddam and Taha, 2005). Consequently, in this research, cellular behavior parameters such as MI, chromosome counts, mean cell and nuclear areas and their ratios, were also studied from *in vitro* and greenhouse grown (*in vivo*) plants, in order to determine if somaclonal variations occurred during the culture protocols. Although the *in vitro* flowering of this species has been previously reported (Xiang and Wang, 2005), changes in cellular behavior during *in vitro* flowering have never been reported before. Therefore, the goals of this study were to establish an efficient regeneration system for this species, to compare cellular behaviors in root meristem cells from *in vivo* and *in vitro* grown plants, to detect whether any somaclonal variation occurred during tissue culture protocols and finally, to observe the changes in cellular behavior parameters during the *in vitro* flowering of this ornamental plant.

## MATERIALS AND METHODS

### Effects of plant growth regulators on *in vitro* studies of *I. balsamina*

*Impatiens balsamina* seeds were obtained from nurseries around Kuala Lumpur, Malaysia, and were sterilized by standard tissue culture protocols (Taha and Tijan, 2002). *Impatiens balsamina* seeds were collected and washed carefully under running water, subsequently treated with Dettol antiseptic liquid (Reckitt Benckiser, Malaysia) and two drops of Tween 20 for 10 minutes, and then rinsed three times in distilled water. The seeds were surface sterilized with a series of sodium hypochlorite solution concentrations (70%, 50% and 30%) for 5 minutes each, and rinsed thoroughly with distilled water. Finally, the *Impatiens balsamina* seeds were treated with 70% ethanol for 1 minute, rinsed with sterile distilled water one more time in a laminar airflow cabinet and then, blotted with sterile tissue paper.

Seeds were cultured aseptically on MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and solidified with 0.25% (w/v) gelrite (Duchefa Biochemie). The pH of the medium was adjusted to 5.8 by using NaOH and HCl. The media were autoclaved at 121 °C for 20 minutes after adjusting the pH. After 4 weeks, stem and shoot explants were excised from aseptically growing *I. balsamina* seedlings that were growing vigorously. Age and size of the used explants were standardized with the purpose to minimize error variation or other factors that would affect the response of the explants. The explants were cultured onto a medium supplemented with plant growth regulators for the different treatments. The used plant growth regulators were GA<sub>3</sub> at the concentrations of 0, 0.5, 1.0, 1.5 and 2.0 mg L<sup>-1</sup> and glutathione at the concentrations of 0, 0.5, 1.0, 1.5 and 2.0 mg L<sup>-1</sup>. The temperature of the culture room was maintained at 25 °C ± 1 °C with 16 hours light with 60 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux density. Each treatment was

conducted on 30 explants with one explant per jam jar. Each treatment was conducted using 30 replications. Subcultures were performed every 2 weeks to supply new and fresh nutrients under the same conditions. The results were recorded after 4 weeks.

### Acclimatization

Three-month-old plantlets of *Impatiens balsamina* obtained from the tissue culture system were transferred to a greenhouse. Initially, the plantlets were removed carefully from the culture vessels and the roots were rinsed with sterile distilled water to remove excess agar from the roots. Plantlets were then transferred into the soil in pots covered with plastic bags and acclimatized in the culture room ( $25 \pm 1$  °C) for one month. The plantlets were then transferred to the greenhouse and their ability to fully adapt to the natural conditions was examined. Plantlets were transferred to three different types of soil, namely black soil, red soil and soil mixture (black soil: red soil; 1:1) and the best transplantation methods with the highest survival rates were identified. The macromorphology of the plantlets was studied during the acclimatization process. Plant height, leaves and flower characteristics were recorded. Observations were conducted for 3 months after the acclimatization. The ability of the plantlets to survive in the natural environment was compared among different treatments, according to their respective culture procedures.

### Cellular behavior

The standard growth of the primary roots of *in vivo* and *in vitro* grown plants was measured using similar methods (Yaacob et al., 2013); only the sources of the used materials were different. Seeds were used for the induction of root growth both *in vivo* and *in vitro*. A total of 100 *Impatiens balsamina* seeds were cultured in 10 petri dishes containing sterilized wet cotton wool. The cotton wool in each petri dish was watered twice every day using sterile distilled water and the cultures were maintained under a cycle of 16 hours of light and 8 hours of dark, at  $25 \text{ °C} \pm 1 \text{ °C}$ . The germination and growth of the primary root were examined daily until most of the primary roots produced secondary roots. The primary root length was evaluated every day at a fixed time and the mean root length of each sample was recorded and plotted against time. The roots with standard age and length were then cut and soaked in fixative (alcohol and acetic acid 3:1) for one hour. Based on the standard curve, the mean root length of the sample showing the greatest growth rate before the appearance of secondary roots was verified. For the following experiments, the mean root length of the samples ( $6.79 \pm 0.45$  mm) was selected, and roots of that size were fixed and made into slides.

The slides were prepared by soaking the root segments in 5M Hydrochloric acid (HCl) for 30 minutes. Then, staining with Feulgen for one hour. Subsequently, root segments were transferred onto slides, add 1 to 2 drops of 45% (v/v) acetic acid, and heated gently by passing over a spirit lamp for a few times. Since the acetic acid dried very fast, nail varnish was used to seal the cover slips for further examination under microscope. Primary roots produced *in vitro* and obtained from plantlets flowering *in vitro* were also made into slides and analyzed as for cellular behaviors, such as MI, chromosome count, nuclear DNA content and ploidy level, mean nuclear and cell areas and their ratios. The slides were observed under an Axioskop Zeiss (Germany) microscope attached to an AxioCamMRc video camera and were then analyzed using the AxioVision 4.7 software obtained from Zeiss, Germany.

The mitotic index was measured by scoring at least 600 cells in a series of random transects across the permanent slides. Data were obtained from three replications from three slides. Meanwhile, 15 metaphase cells of suitable spread were counted from three different slides as for the chromosome number. The observed mean cell and nuclear area images were calculated automatically using the software. The used unit was  $\mu\text{m}^2$ . A total of 150 prophase cells was calculated for each sample and repeated thrice.

The nuclear DNA content and ploidy level were measured using the captured images. Images of 10 cells at anaphase or telophase stages were captured, and their DNA content and mean were calculated. Images of 10 cells at prophase stage were also captured and subjected to DNA

content measurement. This served as the reference cells, where cells at anaphase/telophase have 2C amount of DNA, while cells at prophase stage comprise 4C DNA amount, because chromosomes become thicker and the amount of DNA is doubled during prophase compared to telophase. Subsequently, images of 150 cells at interphase stage were captured from three slides and were subjected to DNA content measurement. The ploidy level of the species was then determined by integrating the optical density of the cells compared to the reference nuclei. The total optical density of the cells of interest was measured using the software. Finally, the DNA C value of each interphase cell is calculated and a histogram is established.

### Statistical data analysis

Data analysis was conducted through analysis of variance (ANOVA) with a completely randomized design. The Duncan's multiple range test (DMRT) at 5% significance level was used to compare the means. The standard errors of the means were calculated.

## RESULTS AND DISCUSSION

### Effects of plant growth regulators on *in vitro* studies of *Impatiens balsamina*

The induction of new shoots of *Impatiens balsamina* from stem and shoot explants were observed after 2 weeks on all media supplemented with PGRs. Shoot induction was successfully induced from both explants; stem and shoot. All treatments were significantly different, except for the treatment with 2.0 mg L<sup>-1</sup> glutathione. The results confirmed that 1.0 mg L<sup>-1</sup> GA<sub>3</sub> was the optimal treatment for *in vitro* regeneration. Figure 1A shows *in vitro* flowering of *I. balsamina* derived from shoot explants cultured on an MS medium fortified with 1.0 mg L<sup>-1</sup> GA<sub>3</sub> after 4 weeks of culture.

Generally speaking, the results showed that a greater number of shoots was produced in media with GA<sub>3</sub> compared with media supplemented with glutathione. The results also showed that the highest mean number of shoots per explant was  $9.00 \pm 0.06$  when shoot explants were cultured on an MS medium supplemented with 1.0 mg L<sup>-1</sup> GA<sub>3</sub> after 4 weeks (Table 1). *In vitro* flowering was obtained after 4 weeks of culture on the same media. As it may be observed in Table 2, the highest mean number of shoots per explant was  $4.40 \pm 0.10$  when stem explants were cultured on an MS medium supplemented with 1.0 mg L<sup>-1</sup> GA<sub>3</sub> for 4 weeks. This result proved that the MS medium supplemented with 1.0 mg L<sup>-1</sup> GA<sub>3</sub> was the ideal medium for shoot formation of this species.



**Figure 1** - (A) *In vitro* flowering of *I. balsamina* derived from shoot explants cultured on an MS medium fortified with 1.0 mg L<sup>-1</sup> GA<sub>3</sub> after 4 weeks of culture. (B) Acclimatization of *I. balsamina* (4 week-old). (C) Number of chromosomes of *I. balsamina* ( $14.27 \pm 0.55$ ) observed from an *in vivo* plant root meristem cell.



**Table 1** - Effects of different concentrations of plant growth regulators on *Impatiens balsamina* shoot explants after 8 weeks

MS + PGR (mg L <sup>-1</sup> )	Mean no. of shoots per explant	Mean height of plantlet (cm)	Mean no. of flowers per explant
Control	0.70±0.16 d	1.50±0.26 d	0.90±0.12 d
0.5GA <sub>3</sub>	5.00±0.05 b	2.80±0.03 bc	1.60±0.10 bc
1.0GA <sub>3</sub>	9.00±0.06 a	4.00±0.02 a	3.50±0.03 a
1.5GA <sub>3</sub>	6.20±0.03 b	2.50±0.04 c	2.00±0.04 b
2.0GA <sub>3</sub>	6.00±0.03 b	3.30±0.08 b	2.50±0.13 b
0.5Glu	1.10±0.22 c	3.00±0.13 b	n/a
1.0Glu	1.00±0.19 c	2.50±0.17 c	n/a
1.5Glu	1.20±0.31 c	2.40±0.12 c	n/a
2.0Glu	0.80±0.24 d	1.80±0.22 d	n/a

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at  $p < 0.05$ , by one-way ANOVA and Duncan's multiple range test (DMRT).

**Table 2** - Effects of different concentrations of plant growth regulators on *Impatiens balsamina* stem explants after 8 weeks

MS + Hormone (mg L <sup>-1</sup> )	Mean no. of shoots per explant	Mean height of plantlet (cm)	Mean no. of flowers per explant
Control	0.63±0.12 d	0.55±0.21 d	n/a
0.5GA <sub>3</sub>	3.80±0.15 b	2.50±0.01 ab	n/a
1.0GA <sub>3</sub>	4.40±0.10 a	2.70±0.04 a	n/a
1.5GA <sub>3</sub>	4.20±0.02 a	3.00±0.05 a	n/a
2.0GA <sub>3</sub>	3.20±0.04 b	2.00±0.06 b	n/a
0.5Glu	1.20±0.25 c	2.80±0.19 ab	n/a
1.0Glu	0.70±0.19 d	1.80±0.11 c	n/a
1.5Glu	1.30±0.23 c	2.40±0.20 ab	n/a
2.0Glu	1.10±0.24 c	2.20±0.28 b	n/a

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at  $p < 0.05$ , by one-way ANOVA and Duncan's multiple range test (DMRT).

## Acclimatization

The success of *in vitro* propagation ultimately depends on the success rate of the plantlets' transplantation to the greenhouse. In this research, complete plantlets with well-developed roots were harvested and selected for acclimatization. *Impatiens balsamina* acclimatization responses are shown in Table 3. Plantlets gave the optimum response when acclimatized in mixed soil (a combination of black soil and red soil at a 1:1 ratio). This treatment gave the highest survival rate for *Impatiens balsamina* acclimatization at  $75.0 \pm 0.6\%$  (Figure 1B). Meanwhile, plantlets acclimatized in black soil had a  $48.0 \pm 0.3\%$  survival rate. Plantlets acclimatized in red soil only showed a  $10.0 \pm 0.2\%$  survival rate. Plantlets were watered every day and observed weekly. Different environmental factors such as temperature, light, soil, humidity and others also affected the acclimatization process.

This research revealed that *in vitro* flowering plantlets regenerated from stem and shoots were successfully acclimatized in the greenhouse. All successfully acclimatized plantlets demonstrated a healthy growth in the culture room and also in the greenhouse. The macromorphology of the plantlets was studied during the acclimatization process. Plant height, leaves and flower characteristics were examined and recorded. The observations were conducted for 3 months after acclimatization. The ability of the plantlets to survive under the natural environment was compared as for the respective culture procedures used before the acclimatization. These characteristics are summarized in Table 4.

**Table 3** - Responses of *in vitro* flowering plantlets after 8 weeks of acclimatization in various sowing media

Type of growth media	Survival rate (%) of plants	Observation
Black soil+red soil (1:1)	75.0±0.6 a	Plantlets survived and showed healthy growth
Red soil	10.3±0.2 c	Plantlets survived and showed slow growth
Black soil	48.0±0.3 b	Plantlets survived and showed healthy growth

Mean ± SE, n=30. Means with different letters in the same column differ significantly at p<0.05, by one-way ANOVA and Duncan's multiple range test (DMRT).

**Table 4** - Comparison of macromorphological characters of *in vivo* and *in vitro* flowering *Impatiens balsamina* plantlets

Characteristic	<i>In vivo</i> plant (control)	<i>In vitro</i> flowering plants
Plant height (cm)	48 ± 0.1	18 ± 0.4
<b>Leaf</b>		
Arrangement	Alternate	Alternate
Type	Simple	Simple
Margin	Serrate	Serrate
Shape	Lanceolate	Linear to Lanceolate
Venation	Pinnate	Pinnate
Length (cm)	5 ± 0.62	3 ± 0.44
Color	Green	Green
No. of leaves per plant	42 ± 0.52	33 ± 0.35
<b>Flower</b>		
No. of flowers per plant	18 ± 0.42	12 ± 0.25
Symmetry	Actinomorphic	Actinomorphic
Color	Red	Red
Flower size (cm)	3 ± 0.57	3 ± 0.24

### Cellular behavior

The standard growth of *Impatiens balsamina* (*in vivo*) was studied in order to determine the growth rate of the primary roots, so as to standardize the root samples for subsequent cytological experiments. The germination and growth of the primary roots were observed and measured every day until most primary roots produced secondary roots. It took 8 days for secondary roots to start emerging. The mean primary root length of *in vivo* samples was determined 14 days after germination. In meristematic cells of *in vivo* roots, the MI value was determined to be  $36.33 \pm 1.75\%$ . The chromosome counts for *in vitro* plants, *in vitro* flowering plantlets and *in vivo* grown plants were  $14.33 \pm 0.68$ ,  $14.07 \pm 0.34$  and  $14.27 \pm 0.55$ , respectively (Table 5). It was found that the chromosome counts for *in vitro*, *in vitro* flowering and *in vivo* grown *Impatiens balsamina*, either with or without plant growth regulators ( $GA_3$ ), were similar. The mean cell and nuclear area were  $248.63 \pm 0.67 \mu m^2$  and  $47.94 \pm 0.54 \mu m^2$ , respectively (Table 6). The MI value for *in vitro* grown plantlets was  $43.0 \pm 0.78$  and the chromosome count was  $14.33 \pm 0.68$  (Table 5). The mean cell and nuclear areas for root meristem cells from *in vitro* grown plants were  $225.45 \pm 0.45 \mu m^2$  and  $49.31 \pm 0.66 \mu m^2$ , respectively, whereas their ratio was  $0.22 \pm 0.12$  (Table 6).

**Table 5** - The Mitotic Index (MI) and mean number of chromosomes from root meristem cells of *in vivo* grown plants, *in vitro* plantlets and *in vitro* flowering plantlets of *Impatiens balsamina*

Growth media	Mitotic Index, MI (%)	Mean no. of Chromosomes
MS basal ( <i>in vitro</i> )	43.0 ± 0.78	14.33 ± 0.68
MS + 1.0 mg L <sup>-1</sup> GA <sub>3</sub> ( <i>in vitro</i> flowering)	53.8 ± 1.34	14.07 ± 0.34
Soil ( <i>in vivo</i> )	36.33 ± 1.75	14.27 ± 0.55.

**Table 6** - Mean nuclear and cell areas and their ratios in root meristem cells of *in vivo*, grown plants, *in vitro* plantlets and *in vitro* flowering plantlets of *Impatiens balsamina*

<i>Impatiens balsamina</i> source	Mean areas ( $\mu\text{m}^2$ )		
	Nucleus (N)	Cell (C)	Ratio (N/C)
MS basal ( <i>in vitro</i> )	49.31 $\pm$ 0.66	225.45 $\pm$ 0.45	0.22 $\pm$ 0.12
MS + 1.0 mg L <sup>-1</sup> GA <sub>3</sub> ( <i>in vitro</i> flowering)	65.18 $\pm$ 0.49	276.35 $\pm$ 0.34	0.24 $\pm$ 0.18
<i>In vivo</i>	47.94 $\pm$ 0.54	248.63 $\pm$ 0.67	0.19 $\pm$ 0.04

In *in vitro* flowering plantlets, the MI was found to be  $53.8 \pm 1.34$ , higher than the MI value of *in vivo* and *in vitro* grown plants. Meanwhile, the chromosome number of *in vitro* flowering plantlets was  $14.07 \pm 0.34$ . There was no significant difference as for the number of chromosomes. The mean nuclear area of root meristem cells from *in vitro* flowering plantlets appeared greater at  $65.18 \pm 0.49 \mu\text{m}^2$ , whereas the mean cell area was  $276.35 \pm 0.34 \mu\text{m}^2$ , which was also greater compared to the *in vivo* and *in vitro* non-flowering samples. The mean ratio of nuclear to cell area for *in vitro* flowering plantlets was the highest compared to cells of *in vivo* grown plants and *in vitro* non-flowering plantlets, with a value of  $0.24 \pm 0.18$ . From Table 6, the ratio of nuclear area to cellular area for *Impatiens balsamina* grown *in vivo* and *in vitro* was low ( $0.19 \pm 0.04$ ,  $0.22 \pm 0.12$ , respectively) indicating that the size of the nucleus was quite small as compared to the cellular area. The ratio of nuclear area to cellular area was higher in *in vitro* flowering *Impatiens balsamina* ( $0.24 \pm 0.18$ ). However, the ratios for *in vitro* flowering plantlets and *in vitro* non-flowering plantlets were not significantly different.

The nuclear DNA content of root meristem cells from *in vivo* grown *Impatiens balsamina* was measured from 150 interphase cells and the DNA C values were used to estimate G1, S, and G2 phases of the cell cycle. DNA C values from 0.0-2.2 C are considered as G1, while 2.2-3.6 C are considered as S, 3.6-4.8 C as G2 and > 4.8 C as polyploid cells (Evans et al., 1981). The percentage of nuclei in various phases of the cell cycle and polyploid cells from *in vivo* grown plants, *in vitro* plantlets and *in vitro* flowering plantlets of *Impatiens balsamina* are shown in Table 7. From the results, it may be suggested that the nuclei were scattered from G1 phase to G2 phase in all the growth types. There were no nuclei in polyploid phase in *in vivo* plants (Figure 2A). However, *in vitro* plantlets of *Impatiens balsamina* had a different ploidy distribution (Figure 2B).

As shown in Table 7, the cell percentage in the G1 phase was 25%, in the S phase it was 40.7% and in the G2 phase it was 20.3%. Meanwhile, there were 14% polyploid cells in *in vitro* plantlets compared with cells of *in vitro* flowering plantlets that had the most polyploid cells (30.7%). For *in vitro* flowering plantlets, the lowest percentage of observed cells was estimated to be in the G1 phase (14%), while the percentage in the S phase it was 21.3 % and in the G2 phase 34% (Figure 2C). More polyploid cells were found in root meristem cells of *in vitro* flowering plantlets than in non-flowering *in vitro* plantlets or in *in vivo* plants. Generally, supplementing the growth media with plant growth regulators such as GA<sub>3</sub> increased the polyploidy level in all the growth types.

Generally speaking, this species was very responsive in the tissue culture system. Shoots were successfully induced from both types of explants. *In vitro* flowering was achieved when shoot explants were cultured on an MS medium supplemented with GA<sub>3</sub> for 4 weeks. Greenhouse grown or *in vivo* plants, *in vitro* plantlets and *in vitro* flowering plants were further used for cellular

**Table 7** - Percentage of nuclei from root tip meristem cells of *in vivo* grown plants, *in vitro* plantlets and *in vitro* flowering plantlets of *Impatiens balsamina* in various cell cycle stages

<i>Impatiens balsamina</i> source	Cell cycle phase (%)			Polyploidy (%)
	G1	S	G2	
MS basal ( <i>in vitro</i> )	25	40.7	20.3	14
MS + 1.0 mg L <sup>-1</sup> GA <sub>3</sub> ( <i>in vitro</i> flowering)	14	21.3	34.0	30.7
<i>In vivo</i>	74	20.6	5.4	0

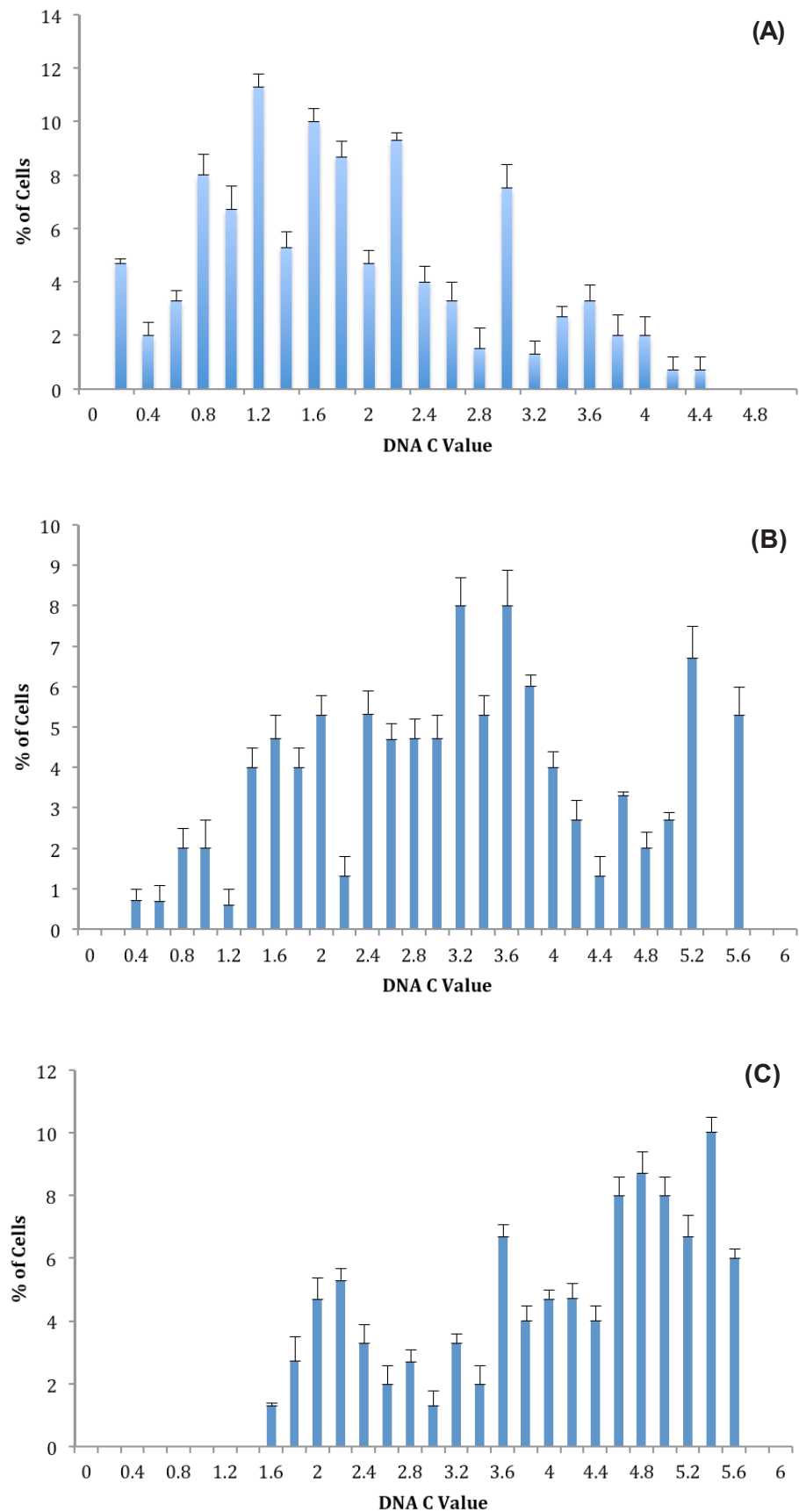


Figure 2 - (A) Histograms showing the distribution of nuclei with DNA C values of cells from root tip meristems of *in vivo* grown *Impatiens balsamina* (B) *in vitro* plantlets of *I. balsamina* (C) *in vitro* flowering plantlets of *I. balsamina*.



behavior studies, in order to understand the changes that occur during regeneration and *in vitro* flowering. The acclimatization of *in vitro Impatiens balsamina* plantlets to the greenhouse was also successfully accomplished. The acclimatization methods of *Impatiens balsamina* followed the methods reported by (Taha and Tijan, 2002).

Most *in vitro* grown species require an acclimatization process to ensure the survival of plantlets (Hazarika, 2003). Therefore, after *in vitro* transplantation, plantlets usually need some weeks of acclimatization with a gradual decrease in air humidity (Bolar et al., 1998). If the transition is gradual, then plantlet survival will increase when they are transferred to a lower relative humidity environment (Smith et al., 1990). A similar acclimatization procedure was also reported in different plant species such as *Eruca sativa* (Sharma et al., 2012).

Two weeks after being transferred to the greenhouse, all the acclimatized plants started to grow normally, like plants grown in the conventional method. After 4 weeks, *Impatiens balsamina*, started to become firmer, healthier and stronger. The leaves also began to grow wider and the leaf color was darker compared to *in vitro* cultured plants in jar. Previous studies have stated that the growth of tissue-cultured plants may seem slow during the initial weeks after transplanting. This is due to the residual presence of exogenous growth regulators to which they were exposed during micropropagation (Perez and Hooks, 2008).

Consequently, cellular behavior parameters in all treatments were analyzed. The standard growth of *Impatiens balsamina (in vivo)* was studied to determine the growth rate of the primary roots, in order to standardize the root samples for subsequent cytological experiments. *Impatiens* is known to have species with different basic chromosome numbers, for example *I. biflora* with  $2n=14$  (Wulff, 1936) and  $2n=10$  (Chinnappa and Gill, 1974). The chromosome number of *Impatiens balsamina* is  $2n=14$ , which has been proven by Khoshoo (1957). This finding, which showed that *Impatiens balsamina* has chromosome number of  $2n=14$ , has also been supported by Raghuvansi and Mahajan (1982). The chromosomes observed in *Impatiens balsamina* were very small and difficult to count. Whitaker and Davis (1962) also indicated that the small size of the mitotic chromosomes in *Cucurbita* makes them troublesome to count accurately, even though they tend to be well separated.

Plant growth regulators such as  $GA_3$  influenced the MI value of *Impatiens balsamina*. The high MI values of *I. balsamina* indicated that the meristematic cells were actively dividing and had a high regeneration potential. In contrast, lower MI values in *in vitro* grown *Vicia faba* root meristem cells compared to *in vivo* grown plants were also reported (Taha and Francis, 1990). Low MI values in *in vitro* grown *Justicia betonica* were also observed (Yaacob et al., 2013). The transfer from *in vivo* to *in vitro* conditions can also affect the genetic constitution of the tissues (Swartz, 1991). Therefore, the MI values were higher in *in vitro* grown plants and in *in vitro* flowering plantlets.

Phase changes from vegetative to flowering are usually accompanied by changes in cellular activities (Gifford and Nistch, 1969), which includes an increase in the DNA synthesis, mitotic activities and cell sizes. The study of *in vitro* flowering is usually useful for breeding improvement programs, to understand the physiology underlying flowering processes and also to develop biochemical and molecular approaches to understand the transition from the juvenile to the mature state of plant development. *Vicia faba*, which was quite difficult to regenerate, appeared to have a very high occurrence of polyploidy compared to *Petunia hybrida* (Taha and Francis, 1990). It was observed that most of the cells were in the S and G2 phase of the cell cycle during flower initiation, whereas in non-flowering plantlets (*in vivo*), most of the cells were arrested in G1 phase of the cycle.

Both mean cell and nuclear areas increased significantly in *in vitro* flowering and *in vitro* non-flowering plantlets. This finding is in contrast with data from a study on *C. cristata*, which had lower mean cell areas but higher nuclear areas in *in vitro* grown plantlets (Taha and Wafa, 2012). The morphogenetic development of the explants has resulted from the interaction of the following factors: existing genetic information in each explant, hormonal balance in nutritive media, and cultivation conditions (Nicuta et al., 2003). This could be due to the use of plant growth regulators, specifically the use of  $GA_3$ , which promotes cell division and cell elongation. Plant growth regulators have been stated to affect the mitotic index of a species. For instance,

the MI values of parenchyma cells of tobacco pith increased after 6 days of culture when supplemented with IAA and kinetin (Das et al., 1957). Extensive studies had been conducted on nuclear and cell sizes of higher plants (Thomas and Davidson, 1983), although very few published work was found for tissue culture-derived plants.

Cytological studies are mainly conducted to evaluate and determine DNA content, chromosome count, genetic stability and cell cycle (Gould, 1984). The transfer from *in vivo* to *in vitro* conditions could result in cellular behavior changes (Armstrong and Francis, 1987). According to Bayliss (1985), plants originated from tissue culture usually have a longer cell cycle, which in turn may cause some changes in DNA content as well as cell and nuclear sizes, due to the transfer from *in vivo* to *in vitro* growth conditions.

*Impatiens balsamina* has gained popularity in the past few years in many countries of the world and there is a great demand in the pharmaceutical, floriculture and medical industries. This study about the *in vitro* regeneration of *Impatiens balsamina* has widened the interest in exploring alternative methods to propagate this commercial ornamental plant. The findings of the present work in the areas of *in vitro* regeneration, *in vitro* flowering, acclimatization and cellular behavior studies will definitely provide new information and knowledge contributing to the production of *Impatiens balsamina* through various methods which could be spread and applied to various fields such as horticulture, floriculture and others. Studies regarding the cellular behavior analysis during *in vitro* flowering will be beneficial to understand the *in vitro* flowering process and for breeding programs, especially to produce early flowering cultivars for this species.

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