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IN VITRO INDUCTION OF TETRAPLOIDS FROM *EXACUM RITIGALENSIS* (GENTIANACEAE)

PCD Perera[1], Nilanthi Dahanayake[1*], SGJN Senanayake[1] and SA Krishnarajah[2]

[1]*Department of Agricultural Biology, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya, Sri Lanka*
[2]*Royal Botanical Gardens, Peradeniya, Sri Lanka*

ABSTRACT

***Exacum ritigalensis* (Gentianaceae) (Sinhalese: Binara or Ginihiriya) has a potential to develop as a potted plant by improving the size of the flowers and inflorescence. The aim of the study was to establish an efficient and effective system for the production of tetraploid plants through colchicine treatment. Single nodal cuttings (0.5 cm) from aseptic plantlets were established on Murashige and Skoog basal medium (MS) supplemented with 2 mgL^{-1}BAP and different colchicine concentrations (0, 10, 20, 30, 40 and 50 mg^{-1}). They were kept in different durations (0, 7, 14 21 and 28 days) and transferred into 10ml of MS basal medium containing 2 mgL^{-1} BAP. After one month, they were transferred to MS medium containing 2mgL^{-1}Indole-3-butyric acid (IBA) for rooting. Then root tips were collected from treated plants to observe chromosome number. Afterwards the selected diploid and tetraploid plants were acclimatized and transferred to field to observe the morphological characters. Factorial Complete Randomized Design (FCRD) with 10 replicates was used for each treatment and statistical analysis was carried out using Duncan's Multiple Range Test of SAS program (9.1.3).The highest percentage of tetraploid plantlets (26.6%) was observed from nodal cuttings treated with MS basal medium with 2mgL^{-1} BAP + 30mgL^{-1} colchicine for seven days. The chromosome number of diploid and tetraploid *E. ritigalensis* determined as 28 and 56 respectively. Cells of the tetraploid plants were comparatively larger cells than diploids plants. The stomata size of the tetraploid (2.96*2.27 µm^2) were larger than those of the diploids (1.94*1.48µm^2). Leaf shape of diploids was lanceolate while it was ovate or narrowly elliptic in tetraploids. Flower petals of diploid plants were overlapping along their entire length while in tetraploids flower petals were not overlapping. The plants height was significantly higher in diploid (67.00±9 cm) than in tetraploid (38.00±4 cm). Newly produced polyploids of *E. ritigalensis* have a potential to popularize as a potted plant in the floriculture trade.**

***Key words*:** colchicine, chromosome, diploid, *Exacum ritigalensis*, tetraploid

INTRODUCTION

Polyploidy breeding holds immense prospect in developing desirable varieties in flowering plants. Currently, at least 50 ornamental plants have already been modified including flower color, fragrance, flower shape, plant architecture, flowering time, postharvest life and resistance for both biotic and abiotic stresses (Chandler and Sanchez, 2012). According to the review of Kazi *et al.,* (2015),polyploidy was successfully induced from *in vitro* and other uncontrolled experiments in Plantanus, Petunia, Pelargonium, Cyclamens, Canna, Zinnia, Hibiscus, Diffenbachia, Colocasia, Iris, Azalea, Phlox,*etc*.using various chemicals.

*Corresponding author: *nilanthi@agbio.ruh.ac.lk*

Exacum ritigalensis (F: Gentianaceae; Tribe: Exaceae) (Sinhalese: Binara or Ginihiriya) is an indigenous wild plant. The family consists of 64 species. It occurs around the Indian Ocean Basin, Africa, Madagascar, Socotra, the Arabian Peninsula, Sri Lanka, India, the Himalayas, mainland Southeast Asia including southern China, Malaysia, and northern Australia (Yuan *et al.*, 2005; Sumanasinghe *et al.*, 2012). This is an annual erect herb 50 – 100 cm in height, producing hermaphrodite flowers from light blue to dark blue with contrasting bright yellow anthers and shiny green foliage. It has a four sided slender stems and upright branching habit. *Exacum ritigalensis* is a horticultural plant with attractive blue col-

ored flowers established in moist soil in forest areas. Chromosome number of *Exacum ritigalensis,* diploid plant is 28 (Perera and Dahanayake, 2017).

Polyploids are organisms with multiple sets of chromosomes in excess of the diploid number (Acquaah, 2007; Chen, 2010; Comai, 2005; Ramsey and Schemske, 1998). Polyploidy is common in nature and provides a major mechanism for adaptation and speciation. Approximately 50-70% of angiosperms, which include many crop plants, have undergone polyploidy during their evolutionary process (Chen *et al.,* 2007).

However, with knowledge of the origins, variations exist and characteristics of different types of polyploids, there are many opportunities for developing and utilizing polyploids in plant improvement programs. Significant opportunities include developing sterile cultivars, overcoming barriers to hybridization, restoring fertility in wide hybrids, enhancing flower size, increasing heterosis and vigor, and improving pest resistance and tolerance to environmental stresses (Thomas, 2000).Ornamental crops such as Snapdragons and Marigolds have been bred through chromosome doubling to improve the quality and size of their blossoms (Emsweller and Ruttle, 1941). The objective of this study was to observe the genome duplication rate using stem explants of diploid *Exacum ritigalensis* treated with colchicine at different concentrations and durations in *in vitro* condition.

MATERIALS AND METHODS

Plant Material

Pods of *Exacum ritigalensis* were collected from Pannala in Kurunegala district, Sri Lanka.

Establishment of aseptic cultures

Pods collected from diploid plants were surface -sterilized by washing under running tap water (1/2 hour) and soapy water (15 minutes). After that pods were immersed in 70% ethanol for 3 minutes and rinsed three times with distilled water. Then seeds were disinfected with 20% (v/v) Clorox (Sodium hypochloride) for 20 minutes in the laminar air flow cabinet. Sterilized seeds were rinsed three times with autoclaved distilled water. They were dried using sterile filter papers. Pod coat was removed with sterile scalpers and pliers. The seeds were cultured in the medium which was prepared with only using0.8% Agar. The pH of the medium was adjusted to 5.8-6.0 with 1N NaOH or 1N HCl solution prior to add Agar and autoclaving at 1.5kgcm^{-2} for 20 minutes. The seeds were cultured under 16 h/8 h photoperiod with white fluorescent light in 1000-1300 Lux intensity in an air-conditioned (22±2ºC) room.

Colchicine treatment and Effect of BAP on shoot proliferation with single node cuttings

The single nodal cuttings (0.5 cm) from aseptic plantlets were established on MS medium (Murashige and Skoog, 1962) (pH-5.8) at 2mgL^{-1}BAP concentrations (Inoka*et al.,* 2014; Tennakoon *et al.,* 2015) with different colchicine concentrations (0, 10, 20, 30, 40and 50mgL^{-1}). They were maintained in above medium for different durations (0, 7, 14 21and 28 days) and transferred to 10ml of MS basal solid medium containing 2 mgL^{-1} BAP (Dahanayake *et al.,*2009).Cultures were incubated at 22±2°C at 16 h/ 8 h photoperiod under cool white fluorescent light with 1000-1300 Lux intensity. One month after establishment, the number of shoots produced from each explant, height of shoots and survival rate of shoots were recorded and analyzed.

Root induction from shoots of *E. ritigalensis*

One month old shoots were selected from above experiments and introduced to MS medium containing 2mgL^{-1} IBA (Tennakoon *et al.,* 2015). Number of roots per shoot, average root lengths and survival rate of plant were recorded at one month after transferring to the above medium. The number of chromosomes was counted using young root tips to verify the ploidy level as describe below.

Observation of Chromosomes

Twenty regenerated plants were randomly selected and from each plant all actively growing root tips of 5–8 mm in length were excised. These root tips were washed with tap water to remove the residues of the medium, and then pre-treated with 0.01% colchicine solution for two hours at room temperature and thereafter

two hours in refrigerator at 4ºC (Perera and Dahanayake, 2017). Then they were washed with tap water and transferred to fixing in Carnoy's solution containing acetic acid: ethanol (1: 3, v/v) for at least 18 hours. The fixed root tips were then hydrolyzed in 1 N HCl for 03 min at 65^0C. After hydrolysis, root tips were rinsed with distilled water for 10 min and cut into about 1.5 mm pieces (Perera and Dahanayake, 2017). These prepared root tips were then placed on slid glass and stained with one drop of carbolfuchsin solution for one minute. After it was squashed under cover slip glass and cell samples of the root tips were observed for chromosomes under a light microscope (Axio Lab A1), and photos (magnification: 630x) were taken with the associated apparatus.

Acclimatization and transferring of plants

After 60 days well elongated plantlets were removed from the media and washed well with water to get rid of any trace of gelling agent, especially on the root to avoid possible contaminations during the acclimatization process. These plants were then placed in the wide mouth glass bottles of about 250ml inner space, which contained 20 ml sterilized distilled water, just to cover the root system of the plants. The bottles were opened gradually to expose plants to natural environment in the plant house over a period of four weeks. The number of plants which survived in each treatment was counted after four weeks. Plantlets were acclimatized on sterilized coir dust and sand medium (1:1 ratio) in culture jars for two weeks, in the culture room (16 h light 1000 lux and at 25±1°C) prior to introduce in to greenhouse. After that plants were transferred into red yellow podzolic soil and maintain in green house condition (Tennakoon *et al.,* 2015).

Stomata analysis

For stomata analysis, the pieces of epidermal layer were torn from the abaxial side of the leaves from the seventh node of tetraploid and diploid plants. These epidermal layers were mounted on glass slide with one drop of distilled water and covered with a cover glass. Sizes and shape were measured and photos of stomata were taken under a light microscope.

Observation of morphological characteristics

Acclimatized tetraploid and diploid plantlets of *E. ritigalensis* were transplanted in pots containing red yellow podzolic soil and maintained in the field. Plants were selected for further identification of characteristics; height of the plant, girth of the plant in seventh node, number of flowers, flower size and colour, girth and leaf length after eight months. After care operations such as watering, fertilizer and inpesticide application were done as necessary.

Experiment design and Statistical analysis

Experiment was arranged according to the Factorial Complete Randomized Design (FCRD). All experiments were conducted three times, each with ten replicates. Statistical analysis was carried out using ANOVA and Duncan's Multiple Range Test of SAS program (9.1.3).

RESULTS AND DISCUSSION

Effects of BAP on shoot proliferation with single node cuttings

The stem cuttings which were treated with colchicine concentration of 50mgL^{-1}were died during the period. When increasing the duration and the concentration of colchicine, the number of shoots per cutting, height of regenerated shoot and survival rate of regenerated shoots were reduced while increasing the number of dates taken to regenerate (Table 1). Similar results were observed by colchicine treated ornamentals (Elrad and Meral, 2010 and Dahanayake *et al.,* 2009). According to the results of Table 1,regenerated shoots except the colchicine treatment of 30mgL^{-1} and 40 mgL^{-1}duration of 21 days were used for root induction to observe the chromosomes.

Root induction from colchicine treated shoots of *E. ritigalensis*

According to the result shown in table 2, the mean number of root and root length was reduced with time when increasing the exposure of stem explants to high colchicine concentration. In contrast with the above results,when

increasing the colchicine concentrations, reduction of rooting and root length was also observed by Dahanayake and Yue-Sheng (2013) and Elrad and Meral (2010).Conspicuously, survival rate also was reduced when increasing the concentration of colchicine and its exposure time. Excluding the treatments of colchicine exposure in 21 days with 20, 30, and 40 $mgL^{-1}$and 14 days with 40 mgL^{-1}were used to observe the chromosome number.

Observation of Chromosomes

For the chromosome counts, the plants were selected considering the observations recorded in Table 2. Tetraploid plants were confirmed by detecting 56 chromosomes in the root tip cells (Figure 1, c). Chimeric plants showed the chromosome numbers different to either diploid plants (2n = 28) or tetraploid plants (4n = 56). The formation of chimeric plants is often a situation that can be found in studies of polyploidy plant (Elrad and Meral, 2010).Evidently, the bigger mean size of cells were observed in tetraploid (56 chromosomes) than that of diploid (28 chromosomes) (Figure 1).

Acclimatization and transferring of plants

Plantlets were acclimatized in the glass bottles containing 20 ml sterilized distilled water had various survival rates after 4 weeks period: 90% in diploid plants, 50% in tetraploid plants and 10% in chimeric plants. Plantlets were acclimatized on sterilized coir dust and sand medium(1:1)in jars for two weeks; 80% diploid plantlets and 30%tetraploidplants were survived except chimeric plantlets. Saikat *et al.,* (2011)and Liu *et al.,* (2007) observed the significantly reduction of the survival rate of tetraploid and chimeric plants compared to diploid plants in their research on *Gerbera jamesonii* and *Platanus acerifolia.*

In stomata analysis, both diploid and tetraploid plant showed stomata in epidermal layer from the abaxial side (Figure 2). Stomata analysis is one of the reliable methods to de-

Table 1: Effect of shoot formation under colchicine treatment

Duration (days)	*Colchicine concentrate (mgL^{-1})*	*Number of days taken to regenerate*	*Mean number of shoot*	*Mean height of regenerated shoot (cm)*	*Survival rate of shoots (%)*
Control		18.80±0.69^{g}	11.67±1.45^{a}	5.20±0.20^{a}	90
7	10	21.16±0.09^{f}	10.67±0.34^{b}	4.16±1.19^{b}	85
	20	21.16±0.69^{f}	9.17±0.69^{c}	3.75±0.10^{c}	85
	30	25.30±0.75^{d}	5.16±0.61^{d}	2.65±0.10^{e}	60
	40	27.00±0.82^{c}	3.00±0.58^{e}	1.23±0.12^{h}	40
14	10	22.00±0.58^{f}	9.00±0.82^{c}	3.47±0.20^{d}	35
	20	24.30±0.94^{e}	5.83±0.69^{d}	3.45±0.08^{d}	15
	30	27.16±0.69^{c}	2.33±0.47^{ef}	2.10±0.08^{f}	10
	40	29.50±0.50^{b}	1.67±0.47^{fg}	0.75±0.14^{i}	5
21	10	30.00±0.82^{ab}	2.67±0.75^{e}	1.43±0.11^{g}	15
	20	30.80±0.69^{a}	1.17±0.69^{g}	0.76±0.20^{i}	5
	30	0.00±0.00^{h}	0.00±0.00^{h}	0.00±0.00^{j}	0
	40	0.00±0.00^{h}	0.00±0.00^{h}	0.00±0.00^{j}	0

Same letter within the columns are not significantly different as determined by Duncan's multiple range test (α=0.05) (n=78).

Table 2: Effects of MS +2mgL^{-1}IBA on root formation of shoots which were selected from Table 1

Duration (days)	*Colchicine concentrate (mgL^{-1})*	*Mean number of root*	*Mean root length (cm)*	*Survival rate of shoots (%)*
Control		11.16±0.69^{a}	3.20±1.34^{a}	85
7	10	6.83±0.69^{b}	2.77±0.09^{ab}	75
	20	5.50±0.50^{c}	2.56±0.09^{bc}	65
	30	3.83±0.69^{d}	1.70±0.08^{d}	40
	40	2.16±0.69^{ef}	0.78±0.07^{e}	10
14	10	3.50±0.50^{d}	2.10±0.14^{cd}	10
	20	2.16±0.69^{ef}	1.90±0.08^{d}	5
	30	2.33±0.40^{e}	0.42±0.07^{ef}	1
	40	0.00±0.00^{g}	0.00±0.00^{f}	0
21	10	1.50±0.50^{f}	0.28±0.12^{ef}	5
	20	0.00±00^{g}	0.00±0.00^{f}	0
	30	0.00±00^{g}	0.00±0.0^{f}	0
	40	0.00±00^{g}	0.00±0.0^{f}	0

Same letter within the columns are not significantly different as determined by Duncan's multiple range test (α=0.05) (n=78).

Table 3: Comparison of the effects of colchicine concentrations and exposure time on chromosome doubling of regenerated plant (n=255)

Exposure time (days)	*Colchicine concentration (mg/L)*	*No. plants sampled*	*No. root tips sampled*	*No. cells observed*	*No. cells with 2x chromosome*	*No. cells with 4x chromosome*	*No. each kind of plants on the base of chromosome counting*			*Tetraploid Percentage (%)*
							2x plants	*Chimeras*	*4x plants*	
Control		30	60	1800	665	0	30	0	0	0.0
7	10	30	60	1750	644	0	30	0	0	0.0
	20	30	60	1820	650	154	20	8	2	6.7
	30	30	60	1720	575	345	16	6	8	26.6
	40	30	40	840	410	183	16	10	4	13.3
14	10	30	60	1700	595	0	30	0	0	0.0
	20	30	40	760	398	10	24	6	0	0.0
	30	15	30	620	388	55	9	4	2	13.3
21	10	30	20	480	276	12	25	4	1	3.3

termine ploidy level (Manawadu *et al.,* 2016; Soloveva, 1990). Therefore the measurement of stomata length could be an effective way to select tetraploid and diploid plants (Manawadu *et al.,* 2016).Differences in size of stomata were identified through microscopic observations (Table 4). Stomataon the lower surface of the tetraploid leaves were larger but fewer compared to that of diploids. Similar results were observed in induced tetraploid of *Echinacea purpurea* (Dahanayake and Yue-Sheng, 2013).

Size of stomata length and width were significantly different between diploid and tetraploid plants (Table 4). It was also observed that the higher stomata density per 9 $\mu m^2$was obtained from the diploid plants (24.60±1.36) than tetraploid plants (18.90±1.14). One of the immediate and obvious consequences of polyploidy in plants is an increase in cell size which in turn leads to enlarged plant organs, a phenomenon termed gigas effect (Acquaah, 2007; Levin, 2002). For example, the volume of tetraploid cells usually is about twice that of their diploid progenitors (Acquaah, 2007; Emsweller and Ruttle, 1941; Levi *et al.,*2002; Schepper *et al.,* 2001).As the enlargement of the stomata in this observation point out the most of the cholchicine treated polyploid plant production compare with the diploids, the length and width of guard cells increased by 48.25% and 52.62%, respectively in previous studies (Przywara *et al.*, 1988; Cohen and Yao 1996; Pansuksan *et al.,* 2014; Quin *et al.,* 2016).

Observation of agronomic characteristics

Overall leaf width, girth of the plant at seventh node, flower diameter and number of flower per branch were significantly different in induced tetraploid plants compared with the diploid plant. Conversely, the diploid plants showed the highest leaf length and height of the plant compare to tetraploid plant (Table 5). However according to the literature based

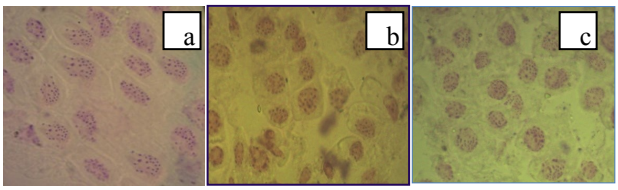

Figure 1. Plant cells a) diploid (2n=28); b) chimera and c) tetraploid (4n=56) (they were taken under the same microscopic conditions; magnification = 630x, Microscope Axio Lab A1)

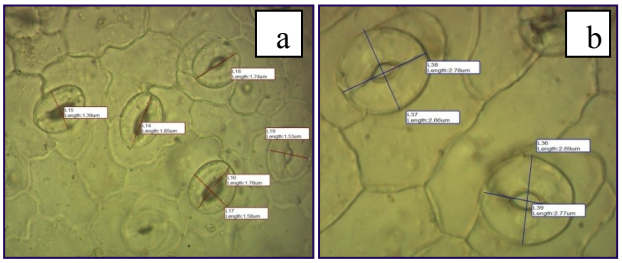

Figure 2. Stomata of *in vitro* grown *E. ritigalensis*; a) diploid plant and b) tetraploid plant (they were taken under the same microscopic conditions; magnification = 630x, Microscope Axio Lab A1)

Table 4: Comparison of stomata size and density among tetraploid and diploid plants of *E. ritigalensis*

Ploidy level	***Diploid***	***Tetraploid***
Stomata length (μm)	1.94±0.10[b]	2.96±0.13[a]
Stomata width (μm)	1.48±0.06[b]	2.27±0.40[a]
Stomata density per 9 μm^2	24.60±1.36[a]	18.90±1.14[b]

***Data in the same row followed by different letters are significantly different by Duncan's multiple range test (α=0.05) (n=30).**

on Hannweg *et al.,* (2013) and Manawadu *et al*., (2016), several reports since the advent of induced polyploidy research describe increases in flower size, alterations in stem length and number of flowers per stem for a wide range of ornamental species and crops.

Increasing ploidy often results in increased cell size that in turn results in thicker, broader leaves and larger flowers and fruit (Thomas, 2000). When screening large numbers of plants, these visual characteristics are often helpful for identifying polyploids. Flow cytometry has also been used as a method for measuring DNA content which can be correlated with ploidy level for a given crop (Sharma and Sharma, 1999).

Observations of leaves showed that it is glossy green in both diploid and tetraploid plant (Figure 3, a). Despite the diploid plant leaf shape is lanceolate but it is ovate or narrowly elliptic in tetraploid *Exacum* (Figure 3, b) and the leaf just below the bud was the same appearance (Figure 3, c). Flowers are blue colour with pale to dark hues. In both types flowers are flattened to cup-shaped and the petals are broadly obovate. However petals of flowers in diploid showed overlapping along their entire length whereas in tetraploid petals did not overlap (Figure 3, d).

Above data were collected from one flowering time in both diploid and tetraploid plant. Therefore additional research is needed to establish possible alterations in vase life of tetraploids compared with the diploids and more phenological studies are required to determine a potential increase or decrease of marketing windows.

CONCLUSIONS

Highest percentage of tetraploid plantlets (26.6%) of *Exacum ritigalensis* was obtained

Table 5: Comparison of morphological characters among tetraploid and diploid

Morphological characters	*Diploid*	*Tetraploid*
Leaf length (cm)	11.95±1.38[a]	9.63±0.79[b]
Leaf width (cm)	2.24±0.16[b]	2.99±0.29[a]
Height of the plant (cm)	67.00±9.26[a]	38.00±4.69[b]
Girth of the plant at 7th node (cm)	3.26±0.48[b]	4.33±0.25[a]
Flower diameter (cm)	5.06±0.23[b]	5.39±0.15[a]
Number of flowers per branch	10.00±1.73[b]	11.70±1.18[a]

*Data in the same rows followed by different letters are significantly different by Duncan's multiple range test (α=0.05) (n=20).

Figure 3. Morphology of *E. ritigalensis* diploid plant(D) and tetraploid plant (T). a, Plant height; b, leaves; c, leaves just after flower bud and d, flowers.

when single nodal cuttings were grown in MS basal medium with $2mgL^{-1}$ BAP + $30mgL^{-1}$ colchicine for seven days. Largest size stomata were gained from tertaploid plants while purposeful morphological characters; highest leaf width, root length and root width were achieved by diploid plants. The leaf shape of diploid plants was lanceolate and it was ovate or narrowly elliptic in tetraploid plants. The leaf just below the bud was showed the same character both in diploid and tetraploid plants. The only difference in flowers was that petals in diploid were overlapping along their entire length and in tetraploid flowers, it was not overlapping. Due to variability in characters present in induced tetraploids, there is a great potential to develop . ritigalensis plant as potted plant having ornamental value.

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