Chromosome Number Increasing in Torenia Hybrid by Application of Colchicine Tablet

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Abstract

The effects of colchicine tablets on Torenia hybrids (*Torenia fournieri* x *Torenia asiatica*) were studied. Nodes and leaf sections were cut and soaked in 15 ppm concentration of colchicine solution for 0, 12, 24, 48 and 72 hours. The highest frequency of polyploid induction was 25% at the soaking duration of 48 hours. The results showed that the plants regenerated from the colchicine-treated explants displayed some of the morphological characteristics of polyploids, such as thick stems and shorter plant height.

Keywords: tetraploid; polyploid; ornamental; mutation

1. Introduction

Torenia species are known by the common names of *Torenia*, Blue Wings and Wishbone Flower because two of the stamens curve inwards to join at the center, resembling a “wish bone,” i.e. the furculum of poultry. *Torenia* is a small bush or herbaceous creeper, with single leaves, spirally or oppositely arranged, leaf margins entire or finely toothed. The flowers are hermaphrodite, bilaterally symmetrical, in the shape of an open pouch with 2 side petal lobes and upper and lower petal lobes. There are 4 stamens, 2 long and 2 short. The bicarpellary ovary is superior,

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situated on a receptacle, with one stigma. *Torenia* has become popular as an ornamental for flower beds and landscaping. It can be used as a potted plant, in beds and in borders. It is especially well suited to partly shady areas with diffuse light and is a star performer for hanging baskets (Starman, 2005). Nineteen species of *Torenia* are found in Thailand (Yamazaki, 1985), and worldwide the genus is composed of about 40 species, most of which are native to tropical and subtropical Asia (Aida, 2000). The plant thus grows and flowers well in Thailand and other warm areas. It can be propagated through several methods including stem cuttings, leaf cuttings and by seed. In the right conditions, *Torenia* flowers profusely with beautifully colored flowers. Some species are annual and must be replanted after flowering, but others are perennial and will continue flowering for many seasons with no need for replanting. At present there is still not enough genetic variability in *Torenia*, because ornamental plant consumers demand new and different varieties constantly. Increasing the chromosome number is one way of creating greater genetic diversity in ornamental plant breeding because polyploids often have different characteristics such as larger flowers, darker colored and thicker leaves, shorter internodes, or thicker stems. One cost effective way of inducing polyploidy in plants is to use colchicine. Colchicine is an alkaloid obtained from the root of *Colchicum autumnale* L., a wild plant native to the Mediterranean region. The bulbs and seeds of Colchicum contain 20 kinds of alkaloids, the main one of which is colchicine. In its isolated form, colchicine takes the form of light yellow needle-shaped crystals. The chemical formula of purified colchicine is $C_{22}H_{25}NO_6$. It is easily soluble in alcohol, chloroform and cool water. Medicinally, it is used to treat gout (Popovice and Gasic, 1993). Today, besides *Colchicum autumnale* L., colchicine can also be extracted from other species in the genus Colchicum, as well as species in the genus Merendera and Gloriosa, such as *Gloriosa superba*, a member of the Liliaceae family. Colchicine can be used to produce polyploid plants because it binds with tubulin and thus interferes with microtubule formation during mitosis. However, pure colchicine can be harmful to humans, causing serious inflammation, and in large amounts it can even be lethal. When used in the laboratory, great caution must be exercised, and the substance must be stored in a secure location. As with many other hazardous chemicals, pure colchicine is not easy to obtain and is expensive. For these reasons, we used colchicine from gout medication tablets (trademark name “Colchicine”) for greater convenience and safety.

In this research we investigated the most suitable duration for soaking Torenia hybrid leaf and node explants in colchicine solution under aseptic conditions, and studied the morphological characteristics of the new plants derived from colchicine-treated explants.

2. Materials and Methods

2.1 Plant material

Diploid hybrid (*Torenia fournieri* x *Torenia asiatica*) lines with purple flowers and a semi-erect, semi-recumbent habit were the subject for this study. The plants were maintained in a greenhouse at a day temperature of 33-35 °C and 60-65 % relative humidity and a night temperature of 29-33 °C and 65-70 % relative humidity.

2.2 Colchicine and tissue culture media

Colchicine was obtained from Colchicine brand oral gout medication (1 tablet contains 0.6 mg colchicine, lactose, magnesium, stearate and starch) purchased over the counter from a local pharmacy. The tissue culture medium was modified MS medium (Murashige and Skoog, 1962) with half the normal content of macronutrients.

2.3 Culture establishment and growth

For the experiment, cuttings were taken from healthy, disease-free Torenia plants. The cuttings were washed in tap water and all leaves were removed, leaving only the apical bud and axillary buds. Next, the cuttings were disinfected in 70 % ethanol for 1 minute, then swished in 10 % Clorox solution with a few drops of Tween 20 detergent for 10 minutes, followed by 5 % Clorox solution with a few drops of detergent for 5 minutes. The containers were agitated to allow the Clorox solution to contact all parts of the cuttings. After that the cuttings were rinsed 3 times in autoclaved water in a laminar flow hood. Plant parts damaged by the surface sterilization chemicals were cut off and the remaining buds were cultured in semi-solid ½ MS medium (Murashige and Skoog, 1962). The culture jars were kept in a temperature-controlled laboratory at 25° C with a 16-hour photoperiod. They were subcultured once per month until there was enough *in vitro* plant material for the next part of the experiment (Chanchula, 2015).

2.4 Polyploid induction

Boonbongkarn (2013) reported that the most effective colchicine treatment for inducing polyploidy in Torenia was 15 ppm colchicine for 3 days, so for this research we decided to use the concentration of 15 ppm colchicines in ½ MS medium and vary the exposure time from 0 to 12, 24, 48 and 72 hours. The explants were nodes and leaves from one-month-old *in vitro* Torenia plants. After the colchicine treatment, they were cultured in semi-solid ½ MS medium as above for 2 culture cycles, or 60 days. The survival rate, number of new shoots, number of roots, root length, plant height, and internode length were recorded.

2.5 Selection of putative polyploids

Putative polyploidy plants began to display morphological changes at about 30 days after colchicine treatment, such as thicker stems, slower growth and darker green leaves (Boonbongkarn, 2013).

2.6 Experimental design

The experiment was planned as a Completely Randomized Design, or CRD, with 20 replications per treatment. The treatments were 15 ppm colchicine for 0, 12, 24, 48 and 72 hours. Each replication consisted of one *Torenia* node section and one *Torenia* leaf

section.

2.7 Preparation of colchicine solution from medicine tablets

The colchicine tablets used contained 0.6 mg colchicine per tablet. We wanted to use a concentration of 15 ppm colchicine, so we calculated thus: 15 ppm (ppm = mg/L) is 15 mg colchicine in 1000 ml solvent. Starting with a concentration of 0.6 mg, we had to add (1000/15) x 0.6 = 40 ml solvent So, the 15 ppm colchicine solution consisted of 0.6 mg colchicine (1 tablet) dissolved in 40 ml solvent.

The solvent was autoclaved distilled water. For the control group, the explants were soaked for the same number of hours in plain distilled water.

3. Results

3.1 Survival rate

Following colchicine treatment (15 ppm colchicine for 0, 12, 24, 48 and 72 hours), the nodes and leaves from one-month-old *in vitro* Torenia plants were cultured in semi-solid ½ MS medium at 25 °C, 60 % RH with a 16-hour photoperiod for 60 days. Looking at the data from the control, 12- hour, 24-hour and 48-hour treatments, it appeared that survival rate tended to decrease with increasing duration of exposure time to colchicine. However, conversely, this did not hold true for the highest concentration, and the survival rate for the 72-hour treatment was 100% for both node sections and leaf sections. For many of the treatment groups, leaf sections tended to have a higher survival rate than node sections, but this was not the case for the control group and the 72-hour group (Table 1). Fluctuation in survival rate obtained in the present study may be due to a small numbers of replications and different performance of explants (including, physiological stages of development, vigor etc.). (Kerdsuwan, 2012)

Table 1 Survival rate (%) of *in vitro* Torenia leaf and node sections 60 days after colchicine treatment

Colchicine concentration	Duration (hours)	Survival rate (%)	
		Node	Leaf
15 ppm	0	100.00	65.00
	12	20.00	70.00
	24	20.00	70.00
	48	10.00	0.00
	72	100.00	100.00

3.2 Growth rate

3.2.1 Plant height and internode length

The mean height of plantlets derived from node and leaf sections from all durations of colchicine treatment was less than the control, with the exception of the plantlets generated from leaf sections from the 24-hour treatment group. As for the mean internode length, for the plantlets generated from leaf sections, those from the 4 colchicine-treated groups were longer than the control, but for the plantlets generated from node sections, the internode length was greater than the control for the 24-hour and 72-hour treatment groups

but less than the control for the 12-hour and 48-hour treatment groups (Table 2 and 3). Overall, plantlets regenerated from node sections were taller and had longer internodes than those regenerated from leaf sections, with the exception of the plantlets generated from leaf sections from the 24-hour treatment group. In general, we can conclude that colchicine has an effect on plant height and internode length, and the stems of colchicine-treated plantlets tended to be thicker (Figure 1 and 2).

Table 2 Plant height and internode length of new shoots generated from *in vitro* Torenia node sections, 60 days after colchicine treatment

Colchicine concentration	Duration (hours)	Height (cm)	Internode length (cm)
15 ppm	0	5.01±1.67 a[1/]	1.26±0.42 b
	12	3.45±2.26 b	1.07±0.76 b
	24	3.87±2.26 ab	1.46±0.85 ab
	48	3.53±2.17 b	1.09±0.74 b
	72	4.49±1.26 ab	1.87±0.47 a
F-test		*	*
C.V. (%)		48.28	49.53

[1/]values in the same column followed by the same letter do not differ to a statistically significant degree when compared using Duncan's Multiple Range Test; *statistically significant difference at 95 % confidence

Table 3 Plant height and internode length of new shoots generated from *in vitro* Torenia leaf sections, 60 days after colchicine treatment

Colchicine concentration	Duration (hours)	Height (cm)	Internode length (cm)
15 ppm	3.39±2.74 ab[1/]	0.70±0.62 b	3.39±2.74 ab[1/]
	3.00±2.73 ab	0.95±0.94 b	3.00±2.73 ab
	4.11±2.83 a	1.58±1.18 a	4.11±2.83 a
	2.28±2.07 b	0.86±0.84 b	2.28±2.07 b
	2.62±1.32 ab	1.20±0.43 ab	2.62±1.32 ab
F-test	*	*	*
C.V. (%)	78.23	79.85	78.23

[1/]values in the same column followed by the same letter do not differ to a statistically significant degree when compared using Duncan's Multiple Range Test; *statistically significant difference at 95 % confidence

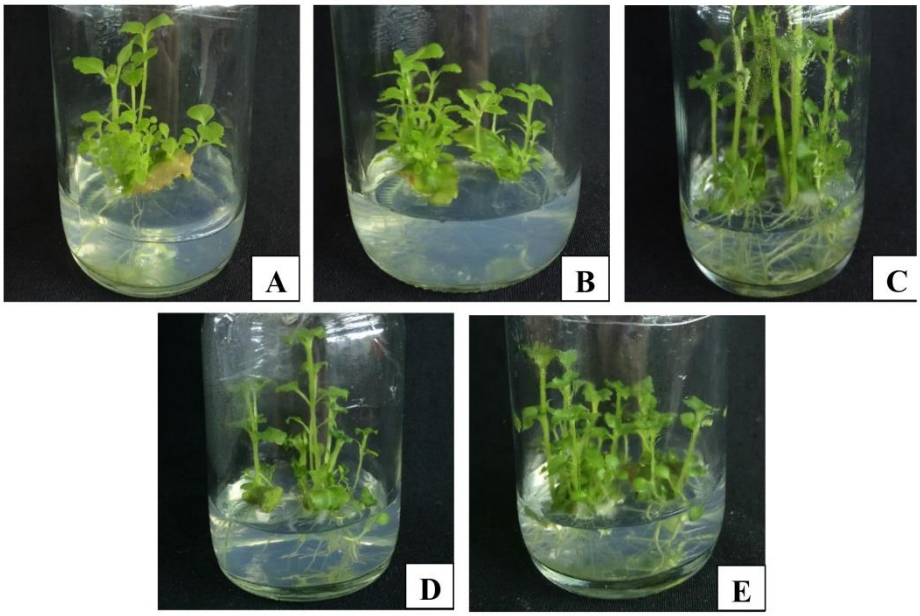

Figure 1 Growth characteristics of new shoots generated from *in vitro Torenia* leaf sections; (A) No colchicine exposure (control); (B) 15 ppm colchicine 12 hours; (C) 15 ppm colchicine 24 hours; (D) 15 ppm colchicine 48 hours; (E) 15 ppm colchicine 72 hours

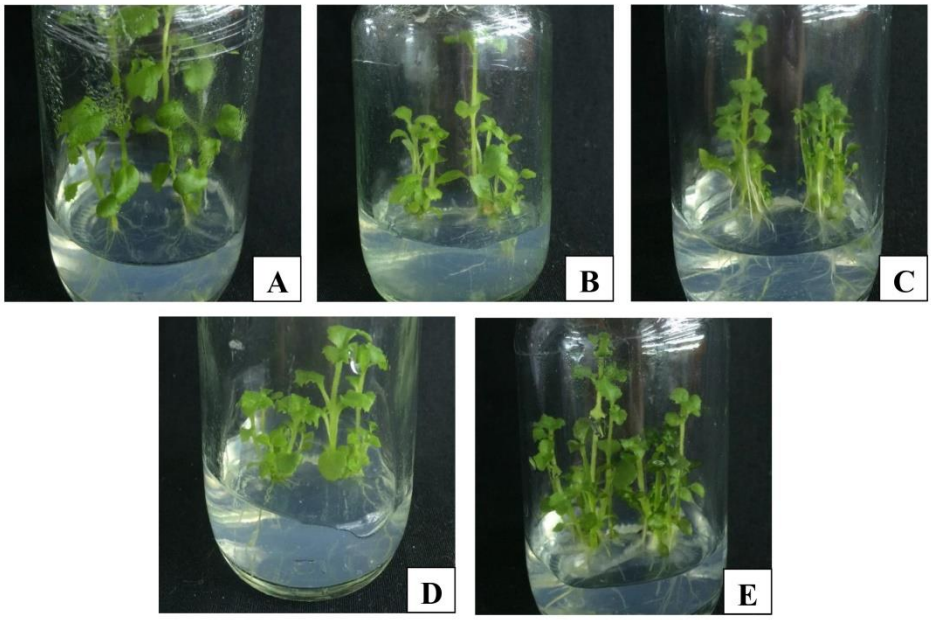

Figure 2 Growth characteristics of new shoots generated from *in vitro* Torenia node sections; (A) No colchicine exposure (control); (B) 15 ppm colchicine 12 hours; (C) 15 ppm colchicine 24 hours; (D) 15 ppm colchicine 48 hours; (E) 15 ppm colchicine 72 hours

3.2.2 Number of roots and root length

Sixty days after colchicine treatment, no statistically significant differences were found in the number of roots and root length of plantlets from the control or the different duration colchicine treatments among plantlets regenerated from node sections (Table 4). However, for plantlets regenerated from leaf sections, there were statistically significant differences in root length between the control and some of the colchicine-treated

Table 4 Number of roots and length of roots generated from *in vitro* Torenia node sections, 60 days after colchicine treatment

Colchicine concentration	Duration (hours)	Number of roots (roots)	Root length (cm)
15 ppm	0	7.55±3.25[1/]	1.88±0.61
	12	5.75±4.29	1.54±0.93
	24	6.90±4.15	1.71±0.93
	48	6.10±3.64	1.56±0.62
	72	7.05±2.06	1.81±0.27
F-test		ns	ns
C.V. (%)		53.53	42.16

[1/]values in the same column followed by the same letter do not differ to a statistically significant degree when compared using Duncan's Multiple Range Test; *statistically significant difference at 95 % confidence

Table 5 Number of roots and roots length generated from *in vitro* Torenia leaf sections, 60 days after colchicine treatment

Colchicine concentration	Duration (hours)	Number of roots (roots)	Root length (cm)
15 ppm	0	5.15±4.94 ab[1/]	1.43±1.14
	12	6.05±5.84 ab	1.87±1.33
	24	7.20±5.36 a	2.16±1.60
	48	3.40±3.05 b	1.37±0.92
	72	6.85±2.43 a	1.88±0.34
F-test		*	ns
C.V. (%)		79.03	66.13

[1/]values in the same column followed by the same letter do not differ to a statistically significant degree when compared using Duncan's Multiple Range Test; *statistically significant difference at 95 % confidence

groups (Table 5). Plantlets regenerated from leaf sections in the control group had an average 5.15±4.94 roots, with mean root length of 1.43±1.14 cm. The greatest number of roots was 7.20±5.36, with mean root length of 2.16± 1.60 cm, observed in the 24-hour colchicine treatment group (Table 5). Meanwhile, for the plantlets regenerated from node sections, the control group had an average 7.55±3.25 roots with mean root length of 1.88±0.61 cm and those from the colchicine-treated groups had slightly fewer and shorter roots (Table 4).

3.2.3 Number of new shoots

When the number of new shoots generated from Torenia leaf and node sections were measured 60 days after colchicine treatment, the greatest number of new shoots generated from node sections was 3.30±0.86 (in the 72-hour colchicine treatment group) and the greatest number of new shoots generated from leaf sections was 3.65±3.39 (in the 12- and 24-hour colchicine treatment groups). For node sections, the number of new shoots increased with increasing duration of colchicine treatment (Table 6), but for leaf sections the number of new shoots was equal to the control in the 72-hour colchicine treatment group, less than the control in the 48-hour colchicine treatment group and greater than the control in the 12-hour and 24-hour colchicine treatment groups (Table 7).

Table 6 Number of new shoots generated from *in vitro* Torenia node sections, 60 days after colchicine treatment

Colchicine concentration	Duration (hours)	Number of shoots
15 ppm	0	1.80±0.83 b[1/]
	12	1.85±1.35 b
	24	2.50±1.67 ab
	48	2.10±1.59 b
	72	3.30±0.86 a
F-test		*
C.V. (%)		56.66

[1/]values in the same column followed by the same letter do not differ to a statistically significant degree when compared using Duncan's Multiple Range Test; *statistically significant difference at 95 % confidence

3.3 Polyploid frequency

Based on visual observation of morphological characteristics, we identified 14 plantlets as putative polyploids. The greatest number came from the 48-hour colchicine treatment group, consisting of 1 plantlet regenerated from a node section and 7 plantlets regenerated from leaf sections, or a

frequency of 5 % for node sections and 25 % for leaf sections. The second greatest number of putative polyploids was observed in the 24-hour colchicine treatment group, consisting of 4 plantlets regenerated from leaf sections, or a frequency of 20 %. Finally, 2 putative polyploids were identified from among the plantlets regenerated from node sections in the 12-hour colchicine treatment group, or a frequency of 10 % (Table 8)

Table 7 Number of new shoots generated from *in vitro* Torenia leaf sections, 60 days after colchicine treatment

Colchicine concentration	Duration (hours)	Number of shoots
15 ppm	0	2.50±2.59 ab[1/]
	12	3.65±3.39 a
	24	3.65±2.98 a
	48	1.30±1.49 b
	72	2.50±1.76 ab
F-test		*
C.V. (%)		93.56

[1/]values in the same column followed by the same letter do not differ to a statistically significant degree when compared using Duncan's Multiple Range Test; *statistically significant difference at 95 % confidence

Table 8 Frequency of putative polyploids observed in colchicine-treated Torenia regenerated *in vitro* from leaf and node sections

Colchicine concentration	Duration (hours)	Putative polyploids		Frequency of polypoidy (%)	
		Node section	Leaf section	Node section	Leaf section
15 ppm	0	0	0	0	0
	12	2	0	10	0
	24	0	4	0	20
	48	1	7	5	25
	72	0	0	0	0

3.4 DNA content analysis by flow cytometry to determine the ploidy level of *Torenia* plantlets

After putative polyploid plants were identified (those with thicker leaves, thicker stems and more indented leaf margins than the control), leaf samples were taken for DNA quantification by flow cytometry, using a Partec II flow cytometer. An increase in nuclear DNA in some of the putative polyploids was confirmed.

The dendrogram for the diploid control leaf sample (2*n*=2*x*) showed a single peak at 200 FL4DNA (Figure 3 and 4), whereas leaf samples from tetraploid Torenia (2*n*=4*x*) showed a second peak at 400 FL4DNA, or double the amount of DNA in the diploid (Figure 5). Flow cytometry also revealed that one of the putative polyploids was an octaploid (2n=8x) with a third peak at 800, or 4 times more DNA than the control (Figure 7). The results showed that 2 tetraploids and 1 octaploid were obtained from the 12-hour and 48-hour colchicine treatments.

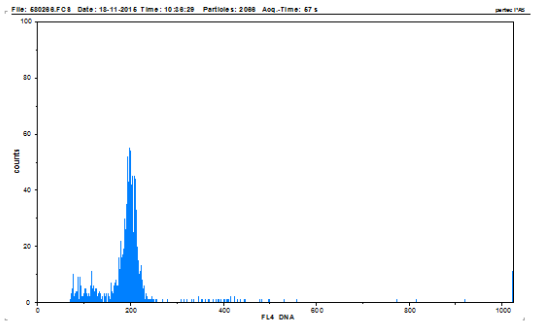

Figure 3 Flow cytometry histogram of diploid Torenia regenerated from a node section (control group)

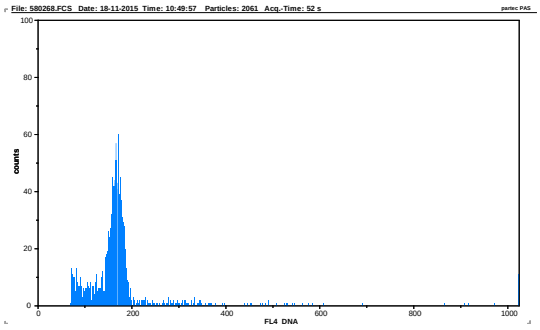

Figure 4 Flow cytometry histogram of diploid Torenia regenerated from a leaf section (control group)

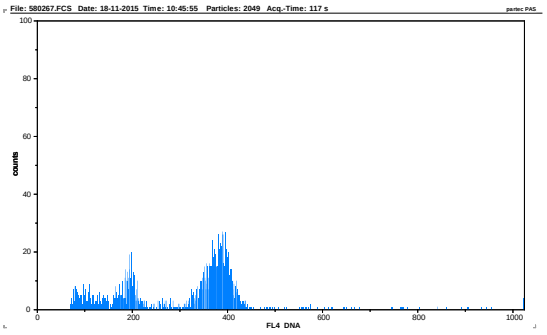

Figure 5 Flow cytometry histogram of tetraploid Torenia regenerated from a node section in the 12-hour colchicine treatment group

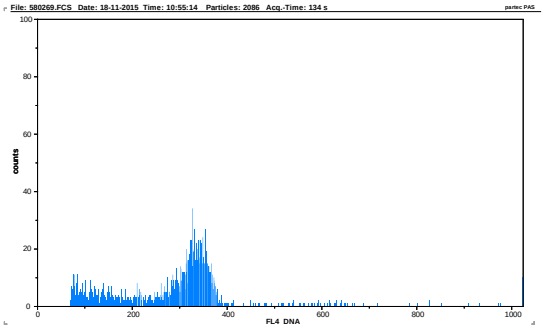

Figure 6 Flow cytometry histogram of tetraploid Torenia regenerated from a node section in the 48-hour colchicine treatment group

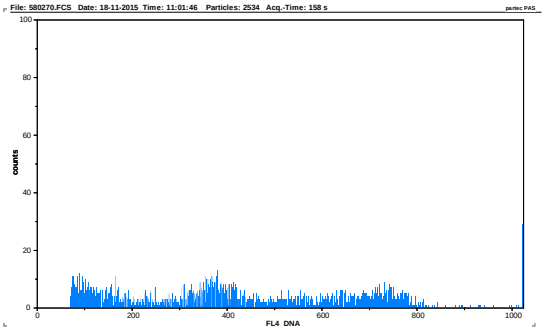

Figure 7 Flow cytometry histogram of octaploid Torenia regenerated from a leaf section in the 48-hour colchicine treatment group

4. Discussion

When survival rate was calculated after 60 days of culture on ½ MS medium, the plantlets regenerated from both node and leaf sections in the control group had the highest survival rate at 100 %, and survival rate tended to decrease with increased duration of exposure to colchicine. This was consistent with the findings of Sungkaew *et al.* (2015), who found in an experiment to induce polyploidy in Lindernia that survival rate tended to decrease with increasing duration of exposure to colchicine. Similarly, Gantait *et al*. (2011) reported that in their research on Gerbera, in which in vitro apical buds were soaked in 0.01, 0.10, 0.5 and 1 % colchicine solution for 2, 4 and 8 hours, survival rate decreased with increasing colchicine concentration and increasing exposure time. Likewise, Ketcharoen (2010) reported that the survival rate of rain lily bulbs decreased when they were exposed to colchicine solution for longer periods. This is probably because when plant tissues are exposed to colchicine solution for a certain period, more colchicine will be absorbed into the plant cells and diffused to various parts of the cell. In addition to impairing normal cell division, colchicine has other toxic effects on plant cells (Derman, 1940). For instance, colchicine has been shown to affect the viscosity of the cytoplasm (Cook and Loudon, 1952).

When the number of shoots, shoot height and internode length of plantlets regenerated from *Torenia* node and leaf sections were compared among the different treatments, statistically significant differences were found. No statistically significant differences were found among the different treatments in terms of number of roots and root length, however. Morphological changes indicative of polyploidy were observed in several of the colchicine-treated plantlets from the 48-hour, 24-hour and 12-hour treatment groups, such as darker green leaves, thicker leaves, indented leaf margins and thicker stems. This was consistent with the findings of Tungkajiwangkoon (2009), who also induced polyploidy in *Torenia* by treating with colchicine solution at several different concentrations for different durations. The polyploids also exhibited thicker stems, and wider, longer and thicker leaves than the diploids. In addition, in the research of Jiranapapan (2011), 4 tetraploid *Torenia* plants were formed following exposure to 15 ppm colchicine solution for 2 days. Chromosome doubling led to increased cell size, and thus to the observable morphological changes (Chandrasekharan *et al*., 1975).

Comparing the responses of node sections versus leaf sections, our results indicated that leaf sections tended to have a greater survival rate following colchicine treatment. Flow cytometry analysis to determine the amount of DNA showed that 2 of the putative polylpoids were tetraploids and one was octaploid. The tetraploid plants were regenerated from node sections from the 12-hour and 48-hour colchicine treatments, and the octaploid was regenerated from a leaf section

from the 48-hour treatment group.

5. Conclusion

Our research intended to find an appropriate type of explant and length of time for exposing *in vitro* explants to colchicine solution from gout medication tablets to induce polyploidy in ornamental Torenia. We tested node sections and leaf sections soaked in 15 ppm colchicine solution for 0, 12, 14, 48 and 72 hours. We found that the survival rate tended to decrease with increasing duration of exposure time to colchicine. The morphological characteristics of colchicine-treated plantlets were different from the control; i.e. they had thicker stems, indented leaf margins, and were darker green. The greatest number of putative polyploids (7) was found in the 48-hour treatment group of leaf sections.

For *in vitro* regeneration of Torenia, the leaf section method might be used because it results in a large number of shoots. In our experiment, a large number of the plantlets regenerated from leaf sections displayed the characteristics of polyploids, so this method can be used to multiply a large number of plantlets to screen for breeding programs in the future.

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