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Petal Color and Petal Form Mutations Observed in *Torenia hybrida* Following Gamma Irradiation *in vitro*

Valerie W. Suwanseree[1]*, Tharathorn Teerakathiti[2], Shermarl Wongchaochant[1] and Thunya Taychasinpitak[1]

ABSTRACT

The aim of this study was to develop new cultivars of *Torenia hybrida* for the ornamental market. Excised nodes of *T. hybrida* plantlets grown *in vitro* were exposed to 0, 0.0025, 0.0075 or 0.0125 mM colchicine or 0, 0.0028 mM, 0.0086 mM or 0.144 mM oryzalin for 48 or 72 h before subsequent *in vitro* multiplication. Four-week-old *in vitro* plantlets from the colchicine treatments were irradiated with 0, 30, 40, or 50 Gy of gamma radiation and those from the oryzalin treatments were irradiated with 0 or 60 Gy of gamma radiation. After subculturing, the plants were transferred to the field and changes in phenotype were noted. Colchicine and oryzalin treatment did not result in any polyploid plants. No variations were observed in leaf shape, color or size but variations were observed in growth habit (compact and creeping), flower color (pink and pale blue, as well as mottled or streaked purple petals), and flower form (erose petal margins). The plants with erose petal margins were selected for possible development of a new cultivar.

Keywords: Torenia, mutation breeding, gamma irradiation, colchicine, oryzalin, tissue culture

INTRODUCTION

Torenia sp., or wishbone flower, is a branching annual in the family Scrophulariaceae that can be propagated by seeds or stem cuttings. It grows to a height of about 15–30 cm with a spread of 30–90 cm. Native to Southeast Asia, it blooms profusely in warm climates, making it useful for borders and hanging baskets. There are approximately 40 species in the genus *Torenia* (Miyazaki *et al.,* 2006), of which a few have been exploited as ornamentals. Most varieties have purple flowers, but breeding work by commercial breeders such as Danziger in Israel, Pan American Seed in the USA and Suntory in Japan, and by researchers such as Sasaki *et al*. (2008), has made available to the market many novel flower colors ranging from white to light blue, dark blue, shades of pink, magenta, and yellow, as well as combinations of the above (Suzuki *et al.,* 2000). At present, to the best of our knowledge, there are no *Torenia* varieties with double flowers or other different flower shapes available in the market. In this study, the variety used for the experiments was a sterile hybrid between *T. concolor* and *T. fournieri*. The flowers are approximately 4 × 3

1 Department of Horticulture, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.

2 National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Phahonyothin Road, Pathum Thani 12120, Thailand.

* Corresponding author, e-mail: vbdfadan@yahoo.com

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cm, lavender in color, with darker lavender on the lateral and ventral petals, and yellow spots in the center of the corolla tube and on the top center of the ventral petal. This variety was selected for development because of its profuse and early flowering, long flowering season and suitability for the Thai climate.

The objective of this research was to develop a new variety of *Torenia* for the ornamental market through the use of mutation breeding (gamma irradiation) and to develop a tetraploid *Torenia hybrida* through the application of colchicine and oryzalin for use in further breeding projects.

Mutation breeding has successfully been used over many decades to create new varieties of ornamental plants in many genera including *Dianthus, Chrysanthemum, Dahlia, Rosa* and *Achimenes* (Sigurbjörnsson and Micke, 1974). A white variety of *Torenia* was reported as a result of mutation breeding using gamma radiation (Brand and Bridgen, 1989). Researchers in Japan reported a wide variety of flower color mutations after exposing *Torenia* varieties to heavy ion beam radiation (Miyazaki *et al*., 2006; Sasaki *et al.,* 2008). In the present study, the gamma radiation dose range was set at 0-60 Gy based on the above findings.

Because polyploid plants are often larger and more robust than plants with the normal chromosome number, it has been deemed desirable to induce polyploidy in many ornamental crops, as the extra set of chromosomes can also stimulate the expression of a greater range of genetic variation, sometimes resulting in valuable changes in flower size or color (Osborn *et al*., 2003). Colchicine and oryzalin are mitotic inhibitors that have been used to induce chromosome doubling in many horticultural species. Colchicine is an alkaloid obtained from the root of *Colchium autumnale* L. or *Iphigenia indica* Kunth et Benth that binds with tubulin and thus interferes with the formation of microtubules during mitosis (Kingsbury, 2009). Colchicine has been used to induce tetraploids in species such as *Cyclamen persicum* Mill. (Takamura and Miyajima, 1996), *Buddleia globosa* (Rose *et al*., 2000), and *Phlox subulata* L. (Zhang *et al.,* 2008). Oryzalin (4-dipropylaminol-3,5-dinitro-benzenesulfonamide) is a herbicide that binds preferentially to plant tubulin and not animal tubulin, so it is less toxic to humans than colchicine (Kermani *et al*., 2003; Dhooghe *et al.,* 2009a). It has been used to induce polyploidy in species such as *Alocasia micholitziana* (Thao *et al*., 2003), *Rosa* sp. (Kermani *et al*., 2003; Allum *et al*., 2007), *Ranunculus asiaticus* (Dhooghe *et al*., 2009a) and *Helleborus* sp. (Dhooghe *et al*., 2009b).

In the present study, different concentrations of colchicine and oryzalin were applied to excised nodes of *Torenia hybrida*, (*T. concolor* × *T. fournieri*) grown *in vitro*, and *in vitro* plantlets were subsequently exposed to 0–60 Gy of gamma irradiation with the objective of inducing chromosome doubling and mutations that could be of commercial value.

MATERIALS AND METHODS

Sterilization of explant material and culture conditions

Shoots of *T. hybrida* approximately 10–15 cm long with 2–3 pairs of fully expanded leaves were cut to a size of 3–7 cm and stripped of leaves. The shoots were washed thoroughly with soap under running tap water for 2 min, rinsed in 70% alcohol, then immersed in 5% Clorox (5.25% w/v sodium hypochlorite solution, Clorox Company, USA.) and 10% Clorox for 5 and 10 min, respectively, and then rinsed six times in sterile distilled water. The nodes (5–10 mm) were excised and placed on a Murashige and Skoog medium (MS medium; Murashige and Skoog, 1962) containing 30 g/L sucrose and 2.5 g/L kelcogel (Kelco, USA). Then 15 mL of medium was dispensed into 25 × 150 mm borosilicate test

tubes. All media were autoclaved at 121 °C at 105 kPa for 17 min. The culture vessels were kept at 25 ± 2 °C with a photoperiod of 16 h/d at a light intensity of 60 ± 5 μmol m^{-2} s^{-1} of fluorescent light (TLD 36W/84 3350 Im Philips, Thailand).

***In vitro* multiplication**

New shoots were subcultured on MS medium containing 30 g/L sucrose and 2.5 g/L kelcogel by excision of nodes, and transferred to new culture vessels (236 mL glass jars containing 25 mL culture medium) every 5–6 wk (three nodes per culture vessel).

Colchicine and oryzalin treatments

Node segments (5–10 mm length) were excised and transferred to liquid MS medium containing 0, 0.0025, 0.0075, or 0.0125 mM colchicine for the colchicine treatments and 0, 0.0028, 0.0086 or 0.144 mM oryzalin for the oryzalin treatments. The node segments were agitated on an orbital shaker for 48 or 72 h (24 node segments for each treatment), after which they were rinsed in sterile distilled water and transferred to semisolid MS medium containing 30 g/L sucrose and 2.5 g/L kelcogel. The treated plantlets were subsequently subcultured after 6 wk and were prepared for gamma radiation treatment.

Gamma irradiation

Four culture vessels (containing three plantlets each, approximately 4 wk old) of plants from the five colchicine treatments (0.0025 mM colchicine for 48 h, 0.0025 mM colchicine for 72 h, 0.0075 mM colchicine for 48 h, 0.0075 mM colchicine for 72 h and 0.0125 mM colchicine for 48 h) were radiated at the rate of 0, 30, 40, and 50 Gy and four culture vessels of plantlets from the five oryzalin treatments (0.0028 mM oryzalin for 48 h, 0.0028 mM oryzalin for 72 h, 0.0086 mM oryzalin for 48 h, 0.0086 mM for 72 h, and 0.144 mM oryzalin for 48 h) were radiated at the rate of 0 and 60 Gy in a Gamma Irradiator Model Mark I-30 delivering 166.5 TBq of Cs-137 at the Gamma Irradiation Service and Nuclear Technology Research Center, Kasetsart University. The irradiated samples and controls were transferred to fresh culture medium within 48 h after exposure to radiation.

Transplantation

Plantlets grown *in vitro* were first taken from the laboratory and placed in the lathe house (50% shade) to acclimatize to ambient temperature for 3-4 d before removal from the tissue culture vessels. The roots were rinsed in tap water to remove the culture medium and then planted in potting soil mixture in 4 cm seedling trays. The seedling tray table was covered with removable clear plastic sheets above and on all four sides to help maintain humidity.

The surviving plants were transferred to 12 cm pots when they were approximately 6–10 cm tall, kept in partial shade, and then later transferred to 20 cm hanging baskets in full sun when they were approximately 16–20 cm tall.

Propagation

Selected mutated plants were propagated by stem cuttings. Stem sections approximately 4–5 cm long were cut with a clean razor blade and placed upright in seedling plug trays filled with a 1:1 mixture of sphagnum moss and rice husk charcoal.

Stomata measurement

Impressions were made of the abaxial leaf surface of randomly sampled leaves (three mature leaves per plant) by applying a coat of clear fingernail polish and waiting 45 min for it to dry, then removing it with a piece of clear adhesive tape and attaching it to a microscope slide, similar to the method used by Cohen and Yao (1996).

The impressions were viewed at 40× under an Axiostar Plus transmitted light microscope (Carl Zeiss). Photographs were taken

using a Sony Cyber-shot DSC-S730 digital camera and viewed on the computer screen for comparison of the guard cell size of stomata. The length of the guard cells and the number of stomata per screen (per microscope field) were recorded for 4 microscope fields for each leaf sample.

Flow cytometry

One young leaf (mature but newly emerged) of *Torenia hybrida* for each specimen to be tested was chopped in a Petri dish with 500 µL of Partec CyStain (a one-step extraction and DAPI stain solution) and filtered through a 30 µ filter before being analyzed in a Partec PAII flow cytometer.

RESULTS

When the colchicine- and oryzalin-treated plants (those that were not subsequently radiated with gamma radiation) were acclimated and removed from tissue culture jars for growing in the lathe house and outdoors, no notable differences in morphology were observed between the treated plants and the control plants. Since no obvious changes in leaf or flower size were noted, it was not possible to identify any putative polyploids among the colchicine- and oryzalin-treated samples of the *Torenia* variety. Thus, leaf surface impressions were made from sample leaves for observation under a light microscope to detect possible differences in the size of stomatal guard cells and the number of stomata per unit of leaf surface area. Analysis of the data on stomatal size and frequency did not reveal a major difference in the size of guard cells in any of the experimental samples compared to the control, although in 7 of the 58 plants treated with colchicine and 2 of the 64 plants treated with oryzalin, the mean guard cell length was 30% longer than the control (data not shown). The results from the measurements of the number of stomata per leaf unit area showed that the mean number of stomata was higher than the control plus two times the standard deviation of the control (the criterion for putative polyploidy selection used by Cohen and Yao (1996)) for 21 of 64 of the oryzalin-treated samples. Based on these results, 28 surviving plants were selected for ploidy analysis by flow cytometry (7 based on having a mean stomatal guard cell length that was greater than the control and 21 for having a mean number of stomata per leaf unit area that was greater than the control). The flow cytometry results revealed that none of the selected samples was polyploid. In summary, the *in vitro* colchicine and oryzalin treatments did not result in polyploid samples of the *Torenia* variety used in this study.

The survival rate of *in vitro* plantlets of the *Torenia* variety 30 d after gamma irradiation was close to 100% and was not different from the control (data not shown). However, for the plantlets that were irradiated with the highest dose of 60 Gy, an observable difference was noted in the growth rate following radiation. At 30 d after radiation, the mean height of the plantlets that were exposed to 60 Gy radiation was 0.71 cm, compared to 1.17 cm for the control (Table 1).

After the irradiated plants were transferred to the field, several variations in flower form and color were observed. The most frequently observed variation was streaked, mottled or variegated petals. This was observed in approximately 33% of the radiated specimens (Table 2). However, it was also observed in the control plants that were not exposed to colchicine, oryzalin or gamma radiation. The occurrence of variegated petals was noted more frequently in the first wave of blooms and less frequently in subsequent blooms from the same plants. However, detailed data were not collected on the frequency of streaked and variegated petals. Aberrant flower forms (extra petals, extra stamens or missing petals) were observed in approximately 22% of the radiated specimens, but these variations were generally observed on only one or a few

Table 1 Mean height of *in vitro* plantlets 30 d after radiation. Comparison of *Torenia hybrida* plantlets previously treated with oryzalin only (the control plants, subcultured on the same day) and plantlets previously treated with oryzalin and subsequently subjected to 60 Gy gamma irradiation.

Treatment	Mean height (mm) ± SD	Number of plantlets with nil growth (not included in mean)
0.0028 mM 48 h oryzalin	1.22 ± 0.71	1
0.0028 mM 48 h oryzalin+ 60 Gy γ	0.76 ± 0.50	6
0.0028 mM 72 h oryzalin	1.12 ± 0.66	0
0.0028 mM 72 h oryzalin + 60 Gy γ	0.51 ± 0.44	1
0.0086 mM 48 h oryzalin	1.48 ± 0.72	1
0.0086 mM 48 h oryzalin + 60 Gy γ	0.47 ± 0.38	6
0.0086 mM 72 h oryzalin	1.22 ± 0.57	1
0.0086 mM 72 h oryzalin + 60 Gy γ	0.62 ± 0.44	3
0.0144 mM 48 h oryzalin	0.79 ± 0.40	0
0.0144 mM 48 h oryzalin + 60 Gy γ	1.20 ± 0.75	3

Table 2 Percentage of mutations observed following gamma irradiation of *Torenia hybrida* plantlets *in vitro*.

Treatment	Gamma radiation dose	Number of rescued plants	Flower color variation (%)	Erose petal margins (%)	Compact growth habit (%)	Creeping growth habit (%)	Variegated Petals (%)
Colchicine							
0.0025 mM 48 h	30 Gy	22	-	-	-	-	40.90
0.0025 mM 72 h	30 Gy	29	-	-	-	-	31.03
0.0075 mM 48 h	30 Gy	34	-	8.82	-	-	14.71
0.0075 mM 72 h	30 Gy	11	-	-	-	-	18.18
0.0125 mM 48 h	30 Gy	20	-	-	-	-	10.00
0.0025 mM 48 h	40 Gy	15	-	-	-	-	26.67
0.0025 mM 72 h	40 Gy	38	2.63	-	-	-	39.47
0.0075 mM 48 h	40 Gy	7	14.29	-	-	-	42.86
0.0075 mM 72 h	40 Gy	11	9.09	-	-	-	27.27
0.0125 mM 48 h	40 Gy	10	-	-	-	-	20.00
0.0025 mM 48 h	50 Gy	21	14.28	-	4.76	-	76.19
0.0025 mM 72 h	50 Gy	18	-	-	-	-	88.88
0.0075 mM 48 h	50 Gy	15	-	-	-	-	40.00
0.0075 mM 72 h	50 Gy	20	-	-	-	-	5.00
0.0125 mM 48 h	50 Gy	18	-	-	5.56	-	16.67
Oryzalin							
0.0028 mM 48 h	60 Gy	14	-	-	-	-	*
0.0028 mM 72 h	60 Gy	20	-	-	5.00	-	*
0.0086 mM 48 h	60 Gy	8	-	-	-	-	*
0.0086 mM 72 h	60 Gy	16	-	-	-	12.50	*
0.0144 mM 48 h	60 Gy	41	-	-	-	-	*
Control	-	31	-	-	-	-	67.74

* data not available

individual blossoms on any given plant. Flower color variations (pink petals and pale blue petals, Figure 1 and Figure 2) occurred in 2.69% of radiated specimens, 12.50% exhibited a creeping habit and 0.69% exhibited a compact growth habit; however the latter was not robust and died early. No variations in leaf color, shape or size were noted. Perhaps the most interesting mutation from a commercial perspective was the appearance of flowers with crinkled or erose petal margins, observed in three plants from the 0.0075 mM 48 h colchicine and 30 Gy irradiation treatment, or 0.59% of the total specimens (Figure 3).

Figure 1 Gamma irradiated *Torenia hybrida* flowers with paler color (control on the right, mutants treated with 0.0075 mM colchicine for 48 h and 40 Gy of gamma irradiation on the left and center). Scale bar in cm.

Figure 2 Gamma irradiated *Torenia hybrida* flowers with pink color (control on the right, mutants treated with 0.0075 mM colchicine for 72 h and 40 Gy gamma radiation center and left). Scale bar in cm.

Figure 3 Gamma irradiated *Torenia hybrida* flowers with erose petal margins (control on the right, mutants treated with 0.0075 mM colchicine for 48 h and 30 Gy gamma radiation center and left). Scale bar in cm.

Stem cuttings were made of the plants with pink and pale blue flowers and those with erose petal margins to determine if the mutations were stable. The mutations remained stable in 100% of the second generation plants, although the sample size was small (5 of 5 regenerated plants with pink petals, 5 of 5 regenerated plants with pale colored petals and 8 of 8 regenerated plants with erose petal margins).

DISCUSSION

The concentrations of colchicine and oryzalin used in the present study were rather low, for the purpose of maintaining a high survival rate. Also, the number of nodes treated was quite low at only 24 nodes per treatment, which may have been the reason that no polyploids were detected. In other studies on the use of anti-mitotic agents *in vitro* to induce polyploidy, large differences in the success rate have been reported depending on the concentration, duration of exposure and species or variety of plant. For example, in *Cyclamen persicum* Mill., 25 mM colchicine for 4 d produced 4.17% tetraploids (Takamura and Miyajima, 1996); in *Zantedeschia* sp., 1.25 mM colchicine for 1, 2 or 4 d resulted in 13–23% tetraploids (Cohen and Yao, 1996); in *Phlox* sp., 0.5 mM colchicine for 30 d resulted in 75% tetraploids (Zhang *et al*., 2008); in *Buddleia globosa,* 0.25 mM colchicine for 3 d resulted in 75% tetraploids (Rose *et al*., 2000); in *Alocasia* sp., 0.289 mM orzyalin for 24 or 48 h resulted in 15.4% tetraploids (Thao *et al*., 2003); in *Rosa rugosa* 0.0025 mM oryzalin for 50 h resulted in 44.4% tetraploids (Allum *et al*., 2007); in *Helleborus* sp., 0.003 mM orzyalin for 12 wk resulted in 2.9% tetraploids (Dhooghe *et al*., 2009a); and in *Ranunculus* sp., 0.0005 mM orzyalin for 4 wk resulted in 14.7% polyploids (Dhooghe *et al*., 2009b). In future studies to induce polyploidy in *Torenia* spp. it would be advisable to increase the concentration of spindle inhibitor and the duration of exposure.

The fact that streaked, mottled or variegated petals were observed in both the control plants and the radiated plants suggests that it could be a physiological response to the tissue culture regimen, especially because many of the same plants also produced flowers with the more typical solid color patterns. It was proposed by Mukherjee and Khoshoo (1970) that the appearance of irregular spots, flecks, and streaks on radiated *Canna* sp. were the result of a physiological or biochemical disturbance caused by the radiation. Further research could be conducted to investigate the rate of occurrence of variegated petals in *T. hybrida* grown in tissue culture.

All the plants (controls and those that were subjected to colchicine, oryzalin and gamma radiation) were multiplied for several cycles *in vitro* before being transferred to the field. Mutations that occurred could have been multiplied through *in vitro* regeneration, resulting in a larger number of mutated plants in the final results. For example, 3 out of 34 plants from the 0.0075 mM colchicine for 48 h and 30 Gy gamma radiation treatment exhibited erose petal margins. Most likely, there was a single mutation event that occurred, but after the mutated tissue was allowed to grow, the plantlet was divided in subsequent subcultures so that three mutated specimens were present by the stage of growing outdoors. Two separate specimens were observed with mutated pink petals, one in the 0.0025 mM colchicine for 72 h and 40 Gy radiation treatment group and one in the 0.0075 mM colchicine for 72 h and 40 Gy radiation treatment group. The two were different shades of pink.

The color mutations observed in the present study were very similar to those reported by Miyazaki *et al.* (2006), who observed pale blue, blue, pale pink and bright pink flower color mutations after exposing *in vitro* leaf tissue and internode segments of *Torenia hybrida* cv. 'Summer Wave Blue' to 5-50 Gy of heavy ion beam radiation. They observed changes in flower

color in 1.06% of irradiated plants. Some of the observed mutations were also similar to those described by Sasaki *et al.* (2008) following heavy ion beam irradiation on one wild type and four transgenic varieties of *Torenia*. Sasaki *et al.* (2008) found the mean original mutation rate for all varieties and all treatments was 8.95%, but only 50.6% of the initially observed mutations proved to be stable after vegetative reproduction. In the present study, stem cuttings were made of the plants with pink and pale blue flowers and those with erose petal margins and the mutations remained stable in 100% of the second generation plants.

Brand and Bridgen (1987) reported leaf variegation as a result of irradiation of *Torenia fournieri* 'Compacta Blue' *in vitro* leaf discs at 0–40 krads. In the present study, neither leaf variegation nor chlorosis was observed, even though these are common effects that have resulted in several new ornamental varieties in other species. It is possible that the sample size was too small to see a wide range of possible mutations.

The occurrence of mutated plants with a creeping habit could be a reversion to the characteristics of *T. concolor* (one of the parents in the *T. hybrida* cross). This mutation is not desirable for a potted plant because it has a straggly appearance rather than being bushy. Likewise, the compact or dwarf mutants that appeared in the present study were not desirable because their leaves were small, and they were not hardy and died before flowering. Compact growth habit has been observed as an induced mutation in genera such as *Begonia, Bidens, Callistephus, Dendranthema, Forsythia, Kalanchoe, Streptocarpus,* and *Weiggela* (Schum and Preil, 1998).

Changes in flower form have been reported in previous induced mutation experiments. In a study on carnations, 2 out of 426 lines that were exposed to 50 Gy of heavy ion beam radiation displayed a change in petal shape from serrate to rounded petals (Okamura *et al.*, 2003). Similarly, a 2 Gy dose of ion beam radiation resulted in a change in ray floret shape to produce double flowers in 1 out of 1,845 plants of chrysanthemum cultivar H13 when *in vitro* leaves were radiated (Matsumura *et al.*, 2010).

As recent advancements in molecular genetics have shown, mutant plants can be useful in elucidating underlying genetic mechanisms. The genome of the genus *Torenia* has not been as thoroughly studied as other model plants, but it would be interesting to perform further genetic analysis on the mutated plants from this study to try to determine the genes responsible for the observed changes in phenotype, such as erose petal margins and the lack of purple color in the petals. The results could also be compared to those of Nishijima and Shima (2006), who produced *Torenia fournieri* Lind. flowers with serrate petal margins by the application of 0.3, 3, or 30 μmol/L forchlorfenuron to developing flower buds (Nishijima and Shima, 2006). They concluded that as CPPU (forchlorfenuron; IUPAC = 1-(2-chloro-4-pyridyl)-3-phenylurea) inhibits cytokinin oxidase and thus induces the accumulation of active endogenous cytokinins, one result was the development of enlarged vascular bundles in the petal tissue. Serrate petal margins formed due to an uneven proliferation of cells around the vascular bundles.

CONCLUSION

Besides possible use in genetic studies, the mutant lines from the present research have potential use for further *Torenia* spp. breeding programs. Although *T. hybrida* is self sterile, the mutant lines, especially the one with erose petal margins, could possibly be developed through biotechnological techniques to create a varietal series of *Torenia* with crinkly-edged flowers. Preliminary assessment suggests that the mutant is robust and flowers profusely.

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