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THESIS

EFFECTS OF GAMMA RADIATION, COLCHICINE AND ORYZALIN ON THE PHENOTYPE OF *Torenia hybrida IN VITRO*

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Valerie Webb Suwanseree 2011: Effects of Gamma Radiation, Colchicine and Oryzalin on the Phenotype of *Torenia hybrida in vitro*. Master of Science (Tropical Agriculture), Major Field: Tropical Agriculture, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Thunya Taychasinpitak, M.S. 51 pages.

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 hours 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.

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Valerie Suwanseree
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INTRODUCTION

Torenia spp., or wishbone flower, is a branching annual in the family Scrophulariaceae that grows to a height of 30 cm in full sun or partial shade. The genus name comes from Reverend Olof Toren, an 18th century Swedish chaplain and apostle of Linnaeus who first described the species on a trip to Asia with the Swedish East India Company circa 1748-1762.

There are about 40 species in the genus *Torenia,* most of which are native to tropical and subtropical Asia (Aida 2008). Two of the species that are the most significant for commercial horticulture and are the ancestors of the hybrid used for this study are *Torenia concolor* and *Torenia fournieri.* Flora of China describes *Torenia fournieri* as an herb, 15-50 cm tall, with erect, subglabrous, quadrangular stem, that is simple or branched above the middle; petioles 1-2 cm; leaf blade oblong-ovate to ovate, 3-5 X 1.5-2.5 cm, subglabrous, margin coarsely serrate; racemes often terminal; bracts linear, 2-5 mm; pedicel 1-2 cm; calyx ellipsoid, 1.3-1.9 X ca. 0.8 cm, green or purple-red at apex and margin, 5-winged; wings decurrent, ca. 2 mm wide, becoming ca. 3 mm wide in fruit; lips subtriangular, 1.5-1.7 cm, sometimes apically lobed. The corolla is 2.5-4 cm, exceeding calyx by 1-2.3 cm; tube pale violet, upper side yellow; lower lip lobes purple-blue, middle lobe with a yellow patch near base, oblong to suborbicular, ca. 10 X 8 mm, subequal; upper lip pale blue, erect, broadly obovate, 1-1.2 X 1.2-1.5 cm, emarginate. The stamens are unappendaged. Capsules are narrowly ellipsoid, ca. 12 X 0.5 mm, and the seeds yellow. In South China it flowers from June to December and is cultivated as well as occurring wild by roadsides or in fields below 1,200 m. It is found in Fujian, Guangdong, Guangxi, Taiwan, Yunnan, Zhejiang, Cambodia, Laos, Thailand, and Vietnam (eFloras 2008).

Torenia concolor is described as a creeping herb with quadrangular stems, rooting from nodes; branches ascending or erect. The petioles measure 2-10 mm; leaf

blades are triangular-ovate, narrowly ovate, or rarely ovate-orbicular, 1-4 X 0.8-2.5 cm, glabrous or sparsely villous, at the base broadly cuneate to subtruncate; leaf margins serrate or crenate and serrate, apex obtuse to acute. The flowers are axillary or terminal,

solitary or in fascicles. The pedicels are 2-3.5 cm, growing to 5 cm in fruit. The calyx measures 1.2-1.7 cm, and up to 2.3 cm in fruit, with decurrent base; 5 wings, slightly more than 1 mm wide; lips narrowly triangular with 5 small lobes evident in fruit. The corolla is blue to blue-purple, 2.5-3.9 cm, exceeding calyx lobes by 1.1-2.1 cm. Anterior stamens have filiform appendages 2-4 mm long. The fruit is a capsule 1.5-1.8 cm long. It flowers from May to November and is found in forests, mountain valleys, and trailsides below 1,500 m in Guangdong, Guangxi, Guizhou, Hainan, Taiwan, Yunnan, Japan (Ryukyu Islands), Laos, and Vietnam (eFloras 2008).

Torenia spp. are valuable as ornamental plants because they bloom profusely during a long growing season in warm climates and can be used to decorate borders or flower beds and for hanging baskets. They can be propagated by seed or stem cutting. Breeding work over the past several decades, mainly by commercial breeders in the USA, Japan and Israel, has resulted in many attractive varieties. For example, Danziger's "Moon" series comes in white, yellow, and different shades of blue, lavender and purple. In 1988 Pan American Seed began selling its "Clown" series with pink, blue and reddish-purple flowers. Suntory's "Summer Wave" series, released in 1995, consists of heat-resistant varieties with flowers in shades of violet, magenta, and white. The "Duchess" series developed by Sakata Seed has a range of colors encompassing light blue, dark blue, burgundy, purple and pink. Likewise, Proven Winners' "Catalina" series features flower colors ranging from white to yellow, pink, violet, blue and amethyst. Rather than solid colors, most of the varieties on the market have two or more shades in each flower. However, there is still a need for more variation because consumers in the ornamental plant market constantly seek flowers that are new and different. As yet, to the best of our knowledge, no breeders have been able to produce Torenia with double flowers or other novel flower forms. One of the objectives of this research was to try to develop a variety with a new

variation in flower shape or form.

The hybrid used in this study was developed at Kasetsart University as a result of the master's of science thesis work of Yupaporn Paphan on Torenia hybridization (Paphan, 2009). It is a complex cross between a commercial *T. concolor* hybrid that was backcrossed with *T. fournieri*. It is somewhat different from other Torenia varieties in that it does not produce fertile seed and must be propagated vegetatively. The plants are average height of 15 cm with average spread of 32 cm. The flowers measure 3.5 X 4 cm on average and are light lavender on the ventral section of the petal, slightly darker lavender on the dorsal petal with a yellow spot in the top center, darker purple on the two side petals, and with yellow and very narrow magenta stripes inside the corolla tube.

The aim of this research was to attempt to produce changes in the morphology of *Torenia hybrida* through the application of anti-mitotic agents combined with gamma radiation, which could possibly result in plants with extra sets of chromosomes or genetic mutations that would be of value for horticultural trade, such as larger flowers, double flowers, or novel flower colors.

OBJECTIVES

1. To induce morphological changes in *Torenia hybrida* that may be of benefit for horticultural commerce

2. To study the effects of gamma radiation on *Torenia hybrida*

3. To compare the effectiveness of colchicine and oryzalin in inducing polyploidy in *Torenia hybrida*

4. To create a polyploid line of *Torenia hybrida* for use in breeding programs

LITERATURE REVIEW

1. Colchicine

Colchicine is an alkaloid obtained from the root of *Colchium autumnale* L. or *Iphigenia indica* Kunth et Benth that can be used to produce polyploid plants because it binds with tubulin and thus interferes with microtubule formation during mitosis (Kingsbury 2009).

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. 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).

Cohen and Yao (1996) produced tetraploid plants in eight cultivars of *Zantedeschia* by adding 0.05% (w/v) colchicine to the multiplication medium for shoot cultures for 1, 2 or 4 days. However, only 20% of the treated shoots survived, irrespective of the length of the colchicine treatment. They reported that for some of the cultivars tested, the tetraploid plants had thicker and larger leaves and spathes than the control (diploid) plants, but for some cultivars tetraploid plants could not be distinguished using morphological characters.

Takamura and Miyajima (1996) treated *in vitro Cyclamen persicum* Mill. 'Kage Yellow' tuber segments with 0, 20, 100 or 500 mg^{-l} colchicine for 1, 2, 4 or 7 days (incubated in the dark) and were able to obtain two solid tetraploids from the 100 mg^{-l} colchicine, 4 days treatment and two mixoploid from the 500 mg^{-l} colchicine, 4 days treatment. They reported that longer duration treatment with the high colchicine concentration was deleterious to plant regeneration but the lower concentrations and shorter durations did not result in polyploidy. In the study, Takamura and Miyajima (1996) found that the mean petal size, guard cell size and pollen diameter of the resultant tetraploids were larger than in their diploid relatives, and the petals had a deeper yellow color.

Rose *et al.* (2000) used colchicine to produce tetraploid *Buddleia globosa* for the purpose of introducing new flower colors through cross breeding. They exposed nodal sections of *B. globosa* grown *in vitro* to 0, 0.01% (0.25 mM), 0.05% (1.25 mM) and 0.1% (2.5 mM) colchicine for 1, 2 or 3 days and ultimately obtained 19 tetraploid and 5 mixoploid plants from the 29 lines that were successfully rooted and weaned. They observed that the tetraploids plants were more compact than diploid and had broader, thicker and more crinkled leaves and inflorescences that were more elliptical than spherical when compared to the diploid control.

In 2005 Escandon *et al.* in Argentina developed a protocol for raising 5 species of the native genus *Scoparia* (another member of the family Scrophulariaceae) via tissue culture and tested 4 concentrations of colchicine (0.001%, 0.01%, 0.05%, and 0.1% v/v) for 24 or 48 hours to try to induce polyploidy in *Scoparia montevididensis.* Upon flow cytometric analysis of the 364 recovered plants, they discovered that 4 were solid tetraploids and 16 were mixoploid chimera. Some of the tetraploids plants had larger flowers and leaves than the diploid control, which was determined to be of benefit for developing *Scoparia* as an ornamental crop.

Working with young flower buds of the *Lilium* cultivars 'Con. Amore' and 'Acapulco,' Wu *et al.* (2007) reported that treatment with 0, 0.02%, 0.05%, 0.1%, and 0.2% colchicine for 3 days *in vivo* (by wrapping greenhouse grown 0.5-cm long flower buds in cotton wool soaked in colchicine solution) resulted in a mutation rate of from 1.2% (the highest concentration of 0.2% colchicine on 'Con Amore') to 25.8% (0.1% colchicine on 'Acapulco'); although the colchicine treatment was quite destructive, with mortality rates of 5.7% to 81.3%. In Wu *et al.'s* study, the mutation rate (induction of diploid gametes) was assessed based on an increase in stigma and pollen size. The diploid gametes were crossed with normal haploid gametes to successfully produce triploid lilies.

In a study on *Phlox subulata* L., Zhang *et al.* (2008) also reported a difference in flower size and leaf width between 2*x* and 4*x* plants after subjecting *in vitro* 1-mm

shoot tips to 0, 0.005%, 0.01%, 0.02%, and 0.04% colchicine in the growing solution for 10, 20 or 30 days. They concluded that the duration of exposure to colchicine had a significant effect on survival rate and the occurrence of mixoploids.

Looking at research accomplished in Thailand, in a study on *Anthurium andraeanum* Lind 'Double Spathe,' callus tissue generated from seeds *in vitro* were exposed to concentrations of 0.25 mM, 1.25 mM and 2.5 mM colchicine for 24 or 48 hours. Some of the resulting regenerated plants had thicker leaves than the control, but none were found to be polyploid (Rungrueng, 1994). Similarly, no polyploid plants were detected in an experiment by Sutuntungjai (2001) in which 4 different concentrations of colchicine (0.2%, 0.4%, 0.6% and 0.8%) were applied to Gomphrena seedlings *in vivo*. Increasing concentrations of colchicine appeared to have a slight effect on days to flowering and flower width, but no changes in ploidy level were observed (Sutuntungjai, 2001).

Colchicine has also been successfully used to induce polyploidy in fruit crops such as citrus, banana, pear, pomegranate, grape and persimmon (Zeng *et al.*, 2006).

Several researchers have concluded that the application of colchicine *in vitro* is more effective than *in vivo*, partly because of the prevalence of chimera and the difficulty of isolating polyploid sectors in large growing plants and partly because of greater absorption through soft tissues when nodes or other plant parts can be bathed in colchicine solution or kept in contact with it under the high humidity conditions of tissue culture (Cohen and Yao 1996, Takamura and Miyajima 1996, Rose *et al.* 2000, Zhang 2008).

2. Oryzalin

Oryzalin (4-dipropylaminol-3,5-dinitro-benzenesulfonamide) is an herbicide that inhibits spindle fiber formation, disrupting normal mitosis. Sree Ramulu (1991) demonstrated its antimitotic activity in tobacco and potato. Van Tuyl (1992) found oryzalin to be more effective than colchicine in inducing polyploidy in *Lilium* spp.

and *Nerine* spp. Notably, oryzalin is less toxic to humans than colchicine because it binds preferentially to plant tubulin and not animal tubulin (Kermani *et al.* 2003, Dhooghe *et al*. 2009a). This is the major advantage of using oryzalin as an anti-mitotic agent to induce polyploidy.

Thao *et al*. (2003) compared the effectiveness of colchicine and oryzalin to induce polyploidy in *Alocasia micholitziana* 'Green Velvet.' They treated *in vitro* shoot tips with 0, 0.01%, 0.05%, or 0.1% colchicine or 0, 0.005%, 0.01% or 0.05% oryzalin for 24, 48, and 71 hours and examined the ploidy levels of the proliferated shoots 3 months later by flow cytometry. The survival rate was higher for shoot tips exposed to oryzalin compared to colchicine. The experiment resulted in 7 tetraploid plants (4.5%) and 13 mixoploid plants (8.4%) from the colchicine treatments and 15 tetraploid (6.8%) and 9 mixoploid (4.1%) plants from the orzyalin treatments. The mean ratio of leaf width to leaf length differed between 2*x* and 4*x* plants.

The variable of leaf width to leaf length ratio was also identified as significantly different among induced polyploids of *Rosa* in a study by Kermani *et al*. (2003). They used the diploid rose cultivar 'Thérèse Bugnet' as the model to attempt to establish the optimal method for inducing polyploidy in *Rosa* by testing oryzalin concentrations of 5 or 15 µM on *in vitro* shoot tips, for 24 h in liquid medium, followed by the same concentration in semi-solid medium for 13, 20 or 27 days or by leaving shoot tips in a solution of 5 or 15 µM oryzalin in liquid medium for a full 14 or 28 days. The researchers also tested cutting nodal section of 1 mm length from the *in vitro* shoots and exposing them to 5 µM oryzalin in solidified regeneration medium for 0-3 days. Based on the results (survival rate and polyploidy induction) the researchers chose to use an oryzalin treatment of 5 µM oryzalin in semi-solid medium for 14-28 days on 2 other diploid cultivars and 2 triploid cultivars of rose. Ploidy was tested using flow cytometry and confirmed following vegetative reproduction. The results showed that chromosome doubled plants had leaves that were thicker, darker green and had a greater leaf breadth/length ratio than their progenitors. The tetraploid 'Thérèse Bugnet' roses had significantly more petals than diploids, but this was not found in the hexaploids derived from triploids.

Allum *et al*. (2007) determined that for the *in vitro* application of oryzalin in *Rosa* spp. (5 µM for 6, 12, 24, or 48 hours) the incidence of polyploidy was significantly higher when the treatment was conducted on 2 mm node sections as opposed to 10 mm node sections. The former treatment resulted in 34.7% tetraploids + mixoploids after 12 hours, compared to 7.4% for the 10 mm node sections. However, the highest percentage of polyploids (67%) was achieved when 2 mm node sections were exposed to just 2.5 µM oryzalin for 48 hours. For the higher concentration of oryzalin (5 µM) the percentage of polyploids was lower with the longer exposure duration of 24 or 48 hours. Kermani *et al*. (2007) concluded that this was because the cell cycle was about 10 hours, and when exposed to high concentrations of oryzalin for longer than 12 hours the cells may have undergone a second spindle-inhibited mitosis, resulting and octoploid cells that caused retarded growth. If the high ploidy levels in reduplicated cells adversely affected growth, then it would give the remaining diploid cells an advantage in growing to be the main sections of the meristem. At the lower concentration, it was deduced that the oryzalin reached a greater proportion of meristematic cells through the cut surface of 2 mm node sections and those cells could grow to new shoots before the chromosomes reduplicated.

In an attempt to develop sterile clones of the ornamental shrub Japanese barberry, which has been identified as an invasive naturalized plant in North America, Lehrer *et al.* (2008) tested both colchicine and oryzalin on pre-germinated seeds of *Berberis thunbergii* var. *atropurpurea* with emerging radicles of 5-7 mm. For their experiments, the concentrations of 0.02%, 0.05%, 0.1% and 0.2% were used for colchicine (dissolved in aqueous solution) and the concentrations of 0.002%, 0.005%, 0.01% and 0.02% were used for oryzalin (dissolved in 1% dimethyl sulfoxide). The seeds were shaken in the solutions for 6, 12 or 24 hours. The results showed that seed survival was depressed by increasing concentrations of both mitotic inhibitors and by increasing exposure time. Overall, the seed survival rates were lower with oryzalin. Flow cytometric analysis revealed that 38% of the colchicine-treated seeds were tetraploid and 61% of the oryzalin-treated seeds were tetraploid. The researchers did not note any significant changes in morphology except for (undesirable) oddly shaped

leaves, irregular sectors or white tissue and thicker leaves on some of the tetraploid plants. Also, 4% of colchicine-treated plants and 2% of oryzalin-treated plants apparently reverted to diploid when re-tested after 52 weeks.

Dhooghe *et al*. (2009a) did a comparison study of the effectiveness of colchicine, oryzalin and trifluralin (another antimitotic agent) to induce polyploidy in *Ranunculus asiaticus* 'Alfa' grown *in vitro*. The survival rates and tetraploid induction rates (based on flow cytometric analysis) were analyzed. The colchicine treatments resulted in only 2 tetraploid plants (from the 100 µM colchicine 16 h treatment and from the 200 µM colchicine 16 h treatment). For oryzalin, 14 mixoploids and 12 tetraploids were produced and the highest percentage of polyploids (32.5%) was found with the use of 0.5 and 1 µM concentrations for the duration of 10 weeks. Dhooghe *et al*. (2009a) reported that oryzalin concentration significantly affected the survival rate, with overall mortality of almost 49% for the lowest concentration of 0.5 µM and 100% at 3 µM.

Dhooghe *et al.* (2009b) also compared the effectiveness of colchicine, oryzalin and trifluralin on 3 species of *Helleborus* grown *in vitro*. The results differed for the 3 different species. For *H. niger*, the treatments of 3 µM oryzalin for 12 weeks and 10 µM trifluralin for 12 weeks resulted in 4 tetraploid plants and 3 mixaploid plants, while 3 µM trifluralin for 12 weeks produced 1 tetraploid and 3 mixaploid. For *H. nigercors*, the treatment of 10 µM trifluralin for 12 weeks resulted in 1 tetraploid plant and 3 mixaploid plants, while 3 µM trifluralin for 12 weeks produced 1 mixaploid. For *H. orientalis*, none of the treatments were successful. The researchers concluded that trifluralin was the preferable mitotic agent because it had a limited effect on viability but a high rate of polyploidization.

Pickens *et al*. (2006) investigated the effects of different concentrations of colchicine and oryzalin on *Euphorbia pulchurrima* 'Winter Rose' leaf sections grown *in vitro* and found that the survival and growth rate was greater with colchicine. However, none of the regenerated shoots treated with colchicine were found to be tetraploid. Of the leaf sections treated with oryzalin, they produced callus but failed to

form adventitious shoots.

3. Gamma Radiation

Gamma rays can induce genetic mutations in plants. Early on, progress in this field was slow due to negative attitudes toward the high percentage of deleterious mutations often encountered (Wongpiyasatid, 2007). However, later researchers proved that it was possible to use gamma rays to advantage in developing many new types of crop plants with desirable characteristics, both food plants and ornamentals.

Examples of ornamental species in which new varieties have been produced from gamma ray induced mutations include *Dianthus* spp., *Chrysanthemum* spp., *Dahlia* spp., *Achimenes* spp., *Streptocarpus* spp., *Alstroemeria* spp., *Rosa* spp., and *Rhododendron* spp. (Sigurbjörnsson and Micke, 1974). In a 2008 report, Lee *et al.* cited 625 as the number of ornamental varieties that were developed through mutagenesis.

Mukherjee and Khoshoo (1970) subjected the rhizomes of four varieties of *Canna generalis* to 1, 2, and 3 kR of acute gamma radiation and found that greater than 62% of the irradiated plants exhibited morphological abnormalities including thinner than normal petals, misshapen petals, extra staminoidia, extra petals, fewer flowers per inflorescence compared to the control or missing flower parts. One color change was noted in the 'Rosamunda Coles' cultivar that was exposed to 1 kR of radiation, a change to a lighter shade of red and with more yellow spots.

Nakornthap (1973) exposed leaf petiole cuttings of *Kalanchoe laciniata* to 0, 10, 20, 30, 40 and 50 Gy of gamma radiation and reported that most of the plants regenerated from leaf petioles exposed to 50 Gy of radiation did not survive the treatment, but some interesting mutations were observed in the lower dose treatments. A dwarf plant with purplish leaves was selected as a possible new variety, while other variations were observed in leaf form (curly, changed from crenate margin to entire margin and changed from the normal ovate shape to nearly round).

In 1985, Kijpaitoon carried out a mutation breeding experiment on Begonia 'Bella Vista,' growing seeds *in vitro* and irradiating them with 0, 20, 40, 60, 80, and 100 Gy of gamma radiation. The most common mutation observed was dwarfism, which was reported in 11.1% of the plants from the 100-Gy treatment group, 6.5% in the 80-Gy group, 2.2% in the 60-Gy group, 2.0% in the 40-Gy group, 1.8% in the 20-Gy group and 0.7% in the control group. The researcher also noted some specimens with paler colored flowers, smaller flowers and larger flowers than the control. One specimen from the 20-Gy treatment group and one from the control group had thicker than normal leaves and these were identified as putative polyploids; however, the researcher was unable to confirm the ploidy level due to technical difficulties and because only a small amount of surviving plant tissue was available (Kijpaitoon, 1985).

In a *Curcuma sparganifolia* improvement project, Krasaechai (1990) subjected dormant rhizomes to 0, 20, 40, 60, 80, and 100 Gy of gamma radiation and found that radiation doses of 40 Gy or more inhibited sprouting entirely. For the second experiment, sprouting rhizomes were subjected to 0, 5, 10, 15, and 20 Gy of gamma radiation. All the radiated specimens sprouted fewer shoots and took longer to flower than the control (mean days to flowering was 57.6 for radiated plants, compared to 36.5 for the control). Two out of 13 surviving plants from the 20 Gy treatment group had striated leaves, and one also had darker colored bracts, but the mutation did not prove stable. The desired characters for commercial development of Curcuma were not achieved.

In a study to develop a variety of *Digitalis obscura* with a higher capacity to synthesize the commercially important secondary metabolite cardenolide, researchers in Spain grew digitalis *in vitro* and exposed the shoot tips to 0, 20, 40, 60, 80, and 100 Gy gamma radiation. The LD_{50} was found to be 60 Gy. No changes in morphology were observed. However, plantlets developed from irradiated shoot tips displayed a high variability in cardenolide production, and although 54% of them produced less than the control, some radiated specimens exhibited higher production than the control (Gavidia and Pérez-Bermúdez, 1999).

In a study on *Chrysanthemum moriflorum*, ray florets were cultured on MS medium supplemented with 10 mg/l BA to induce shoot formation, and the shoots were exposed to up to 100 Gy gamma radiation. All the plants from the treatment groups exposed to 50 Gy or higher subsequently died, but mutations were observed in the surviving plants from the 10 Gy and 30 Gy treatment groups. The mutations included changes in flower color (from medium purple to dark and lighter shades of purple, and yellowish) and flower form (flower size and number of ray florets). Interestingly, some changes in flower characters were also observed in the control plants that were multiplied *in vitro* but not exposed to radiation (Lamseejan *et al.*, 2000).

Three new varieties of perennial *Portulaca grandiflora* were developed at Kasetsart University as a result of a gamma radiation study (Wongpiyasatid and Hormchan, 2000). Stem cuttings of 2 double-flowered varieties were exposed to 0, 10, 20 and 40 Gy gamma radiation and the survival rate was 100% for all treatments. Several mutations were observed in flower color and flower form, but many of the changes did not prove to be stable when propagated, including an unstable mutation to undulate petal margins. The researchers noted that in *Portulaca*, mutations from orange to pink flowers were common, but not vice versa.

Koh and Davies (2001) experimented to develop a new variety of the Bromeliade *Tillandsia fasciculate* Swartz var. *fasciculate* with different colored or variegated leaves by subjecting seeds to 10-29 kR gamma radation, 0.1-3.2 kR combined thermal neutron and gamma radiation, 1.2% EMS×3 h and 0.4% EMS×5 h, then growing the seeds *in vitro*. They found that from 0.4% to 3.2% of seeds exposed to gamma radiation (12 kR to 27 kR) produced seedlings with yellowish-green leaves and from 1.2% to 4.4% produced seedlings with variegated leaves (15 kR to 27 kR). The mortality rate was 26.8% at the 27 kR dose, but 100% at 29 kR. For the combined thermal neutron and gamma radiation treatments, 0.4% to 2.4% of specimens produced seedlings with yellowish-green leaves (0.1 kR to 3 kR) and 0.4% to 1.6% produced seedlings with variegated leaves (0.1 kR to 3.1 kR). The mortality rate ranged from 8.8% at 0.1 kR up to 24% at 3.1 kR. However, Koh and Davies

reported that the radiated *Tillandsia* seedlings with variegated leaves were all sectorial or mericlinal mutants and the variegation was not preserved in subsequent leaf development.

Wongpiyasatid *et al*. (2007) achieved interesting results by subjecting leaf cuttings of African violet (*Saintpaulia ionantha*) to 0, 10, 20, 40 and 60 Gy of acute gamma irradiation. The mutation rate ranged from 5% at 10 Gy to 11.67% at 40 Gy and up to 18.33% at 60 Gy. In all, 23 mutations were observed, including white flowers (20.7%), darker violet flowers (19.0%), light blue flowers (13.8%), double flowers (3.5%) and pink flowers (1.7%). Some streaked and blotched colored petals were observed. Changes in leaf color, leaf margin, leaf thickness, leaf shape and leaf size were also noted. All of the mutations proved to be stable following subsequent propagation.

When *in vitro* stem segments of *Dendranthema grandiflorum* ‘Argus’ were exposed to 0, 30, 40 and 50 Gy gamma radiation Lee *et al*. (2008) identified 5 mutants (17%) that had different colors of disc and ray florets compared to the control as well as differences in flower diameter. Following vegetative reproduction, the mutations remained stable after 2 years in greenhouse conditions.

MATERIALS AND METHODS

1. Sterilization of explant material

Shoots of *T. hybrida* approximately 6 weeks old were cut to a size of 3-7 cm and stripped of leaves. The shoots were washed with soap (Teepol) and water, rinsed in 70% ethyl alcohol, then immersed in 5% sodium hypochlorite solution (with 1 drop Tween per 100 ml) and agitated for 5 minutes, and finally immersed in 10% sodium hypochlorite solution (with 1 drop Tween per 100 ml) and agitated for 10 minutes. The shoots were then rinsed in distilled water 6 times and the nodes (5-10mm) were excised and placed in test tubes with Murashige and Skoog (MS) medium (Murashige and Skoog, 1962).

2. *In vitro* multiplication

The nodes were kept at 25 $\pm$ 2° C with light for 16 hours/day at intensity 60 $\pm$ 5 μmol/m^2/s from a fluorescent bulb (TLD 36W/84 3350 Im Philips Thailand). After new shoots had formed the plantlets were subcultured by excision of nodes (5-10 mm) and transferred to new culture vessels (8-ounce glass jars containing 25ml culture medium) every 5-6 weeks (3 nodes per culture vessel).

3. Colchicine and oryzalin treatments

The methods for treating excised nodes with colchicine and oryzalin were performed in the same way.

Stock solutions were prepared with a concentration of 100 ppm colchicine or oryzalin (the colchicine was obtained from tablets for medicinal use). The solutions were filtered through 0.2 μm sterile filters added to liquid MS medium in 250-ml glass flasks with a micropipette to form final concentrations of 0.0025 mM, 0.0075 mM and 0.0125 mM colchicine or 0.0028 mM, 0.0086 mM and 0.0144 mM oryzalin. Liquid MS medium with nothing added was used for the control. Node segments (5-

10 mm length) of *in vitro T. hybrida* shoots were excised and transferred to the colchicine or oryzalin solutions in liquid medium (or control) and left on a shaker table to be agitated for 48 or 72 hours. The treatments were as follows:

Colchicine treatments

Treatment 1 0.0025 mM colchicine 48 hours
Treatment 2 0.0025 mM colchicine 72 hours
Treatment 3 0.0075 mM colchicine 48 hours
Treatment 4 0.0075 mM colchicine 72 hours
Treatment 5 0.0125 mM colchicine 48 hours

Oryzalin treatments

Treatment 1 0.0028 mM oryzalin 48 hours
Treatment 2 0.0028 mM oryzalin 72 hours
Treatment 3 0.0086 mM oryzalin 48 hours
Treatment 4 0.0086 mM oryzalin 72 hours
Treatment 5 0.0144 mM oryzalin 48 hours

Following exposure to colchicine and oryzalin solutions (or liquid MS medium with no additives for the control), the nodes were rinsed in sterile distilled water and aseptically transferred to fresh MS medium. After development of new shoots for 6-8 weeks after treatment, plantlets were subcultured as described above and multiplied in preparation for gamma radiation treatment or planting out.

4. Gamma radiation

For the initial gamma radiation test, 12 samples from the 0.0025 mM/48h colchicine treatment (subcultured twice after treatment) were subjected to chronic gamma radiation from a $Cobalt^{60}$ source at the Kasetsart University Gamma Radiation and Nuclear Technology Research Service Center at the following rates: 12 Gy (2 days), 18 Gy (3 days), 24 Gy (4 days), 32.6 Gy (5 days), 38 Gy (6 days) and 44.7 (7 days). The irradiated samples were transferred to fresh culture medium within

48 hours after exposure to radiation. The LD_{50} was calculated based on the survival rate after 30 days.

For the acute gamma radiation test, 4 culture vessels (containing 4 plantlets each) from each of the 5 colchicine treatments were radiated at the rate of 0, 30, 40, and 50 Gy in a Gamma Irradiator Model Mark I-30 delivering 4500 Ci of Cs-137 at the Gamma Irradiation Service and Nuclear Technology Research Center, Kasetsart University. The irradiated samples were transferred to fresh culture medium within 48 hours after exposure to radiation and control plantlets were subcultured at the same time. The LD_{50} was calculated based on the survival rate after 30 days.

The initial gamma radiation trials were made using samples from the colchicine treatments. Based on the results, additional samples from the oryzalin treatments were exposed to 60 Gy of acute radiation following the same method.

5. Transplantation

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

The surviving plants were transferred to 12 cm pots when they were approximately 6-10 cm tall, and later to 20 cm hanging baskets when they were approximately 16-20 cm tall.

6. Stomata measurement

Impressions were made of the abaxial leaf surface of randomly sampled leaves (3 mature leaves per plant) by applying a coat of clear nail varnish and waiting

45 minutes 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 40X 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 stomata guard cell size. The length of guard cells and the number of stomata per screen (per microscope field) were recorded for 4 microscope fields for each leaf sample.

Putative polyploids were selected for confirmation of DNA content analysis by flow cytometry based on having mean stomata guard cell length that was 30% ≥ the mean of the controls or based on having mean number of stomata per screen greater than the mean of the controls plus twice the standard deviation of the controls.

7. 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 microlitres of Partec CyStain (a one-step extraction and DAPI stain solution) and filtered through a 30µ filter before being analyzed in a Partec PAII flow cytometer.

8. Statistical analysis

Statistical analysis was performed using Microsoft Excel (mean, range, standard deviation, *t*-test).

PLACE AND DURATION

The experiments were conducted at the National Genetic Engineering and Biotechnology Center (BIOTEC), Thailand Science Park, Pathum Thani, Thailand and at Kasetsart University, Bangkhen Campus, Bangkok, Thailand from May 2008-October 2010.

Flow cytometry was performed at the plant biotechnology lab of Associate Professor Dr. Julapak Khunwongs, Department of Horticulture, Kasetsart University, Kamphaeng Saen Campus.

RESULTS

1. Colchicine and oryzalin treatments

The growth rates of *in vitro* plantlets regenerated from excised nodes that were exposed to colchicine (5 treatments) and oryzalin (5 treatments) and controls were compared at 2, 4, and 6-week intervals (Table 1).

Table 1 Comparison of *in vitro Torenia hybrida* shoot growth rates at 2, 4, and 6 weeks following treatment with different concentrations/durations of colchicine and oryzalin

Treatment	Mean height 2 weeks (cm)	Mean height 4 weeks (cm)	Mean height 6 weeks (cm)
0.0025 mM colchicine 48 h	0.44 ± 0.22*	1.19 ± 0.66	2.46 ± 1.12
0.0025 mM colchicine 72 h	0.34 ± 0.14	0.77 ± 0.43	1.49 ± 0.84*
0.0075 mM colchicine 48 h	0.50 ± 0.24	1.18 ± 0.86	2.17 ± 0.88
0.0075 mM colchicine 72 h	0.49 ± 0.22*	1.15 ± 0.61	2.60 ± 1.11
0.0125 mM colchicine 48 h	0.68 ± 0.20	1.47 ± 0.46	2.89 ± 1.83
0.0028 mM oryzalin 48 h	0.76 ± 0.27	1.19 ± 0.80	3.56 ± 2.13*
0.0028 mM oryzalin 72 h	0.57 ± 0.22*	1.02 ± 0.74*	3.20 ± 1.45
0.0086 mM oryzalin 48 h	0.64 ± 0.30	0.87 ± 0.59*	2.50 ± 1.78
0.0086 mM oryzalin 72 h	0.75 ± 0.36	1.31 ± 0.56	3.61 ± 2.38*
0.0144 mM oryzalin 48 h	0.58 ± 0.32*	0.77 ± 0.40	2.99 ± 1.53
Control	0.77 ± 0.41	1.47 ± 0.86	2.67 ± 1.85

Note: All figures indicate the mean height plus or minus standard deviation.

* Indicates that the value differs from the control to a statistically significant degree according to independent samples t-test, $\alpha = 0.05$.

As seen in Table 1, when shoot height was measured after 2 weeks and after 4 weeks, all the plants treated with colchicine or oryzalin had a slower growth rate than the control, although the difference was statistically significant only for 4 of the

treatments at 2 weeks (0.0025 mM colchicine 48 h, 0.0075 mM colchicine 72 h, 0.0028 mM oryzalin 72 h and 0.0144 mM oryzalin 48 h) and 2 of the treatments at 4 weeks (0.0082 mM oryzalin 72 h and 0.0086 mM oryzalin 48 h).

However, at 6 weeks there was difference in growth rate between the plants treated with colchicine and those treated with oryzalin. Almost all the plants treated with colchicine had a mean height that was less than the control, with the exception of the 0.0125 mM colchicine 48 h treatment, which had a mean height that was greater than the control, but not to a statistically significant degree. By contrast, almost all the plants treated with oryzalin showed a mean height that was greater than the control at 6 weeks, with the exception of the 0.0086 mM oryzalin 48 h treatment, but the difference in height for that treatment was not statistically significant.

When the colchicine- and oryzalin-treated plants (those that were not subsequently radiated with gamma radiation) were acclimated and removed from sterile 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 Torenia. Thus, leaf samples were taken and leaf surface impressions were made to observe under a light microscope to detect possible differences in the size of stomata guard cells and the number of stomata per unit of leaf surface area, which can be an indicator of polyploidy (Cohen and Yao 1996).

Analysis of the data on stomata size and frequency did not reveal a great difference in the size of stomata guard cells in any of the experimental samples compared to control. For 18 of 122 samples (2 from the 0.0025 mM colchicine 48 h treatment, 1 from the 0.0075 mM colchicine 48 h treatment, 4 from the 0.0075 mM colchicine 72 h treatment, 2 from the 0.0125 mM colchicine 48 h treatment, 2 from the 0.0028 mM oryzalin 48 h treatment, 2 from the 0.0028 mM oryzalin 72 h treatment, 3 from the0.0086 mM oryzalin 48 h treatment, and 2 from the 0.0086 mM

oryzalin 72 h treatment), the mean guard cell length was 20% longer than the control and for 9 of 122 samples (1 from the 0.0025 mM colchicine 48 h treatment, 4 from the 0.0075 mM colchicine 72 h treatment, 2 from the 0.0125 mM colchicine 48 h treatment, and 2 from the 0.0086 mM oryzalin 48 h treatment) the mean guard cell length was 30% longer than the control. It should be noted that in other studies, stomata guard cell length in polyploid plants has been found to be approximately double or more than that of the control diploid plants.

As for the measurements of number of stomata per leaf unit area, the results showed that the mean number of stomata recorded was higher than the control for 39 of 58 colchicine-treated samples and 63 of 64 oryzalin-treated samples. Of those, the mean number of stomata was higher than the control plus one standard deviation of the control for 39 of 58 colchicine-treated samples and 40 of 64 oryzalin-treated samples. 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 (1 from the 0.0028 mM oryzalin 48 h treatment, 6 from the 0.0028 mM oryzalin 72 h treatment, 2 from the 0.0086 mM oryzalin 48 h treatment, 5 from the 0.0086 mM oryzalin 72 h treatment and 7 from the 0.0144 mM oryzalin 48 h treatment). It should be noted that the oryzalin-treated plants were transferred to the field later than the colchicine-treated plants, meaning most of them were grown outdoors starting at the end of the dry season or the beginning of the rainy season, as compared to the colchicine-treated plants that were grown outdoors during the dry season. Control plants were transferred to the field during both time periods.

Based on these results, 6 of the surviving experimental plants were selected for ploidy analysis by flow cytometry based on having mean stomata guard cell length that was 30% greater than control and 21 plants were selected for having mean number of stomata per leaf unit area that was greater than the mean of the control plus 2 times the standard deviation of the control. Flow cytometry results revealed that none of the selected samples were polyploid. Only one sample (from the 0.0028 mM 48 h oryzalin treatment) was identified as possible aneuploid or mixoploid from

having a fluorescence peak that was broader than control.

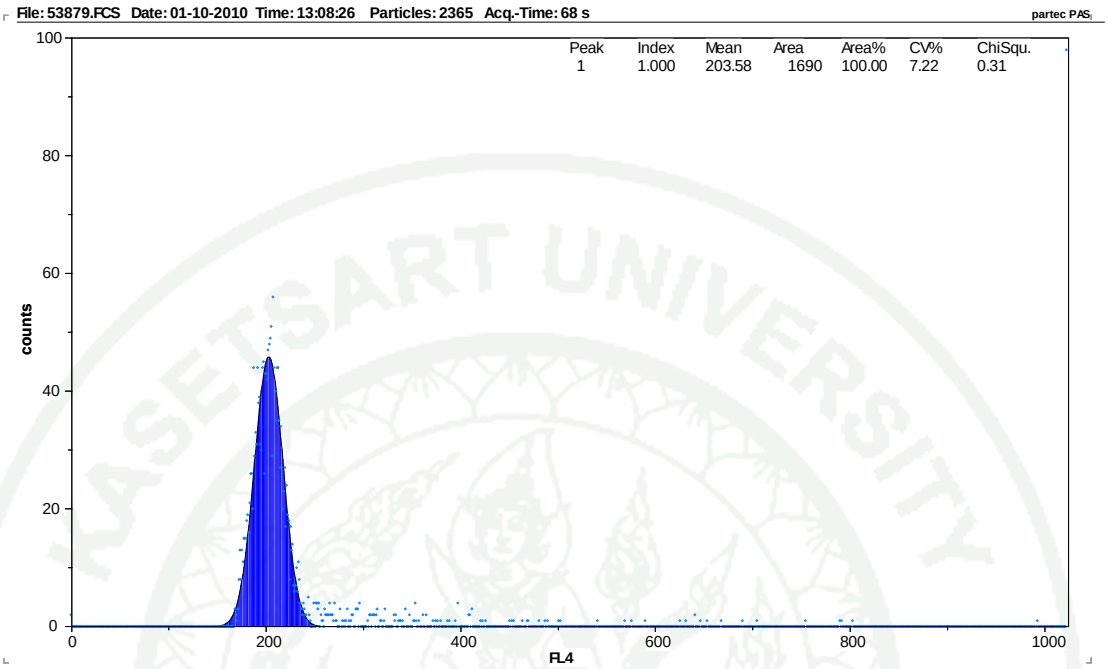

Figure 1 Flow cytometry histogram of control (diploid) *Torenia hybrida* showing a single peak at 203 nm.

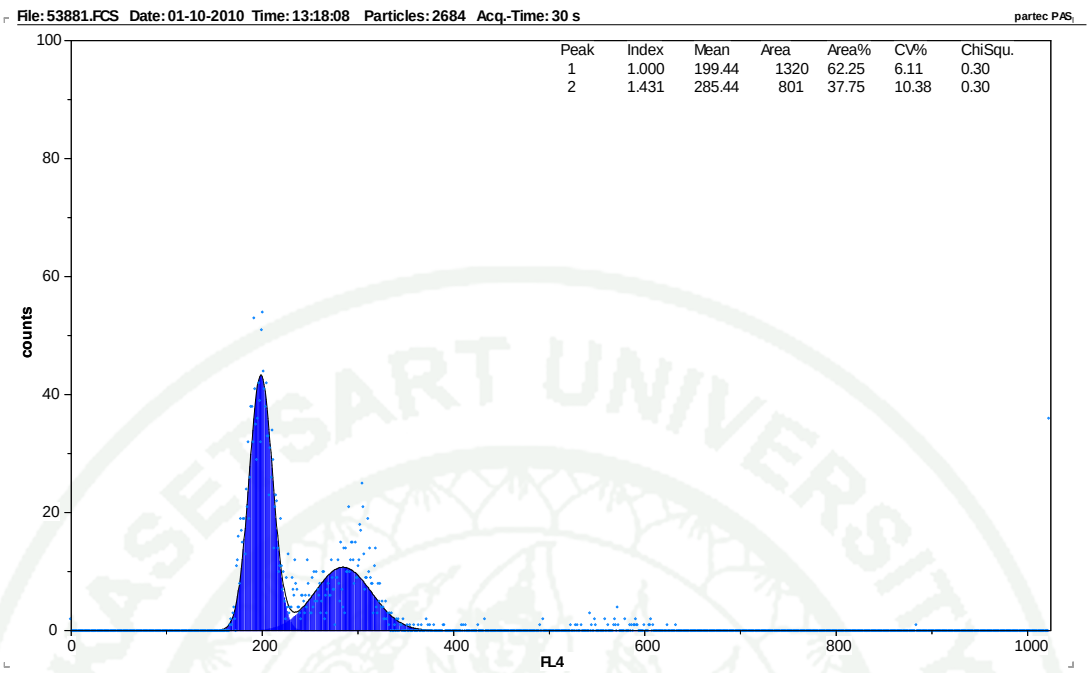

Figure 2 Flow cytometry histogram of probable aneuploid *Torenia hybrida* subjected to 0.0028 mM oryzalin for 48 hours showing a second, lesser peak at 285 nm.

In summary, the *in vitro* colchicine and oryzalin treatments did not result in polyploid Torenia in this study.

2. Gamma radiation

Results could not be properly recorded for the initial trial of chronic gamma radiation of 0, 12, 18, 24, 32.6, 38 and 44.7 Gy because when the plants were taken from the laboratory, removed from culture vessels and grown in the lathe house their creeping stems sent down roots in adjacent pots, so it was not possible to distinguish which stems originally came from which specimens. This was a problem that was rectified in the later stages of the project by keeping the pots of plants from each experimental treatment group farther apart, and by growing them in hanging pots.

For the acute gamma radiation experiments, 4-week-old *in vitro* plantlets that had been earlier treated with colchicine were exposed to 30 Gy, 40 Gy and 50 Gy gamma radiation and 4-week-old *in vitro* plantlets that had been earlier treated with oryzalin were exposed to 60 Gy gamma radiation. They were subcultured within 48 hours following the radiation, and control plants that had been treated with the same colchicine or oryzalin treatments but had not been irradiated were subcultured on the same day. The survival rate was recorded after 30 days (Tables 2, 3, 4 and 5).

Table 2 Survival rate of *T. hybrida* plantlets *in vitro* 30 days after acute gamma irradiation at the rate of 30 Gy compared to controls that were subcultured on the same day.

No. nodes day 1	Nodes Surviving day 30	Survival rate (%)	No. nodes day 1	Nodes surviving day 30	Survival rate (%)
0.0025 mM 48 h colchicine (no radiation)			0.0025 mM 48 h colchicine + 30 Gy γ		
15	15	100	15	15	100
0.0025 mM 72 h colchicine (no radiation)			0.0025 mM 72 h colchicine + 30 Gy γ		
15	12	80	15	9	60
0.0075 mM 48 h colchicine (no radiation)			0.0075 mM 48 h colchicine + 30 Gy γ		
15	14	93	15	15	100
0.0075 mM 72 h colchicine (no radiation)			0.0075 mM 72 h colchicine + 30 Gy γ		
15	13	87	15	12	80
0.0125 mM 48 h colchicine (no radiation)			0.0125 mM 48 h colchicine + 30 Gy γ		
15	15	100	15	15	100

Table 3 Survival rate of *T. hybrida* plantlets *in vitro* 30 days after acute gamma irradiation at the rate of 40 Gy compared to controls that were subcultured on the same day.

No. nodes day 1	Nodes surviving day 30	Survival rate (%)	No. nodes day 1	Nodes surviving day 30	Survival rate (%)
0.0025 mM 48 h colchicine (no radiation)			0.0025 mM 48 h colchicine + 40 Gy γ		
15	15	100	15	13	87
0.0025 mM 72 h colchicine (no radiation)			0.0025 mM 72 h colchicine + 40 Gy γ		
15	15	100	15	15	100
0.0075 mM 48 h colchicine (no radiation)			0.0075 mM 48 h colchicine + 40 Gy γ		
15	15	100	15	15	100
0.0075 mM 72 h colchicine (no radiation)			0.0075 mM 72 h colchicine + 40 Gy γ		
15	15	100	15	14	93
0.0125 mM 48 h colchicine (no radiation)			0.0125 mM 48 h colchicine + 40 Gy γ		
15	15	100	15	15	100

Table 4 Survival rate of *T. hybrida* plantlets *in vitro* 30 days after actue gamma irradiation at the rate of 50 Gy compared to controls that were subcultured on the same day.

No. nodes day 1	Nodes surviving day 30	Survival rate (%)	No. nodes day 1	Nodes surviving day 30	Survival rate (%)
0.0025 mM 48 h colchicine (no radiation)			0.0025 mM 48 h colchicine + 50 Gy γ		
16	15	94	16	14	88
0.0025 mM 72 h colchicine (no radiation)			0.0025 mM 72 h colchicine + 50 Gy γ		
16	12	75	16	13	81
0.0075 mM 48 h colchicine (no radiation)			0.0075 mM 48 h colchicine + 50 Gy γ		
16	15	94	16	15	94
0.0075 mM 72 h colchicine (no radiation)			0.0075 mM 72 h colchicine + 50 Gy γ		
16	15	94	16	16	100
0.0125 mM 48 h colchicine (no radiation)			0.0125 mM 48 h colchicine + 50 Gy γ		
16	13	81	16	16	100

Table 5 Survival rate of *T. hybrida* plantlets *in vitro* 30 days after acute gamma irradiation at the rate of 60 Gy compared to controls that were subcultured on the same day.

No. nodes day 1	Nodes surviving day 30	Survival rate (%)	No. nodes day 1	Nodes surviving day 30	Survival rate (%)
0.0028 mM 48 h oryzalin (no radiation)			0.0028 mM 48 h oryzalin + 60 Gy γ		
24	23	96	24	18	75
0.0028 mM 72 h oryzalin (no radiation)			0.0028 mM 72 h oryzalin + 60 Gy γ		
24	24	100	24	23	96
0.0086 mM 48 h oryzalin (no radiation)			0.0086 mM 48 h oryzalin + 60 Gy γ		
24	23	96	24	18	75
0.0086 mM 72 h oryzalin (no radiation)			0.0086 mM 72 h oryzalin + 60 Gy γ		
24	23	96	24	21	88
0.0144 mM 48 h oryzalin (no radiation)			0.0144 mM 48 h oryzalin + 60 Gy γ		
24	24	100	24	21	88

The survival rate was well above 50% for almost all of the radiated plants, so it was not possible to calculate the LD_{50}. The survival rate was noticeably lower than the control at the radiation dosage rates of 40 Gy and 60 Gy.

Data on growth rates of radiated plants was not recorded for those treated with colchicine and radiated with gamma radiation at the rates of 30, 40 and 50 Gy. However, for the plantlets that were treated with oryzalin first and then irradiated with 60 Gy of gamma radiation, during the tissue culture multiplication stage an observable difference was noticed in the growth rate following radiation and the plant height was measured at 30 days (Table 6).

Table 6 Mean height of *in vitro* plantlets after 30 days: comparison of plantlets previously treated with oryzalin only (the control plants, subcultured on the same day) and plantlets previously treated with oryzalin and subsequently subjected to gamma irradiation at the rate of 60 Gy.

Treatment	Mean height (mm) ± SD	Mean height (mm) ± SD
	Radiated at 60 Gy	Not radiated (control)
0.0028 mM 48 h oryzalin	0.76 ± 0.50	1.22 ± 0.71
0.0028 mM 72 h oryzalin	0.51 ± 0.44	1.12 ± 0.66
0.0086 mM 48 h oryzalin	0.47 ± 0.38	1.48 ± 0.72
0.0086 mM 72 h oryzalin	0.62 ± 0.44	1.22 ± 0.57
0.0144 mM 48 h oryzalin	1.20 ± 0.75	0.79 ± 0.40

For the plants that were treated with colchicine and oryzalin and subsequently exposed to acute gamma radiation, after they 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 7, Figure 10). 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 on subsequent blooms from the same plants. However, detailed data were not collected on the frequency of streaked and variegated petals.

No variations in leaf color, shape or size were noted. 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 individual blossoms on any given plant. Flower color variations (pale blue petals and pink petals, Figures 6, 7 and 8) occurred in 2.69% of radiated specimens

and 0.69% exhibited a compact growth habit (Figure 3). However, the latter was not robust and died early. A few specimens exhibited a creeping growth habit (Figure 4), which could be a reversion to the habit of *T. concolor*, one of the ancestors of the hybrid. 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 9).

Stem cuttings were made of the plants with pink and pale blue flowers and those with erose petal margins to determine if the mutation was stable. After the first cycle, the mutations remained stable in 100% of the second generation plants (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).

Table 7 Percentage of mutations observed following gamma irradiation of *Torenia hybrida* plantlets *in vitro*.

Antimitotic treatment	**Gamma radiation dose**	**Number 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

Figure 3 *Torenia hybrida* with compact growth habit following exposure to 0.0125 mM colchicine for 48 h and 50 Gy gamma radiation (control plant on the right).

Figure 4 *Torenia hybrida* with creeping growth habit following exposure to 0.0086 mM oryzalin for 72 h and 60 Gy gamma radiation (control plant on the left).

Figure 5 Mutated chimera of *Torenia hybrida* with some pink flowers following exposure to 0.0025 mM colchicine for 72 h and 40 Gy gamma radiation.

Figure 6 Mutated *Torenia hybrida* flowers with paler color following exposure to 0.0075 mM colchicine for 48 h and 40 Gy gamma radiation, left and center; control on the right.

Figure 7 Mutated *Torenia hybrida* flowers with pink color following exposure to 0.0075 mM colchicine for 72 h and 40 Gy gamma radiation, left and center; control on the right.

Figure 8 Mutated *Torenia hybrida* flowers with pink color following exposure to 0.0025 colchicine for 72 h and 40 Gy gamma radiation, left and center; control on the right.

Figure 9 Mutated *Torenia hybrida* flowers with erose petal margins following exposure to 0.0075 mM colchicine for 48 h and 30 Gy gamma radiation (control on the right).

Figure 10 Examples of *Torenia hybrida* flowers with streaked, speckled or mottled petals. This variation was observed in plants from all colchicine, oryzalin and gamma radiation treatments as well as the control.

DISCUSSION

Colchicine and oryzalin

In this study, neither the colchicine nor the oryzalin treatments succeeded in inducing chromosome doubling in *Torenia hybrida*. One possible reason may be that the concentrations of colchicine and oryzalin used were rather low and the durations rather short compared to those used by other researchers. The purpose for using relatively low concentrations was to maintain a high survival rate. In this study, we used 0.0025 mM (1 ppm) for 48 or 72 h, 0.0075 mM (3 ppm) for 48 or 72 h, and 0.0125 mM (5 ppm) for 48 h for the colchicine treatments and 0.0028 mM (1 ppm) for 48 or 72 h, 0.0086 mM (3 ppm) for 48 or 72 h and 0.0144 mM (5 ppm) for 48 h for the oryzalin treatments.

By comparison, Takamura and Miyajima (1996) succeeded in producing tetraploids Cyclamen using a concentration of 25 mM colchicine with 4 day exposure time and reported that lower concentrations and shorter duration treatments were not effective.

Rose (2000) used concentrations of 0.25 mM colchicine with 48 h exposure time and 1.25 mM colchicine for 48 h to induce polyploidy in Buddleia, but a concentration of 2.5 mM colchicine for 72 h was reported as the most effective.

In the research on Zantedeschia undertaken by Cohen and Yao (1996), 19.5% of samples were identified as tetraploids following exposure to a concentration of 1.25 mM colchicine for 1 to 4 days.

Zhang *et al*. (2008) reported that for Phlox, up to 75% of their specimens were shown to be tetraploid after application of 0.5 mM colchicine for 30 days, 66.7% tested as tetraploids after application of 0.25 mM colchicine for 30 days, 66.7% tested as tetraploids after application of 1.00 mM colchicine for 20 days and 26.7% tested as

tetraploids after application of 0.125 mM colchicine for 10 days.

Escandon *et al*. (2005) used concentrations of as low as 0.00125 mM colchicine (the highest concentration used in the present study) for 24 h and .025mM for 24 and 48 h to induce polyploidy in Scoparia.

As for oryzalin, Thao *et al.* (2002) used concentrations of 0.144 mM and 0.289 mM (both for 48 h) to induce chromosome doubling in Alocasia.

Lehrer *et al*. (2008) reported success in inducing tetraploidy in Berberis seeds after exposure to concentrations of 0.058 mM, 0.144 mM, 0.289 mM and 0.58 mM for 6, 12 and 24 h but found that the concentration of 0.289 mM was the most effective.

Allum *et al.* (2007) were successful in inducing polyploidy in Rosa when node sections were treated with 0.0025 mM oryzalin *in vitro*. They noted that the treatment was more effective on 2-mm node sections than 10-mm node sections, due to greater absorption. The node sections used in the present study were of variable length, but close to 8-10 mm long. This may have been a factor affecting the success of polyploidy induction, and it would be advisable to cut the node sections to a shorter length in any subsequent experiment on Torenia.

Kermani *et al*. (2003) found that placing Rosa nodes in semi solid tissue culture medium containing 0.005 mM oryzalin for 28 days resulted in 20% tetraploids but the same concentration for 14 days resulted in 40% tetraploids.

Dhooghe *et al*. (2009 a and b) used concentrations of only 0.0005 mM and 0.001 mM oryzalin, but for a longer duration of 10 weeks, to induce polyploidy in Ranunculus and 0.003 mM for 12 weeks to induce polyploidy in Helleborus.

Pickens *et al.* (2006) attempted to induce polyploidy in Euphorbia using concentrations of 0.0289 to 0.144 mM, but the experiment did not result in any

polyploid plants, just as in the present study.

Other researchers have also reported negative results (no polyploidy), such as Rungrueng (1994), who did not detect any polyploid plants after exposing Anthurium 'Double Spathe' callus tissue to 0.25 mM, 1.25 mM and 2.5mM colochicine for 24 or 48 hours.

Another explanation as to why the colchicine and oryzalin treatments failed to induce any tetraploids in this study could be simply that the sample size was too small. Due to limited lab space and time, the number of *in vitro* plantlets available for treatment was relatively small, at approximately 24 excised nodes per treatment. This reduced the chances of success with the colchicine and oryzalin treatments.

For further studies on *Torenia hybrida* it would be advisable to increase the concentration of anti-mitotic agents (because the survival rate was very high in this study) and increase the exposure time as well as cutting the nodes to shorter than 8 mm.

Gamma radiation

It is very difficult to conclude if the observed variations in phenotype were a result of the gamma irradiation only, the colchicine or oryzalin treatments, a combination of the two, or possibly somaclonal variation due to the extended tissue culture period of up to seven months. In addition to inducing polyploidy, colchicine has been reported to cause mutations such as changes in flower color in several ornamental plants (Wongpiyasatid, 2007). For instance, in a study on Cyclamen, Takamura *et al.* (1996) noted that some of the colchicine-treated plants had a deeper yellow shade of flowers. Rungrueng (1994) reported that some of the specimens of Anthurium 'Double Spathe' regenerated from callus tissue that were exposed to 1.25 mM – 2.5 mM colchicine for 24 and 48 h developed thicker leaves than the control and yellow blotches on the leaves, but the plants did not test positive as tetraploids.

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. This finding is similar to a report on gamma-ray induced morphological changes in Chrysanthemum by Lamseejan *et al.* (2000), in which ray florets were cultured *in vitro,* exposed to 10 or 30 Gy gamma radiation, and subcultured 3 more times before regenerated plants were planted out. Lamseejan *et al.* (2000) reported that changes in flower characters were observed in both the controls and the treated plants. Some of the control plants exhibited changes in flower size, number of ray florets and petal color (from light purple to darker purple). To elucidate the findings in the present study, further research could be conducted to investigate the rate of occurrence of variegated petals in *T. hybrida* following tissue culture.

All the plants, both 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 hours 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 3 mutated specimens were present by the stage of growing outdoors. As for the mutation to pink petals, two separate specimens were observed, one in the 0.0025 mM colchicine for 72 hours and 40 Gy radiation treatment group and one in the 0.0075 mM colchicine for 72 hours and 40 Gy radiation treatment group. The two were different shades of pink.

Some of the observed mutations in this study were similar to those described by Sasaki *et al.* (2008) following heavy ion beam irradiation on one wild type and four transgenic varieties of *Torenia*. They reported a wide range of color and coloration patterns in the radiated plants, including “tone-shifted, bordered, gradated, streaked, and tie-dyed” patterns. Interestingly, Sasaki *et al.* (2008) excluded the first

phenotypes at the beginning of the flowering period are sometimes unstable in 10 blooms from each plant in their data collection because they noted that flower Torenia, especially with regard to petal number and shape. Unfortunately, in the present study the data collection method for noting flower mutations was not as well refined. 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 this study, the mutations remained stable in 100% of the second generation plants. A third generation was not produced, however, due to limited time.

The color mutations observed in this study were also 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.

Besides the variations in flower color, in this study 8.82% of the plants from the 0.0075 mM colchicine for 48 h and 30 Gy gamma radiation treatment had crinkled or erose petal margins. Similar mutations have been observed in previous work. For example, in a study on carnation, 2 out of 426 lines that were exposed to 50 grays of heavy ion beam radiation displayed a change in petal shape from serrate to rounded petals, while the overall mutation rate on all 1,150 lines tested came out to 2.3% (Okamura *et al.*, 2003). Similarly, a 2-gray 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). Wongpiyasatid and Hormchan (2000) reported that when stem cuttings of perennial *Portulaca grandiflora* were exposed to 10, 20, and 40 Gy of gamma radiation, some of the plants reverted from double flowers to semi-double or single flowers. However, the mutation did not prove stable. In addition, in Wongpiyasatid and Hormchan's study, they observed some plants with undulate petal margin, and this mutation also did not remain stable following vegetative propagation.

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 rather than bushy appearance. Likewise, the compact or dwarf mutants that appeared in this 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).

In 1987, Brand and Bridgen 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, no leaf variegation nor chlorosis were observed, even though it is a common effect that has 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.

Another limitation of this study was that the irradiated plants were not tested for possible mutations that could affect their disease resistance, insect resistance, heat or cold tolerance or photoperiodic sensitivity.

As recent advancements in molecular genetics have shown, mutant plants can be useful in elucidating underlying genetic mechanisms. The genome of *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 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 (CPPU) to developing flower buds. They concluded that as CPPU 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

The objectives of this study were to induce morphological changes in *Torenia hybrida* that could be of benefit for horticultural commerce, to compare the effectiveness of colchicine and oryzalin in inducing polyploidy in *Torenia hybrida* and to create a polyploid line of *Torenia hybrida* for use in breeding programs. The results of the latter two objectives were inconclusive because no polyploid plants were detected.

However, the first objective was at least partially achieved because *Torenia hybrida* lines with two shades of pink flowers, one shade of pale blue flowers, and with crinkled or erose petal margins were identified. The lines with pink flowers and pale blue flowers are of dubious or little value for horticultural commerce for the reason that pink and light blue Torenia varieties are already available on the market. As for the line with erose petal margins, it is possible that it could be developed as a new variety with a novel flower form. A preliminary assessment suggests that it is robust and flowers profusely. It could possibly be crossed with other Torenia varieties using biotechnological techniques to create a series of Torenia with crinkly-edged flowers.

Horticultural value aside, the mutant lines from this research could also be useful for genetic research. Genetic analysis and comparison with other Torenia varieties could help elucidate the genes responsible for the phenotypes observed.

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