THESIS

BIOTECHNOLOGICAL TECHNIQUES FOR IMPROVEMENT OF NATIVE TORENIA AND THEIR HYBRIDS

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THESIS

BIOTECHNOLOGICAL TECHNIQUES FOR IMPROVEMENT OF NATIVE TORENIA AND THEIR HYBRIDS

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Torenia is considered as an important economic pot plant in Japan. Sterile or less fertile pollen occurred after interspecific breeding, so F1 pollen could not be used to breed. Hence, the objective of this experiment was to induce tetraploid and mutant induction by biotechnology techniques. Axillary buds (1 node cuttings) of new Thai native *Torenia fournieri* Lind. were exposed to acute (Cs-137) gamma ray irradiation at 0, 20, 40, 60, 80 and 100 grays. The results showed that GR_{50} was 68.83 gray, so axillary buds from the selected mutated plants from the first generation were then irradiated again at 0, 60, 65, and 70 grays. Morphological screening for mutations revealed 3 mutated phenotypes (pink, dark and wavy shaped flowers). Genetic variation was analyzed by HAT-RAPD technique. Polymorphisms revealed by the D30 and F29 random primers could be used to differentiate between the samples, but chromosome numbers (2n=2x=18) were not changed when the chromosome were observed by fluorescence *in situ* hybridization (FISH) analysis. The pollen of some mutants (wavy flower shape) was sterile. The plants with pink, dark and wavy petals were selected for possible development of new cultivars. Leaves were cut from F1 hybrids of *Torenia* for the different treatments. The petioles were soaked in 15 mgl^{-1} colchicine solution for 0, 12, 24, 48 and 72 h (32 leaves per treatment), after which they were placed upright in peat moss for rooting. The survival rate decreased when treatment duration was increased. The results showed that tetraploidy was induced in 3 clones from *T. asiatica* x *T. ranongensis*, 15 clones from *T. bentamiana* x *T. asiatica*, 24 clones from *T. asiatica* x *T. pierriana* and 18 clones from *T. fournieri* x *T. asiatica*. The leaf explants cultured on MS medium with Picloram added at every concentration tested formed soft, loosely aggregated callus tissue. Callus tissue was induced from leaves of diploid *Torenia* to the greatest degree (95 % callus formation at 3 weeks). For polyploid *Torenia*, the highest percentage of callus formation (92.5 %) was observed on leaves cultured on MS medium containing Picloram at the rates of 1.0 and 1.5 mgl^{-1} for 3 weeks in the dark. Following transfer of the embryogenic callus tissue to hormone-free MS medium under light conditions (16-h photoperiod), the greatest rate of somatic embryo formation was observed in the callus derived from the 1.5 mgl^{-1} Picloram treatment, in the case of both diploid and polyploid *Toreni*a accessions. The LD_{50} were 63.65 grays in diploid plants and 72.00 grays in polyploid plants from tissue culture.

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Nattapong Chanchula

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

MS medium	=	Murashige and Skoog medium
2,4-D	=	2,4-dichlorophenoxyacetic acid
BA	=	Benzyladenine
rRNA	=	Ribosomal ribonucleic acid
DNA	=	Deoxyribonucleic acid
HAT-RAPD	=	High annealing temperature-random amplified polymorphic DNA
FISH	=	Fluorescence *in situ* hybridization
DAPI	=	4',6-diamidino-2-phenylindole
LD_{50}	=	50 percent lethal dose
GR_{50}	=	50 percent growth reduction
Gy	=	Gray
Cs-137	=	Cesium-137
EMS	=	Ethyl methyl sulfonate

BIOTECHNOLOGICAL TECHNIQUES FOR IMPROVEMENT OF NATIVE TORENIA AND THEIR HYBRIDS

INTRODUCTION

Torenia spp., or wishbone flower, is an ornamental plant that is widely used as a summer garden plant. It is a branching annual in the family Linderniaceae that grows to a height of 30 cm in full sun or partial shade. The center of origin of the genus *Torenia* is uncertain (Fischer, 2004) but *Torenia* species are found growing as native vegetation in Southeast Asia, Africa and Madagascar (Yamazaki, 1985) and almost all species have been traced to tropical and subtropical areas in Asia, Africa and Madagascar. 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 are *T. concolor* and *T. fournieri.* At first, ornamental *Torenia* was only available with purple flowers for quite a long time. Then, in 1988, Pan America Seed Company in Illinois, USA, produced the Clown series with new pink, white and reddish-purple flowers. Later, several other series came out, including Summer Wave, Moon, Catalina, Panda and Lovely. These cultivars are very popular as potted plants and hanging basket plants in Japan, the USA and Australia (Aida *et al*., 2000). In Thailand, there is still a lack of new flower colors because the varieties available have only white, dark blue, purple and pink flowers. New cultivars have been developed through conventional breeding methods, such as inter-specific crosses, i.e. *T. benthamiana* with *T. asiatica*, *T. asiatica* with *T. pierreana*, *T. fournieri* with *T. pierreana* and *T. asiatica* with *T. concolor.* However, most of the inter-specific hybrids are sterile or have low fertility so they cannot be used for further breeding attempts. Many of them have small sized flowers with colors that are not bright. Thus, in this research, colchicine from gout medication tablets was utilized to try to induce polyploidy in hybrid *Torenia*. In addition, a new wild variety of *T. fournieri,* discovered in a survey of Thep Sathit District, Chaiyaphum Province, was selected for breeding because it carries genes for resistance to powdery mildew, a very common disease that most varieties of *T. fournieri* are susceptible to. Since the hybrids developed from this disease-resistant variety did not express disease resistance, mutation breeding through acute gamma radiation treatment and was utilized to try to induce mutations that could affect disease resistance as well as plant form, flower color and other characters. The single bud cutting method (propagating new plants from node sections with just one axillary bud) was used to facilitate selection of mutant sectors and avoid chimera. Lastly, an embryogenesis technique via tissue culture was also employed in this research as another method of mutation induction.

OBJECTIVES

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

2. To create new color of *Torenia fournieri* for use in breeding programs

3. To study the effects of gamma irradiation on *Torenia fournieri*

4. To study effects of 2,4-D and Picloram on *Torenia fournieri in vitro*

5. To create a tetraploid line of *Torenia hybrida* for use in breeding programs

6. To make an anatomical comparison of diploid and tetraploid *Torenia hybrida* and mutant of *Torenia fournieri*

LITERATURE REVIEW

Botanical characteristics of *Torenia* spp.

There are two kinds of *Torenia* species – annual and perennial. Annual species include *Torenia fournieri*, which has red, pink, dark purple and light purple flowers and often a yellow spot on the lower white petal, *T. flava*, which has yellow petals that are purple at the bases, and *T. violacea* (Azaola ex Blanco) Pennell, which has white flowers with purple spots on the sides. Annual species are mostly found growing in moist meadows or areas with sandy soil or damp conditions such as Phu Kradeung Mountain, Phu Luang Mountain and Phu Miang Mountain in Thailand. The most notable perennial species is perhaps *T. concolor* (Weesommai *et al*., 1997; notable perennial species is perhaps *T. concolor* (Weesommai *et al*., 1997; Yamazaki, 1985; Boufford *et al*., 1998; Taychasinpitak, 2002; Boufford *et al*., 1998).

Torenia spp. grow in small clumps with green to bronze leaves, and finely toothed leaf margins. The trumpet-shaped flowers are about one inch long and come out in racemes with 3-7 flowers per stalk, each inflorescence measuring 1.5-2.5 cm. The 5 unevenly sized petal lobes are fused at the base, forming a tube (Kaiyanam, 2008). The top petal lobe is usually light violet or white and the bottom 3 petal lobes may be light violet, dark purple or red. The corolla tube is white to yellow and there is a yellow dot in the middle of the bottom petal lobe. Two yellow stamens arise from either side of the base of the corolla tube and curve in towards each other in the center to form a “Y” or wishbone shape, which is the origin of the common name “wishbone flower” (Wanthanaput, 2002).

Cultivation and utilization

Torenia can flower continuously throughout the year and is not sensitive to seasonal triggers or day length (Wanthanaput, 2002). It prefers shade to partial shade and grows well in moist to wet soil, pH 5.5-6.5, in areas with sufficient drainage. *Torenia* requires warm temperatures for growth with the optimal flowering at temperatures ranging from 21-25 ˚C. It is heat resistant and resistant to many kinds of pests and some diseases, but cannot withstand saline soil, drought and very cold temperatures (Miyazaki, 2006; Fischer, 2004). *Torenia* is a popular ornamental for greenhouses, home gardens, and various landscaping situations such as in beds, borders, and pots, especially in shady areas. It can be planted along roads or paths, around buildings and is very useful in hanging baskets. It can be used as a ground cover and a trailing plant that can decorate rock gardens, rock walls and artificial mountain arrangements (Weesommai *et al*., 1997; Chaibreecha, 2001; Fischer, 2004; Kikuchi *et al*., 2000). Because the plant is adapted to living in shady, moist areas, it is often planted in partially shaded areas under trees or in pots placed in areas with partial sunlight or morning sunlight exposure (Kaiyanam, 2008). A single plant may be planted in a 3- or 4-inch diameter pot, 3-4 plants may be planted together in a 6-inch or larger pot, or several plants may be planted in larger containers (Wanthanaput, 2002).

Chromosome number of *Torenia*

Most *Torenia* species are diploid but the chromosome number varies with the species. As far as has been reported, the chromosome count of *T. fournieri* is 2n=2x=18; of *T. bailonii* is 2n=2x=16; of *Torenia* hybrid is 2n=2x=26 (Kikuchi *et al*., 2007); and of *T. benthamiana* is 2n=2x=36 (Hsieh and Yang, 2002). There is disagreement as to the standard chromosome count of *T. concolor*. Kikuchi *et al*. (2007) reported that is hybrid between those of *T. fournieri* and *T. concolor* has 2n=2x=26 but Hsieh and Yang (2002) reported that *T. concolor* has 2n=2x=34. The chromosome number of other *Torenia* species has not yet been reported.

***Torenia* spp. breeding**

In 1988 Pan American Seed (Illinois, USA) introduced the Clown series of cultivars with pink, white and purplish-red flowers. Breeders in other countries subsequently introduced many other series of cultivars including Summer Wave, Moon, Catalina, Panda and Lovely, which were well received by nurseries, growers and consumers in Japan, the USA, Canada and Australia, because before that *Torenia* was primarily available in purple only (Australian Government, 2006). There are many ways in which new cultivars have been developed, such as through conventional breeding, mutation breeding and biotechnology. The primary goals of breeders working through conventional methods are diversity, new flower colors, earlier flowering, longer blooming time and disease resistance (Australian Government, 2008). Mutation breeding has mainly been done with radiation. For instance, acute gamma radiation was used to induce changes in flower color and form (Jiranapapan, 2007). As for biotechnology approaches, Aida *et al.* (2000) stated that genetic engineering is an important technique for bringing about alterations in flower shape and color.

Commercial cultivars

Torenia is useful as a potted plant, and potted plants should have an appropriate height and growth habit that is in balance with the containers in which they are grown. Normally, they should reach a height of one to one and a half times the height of the pot, or about 20-25 cm in most cases, depending on the type of container used (Phapan, 2007).

There are several commercial cultivars of *Torenia* on the market today for use in borders, hanging baskets and other applications (Phapan, 2007), for example:

1. ‘Summer Wave’ annual, trailing, spread 70 cm, height 15-25 cm, heat resistant, disease resistant, easy to care for, fast growing, can be grown in the shade, in hanging pots or for landscaping

2. ‘Moon’ annual, trailing to semi-recumbent, large flowers, early flowering, mostly used in hanging baskets

3. ‘Duchess’ annual, erect habit, 15-20 cm tall, flowers starting at 60 – 70 days from germination, resistant to mealy bugs and powdery mildew, used for pots or landscaping

4. ‘Catalina’ annual, trailing, spread 25-40 cm, height 25 – 30 cm, can be grown in low-sunlight areas or sunny locations, good heat and disease resistance, easy to care for, used for hanging baskets or in beds.

Inter-specific hybridization

Artificial crossing by hand fertilization between plants of different species (inter-specific hybridization) or even different genera (inter-generic hybridization) has caught the attention of plant breeders ever since Thomas Fairchild successfully crossed a carnation with a sweet william (Sriwatanapong, 2009).

Inter-specific hybridization has yielded good results in ornamental plants and certain kinds of fruit trees that can be propagated by bud grafting, layering or cuttings. For plants that are mainly propagated by seed, inter-specific hybridization usually results in infertile hybrids, so it is not used as widely. Examples of some kinds of plants in which new cultivars have been developed through inter-specific hybridization are orchids, gladiolus, roses and tulips (Sriwatanapong, 1985).

Kikuchi *et al.* (2007) studied the reproduction and pollen tube growth of *T. fournieri, T. baillonii* and *T. concolor* by performing all possible crosses between the 3, and found that only 4 of the crosses resulted in fertile offspring, namely, the selfed offspring of the 3 species and the hybrid between *T. fournieri* and *T. baillonii.* The inter-specifid hybrid had flower color and form that were intermediate between the two parent plants and was a novel flower color.

Polyploidy in plants

Polyploidy is the phenomenon of a living thing having a double of the normal set of chromosomes, or even more than twice the normal set of chromosomes (3x, 4x, 6x, …). Polyploidy is much more common in the plant kingdom than in the animal kingdom (Sripijit, 2000).

There are 2 kinds of polyploidy organisms. Autopolyploid have more than one set of chromosomes from the same genome, and in cell division more than 2 pairs of homologous chromosomes match up. For instance, if the diploid genome was AA the autopolyploid will have a genome AAAA. This usually results in increased expression of all the genes and increased production, such as plant organ size. The other type of polyploidy is allopolypoid, which means the organism has incorporated another set of chromosomes from a different genome, usually from another species that is very closely related through evolution. Allopolyploid plants have arisen in nature through natural inter-specific hybridization. In most cases, inter-specific hybrids are infertile because the chromosomes from different genomes cannot match up properly during cell division. However, if there is an irregularity during meiosis or any other cause that results in chromosome doubling, then the organism will have homologous chromosomes and will be able to undergo cell division normally once again. Allopolyploids are also commonly more productive than their diploid ancestors (Kampiranon, 1993).

Polyploids are interesting to plant breeders because they tend to be larger, with some desirable traits such as thicker and longer leaves and larger flowers. Over the years, plant breeders have devised ways to artificially induce polyploidy in plants. These include:

- Decapitation: the apical meristem and axillary meristems are removed and the wounded cut areas are kept very moist by wrapping with peat moss. This may result in double shoots arising from the cut areas, which may be polyploidy.

- Heat treatment: in some cases, when plants are placed in a growth chamber heated to 38-45º C, or when the apical meristem is wrapped in hot cloth, it will induce a new shoot to arise that has double the normal chromosome number.

- Chemical treatment: several chemical substances have been discovered to induce polyploidy in plants, including colchicine, oryzalin, dimetyl sulfoxide or DMSO, germicide and phenylmerculic-p-toluensulfonanilide (Prapa, 2000). When plant cells are exposed to these substances for longer than one cell cycle, the chromosomes may double without normal cell division occurring (Griesbach, 1987). The chemicals most commonly used for inducing polyploidy are colchicine and oryzalin.

Colchicine

Colchicine is an alkaloid obtained from the root of *Colchium autumnale* L., a wild plant native to the Mediterranean region. The bulbs and seeds of *Colchicum* contain 20 kinds of alkaloids, the main one of which is colchicine. In its isolated form, colchicine takes the form of light yellow needle shaped crystals. The chemical formula of purified colchicine is $C_{22}H_{25}NO_6$. It is easily soluble in alcohol, chloroform and cool water. Medicinally, it is used to treat gout (Popovice and Gasic, 1993). Today, besides *Colchium autumnale* L., colchicine can also be extracted from other species in the genus *Colchicum,* as well as species in the genus *Merendera* and *Gloriosa,* such as *Gloriosa superba,* a member of the Liliaceae family. Colchicine can be used to produce polyploid plants because it binds with tubulin and thus interferes with microtubule formation during mitosis (Kingsbury 2009).

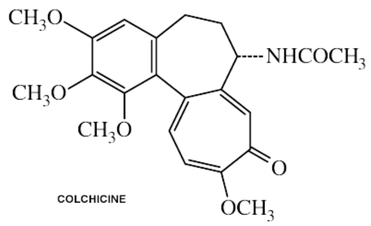


Figure 1 Chemical formula and structure of colchicine

Pure colchicine has a toxic effect similar to that of arsenic. Contact with it can be dangerous, and it causes extreme irritation if it gets in a person's eyes. If ingested it will have a strong toxic effect on the digestive system, in particular the stomach and intestines. In high doses, colchicine can even be fatal to humans (Wimol, 1984). In the solution preparation process, working with colchicine can be hazardous to plant breeders or researchers if it is inhaled or comes into contact with skin. In addition, pure colchicine is not that easy to obtain and has a high cost. For that reason, colchicine for this *Torenia* breeding research was obtained in the form of imported Colchicin brand gout medication tablets, which are available for sale over the counter in most pharmacies in Thailand. The product consists of 0.6 mg of colchicine per tablet. Tetraploid plants can be obtained by soaking *Torenia* leaves in colchicine solution made from the tablets, and they have good characteristics compared to the diploid plants, such as larger leaves and flowers, thicker leaves and stems, higher fertility and heartiness (Jiranapapan, 2011). It has also been reported that polyploidy *Torenia* obtained through colchicine treatment have larger stomata guard cells, larger pollen cells and higher pollen viability (Tungkajiwangkun, 2009).

Tungkajiwangkun (2009) studied the effects of colchicine solution from dissolved gout medication tablets on morophological changes in a hybrid between *T. concolor* and *T. fournieri* and on a local native species. Leaves were cut and the petioles were soaked in solution containing 0, 5, 10, 15 and 20 ppm colchicine for 0, 1, 2 and 3 days. The survival rate and growth rate (plant height, spread and number of branches) tended to decrease with increasing concentration of colchicine and increasing exposure time. Some of the colchicine-treated plants developed flowers with different colors and shapes, with larger flowers, stronger stems, and larger and thicker leaves. In addition, Santida (2009) reported that 4 of the colchicine-treated hybrids (*T. concolor* X *T. fournieri*) were tetraploid (2n=4x=36), one was hexaploid (2n=6x=54), and 6 of the colchicine-treated native *Torenia* plants were tetraploid (2n=4x=36). The polyploidy plants had larger stomata guard cells and larger pollen than their diploid progenitors. The percentage of viable pollen was lower in the polyploidy plants, however, the per cent that were infertile was less.

Similarly, Jiranapapan (2011) treated excised leaves of hybrids between *T. fournieri* and *T. baillonii* and a mutated yellow-flowered strain of *Torenia* with colchicine from gout medication tablets at the concentrations of 0, 5, 10, 15 and 20 ppm for 0, 1, 2 and 3 days and found that the survival rate and growth rate (plant height, spread and number of branches) tended to decrease with increasing concentration of colchicine and increasing exposure time. Fourteen putative polyploids were selected from the colchicine-treated hybrid *Torenia* and 7 putative polyploids from the colchicine-treated yellow-flowered mutant *Torenia*. Microscopic inspection revealed that all of them were indeed tetraploids with 2n = 4x=34 chromosomes. The treatment group that resulted in the highest rate of tetraploid induction (0.08 %) for the hybrid *Torenia* was 15 ppm colchicine for 2 days, while the treatment group that resulted in the highest rate of tetraploid induction (0.06 %) for the mutant yellow-flowered *Torenia* was 20 ppm colchicine for 1 day. The tetraploid hybrid *Torenia* plants were taller, wider, and had more branches than the diploid ones. The tetraploid yellow-flowered mutant *Torenia* plants were shorter, and less wide than the diploid ones, but had more branches. All the tetraploids plants had thicker stems, wider leaves, longer leaves and thicker leaves than the diploid plants.

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.

Gu *et al*. (2005) was able to induce tetraploidly in *Zizyphus jujube* Mill. cv. Zhanhua using colchicine solution at the concentrations of 0.01, 0.03, 0.05, 0.1 and 0.3% incorporated into liquid growth medium. Jujube meristems were agitated in the colchicine-containing medium for 24, 48, 72, and 96 hours in the dark. The polyploidy induction rate was greater than 3 % at the concentration of 0.05 % colchicine for 48 and 72 hours and the concentration of 0.1% colchicine for 24 and 48 hours. The tetraploids plants had larger stomata guard cells and more chloroplasts than diploid plants. When the plants were transferred from the laboratory to the greenhouse, the tetraploids had large, round stems, thicker and rounder shaped leaves, and larger flowers than the diploids.

Seneviratne *et al*. (2007) induced mutations in African violet (*Saintpaulia*) by dipping the petioles in colchicine solution at concentrations of 0, 0.04, 0.06, and 0.09 % for 21.5, 22.5, 23.5 and 48 hours before planting the cut leaves in planting material. Some of the plants that developed from the leaves subjected to the 0.06% colchicine treatment for 22.5 hours developed mutated flowers with white petals that were purple at the edges. However, 7 days after blooming, the African violet flowers reverted to the usual all purple 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.

In a recent work, Boonbongkarn (2013) reported on the an experiment in which leaves of native *Torenia fournieri* were exposed to colchicine from gout medication tablets at the concentrations of 0, 5, 10, 15, and 20 ppm for 0, 1, 2, and 3 days. The survival rate and growth rate (plant height, spread and number of branches) tended to decrease with increasing concentration of colchicine and increasing exposure time. Seven putative polyploids were selected from the plants that grew from the colchicine-treated leaves, based on the characteristics of slower growth, darker green and thicker leaves, larger leaves and larger flowers. The stomata guard cells of these putative polyploids were larger than those of the diploid control plants. When the amount of DNA was analyzed by flow cytometry, it showed that the amount of nuclear DNA of the putative polyploids was twice that of the diploid plants. Microscopic analysis confirmed that the number of chromosomes had doubled to 2n=4x=36.

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).

Gamma Radiation

Gamma rays are a form of high-frequency electromagnetic radiation with no mass, no charge, and high penetrating power. Plant breeders use two forms of gamma radiation to induce mutations in plants-- acute irradiation, which means a high dose of radiation administered over a short period of time (measured in minutes or hours) and chronic irradiation, which means a lower amount of radiation administered over a long period of time (weeks, months or years) (Arunee, 2007).

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. Following are some examples of gamma radiation breeding projects.

Harten *et al.* (1981) grew sweet potato in tissue culture and applied X-rays and the adventitious bud technique to induce mutations. They used *in vitro* petioles and leaves as the explants for radiation treatment, applying X-rays at the rates of 0, 15, 17.5 and 20 grays to the petioles sections and at the rates of 0, 22.5, 25 and 27.5 to the leaf sections, and were able to obtain mutant plants that had yellow tubers instead of the normal red. The mutation rate was high and with the adventitious bud technique and the chimera rate was low. Yellow tuber tissue was found in the L1 layer while cells in the L2 and L3 layer were still red.

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.

Okamura *et al.* (2003) applied radiation and tissue culture techniques to induce mutations in carnation. They compared the effects of ion beams, X-rays and gamma rays, using Carbon ion beams at the rates of 0, 10 and 15 grays, gamma rays at the rates of 0, 30, 50, 70 and 100 grays and X-rays at the rates of 0, 40, 80, 90 and 130 grays. The experiment showed that the number of new shoots that developed from carnation leaves exposed to Carbon ion beams decreased with increasing dosage and that ion beam treatment resulted in the highest frequency of mutations in flower color and form, followed by gamma radiation treatment and X-ray treatment, in that order.

Mutsumura *et al.* (2010) used ion beams to induce mutations in two cultivars of chrysanthemum-- H13, with purplish-red flowers, which they hoped to change to shades of yellow to white, and Shiroyamate, with white flowers, which they hoped to change to different colors. They applied ion beams at the rate of 1, 2, 4 and 8 grays to leaf and flower petal sections. The number of buds and new shoots formed from the leaf and flower petal sections decreased with increasing doses of ion beam treatment. The most suitable ion beam dose rate for mutation induction and development of new shoots from leaf and flower petal sections was 1 or 2 grays for H13 cultivar and 1, 2 or 4 grays for Shiroyamate cultivar. The flowers of one of the H13 plants from the 1-gray treatment group changed to reddish-orange with reddish-orange and white petals in spray type inflorescences, and the flowers of some of the H13 plants from the 2-gray treatment group changed to orangish-red, dark red, pink, and red and white streaked, with spray type inflorescences, while some flowers also exhibited doubling of petal layers. As for the Shiroyamate plants, yellow-flowered plants were found from the 1, 2 and 4-gray treatment groups, with the largest number of mutants in the 4-gray treatment group.

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.

Embryogenesis

Embryogenesis is the process of formation of a complete embryo from a zygote. However, there are also tissue culture techniques that can stimulate the formation of a complete embryo from another somatic cell that is not a fertilized zygote. This shows that plant cells have real totipotency. The formation of a complete embryo for a single plant cell or undifferentiated callus tissue is called somatic embryogenesis. Besides "somatic embryo," other terms are also sometimes used, such as embryoid, adventitious embryo, vegetative embryo, or embryo-like structure. The plant tissues that are the most conducive for forming somatic embryos are floral organs, zygotic embryos, anthers, pollen, endosperm and the nucellar tissue in the case of *Citrus* spp. The typical stages in somatic embryogenesis are the single cell stage, the cell aggregate stage, globular stage, heart-shaped stage, torpedo stage, and seedling (Siwapong, 2005).

Somatic Embryogenesis (Suprasanna, 2012)

In vitro plant regeneration occurs *via* somatic embryogenesis and organogenesis. Somatic embryogenesis is an excellent system for clonal propagation and mutation induction. The fact that somatic embryos originate from single cell prevents chimeras among regenerated *in vitro* plants and makes them an ideal subject for mutagenesis. Also, direct mutant somatic embryos can be developed from mutagenized somatic embryo cells and germinated into whole plants. This system is suitable for vegetatively propagated crops, including fruits, citrus, date palm, mangos and others. Therefore, if plants can be regenerated directly through somatic embryogenesis, the number of rounds of sub-culture required could be minimized.

The bipolar structure of the somatic embryo contains both shoot and root meristems. Somatic embryos develop and progress through distinct developmental stages. These are specific to the species, but include: globular, heart, torpedo, cotyledon and mature stage. Direct somatic embryogenesis is the formation of somatic embryos or embryogenic tissue directly from explants without the formation of an intermediate callus phase. The trend is to develop procedures that are directly embryogenic, as time in culture (and particularly callus culture) is proportional to the extent of somaclonal variation in regenerated plants, and this is often undesirable.

While the induction of secondary embryogenesis or repetitive embryogenesis can further increase the number of plants, it should not be used for *in vitro* mutagenesis programs after mutagenic treatment, since it will increase the number of plants of the same genetic make-up. This should be taken into consideration when somatic embryogenesis occurs in callus cultures. Callus is coherent, but unorganized and amorphous tissue, formed by the vigorous division of plant cells. In this case, limited numbers of regenerative plants from the same callus should be sampled in making up the population for mutant screening. On the other hand, embryogenic cells are particularly preferred for in vitro mutagenic treatment, though they have some disadvantages.

Advantages and disadvantages of embryogenic cells for *in vitro* mutagenesis

Advantages

- Somatic embryos originate from a single cell and minimize or eliminate chimera.

- Somatic embryogenic cell suspension is ideal for mutation induction due to the production of mutant somatic embryos.

- Somatic embryos can be produced in a bioreactor for their large-scale production for selection.

- Embryogenenic cells are suitable for long-term storage by cryopreservation to enable preservation of mutants.

Disadvantages

- Somatic embryogenesis is highly species- and genotype-dependent and therefore requires specific culture media formation.

- Germination rate of somatic embryos is very poor in most crops.

- Somatic embryogenic cultures can lose their embryogenic property if they are not sub-cultured regularly.

Organogenesis

Organogenesis is used for plant multiplication mainly by shoot tip culture, which is a common practice adopted by commercial laboratories. With this method, mutant plants can be multiplied in large numbers. Normally, the germination rate of somatic embryos is very low, which makes it difficult to exploit commercially for large scale plant production. The combination of somatic embryogenesis and organogenesis would therefore be suitable for mutation induction and subsequent large-scale multiplication of mutant plants, respectively. If shoot tips are used in gamma radiation treatment, this would generate chimerical plants depending on the occurrence of mutations in L1, L2 and/or L3 meristematic layers. The regenerated mutant plants will be unstable due to segregation in subsequent vegetatively propagated generations. Therefore, it is highly desirable to dissociate chimeras by multiplying plants up the M1V3 - M1V4 generations. This is usually done by axillary bud culture, as this represents the simplest type of *in vitro* plant propagation system, extensively applied in several ornamental plants and woody species. The axillary bud proliferation system can yield an average tenfold increase in shoot number in a four to six week culture passage. For annual and other fast-growing species a large population of plants can be generated within a year. Adventitious shoot proliferation is the most frequently used multiplication technique in micropropagation systems, as seen in the following examples:

Warch *et al.* (1989) induced callus in leaves, petioles and stems of *Solanum tuberosum* L. by culturing in MS medium with 2,4-D at the concentration of 3 $mg.l^{-1}$.

Nigra *et al.* (1989) induced callus from hypocotyls of *S. eleagnifolium* Cav. by culturing on MS supplemented with 4.5 mM 2,4-D.

Cuenca *et al.* (1989) induced callus from hypocotyls from pepper seedlings (*Capsicum annuum* L.) by culturing on MS with 2,4-D at the concentration of 1 $mg.l^{-1}$.

Leuangthongaram (2000) induced callus from cotyledons of soybean (*Glycine max*) by culturing on MS with 2,4-D at the concentration of 2 $mg.l^{-1}$.

Apisittiwanit (1988) induced callus from epicotyls of soybean variety Nakhon Sawan 1 by culturing on MS with 2,4-D at the concentrations of 2, 4, 6, 8 and 10 $mg.l^{-1}$.

6. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a laboratory technique for the rapid duplication of specific sequences of DNA that relies on reactions between (1) the enzyme DNA polymerase, used in the synthesis of DNA molecules; (2) 2 DNA primers, or short chains of DNA that are used as the starting points for the synthesis of new DNA molecules and that define the length of the DNA to be synthesized; (3) free nucleotides; and (4) the target DNA to be duplicated. After the chain reaction has run many times, it is possible to retrieve a large amount of newly synthesized DNA molecules in a short period of time, and the length of the DNA sections produced is determined by the distance between the primers as they pair up with the single strand of the target DNA. There are two kinds of DNA markers that have been developed from the PCR techniques.

1. Specific primer – this kind of marker attaches at a specific site on the DNA of interest, and consists of sequence-tagged sites (STS) and SSLP (simple sequence length polymorphisms) or microsatellites

2. Random primer – this kind of marker can attach at several different sites, and consists of RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism)

The technique results in newly synthesized strips of DNA of many different lengths. The sizes depend on the sites at which the primers attach to begin DNA synthesis.

Molecular genetic studies look in depth at the genes that control the expression of different phenotypes in living things. DNA marker techniques have been applied for many purposes, such as DNA fingerprinting, varietal identification, genetic mapping, gene tagging and other uses that are beneficial for plant and animal breeding.

Random Amplified Polymorphic DNA (RAPD)

RAPD is a technique based on Polymerase Chain Reaction (PCR) that utilizes random primer or arbitrary primer matching in the multiplication of DNA. This is based on the principle that all living things contain DNA with different codes made from the arrangement of the nucleotide bases adenosine (A), thymine (T), guanine (G), and cytosine (C). Different sections of the DNA code sequence are randomly duplicated through the same mechanisms as ordinary cellular DNA replication, and the replicated DNA strands are then separated using the process of gel electrophoresis. The resulting gel sheets can be analyzed to see the differences among DNA from different samples in the technique known as DNA fingerprinting. Organisms with different genetic codes or different sequences of bases will have DNA fingerprints that can be differentiated, and organisms that are closely related will have very similar DNA fingerprints. In RAPD, usually the temperature for the annealing step is about

35-42 ˚C, but for HAT-RAPD, which gives higher resolution reproducibility and polymorphism detection, the annealing temperature is 46-62 ˚C. (Piyachokhanakul, 2012)

Anatomical characteristics of *Torenia*

The anatomical nature of *Torenia* resembles that of dicotyledons in general, but there are notable ridges to the stem on all 4 corners. Sclerenchyma fiber, a structure that dyes red with Saflanin O, can be observed in cross section, serving to add strength to the stem. There were no previous reports of research studies on the anatomy of the plant stem, which is composed of the following: epidermis, vascular bundle, cortex, tricome and pith, as shown

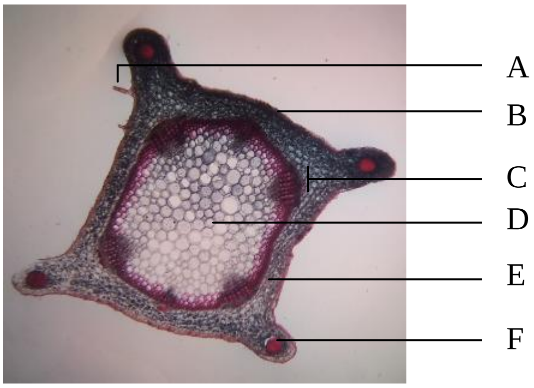


Figure 2 Anatomy of *Torenia* spp. stem section

A. Tricome

B. Epidermis

C. Vascular bundle

D. Pith

E. Cortex

F. Sclerenchyma fiber

MATERIALS AND METHODS

1. Plant material

1.1 Four inter-specific hybrids of *Torenia* e.g. *T. benthamiana* × *T. asiatica*, *T. asiatica* × *T. pierreana*, *T. fournieri* × *T. pierreana* and *T. asiatica* × *T. concolor* (cooperation research from Kanno (2013) production of inter-specific hybrids, Chiba University)

1.2 Diploid and polyploid (from Ms. Sasiree Boonbongkarn experiment) purple-flowered native Thai Torenia (*Torenia fournieri* Lind.) with a semi-recumbent, semi-erect habit. The plants were maintained in a greenhouse at a day temperature of 33–35 ˚C and 60-65 % relative humidity and a night temperature of 29-33 ˚C and 65-70 % relative humidity.

2. Sterilization of explant material

Axillary buds of *Torenia fournieri* were used as explants. They were first surface cleaned by washing with Tepol® detergent (Jala, 2014), rinsed with tap water and dipped in 70 % alcohol for 1 minute. Next, the explants were surface sterilized by soaking in 20 % (v/v) Clorox® for 10 minutes, followed by 5 % (v/v) Clorox® for 10 minutes, then rinsed 3 times with sterile distilled water for 5 minutes each time. Explants were cultured on modified solid MS medium (Murashige and Skoog, 1962) supplemented with BA 0.25 mg/l, activated charcoal and 3% sucrose. Cultures were incubated at 25±2 °C under a 16-hour photoperiod with illumination provided at 60 $\mu mol m^{-2} sec^{-1}$(TLD 18 w/18 lm Phillips, Holland). The leaves obtained after 8 weeks of culture were then used as the source of explants for the experiments.

3. *In vitro* multiplication

The nodes were kept at 25 ± 2 ˚C with light for 16 hours/day at intensity 60 ± 5 $\mu mol/m^2/s$ 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 mm) and transferred to new culture vessels (4-ounce glass jars containing 10 ml culture medium) every 4 weeks (1 node per culture vessel).

4. Somatic embryogenesis

Leaves *Torenia fournieri* (diploid and polyploid) were cultured on MS medium supplemented with 0, 0.5, 1.0, 1.5 and 2.0 mg/l of Picloram or 0.5, 1.0, 1.5, 2.0 mg/l of 2,4-D and 3 % of sucrose and 0.25 % of phytagel. The cultures were maintained at 25 ± 2 ˚C under dark condition. After 0, 1, 2, 3 and 4 weeks of culture, the callus tissue formed was transferred to MS medium and cultured under light condition (16 hour photoperiod with illumination provided by cool fluorescent lamps at an intensity of 60 $\mu molm^{-2}sec^{-1}$).

5. Colchicine treatment

Leaves were cut from F1 hybrids of Torenia for the different treatments. The petioles were soaked in 15 mg/l colchicine solution for 0, 12, 24, 48 and 72 h. (32 leaves per treatment), after which they were placed upright in peat moss for rooting (modified from Boonbongkarn, 2013). Once roots were established, the leaf cuttings were transferred to 4-inch pots containing a mixture of sand, rice husk charcoal, coconut coir, coconut husk chips, and manure 1:1:1:1:1/2. Slow-release fertilizer of formula 14-14-14 was added at the rate of 5 g per pot, and liquid fertilizer of formula 21-21-21 was also provided once a week at the concentration of 30 g per 20 l of water. The survival rate was recorded at 60 d.

6. Anatomical observation

The paraffin technique (Johansen, 1940) with fast green and Safanin o staining was used to study the size and characteristics of epidermis, cortex, pith and sclerenchyma tissues and vascular bundles in samples of diploid and polyploidy *Torenia* as well as mutated *Torenia*.

7. Gamma ray irradiation in tissue culture

For the acute γ-ray irradiation test, leaves were cultured on modified solid MS medium (Murashige and Skoog, 1962) supplemented with BA at 0.25 mg/l, and 3% sucrose (in petri-dishes), and exposed to gamma ray irradiation at doses of 0, 20, 40, 60, 80, 100 and 120 grays in a Gamma Irradiator Model Mark I machine (J.L. Shepherd & Associates, San Fernando, CA) delivering 4500 Ci of Cs-137 at the Gamma Irradiation Service and Nuclear Technology Research Center, Kasetsart University.

One hundred leaves per replication were irradiated at each exposure dose, with 3 replications. Shoots were regenerated by culturing irradiated leaves on MS medium supplemented BA 0.25 mg/l, activated charcoal and 3 % sucrose. The surviving plants, number of plants regenerated, plant height and length of root were measured 60 d after irradiation. The LD_{50} was calculated based on the survival rate after 60 days.

8. Gamma ray irradiation of axillary buds

For the acute γ-ray irradiation test, axillary buds (1 node cutting) were irradiated at doses of 0, 20, 40, 60, 80, and 100 grays using a gamma irradiator Mark I machine (J.L. Shepherd & Associates, San Fernando, CA) with a Cs-137 source and then planted in peat moss.

In total, 100 axillary buds per replication (3 replications) were irradiated at each exposure dose. Regenerated shoots appeared by 30 days. The surviving plants and plant height were measured 60 d after irradiation. The GR_{50} was calculated based on the plant height after 60 days.

1,000 axillary buds from the M_1V_2 generation were irradiated at doses of 0, 60, 65, and 70 grays using a gamma irradiator Mark I machine (J.L. Shepherd & Associates, San Fernando, CA) with a Cs-137 source and then planted in peat moss.

9. Phenotype evaluation and FISH analysis

The plant height, plant spread and flower size of irradiated plants 60 d after exposure were measured with ruler and vernier caliper.

Chromosome preparation from flower buds and the use of FISH for meiotic chromosomes were performed according to the procedures described in Kikuchi *et al*., 2008. A *Torenia* centromeric repetitive DNA sequence (TCEN) was labeled with either biotin-dUTP or digoxigenin-dUTP by PCR or DIG-High Prime (Roche). All images were captured with an Olympus BX61 fluorescence microscope equipped with a cooled-CCD camera (Photometrics CoolSNAP fx: Roper Scientific) and processed using the Meta imaging series 5.0 software (Universal Imaging Corporation) (Jiranapapan *et al*., 2011).

10. HAT-RAPD

DNA extraction followed the Doyle and Doyle (1987) protocol. DNA solution was diluted to a concentration of 100 ng per microliter and placed with 10 microliters of DNA sample (Mixed DNA) in a centrifugation tube. The solution derived from mixed DNA was used as the template in PCR, using 72 pairs of primers (A2, B2, C2, D2, E2 and F2 groups of 12 pairs of primers from Waka Company, Japan) as follows:

The HAT-RAPD DNA amplification reactions were performed in a total volume of 20 µl containing 100 ng of template DNA, 250 ng random primer, buffer solution (50 mM KCl, 20 mM Tris-HCl pH 8.4, 0.25 mM $MgCl_2$), 200 µM dNTP, 1 unit of *Taq* DNA polymerase (5 U/µl) (RBCBioscience, Taiwan) and deionized water. PCR were performed a thermal cycler (Eppendorf AG, Germany). Amplification was as follows: initial denaturation was executed at 94 °C for 3 min before the start of cycling followed cycle consisted of 45 second at 94 °C, 45 second at 46 °C and 1 min at 72 °C. A total of 40 cycles were performed and the cycling ended with a final extension at 72 °C for 5 min. After completion of amplification, DNA fingerprint was detected and analyzed in 1.5 % gel electrophoresis, which was repeated 3 timed for robustness.

Data analysis was done using UPGMA (unweighted pair group method with arithmetic mean); the solution in score bands was observed and a phylogenetic tree was constructed (Rohlf, 2002).

11. 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 Canon 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.

12. 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.

13. Chromosome counting

Chromosome preparation with flower bud and the use of FISH for meiotic chromosomes were performed according to the procedures described in Kikuchi *et al*., 2008. The slides were mounted with Vectashield (Vector) containing 5 mg/mL 4’, 6-

diamidino-2-phenylindole (DAPI) for staining of chromosomes. All images were captured with an Olympus BX61 fluorescence microscope equipped with a cooled-CCD camera (Photometrics CoolSNAP fx:Roper Scientific) and processed using the Meta imaging series 5.0 software (Universal Imaging Corporation).

14. Pollen viability testing and size determination

The pollen of mutated *Torenia* and polyploidy *Torenia* were tested for percent viability and size in comparison to the pollen of control plants using the following method

1. Immature flowers were plucked from plants growing in pots

2. Pollen grains were scraped from the anthers onto a microscope slide

3. A few drops of aceto-carmine stain were applied to the slide

4. The pollen grains were spread throughout the drop of stain using a needle and a cover slip was placed on top

5. The size of pollen grains was recorded using a microscope with an ocular micrometer and camera

6. The number of normal and malformed pollen were counted; those that were darkly stained were considered normal and those that were unstained, pale or unusually small were counted as malformed

7. The total number of pollen gains visible and the number of malformed pollen grains visible in each microscope field was recorded, and enough different fields were viewed to bring the total pollen count up to at least 300 for each sample. Percent pollen viability was calculated using the following formula:

$$\text{Percent pollen viability} = \frac{\text{number of malformed pollen}}{\text{total number of pollen}} \times 100$$

15. Data recording

15.1 Survival rate

15.2 Growth (plant height, spread, number of side shoots)

15.3 Leaf width and length

15.4 Flower width and length

15.5 Morphological changes compared to control, such as plant habit

15.6 Size of stomata guard cells

15.7 Number of chromosomes

15.8 Pollen size

15.9 Percent pollen viability

16. Statistical analysis

Statistical differences were tested using Duncan’s new multiple range test at the $P \leq 0.01$ level.

Places and Duration

The experiments were conducted at Department of Horticulture, Kasetsart University, Department of Biotechnology, Thammasat University, and Graduate School of Horticulture, Chiba University, from May 2012-October 2014.

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

Chromosome observation was performed at the genetic and plant breeding laboratory of the Graduate School of Horticulture, Department of Horticulture, Chiba University, Japan.

RESULTS AND DISCUSSION

Gamma ray irradiation from axillary buds

Effects of γ-ray irradiation on plant growth

Torenia spp. are known to be easily propagated in tissue culture (Takeuchi *et al*., 1985). In the present study axillary buds were cultured in peat moss and regenerated new shoots from axillary buds. This technique omits several *in vitro* work steps and reduces the regeneration time and acclimatization time required in the production of M_1 plants. After γ-ray irradiation, the regenerated shoots were grown for 60 d, and the mean plant height was recorded as 15.2, 13.8, 10.8, 9.13, 5.73 and 3.86 cm at 60 d for the lateral shoots exposed to 0, 20, 40, 60, 80 and 100 gray of irradiation, respectively. Internode length, crown spread and number of flowers similarly decreased with exposure to increasingly higher rates of gamma irradiation (Table 1). The half growth reduction dose (GR_{50}) of acute γ-rays was 68.83 grays (Figure 3). The effect of γ-rays on plant height is dependent on the exposure dose, irrespective of the irradiation method. A similar gradual reduction in plant growth of irradiated plants was noted in interspecific hybrids between *T. fournieri* and *T. bailonii* (Sawangmee *et al.*, 2011). In the present study, one difference in the morphological characteristics of *T. fournieri* Lind. observed 60 d after exposure to varying doses of acute gamma irradiation compared with the control was smaller canopy (Figure 4). Apparent mutations in the M_1V_1 plants after acute gamma irradiation were not stable. No solid mutants were obtained after using the cutting back technique (Figure 5). After the GR_{50} was calculated, for the next step of the research 1,000 axillary buds collected from among *T. fournieri* Lind. mutants from M_1V_2 generation were subjected to irradiation at doses of 0, 60, 65, and 70 grays. The major morphological changes observed in the mutant plants were pink flower color (from γ-ray irradiation at 60 grays) (Figure 5B), dark flower color (from γ-ray irradiation at 65 grays) (Figure 5C) and wavy shaped flower (from γ-ray irradiation at 65 grays) (Figure 5D). Mutant *Torenia* that show changes in petal color may come from the mutation of several genes involved in anthocyanin synthesis. Suzuki *et al*. (2000) and Sawangmee *et al*.

(2011) produced yellow flowered plants from transgenic lines of *Torenia* hybrid and γ-ray irradiation in interspecific hybrids between *T. fournieri* and *T. bailonii*, respectively. The yellow colored petals are probably due to the cosuppression of chalcone synthase (CHS) or dihydroflavonol-4-reductase (DFR) genes in anthocyanin synthesis. These mutants did not display any other changes in phenotype. Boonbongkarn *et al*. (2013) showed that the character of the mutation in the plant could be maintained by vegetative cutting without the mutation reverting back to the original character. Table 2 shows the plant height, internode length, spread, number of branches, number of flowers, size of flowers, survival rate of pollen and size of pollen 60 d after selected mutant clones from the experiment were irradiated at different doses (0, 60, 65, 70 grays). Differences in plant height, internode length, crown spread, and number of branches were non-significant, but leaf size was significantly smaller and the number of flowers in some irradiated plants was significantly less than in wild type. The pink color mutant had the smallest flowers and the wavy shaped mutant had sterile pollen (Figure 7E). The color mutants and crown spread observed in the present study were very similar to some reported by Suwanseree *et al*. (2011), who observed pale blue and pink flower color mutations and some mutants with compact spread after exposing *in vitro* node segments of *Torenia hybrida* to 0-50 Gy of gamma irradiation. Miyazaki *et al.* (2006) 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 irradiation. In the present study, stem cuttings were made of mutant plants and the mutations remained stable in 100 % of the next generation plants (M_1V_3). Similar morphological changes have been reported in other research; for example, in an induced mutation experiment in carnation, 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 petal (Okamamura *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).

HAT-RAPD analysis

HAT-RAPD analysis of was done using 72 arbitrary primer pairs in order to determine the genetic relationship between the original wild type of *Torenia fournieri* Thai and 3 mutant types selected from the irradiation experiment. Two HAT-RAPD markers, primer D30 (5´-GAGACTACCGAA-3´) and primer F29 (5´-GCCGCTAATATG-3´), exhibited polymorphisms, and the distinct banding patterns could be used to distinguish and identify the mutant strains from the wild type. Similar results have been obtained with this method in other ornamental plants, such as chrysanthemum, petunia and rose (Krasaechai, 2009) and in other crops such as rice, in which HAT-RAPD was used to characterize a mutant strain produced by ion beam radiation of the Hom Mali 105 cultivar (Phanchaisri, 2007). The two primers mentioned above were used to identify the mutant *Torenia fournieri* Lind. strains and to indicate their genetic relationship with the wild type (Figure 8). The HAT-RAPD technique was adapted from the RAPD technique by raising the annealing temperature and PCR temperatures so that the random primers can attach more efficiently to specific sequences, giving a more accurate DNA fingerprint (Thananantha *et al.,* 2012). This technique has been used in studies on jasmine rice (Thananata *et al*., 2014), mung bean (Sumrith *et al*., 2014), *Michelia* spp. (Tansanhga *et al*., 2014) and banana (Thomsopa *et al.*, 2014). HAT-RAPD is effective for identification and discriminating among different cultivars that are used for breeding purposes in agriculture and horticulture. It can demonstrate the similarities and differences among genotypes of new cultivars by showing the shared bands and different bands in the DNA fingerprint that originate mainly from somatic mutation and nucellar variation. It indicates altered phenotype of new cultivar resulting from modified intra-genotype of crops.

FISH analysis

The chromosome number of 3 types of *Torenia fournieri* Lind. mutants from the irradiation experiments was investigated by FISH analysis to confirm that the morphological differences observed were not due to hybridization with other species. Red signals shown in Figure 9 (A-D) correspond to the TCEN repeat on the centromeric region of *T. fournieri* Lind. chromosomes (A-D). The number of chromosomes was not changed from the wild type (2n=2x=18) (Figure 9).

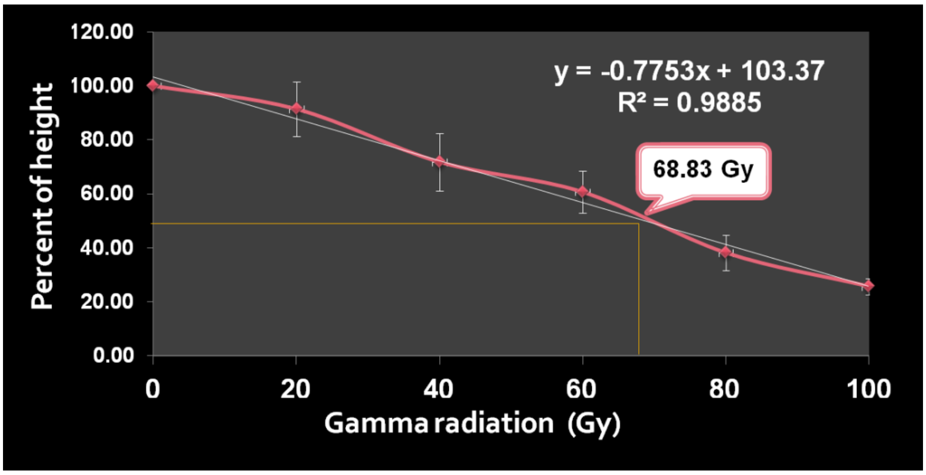


Figure 3 Difference in mean plant height (shown as percentage) of *T. fournieri* Lind. plants 60 d after exposure to varying doses of acute gamma I rradiation compared to the control, showing the GR_{50} level.

Table 1 Plant height, internode length, spread, number of branches, number of flowers and observed mutations of *Torenia fournieri* Lind. 60 d after gamma irradition

Dose (Gy)	**Total plants**	**Plant Height (cm)**	**Internode length (cm)**	**Crown spread (cm)**	**Number of flowers**	**Number of floral mutants**	**Mutation rate (%)**	**Number of Solid Mutants**
0	300	15.2 ± 1.71^{a}	3.00 ± 0.20^{a}	17.83 ± 0.29^{a}	46.33 ± 3.21^{a}	0	0	0
20	300	13.8 ± 0.92^{a}	2.80 ± 0.20^{ab}	15.50 ± 0.50^{b}	43.33 ± 1.53^{a}	0	0	0
40	300	10.8 ± 0.60^{b}	2.46 ± 0.06^{bc}	13.83 ± 0.76^{b}	23.67 ± 1.15^{b}	1	0.33	0
60	300	9.13 ± 0.42^{b}	2.40 ± 0.10^{c}	11.00 ± 1.00^{c}	14.33 ± 4.04^{c}	2	0.67	0
80	300	5.73 ± 0.61^{c}	2.10 ± 0.15^{c}	7.33 ± 0.58^{d}	0.00 ± 0.00^{d}	0	0	0
100	300	3.86 ± 0.12^{c}	1.47 ± 0.06^{d}	7.16 ± 1.04^{d}	0.00 ± 0.00^{d}	0	0	0
F-test	-	**	**	**	**	-	-	-
% C.V.	-	9.05	5.95	6.15	10.56	-	-	-

**Means ± SD within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

ns = non significant

Table 2 Mean plant height, internode length, spread, number of branches, number of flowers, flower size, pollen survival rate and size of pollen of *Torenia fournieri* Lind. 60 d after selected mutant clones were exposed to different doses of gamma irradiation

Plants	Plant height (cm)	Internode length (cm)	Crown spread (cm)	Number of branches	Size of leaves (cm)	Number of flowers	Size of flowers (mm)	Survival of pollen (%)	Size of pollen (µm)
Control	14.33 ± 1.53	2.33 ± 0.29	27.50 ± 4.82	11.33 ± 1.15	2.28 ± 0.29^{a}	42.00 ± 5.29^{a}	23.79 ± 0.40^{b}	78.57 ± 0.91^{a}	32.00 ± 2.00^{a}
Pink	12.67 ± 0.58	1.93 ± 0.12	22.17 ± 2.89	13.00 ± 1.00	1.72 ± 0.22^{b}	35.67 ± 4.04^{a}	17.23 ± 0.21^{c}	81.10 ± 6.52^{a}	23.33 ±1.15^{b}
Dark	16.33 ± 2.31	2.50 ± 0.00	24.50 ± 1.80	13.67 ± 1.53	1.95 ± 0.11^{ab}	23.00 ± 1.00^{b}	24.52 ± 0.03^{b}	40.57 ± 1.44^{b}	24.00 ± 2.00^{b}
Wavy	13.33 ± 1.15	2.17 ± 0.29	23.00 ± 2.30	10.67 ± 1.53	1.64 ± 0.06^{b}	22.00 ± 2.00^{b}	27.43 ± 0.54^{a}	0.00 ± 0.00^{c}	12.66 ± 1.15^{c}
F-test	ns	ns	ns	ns	**	**	**	**	**
% C.V.	10.78	9.50	13.03	10.87	10.04	11.45	1.52	6.72	7.10

**Means ± SD within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

ns = non significan

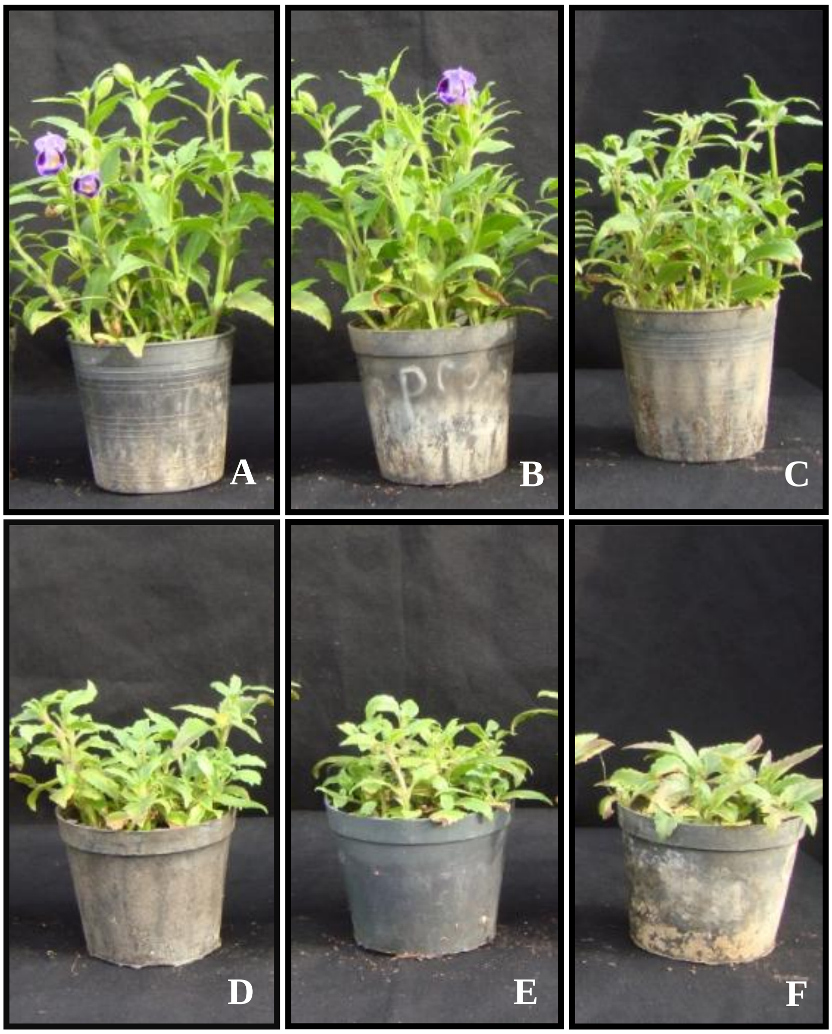


Figure 4 Characteristics of *T. fournieri* Lind. 60 d after exposure to varying doses of acute gamma irradiation compared to the control: (A) 0 gray; (B) 20 grays; (C) 40 grays; (D) 60 grays; (E) 80 grays; and (F) 100 grays.

Figure 5 Flower shape of M_1V_1 generation of *T. fournieri* Lind. after acute gamma irradiation: (A) 0 gray; (B) 20 grays; (C) 40 grays and (D) 60 grays.

Figure 6 Flowers from the M_1V_3 generation of *T. fournieri* Lind. plants propagated by cutting back technique; (A) 0 Gray; (B) pink flower from 60-gray treatment group; (C) dark flower from 65-gray treatment group; (E) wavy shaped flower from 65-gray treatment group.

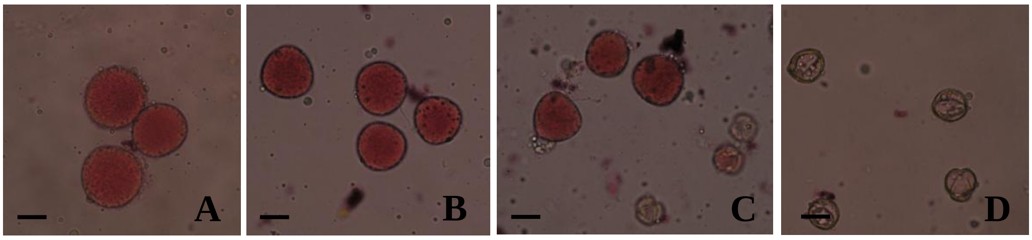


Figure 7 Pollen of *T. fournieri* Lind. mutant after staining with acetocarmine; (A) Control ; (B) pink flower from 60 gray; (C) dark flower from 65 gray; (E) wavy shape flower from 65 gray. (Bar = 10 µm)

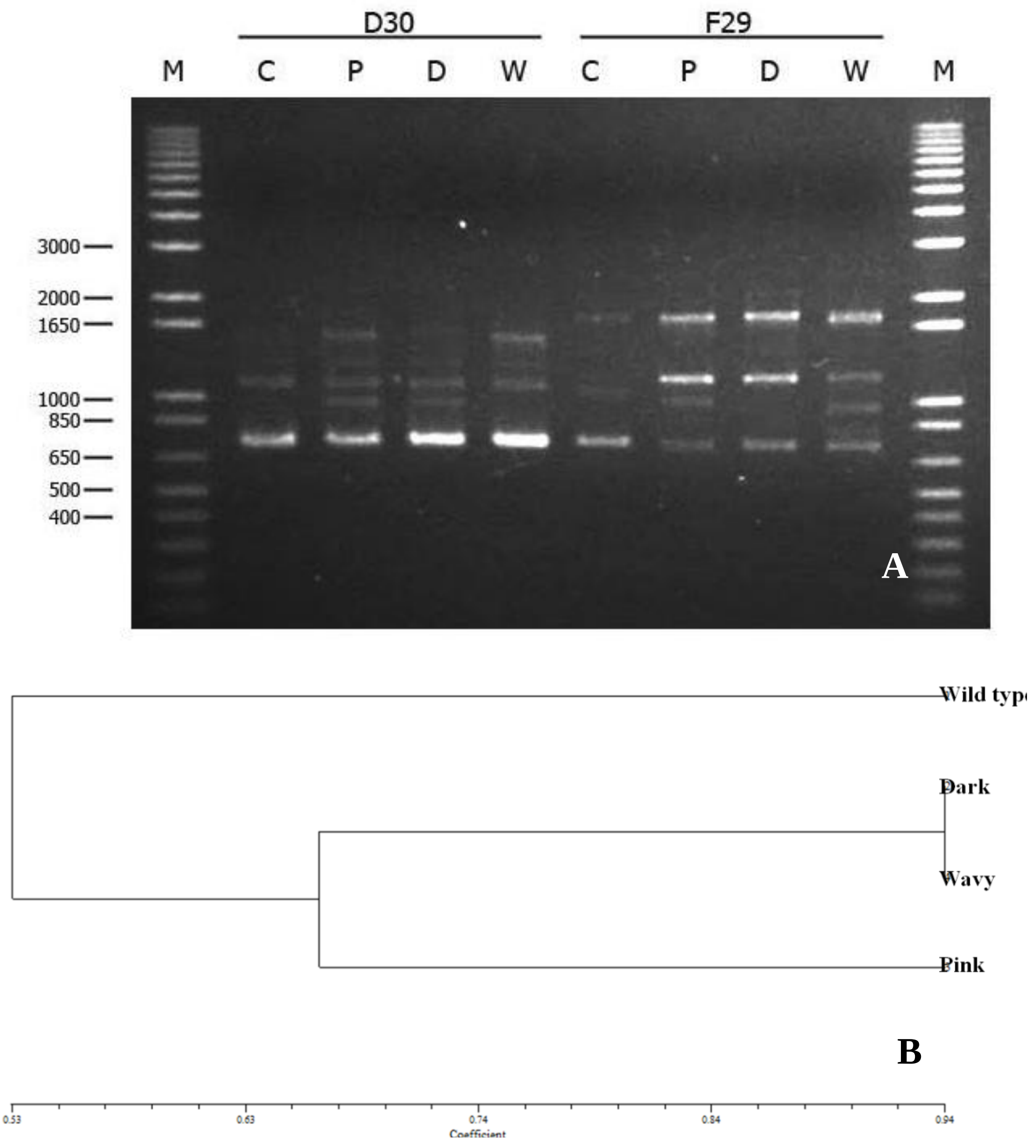


Figure 8 A: DNA fingerprinting from 4 types of *T. fournieri* Lind generated by using primer number D30 (5´-GAGACTACCGAA-3´) and primer number F29 (5´-GCCGCTAATATG-3´) [M was 1 Kb DNA plus Ladder (Invitrogen™ Life Technology, USA)] (C) Wild type; (P) pink flowered plant from 60-gray; (D) dark flower plant from 65-gray; (W) wavy shaped flower plant from 65-gray.

B : Dendrogram of the 4 analyzed types of *T. fournieri* Lind. from HAT-RAPD technique.

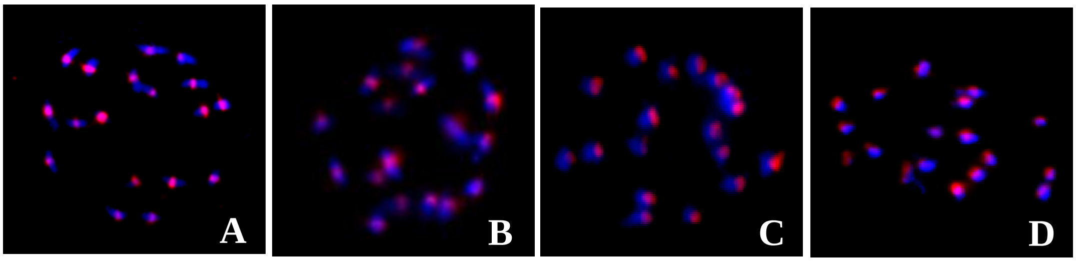


Figure 9 Chromosomes of 4 types of *T. fournieri* Lind. by FISH analysis confirming that the mutations are not due to hybridization with other species. Red signals: TCEN repeat on *T. fournieri* Lind. (A-D) The number of chromosome were not changed

Table 3 Mean size of epidermis, vascular bundle, cortex, pith and sclerenchyma fiber of *Torenia fournieri* Lind. 90 d after selected mutant clones (Control, Pink, Dark and Wavy petal)

Plants	Epidermis (µm)	Vascurlar bundle (µm)	Cortex (µm)	Pith (µm)	Sclerenchyma fiber (µm)
Control	5.52 ± 0.21[c]	57.17 ± 2.27[a]	43.22 ± 0.11[b]	238.85 ± 1.52[c]	30.22 ± 0.78[b]
Pink	7.25 ± 0.25[a]	54.33 ± 2.65[a]	55.33 ± 0.88[a]	316.17 ± 5.40[a]	25.08 ± 2.08[c]
Dark	6.27 ± 0.02[b]	34.44 ± 0.10[c]	37.00 ± 0.38[c]	271.67 ± 7.75[b]	24.88 ± 0.10[c]
Wavy	4.40 ± 0.10[d]	43.42 ± 2.92[b]	34.66 ± 0.77[d]	244.98 ± 0.91[c]	34.82 ± 1.00[a]
F-test	**	**	**	**	**
%C.V.	2.96	4.81	1.45	1.79	4.25

**Means ± SD within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

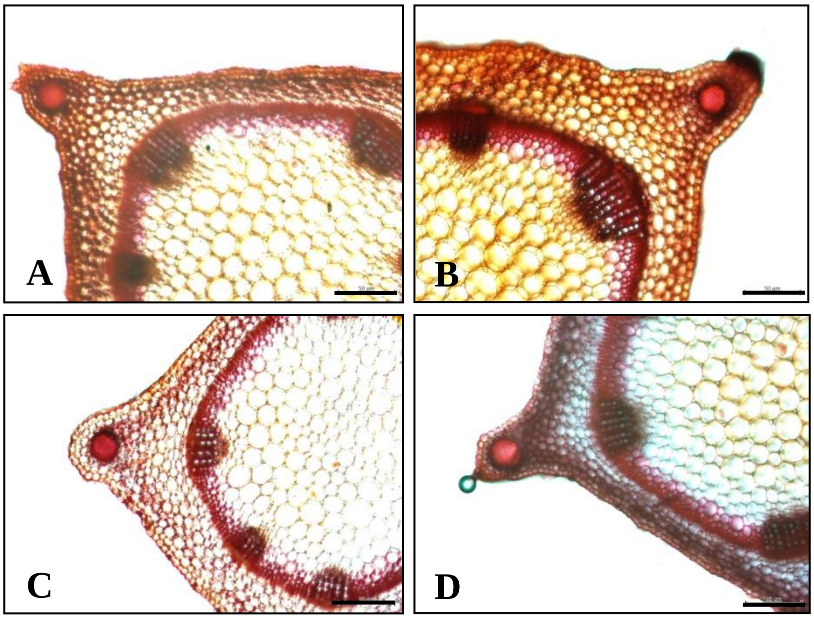


Figure 10 Anatomy of stem in *Torenia fournieri* Lind. 90 d after selected mutant clones. (A) Wild type; (B) pink flower from 60-gray; (C) dark flower from 65-gray; (E) wavy shaped flower from 65-gray. (Bar = 50 µm)

Somatic Embryogenesis

The results showed that *Torenia* young leaf explants that were cultured in media containing no exogenous plant growth regulators in the dark grew and developed into somatic embryos without passing through an intermediate step of embryogenic callus development. Young leaf explants that were cultured on semi-solid MS medium containing 2,4-D at the concentrations of 0.5, 1.0, 1.5 and 2.0 mg/l turned brown and stopped growing at a greater rate than the control group. Embryogenic callus tissue developed the most on young leaf explants that were cultured on MS medium containing Picloram at the rates of 0.5, 1.0, 1.5 or 2.0 mg/l. Callus was first detected after one week of culture. The callus consisted of white to yellowish loosely aggregated clumps of cells (Figure 11). After 3 weeks of culture in the dark, the highest percentage of callus formation (95 %) from young leaves of diploid Torenia was observed in the treatment on MS supplemented with Picloram at the rate of 1.5 mg/l. When the cultures were left for longer than 3 weeks, the percentage of tissue turning brown and dying increased with increasing time, especially with higher concentrations of Picloram. For the polyploid accession of *Torenia*, the treatments cultured in MS medium with Picloram added at the rates of 1.0 and 1.5 mg/l exhibited the highest percentage of callus formation (92.5 %) after culture in the dark for 3 weeks. Again, after 3 weeks, the percentage of tissue browning increased with increasing time and with higher concentrations of Picloram (Table 4). These results are consistent with those in several previous reports in which callus was induced from leaf explants of other species, for instance *Saintpaulia* (Sunpai and Kanchanapoom, 2002), *Curcuma amada* (Prakash *et al*., 2004), *Curcuma aromatica* (Mohanty *et al*., 2008), and *Kaempferia galanga* (Rahman *et al*., 2004).

When embryogenic callus from the callus induction step was transferred to hormone-free semi-solid MS medium and introduced to light conditions (16-hour photoperiod), somatic embryos and new shoots developed within 3 weeks. For both diploid and polyploid Torenia, the number of somatic embryos generated was the greatest in the callus that came from the Picloram 1.5 mg/l treatment (Table 5). As expected, the new shoots developed from polylpoid Torenia tissue tended to be larger than those from diploid Torenia (Figure 12) As previous researchers have pointed out, the correlation between the surface-to-volume ratios of the nucleus and the cell indicates that polyploid nuclei might be required for the formation of large plant cells. The physiological role of genetically programmed polyploidy is, however, elusive as it might contribute to or be a consequence of cell-differentiation programs. Multiplication of the genome has been proposed to increase metabolic activity, rRNA synthesis and transcriptional activity (Nagl, 1976; Baluska and Kubica, 1992).

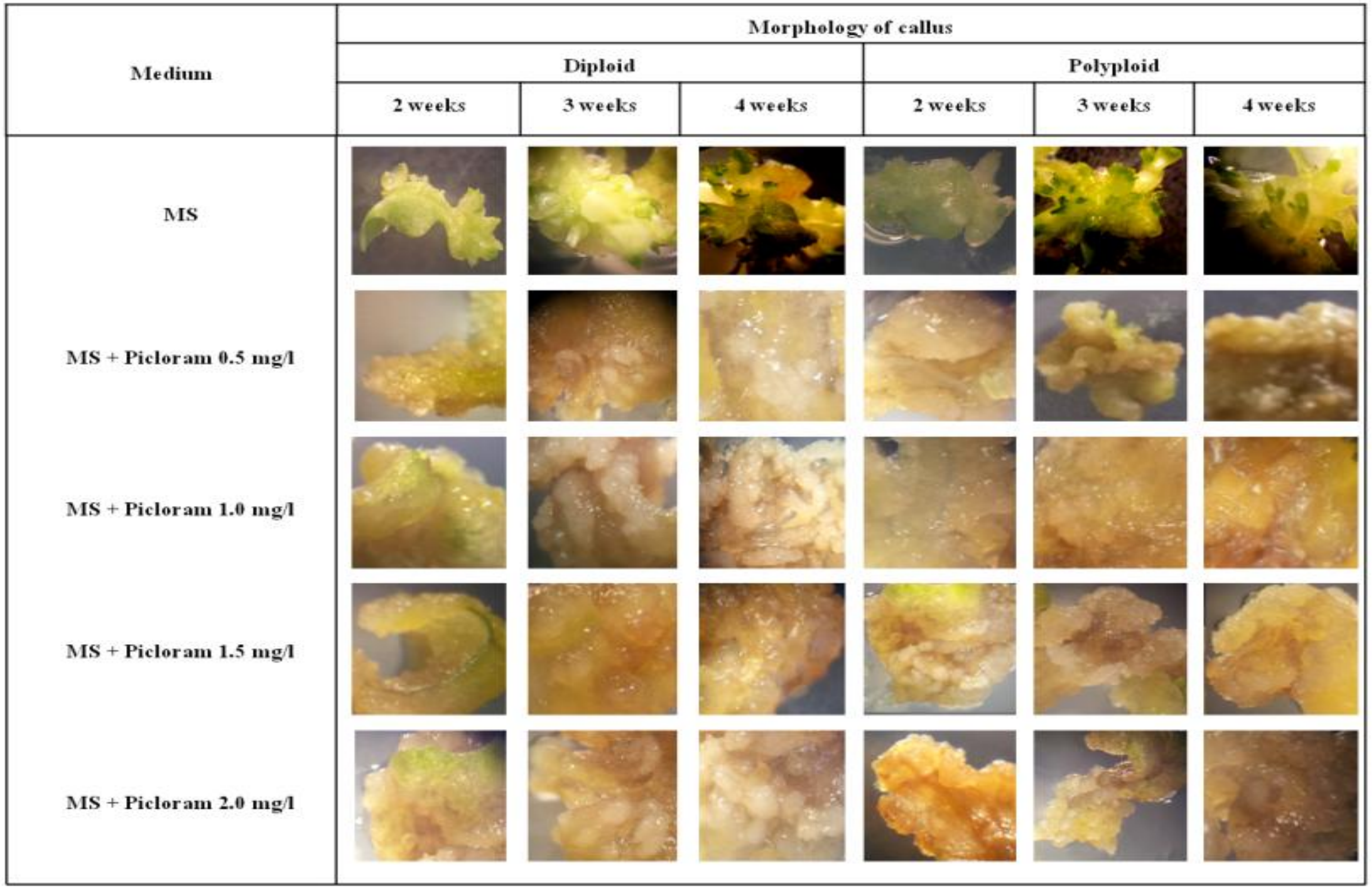


Figure 11 Morphology of *Torenia fournieri* on MS medium and different growth regulators after 2, 3 and 4 weeks

Table 4 Effect of different growth regulators on induction of callus of *Torenia fournieri* Lind. on MS medium after 1, 2, 3 and 4 weeks

Medium (MS + Picloram)	**Callus formation (%)**							
	Diploid				**Polyploid**			
	1 week	**2 weeks**	**3 weeks**	**4 weeks**	**1 week**	**2 weeks**	**3 weeks**	**4 weeks**
MS + Picloram 0.5 mg/l	N	27.5 ± 0.96^{b}	80.0 ± 0.82^{b}	60.0 ± 0.82^{a}	N	55.0 ± 0.58^{bc}	75.0 ± 0.58^{a}	75.0 ± 0.58^{a}
MS + Picloram 1.0 mg/l	N	40.0 ± 0.81^{ab}	85.0 ± 0.58^{ab}	67.5 ± 1.26^{a}	N	65.0 ± 0.58^{ab}	92.5 ± 0.96^{a}	80.0 ± 0.82^{a}
MS + Picloram 1.5 mg/l	N	45.0 ± 0.58^{a}	95.0 ± 0.58^{a}	55.0 ± 1.29^{ab}	N	70.0 ± 0.82^{a}	92.5 ± 0.96^{a}	85.0 ± 0.58^{a}
MS + Picloram 2.0 mg/l	N	52.5 ± 0.50^{a}	55.0 ± 0.58^{c}	37.5 ± 0.50^{b}	N	50.0 ± 0.82^{c}	55.0 ± 1.29^{b}	42.5 ± 0.50^{b}
F-test	-	**	**	**	-	**	**	**
% CV	-	19.95	9.16	20.75	-	19.97	13.90	9.78

**Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

N = not callus

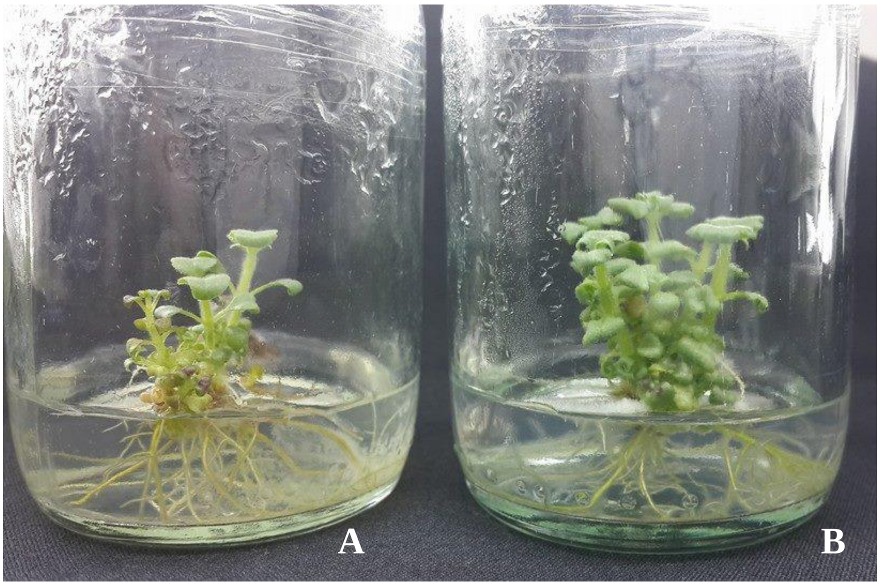


Figure 12 Plant regenerated from callus in *Torenia fournieri* on MS medium after 4 weeks. Diploid (A) and polyploidy (B**)**

Table 5 Effect of different growth regulators on induction of shoots of *Torenia fournieri* Lind. on MS medium after 30 days

Medium	Number of plant from callus							
	Diploid				Polyploid			
	1 week	2 weeks	3 weeks	4 weeks	1 week	2 weeks	3 weeks	4 weeks
MS + Picloram 0.5 mg/l	1.25 ± 0.50c	1.00 ± 0.82^{b}	4.75 ± 0.50	4.50 ± 0.58	1.75 ± 0.96	3.50 ± 1.29^{a}	1.00 ± 0.82^{b}	4.25 ± 0.50^{a}
MS + Picloram 1.0 mg/l	1.75 ± 0.50^{bc}	1.00 ± 0.82^{b}	5.00 ± 0.00	4.25 ± 0.50	1.75 ± 0.96	1.50 ± 0.58^{ab}	1.00 ± 0.82^{b}	4.50 ± 0.58^{a}
MS + Picloram 1.5 mg/l	2.75 ± 0.50^{ab}	2.50 ± 0.58^{a}	5.50 ± 0.58	3.75 ± 0.96	1.50 ± 0.58	1.25 ± 0.96^{a}	5.25 ± 0.96^{a}	3.50 ± 0.58^{ab}
MS + Picloram 2.0 mg/l	3.75 ± 0.50^{a}	3.25 ± 0.50^{a}	4.75 ± 0.50	3.75 ± 0.96	1.56 ± 0.82	3.00 ± 0.82^{ab}	4.75 ± 0.50^{a}	2.50 ± 0.58^{b}
F-test	**	**	ns	ns	ns	**	**	**
% CV	21.05	35.72	9.12	19.13	48.09	40.92	26.35	15.15

**Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

ns = non significant

Gamma ray irradiation in tissue culture

Effect of gamma irradiation on plant growth

Torenia spp. are known to be easily propagated in tissue culture (Takeuchi, 1985) and 60 d after gamma irradiation, new shoots were regenerated at the surface of leaves cultured on MS medium supplemented BA 0.25 mg/l, activated charcoal and 3 % sucrose. In this study, a comparison was made between irradiation of diploid and polyploid *Torenia fournieri*. For diploid *Torenia fournieri*, the survival rate was recorded as 100.00, 86.67, 70.00, 56.67, 36.67, 20.00 and 1.11 % for the leaves exposed to 0, 20, 40, 60, 80, 100 and 120 grays of irradiation, respectively (data not shown). The half lethal dose (LD_{50}) was 63.65 grays (Figure 13). The effect of gamma rays on plant survival depends on the exposure dose, irrespective of the irradiation method. A similar reduction in the survival rate has been observed in many species, for example, γ-ray irradiation in *Torenia* hybrida (Sawangmee, 2011) and X-ray irradiation of wheat (Kikuchi, 2009). Table 6 shows the average number of shoots, plant height and root length 60 d after acute gamma irradiation. The plant height and root length were reduced with increasing doses of gamma ray irradiation (Figure 15). A similar result was observed in leaf explants after treatment with gamma rays in *Torenia fournieri* (Jala, 2011). For polyploid *Torenia fournieri*, the survival rate was recorded as 100.00, 83.33, 73.33, 60.00, 47.78, 33.33 and 11.11 % for the leaves exposed to 0, 20, 40, 60, 80, 100 and 120 grays of irradiation, respectively (data not shown). The half lethal dose (LD_{50}) was 72.00 grays (Figure 14). Table 7 shows the average of number of shoots, plant height and root length 60 d after treatment with gamma irradiation. The plant height and root length were reduced with increasing radiation dose (Figure 16). These results are consistent with those reported for *Curcuma alismatifolia* (Thohirah, 2009). The leaf color 60 d after treatment with acute gamma irradiation changed to red in some treatment groups, i.e. the 20 gray group for diploid *Torenia fournieri* (Figure 17B) and the 80 gray group for polyploid *T. fournieri* (Figure 17D). A similar result was observed in African violet, where leaf and flower color changed due to radiation treatment (Wongpiyasatid, 2007). Research comparing a radioresistant with a radiosensitive cultivar of *Cicer arietinum* L.

revealed differences in the number of chromosome aberrations at the same dose. The 2 cultivars had already been shown by growth inhibition studies to be respectively sensitive and resistant to ionizing radiation. Chromosomal damage at the same doses, over a range from 20 to 150 kR, was determined by scoring percentages of anaphases with bridges at the first root-tip anaphase from dormancy and at anaphase I of meiosis. Mitotic delay with dose was also determined. The results indicated that more chromosomal damage at the same dose was produced in the cultivar which showed more growth-inhibition, lower survival and more dose-delay at mitosis. It could not be determined that a purely allelic difference accounted for sensitivity and resistance (Ahmad, 1981).

Table 6 The average of number of shoots, plant height and root length of diploid *Torenia fournieri* 60 d after treatment with gamma irradiation

Dose (Gy)	**Number of plants**	**Plant height (cm)**	**Root length (cm)**
0	16.10 ± 3.87^{c}	5.82 ± 0.54^{a}	9.40 ± 0.87^{a}
20	16.20 ± 0.91^{c}	5.30 ± 0.58^{b}	6.75 ± 0.67^{b}
40	17.50 ± 1.64^{c}	4.30 ± 0.53^{c}	3.85 ± 0.78^{b}
60	37.90 ± 2.72^{a}	3.00 ± 0.52^{d}	0.40 ± 0.39^{d}
80	29.60 ± 1.89^{b}	0.70 ± 0.25^{e}	0.00 ± 0.00^{d}
100	12.90 ± 2.42^{d}	0.42 ± 0.12^{ef}	0.00 ± 0.00^{d}
120	0.10 ± 0.31^{e}	0.02 ± 0.06^{f}	0.00 ± 0.00^{d}
F-test	**	**	**
% C.V.	12.10	15.40	17.37

**Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

Table 7 The average of number of shoots, plant height and root length in polyploid *Torenia fournieri* 60 d after treatment with gamma irradiation

Dose (Gy)	**Number of plants**	**Plant height (cm)**	**Root length (cm)**
0	5.10 ± 0.73^{b}	5.60 ± 0.56^{a}	8.05 ± 0.76^{a}
20	5.40 ± 0.69^{b}	4.75 ± 0.58^{b}	5.25 ± 0.48^{b}
40	13.00 ± 1.82^{a}	3.20 ± 0.53^{c}	3.75 ± 0.42^{c}
60	14.40 ± 2.27^{a}	2.90 ± 0.45^{c}	3.70 ± 0.42^{c}
80	14.20 ± 1.75^{a}	2.00 ± 0.26^{d}	3.05 ± 0.92^{d}
100	5.40 ± 0.84^{b}	0.95 ± 0.38^{e}	0.15 ± 0.24^{e}
120	0.40 ± 0.51^{c}	0.20 ± 0.25^{f}	0.00 ± 0.00^{e}
F-test	**	**	**
% C.V.	16.83	18.81	15.97

**Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

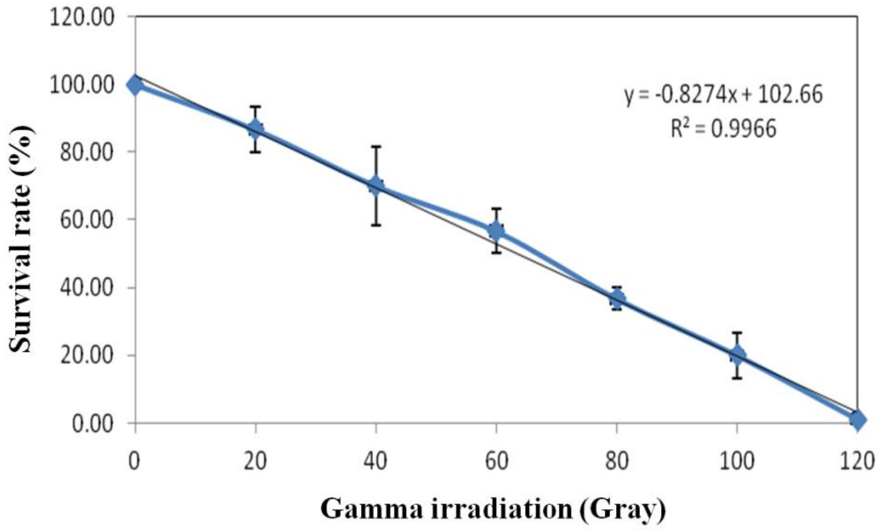


Figure 13 Survival rate (%) of diploid *Torenia fournieri* 60 d after exposure to varying doses of acute gamma radiation compared to the control, showing the LD_{50} level. (LD_{50} = 63.65 Gy)

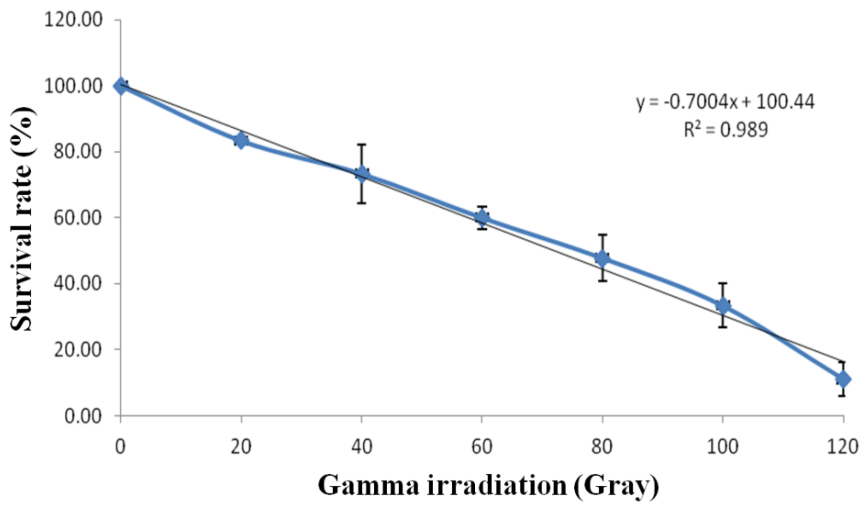


Figure 14 Survival rate (%) of polyploid *Torenia fournieri* 60 d after exposure to varying doses of acute gamma radiation compared to the control, showing the LD_{50} level. (LD_{50} = 72.00 Gy)

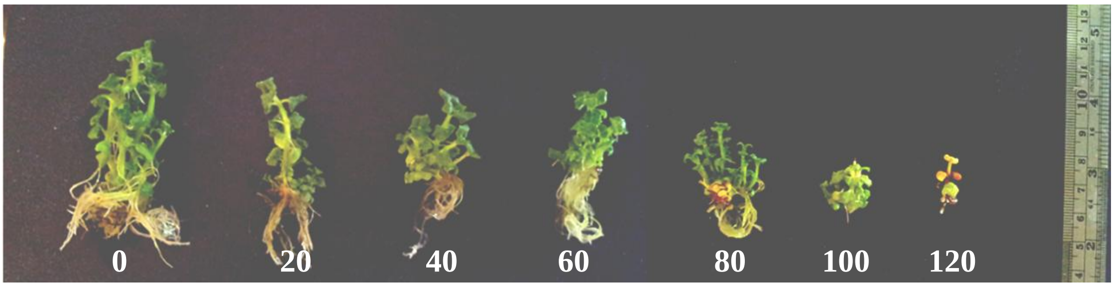


Figure 15 Morphology of diploid *Torenia fournieri* 60 d after exposure to varying doses of acute gamma radiation compared to the control (0, 20, 40, 60, 80, 100 and 120 Gy from left to right, respectively)

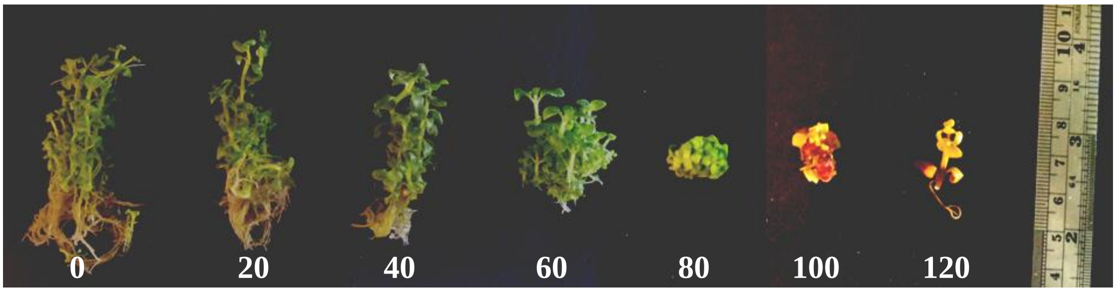


Figure 16 Morphology of polyploid *Torenia fournieri* 60 d after exposure to varying doses of acute gamma radiation compared to the control (0, 20, 40, 60, 80, 100 and 120 Gy from left to right, respectively)

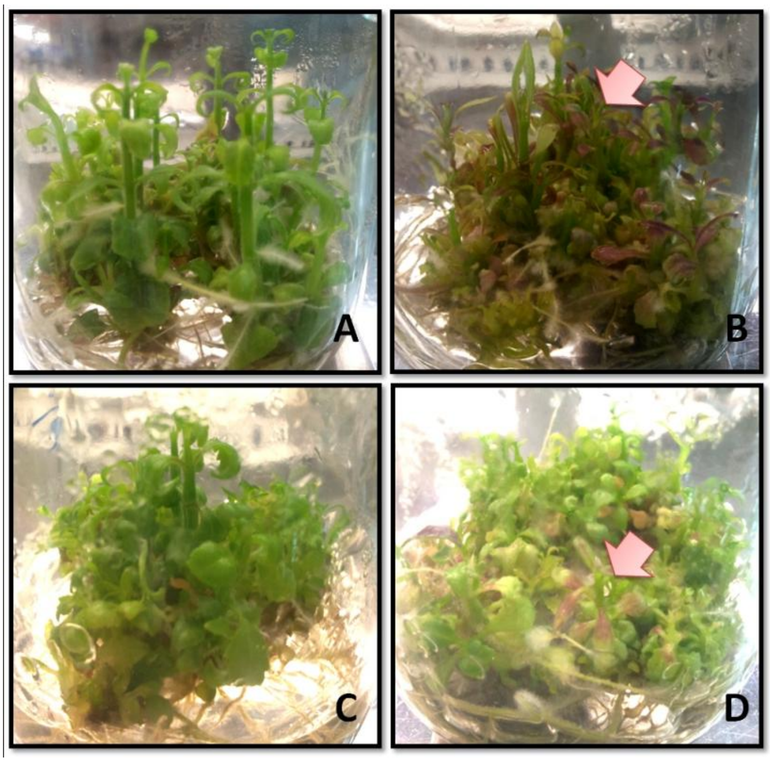


Figure 17 Red color of leaves in *Torenia fournieri* 60 d after treatment with acute gamma irradiation

(A) Diploid control.

(B) Diploid treated with 20 Gy of acute gamma irradiation.

(C) Polyploid control.

(D) Polyploid *T. fournieri* treated with 80 Gy of acute gamma irradiation

When the *T. fourneri* seedlings were planted out after 30 days, the survival rate of the polyploid plants was generally higher than that of the diploid plants, and at the highest radiation dose of 120 grays, the survival rate was zero for both diploid and polyploid *Torenia* (Table 8).

After the diploid seedlings were transplanted to pots after 60 days, there were no statistically significant differences in plant height, number of branches, internode length, or crown spread between the different treatments and the control, but there were statistically significant differences in leaf size and number of flowers. One mutated plant was observed from the 80-gray treatment group, with reddish flowers and leaves (Figure 20B) compared to the usual green (Figure 20A). Thus for the entire group of 250 specimens, only one mutant was observed, or a mutation rate of 0.4 %. However, when the cutting back method was used for propagation, the mutation to red leaves was not stable and reverted to green. This could be because the morphological change observed was a result of gamma irradiation damage to plant tissues rather than a genetic mutation, so the plant was able to recover and revert to its normal appearance (Table 9).

When a comparison was made between the growth characteristics of the sole mutant plant from the diploid 80-gray irradiated treatment group and the control, there were no statistically significant differences in plant height, number of branches, internode length, crown spread, size of leaves, number of flowers or flower size, but the color of the leaves was darker when compared using the SPAD method (Table 10).

Looking at the irradiated polyploid *Torenia*, after transplanting at 60 days, there was no statistically significant difference in number of flowers between the different treatments, but the values for plant height, number of branches, internode length, crown spread, and size of leaves were lower for all the irradiated groups compared to the non-irradiated control group. One mutant plant was observed out of the 15 surviving plants in the 100-gray treatment group, or a mutation rate of 6.67 %. This mutant had smaller leaves, slow growth, was stunted and was more susceptible to insect pests and plant diseases. Cuttings of the mutant were taken for propagation through tissue culture, but it was found that *in vitro,* the mutant was also more slow-growing than the control plants of the same age (Figure 21). When the mutant was propagated by the cutting back method, the deleterious mutation remained stable (Table 11).

Compared to the other polyploid plants, the mutant from the 100-gray treatment group had approximately the same number of branches, but the plant height, internode length, crown spread, size of leaves, number of flowers and flower size of the mutant were all significantly lower. The leaf color of the mutant plant was also darker when compared using the SPAD method (Table 12).

Microscopic analysis revealed that there were no significant differences in size of vascular bundle and sclerenchyma fiber between the mutant diploid *Torenia* and the control diploid *Torenia*, but the thickness of the epidermis and pith tissues was greater in the mutant, and cortex was thinner (Table 13 and Figure 18). As for the single polyploid mutant, its epidermis and vascular bundle cortex were similar to the control groups', but the pith layer was smaller and the sclerenchyma fiber on all 4 corners was larger (Table 14 and Figure 19).

Table 8 Survival rate of *Torenia fournieri* diploids and polyploids after 30 d transplanting.

Dose (Gy)	Diploid			Polyploid		
	No. of plant	Survival	Survival (%)	No. of plant	Survival	Survival (%)
0	150	140	93.33	40	38	95
20	150	145	96.67	40	35	87.50
40	150	145	96.67	100	84	84
60	270	200	74.07	100	70	70
80	250	200	80.00	100	70	70
100	90	70	77.78	30	15	50
120	-	-	-	-	-	-

Table 9 Plant height, internode length, spread, number of branches, number of flowers and observed mutations of *Torenia fournieri* Lind. (diploid) 60 d. after transplanting

Dose (Gy)	Total plants	Plant Height (cm)	Number of brances	Internode length (cm)	Crown spread (cm)	Size of leaf (cm)	No. of flowers	No. of floral mutants	Mutation rate (%)	No. of Solid Mutant	% solid mutant
0	150	15.50 ± 0.54	12.5 ± 2.50	2.88 ± 0.37	23.33 ± 2.33	2.37 ± 0.14^{ab}	27.17 ± 7.02^{ab}	0	0	0	0
20	150	16.33 ± 1.75	11.17 ± 3.60	2.61 ± 0.31	20.00 ± 4.66	2.46 ± 0.38^{ab}	14.50 ± 6.74^{b}	0	0	0	0
40	150	16.00 ± 1.41	13.53 ± 2.73	2.41 ± 0.20	24.08 ± 3.38	2.68 ± 0.12^{a}	29.16 ± 12.62^{a}	0	0	0	0
60	270	15.57 ± 1.75	11.30 ± 1.51	3.00 ± 0.44	20.58 ± 4.30	2.81 ± 0.23^{a}	18.67 ± 7.99^{ab}	0	0	0	0
80	250	16.33 ± 1.36	12.50 ± 2.16	2.46 ± 0.40	24.75 ± 2.78	2.37 ± 0.14^{ab}	23.00 ± 6.26^{ab}	1	0.4	0	0
100	90	13.50 ± 0.83	9.67 ± 1.03	2.50 ± 3.39	21.5 ± 3.39	2.02 ± 0.21^{b}	15.5 ± 2.81^{b}	0	0	0	0
F-test	-	ns	ns	ns	ns	**	**	-	-	-	-
% C.V.	-	8.52	20.45	15.81	15.96	9.31	36.57	-	-	-	-

**Means ± SD within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

ns = non significant

Table 10 Mean plant height, internode length, spread, number of branches, number of flowers, flower size, pollen survival rate and size of pollen of *Torenia fournieri* Lind. (diploid) 60 d after selected mutant clones were transplanted (0, 80 grays)

Dose (Gy)	Plant height (cm)	Internode length (cm)	Crown spread (cm)	Number of branches	Size of leaves (cm)	Color of leaf (spad unit)	Number of flowers	Size of flowers (mm)
Control	15.36 ± 0.85	2.76 ± 0.26	13.95 ± 1.64	8.6 ± 0.54	2.65 ± 0.37	45.62 ± 2.17[b]	45.85 ± 5.11	1.24 ± 0.04
80	15.10 ± 0.65	2.86 ± 0.26	13.20 ± 0.99	8.8 ± 0.83	3.15 ± 1.35	57.75 ± 2.43[a]	43.40 ± 3.20	1.22 ± 0.04
t-test	ns	ns	ns	ns	ns	**	ns	ns
% C.V.	4.97	9.10	9.99	8.12	34.26	6.52	9.57	3.52

**Means ± SD within the same column followed by different superscripts are significantly different using DMRT, $P \leq 0.01$

ns = non significant

Table 11 Plant height, internode length, spread, number of branches, number of flowers and observed mutations of *Torenia fournieri* Lind. (polyploid) 60 d after transplanting

Dose (Gy)	Total plants	Plant Height (cm)	Number of brances	Internode length (cm)	Crown spread (cm)	Size of leaf (cm)	Number of flowers	Number of leaf mutants	Mutation rate (%)	Number of Solid Mutants	Solid mutant (%)
0	38	19.83 ± 1.47	10.17 ± 1.60 ^{a}	2.91 ± 0.20^{a}	18.91 ± 0.66^{a}	4.07 ± 0.12^{a}	32.00 ± 2.75	0	0	0	0
20	35	19.33 ± 1.40	9.33 ± 1.63^{ab}	2.51 ± 0.29^{b}	17.75 ± 1.03^{a}	4.07 ± 0.09^{a}	32.17 ± 1.72	0	0	0	0
40	84	18.84 ± 2.16	9.33 ± 1.63^{ab}	2.16 ± 0.25^{c}	17.41 ± 1.46^{ab}	3.55 ± 0.16^{b}	29.00 ± 2.36	0	0	0	0
60	70	18.94 ± 1.06	7.83 ± 0.75^{bc}	1.80 ± 0.14^{d}	15.25 ± 1.03^{c}	3.52 ± 0.14^{b}	27.83 ± 1.47	0	0	0	0
80	70	17.33 ± 1.21	6.33 ± 1.03^{cd}	1.68 ± 0.14^{d}	16.08 ± 0.91^{bc}	3.20 ± 0.12^{c}	24.50 ± 2.07	0	0	0	0
100	15	17.33 ± 1.21	4.67 ± 0.51^{d}	1.80 ± 0.14^{d}	16.00 ± 0.63^{bc}	3.00 ± 0.10^{d}	24.67 ± 1.36	1	6.67	1	6.67
F-test	-	ns	**	**	**	**	ns	-	-	-	-
% C.V.	-	7.88	4.62	11.96	5.90	3.35	7.12	-	-	-	-

**Means ± SD within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

ns = non significant

Table 12 Mean plant height, internode length, spread, number of branches, number of flowers, flower size, pollen survival rate and size of pollen of *Torenia fournieri* Lind. (polyploid) 60 d after selected mutant clones were transplanted (0, 100 grays)

Dose (Gy)	Plant height (cm)	Internode length (cm)	Crown spread (cm)	Number of branches	Size of leaves (cm)	Color of leaf (spad unit)	Number of flowers	Size of flowers (cm)
0	20.88 ± 1.39[a]	2.72 ± 0.21[a]	19.72 ± 0.64[a]	8.60 ± 0.54	3.99 ± 0.21[a]	52.38 ± 2.61[b]	51.00 ± 0.19[a]	1.74 ± 0.19[a]
100	12.66 ± 1.63[b]	1.24 ± 0.18[b]	12.05 ± 0.77[b]	8.80 ± 0.83	1.72 ± 0.30[b]	64.48 ± 1.33[a]	0.84 ± 0.23[b]	0.84 ± 0.23[b]
t-test	**	**	**	ns	**	**	**	**
% C.V.	9.08	10.10	4.49	8.13	9.10	3.54	6.67	16.53

**Means ± SD within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

ns = non significant

Table 13 Mean size of Epidermis, Vascular bundle, Cortex and Pith *Torenia fournieri* Lind. (diploid) 90 d. after leaves cutting.

Plants	**Epidermis (µm)**	**Vascular bundle (µm)**	**Cortex (µm)**	**Pith (µm)**	**Sclerenchyma Fiber (µm)**
Control	5.52 ± 0.23^{b}	57.17 ± 2.27	43.20 ± 0.11^{a}	238.85 ± 1.52^{b}	30.225 ± 0.78
Mutant	8.24 ± 0.03^{a}	53.42 ± 2.43	38.44 ± 0.36^{b}	296.17 ± 2.47^{a}	30.500 ± 0.43
t-test	**	ns	**	**	ns
%C.V.	2.34	4.25	0.65	0.76	2.37

**Means ± SD within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

ns = non significant

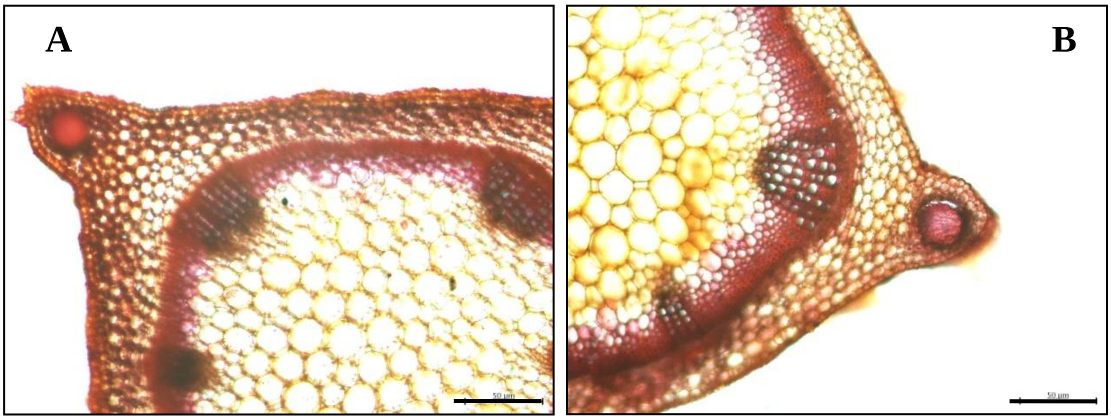


Figure 18 Comparison of anatomical of stem between *Torenia fournieri* (diploid) (A) and *Torenia fournieri* (Mutant) (B) 90 d. after cutting. (Bar = 50 µm)

Table 14 Mean size of Epidermis, Vascular bundle, Cortex and Pith *Torenia fournieri* Lind. (polyploid) 90 d. after leaves cutting**.**

Plants	Epidermis (µm)	Vascular bundle (µm)	Cortex (µm)	Pith (µm)	Sclerenchyma Fiber (µm)
Control	8.66 ± 0.10	72.67 ± 0.76	46.00 ± 0.09	383.17 ± 0.76[a]	23.83 ± 0.88[b]
Mutant	8.95 ± 0.42	59.69 ± 9.64	45.90 ± 4.19	296.83 ± 3.59[b]	27.32 ± 2.79[a]
t-test	ns	ns	ns	**	**
%C.V.	3.45	10.33	6.59	4.90	8.10

**Means ± SD within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

ns = non significant

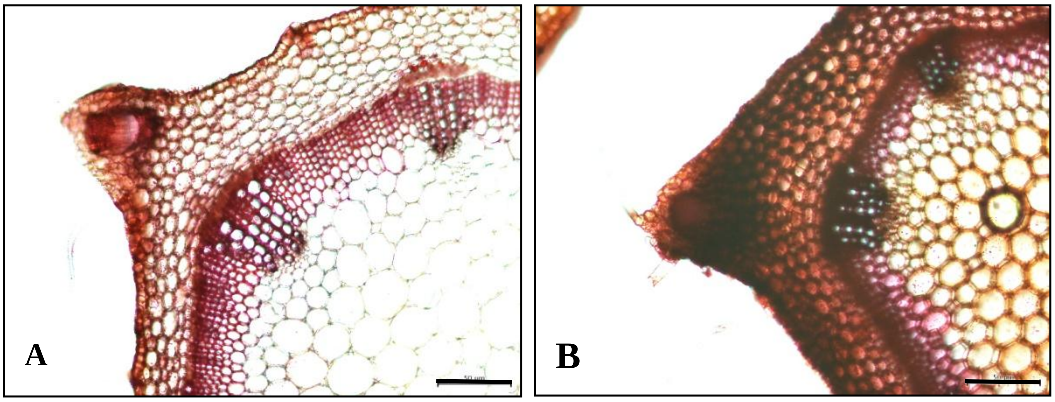


Figure 19 Comparison of anatomical of stem between *Torenia fournieri* (polyploid) (A) and *Torenia fournieri* (Mutant) (B) 90 d. after cutting. (Bar = 50 µm)

Figure 20 Mophological appearance of *Torenia fournieri* (diploid) (A) and *Torenia fournieri* (Mutant) from gramma irradiation 80 Gy. (B) 60 d. after transplanting

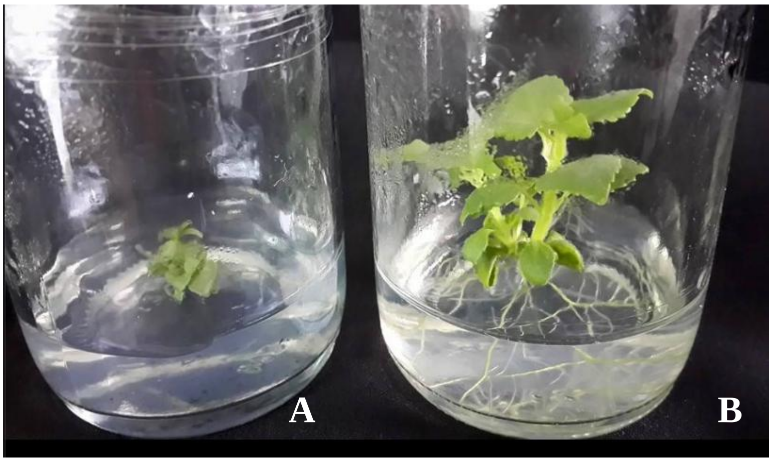


Figure 21 Mophological appearance of *Torenia fournieri* (Mutant) (A) and *Torenia fournieri* (polyploid) control (B) 30 d. after culturing

Tetraploid induction

Leaves were cut from F1 hybrids of Torenia for the different treatments. The petioles were soaked in 15 mg/l colchicine solution for 0, 12, 24, 48 and 72 h (32 leaves per treatment), after which they were placed upright in peat moss for rooting (modified from Boonbongkarn, 2013). The survival rate decreased when treatment duration was increased. The results showed that 3 clones in *T. asiatica* x *T. ranongensis* (1 plant from 48 h, 2 plants from 72 h) (Table 15), 15 clones in *T. bentamiana* x *T. asiatica* (8 plants from 12 h, 1 plant from 24 h, 3 plants from 48 h, 3 plants from 72 h) (Table 16), 24 clones from *T. asiatica* x *T. pierriana* (6 plants from 12 h, 6 plants from 24 h, 6 plants from 48 h, 6 plants from 72 h) (Table 17) and 18 clones in *T. fournieri* x *T. asiatica* (7 plants from 12 h, 6 plants from 24 h, 3 plants from 48 h, 2 plants from 72 h (Table 18) were tetraploid.

Table 15 Frequency of tetraploids induced in hybrids between *T. asiatica* and *T. ranongensis* 60 days after colchicine treatment.

Duration (hours)	Number of leaves	Number of survival	Tetraploid	% Tetraploid
0	32	32	0	0
12	32	32	0	0
24	32	24	0	0
48	32	29	1	3.12
72	32	31	2	6.25

Table 16 Frequency of tetraploids induced in hybrids between *T. bentamiana* and *T. asiatica* 60 days after colchicine treatment .

Duration (hours)	Number of leaves	Number of survival	Tetraploid	% Tetraploid
0	32	32	0	0
12	32	32	8	25
24	32	32	1	3.12
48	32	32	3	9.37
72	32	32	3	9.37

Table 17 Frequency of tetraploids induced in hybrids between *T. asiatica* and *T. pierriana* 60 days after colchicine treatment.

Duration (hours)	Number of leaves	Number of survival	Tetraploid	% Tetraploid
0	32	32	0	0
12	32	32	6	18.75
24	32	32	6	18.75
48	32	31	6	18.75
72	32	31	6	18.75

Table 18 Frequency of tetraploids induced in hybrids between *T. fournieri* and *T. asiatica* 60 days after colchicine treatment.

Duration (hours)	Number of leaves	Number of survival	Tetraploid	% Tetraploid
0	32	32	0	0
12	32	32	7	21.87
24	32	32	6	18.75
48	32	29	3	9.37
72	32	22	2	6.25

The growth rates after treatment with colchicine tablet solution on plant height, internode length, crown spread, number of branches, number of flowers were observed in 60 days. In *T. asiatica* x *T. ranongensis*, there were no statistically significant differences in growth rates among all the plants treated with colchicine (Table 19). In *T. bentamiana* x *T. asiatica*, internode length was not statistically significant, but, number of branches decreased after treatment with colchicine (Table 20). In *T. asiatica* x *T. pierriana*, differences in crown spread, number of branches, and number of flowers were not statistically significant. But, internode length of all the plants decreased and plant height was different (Table 21). In *T. fournieri* x *T. asiatica*, differences in crown spread and number of branches were not statistically significant (Table 22).

When studied size of epidermis, vascular bundle, cortex and pith 90 days after cutting, all tetraploid hybrids were bigger than diploid; *T. asiatica* x *T. ranongensis* is shown in Table 23 and Figure 22, *T. bentamiana* x *T. asiatica* is shown in Table 24 Figure 23, *T. asiatica* x *T. pierriana* is shown in Table 25 Figure 24 and *T. fournieri* x *T. asiatica* is shown in Table 26 Figure 25.

Table 19 Plant height, internode length, crown spread, number of branches, and number of flowers observed in hybrids between *T. asiatica and T. ranongensis* 60 d after treated with colchicine.

Duration (hours)	**Plant height (cm)**	**Internode length (cm)**	**Crown spread (cm)**	**Number of brances**	**Number of flowers**
0	31.78 ± 1.71	3.90 ± 0.37	42.00 ± 8.03	1.33 ± 0.54	0.00 ± 0.00
12	30.30 ± 1.70	3.60 ± 0.44	33.75 ± 5.64	1.83 ± 0.40	0.00 ± 0.00
24	30.27 ± 1.39	3.14 ± 0.20	30.42 ± 5.79	1.66 ± 0.91	0.00 ± 0.00
48	30.58 ± 2.03	4.40 ± 0.37	35.50 ± 3.51	1.33 ± 0.51	0.00 ± 0.00
72	34.00 ± 3.72	4.06 ± 0.39	32.92 ± 1.74	1.50 ± 0.54	0.00 ± 0.00
F-test	ns	ns	ns	ns	ns
% C.V.	7.28	10.25	15.03	7.65	0.00

** Means within the same column followed by different superscripts are significantly different using DMRT, $P \leq 0.01$

ns = non significant

Table 20 Plant height, internode length, crown spread, number of branches, number of flowers observed in hybrids between *T. bentamiana* and *T. asiatica* 60 d after treated with colchicine.

Duration (hours)	Plant height (cm)	Internode length (cm)	Crown spread (cm)	Number of brances	Number of flowers
0	16.07 ± 1.39[b]	3.10 ± 0.10	19.08 ± 1.82[b]	3.67 ± 1.21[a]	0.00 ± 0.00[b]
12	20.25 ± 3.83[a]	3.11 ± 0.20	28.67 ± 2.44[a]	2.00 ± 0.89[b]	1.33 ± 0.51[a]
24	20.82 ± 1.88[a]	3.28 ± 0.40	29.42 ± 3.42[a]	1.83 ± 0.75[b]	1.33 ± 0.51[a]
48	12.45 ± 2.21[b]	3.05 ± 0.08	21.08 ± 2.39[b]	1.33 ± 0.51[b]	1.50 ± 0.54[a]
72	7.48 ± 1.18[c]	3.13 ± 0.19	16.59 ± 1.90[c]	1.50 ± 0.54[b]	0.83 ± 0.75[ab]
F-test	**	ns	**	**	**
% C.V.	14.93	7.28	10.74	39.00	52.00

** Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

ns = non significant

Table 21 Plant height, internode length, spread, number of branches, and number of flowers in hybrids between *T. asiatica* and *T. pierriana* 60 d after treated with colchicine.

Duration (hours)	**Plant height (cm)**	**Internode length (cm)**	**Crown spread (cm)**	**Number of brances**	**Number of flowers**
0	26.17 ± 1.29[b]	3.28 ± 0.24[a]	37.00 ± 2.58	2.00 ± 0.63	1.16 ± 0.98
12	39.83 ± 7.50[a]	2.83 ± 0.18[b]	38.00 ± 5.98	1.83 ± 0.75	1.16 ± 0.75
24	29.17 ± 10.02[ab]	2.90 ± 0.08[b]	36.42 ± 3.62	1.66 ± 0.51	1.66 ± 0.53
48	39.50 ± 6.00[a]	2.81 ± 0.11[b]	36.58 ± 0.86	1.35 ± 0.83	1.50 ± 0.83
72	39.42 ± 2.05[a]	2.71 ± 0.14[b]	34.50 ± 3.24	1.66 ± 0.51	1.66 ± 0.51
F-test	**	**	ns	ns	ns
% C.V.	18.32	5.74	10.02	38.25	51.89

** Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

ns = non significant

Table 22 Plant height, internode length, spread, number of branches, number of flowers observed in hybrids between *T. fournieri and T. asiatica* 60 d after treatment with colchicine.

Duration (hours)	**Plant height (cm)**	**Internode length (cm)**	**Crown spread (cm)**	**Number of brances**	**Number of flowers**
0	24.23 ± 1.18^{ab}	2.6 ± 0.35^{a}	30.08 ± 1.35	3.00 ± 0.75	6.17 ± 1.16^{b}
12	22.17 ± 4.91^{ab}	2.65 ± 0.20^{a}	30.00 ± 0.94	2.20 ± 0.51	6.00 ± 1.78^{b}
24	22.00 ± 1.41^{ab}	2.26 ± 0.29^{ab}	26.17 ± 4.28	2.40 ± 0.51	6.83 ± 1.47^{b}
48	19.98 ± 2.94^{b}	2.01 ± 0.18^{ab}	28.33 ± 1.80	3.20 ± 0.89	11.67 ± 2.58^{a}
72	25.73 ± 5.09^{a}	2.00 ± 0.19^{c}	26.83 ± 1.25	2.60 ± 0.98	8.83 ± 1.47^{ab}
F-test	**	**	ns	ns	**
% C.V.	13.26	11.26	8.05	26.38	22.35

** Means within the same column followed by different superscripts are significantly different using DMRT, $P \leq 0.01$

ns = non significant

Table 23 Mean size of Epidermis, Vascular bundle, Cortex and Pith between *T. asiatica* and *T. ranongensis* 90 d. after cutting.

Plants	Epidermis (μm)	Vascular bundle (μm)	Cortex (μm)	Pith (μm)	Sclerenchyma fiber (μm)
Diploid	5.12 ± 0.10^{b}	35.08 ± 1.18^{b}	34.91 ± 0.52^{b}	235.83 ± 1.25^{b}	25.08 ± 0.52^{a}
Tetraploid	7.04 ± 0.05^{a}	45.85 ± 0.11^{a}	38.75 ± 0.00^{a}	260.83 ± 1.04^{a}	20.19 ± 0.38^{b}
t-test	**	**	**	**	**
%C.V.	1.36	2.05	1.00	0.46	2.01

** Means within the same column followed by different superscripts are significantly different using DMRT, $p \leq 0.01$

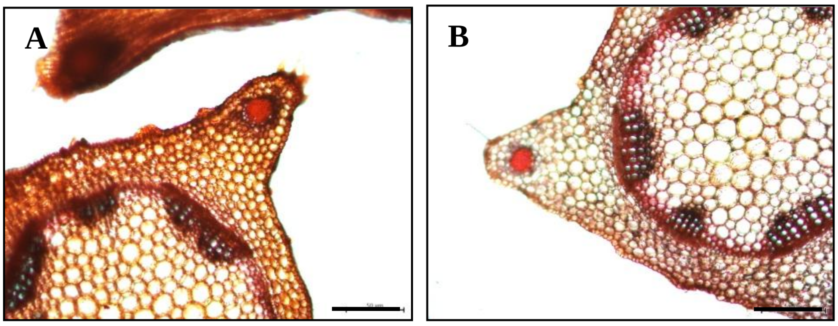


Figure 22 Comparison of anatomical of stem between *T. asiatica* and *T. ranongensis* 90 d. after cutting (Bar = 50 μm)

Table 24 Mean size of Epidermis, Vascular bundle, Cortex and Pith between *T. bentamiana* and *T. asiatica* 90 d. after cutting.

Plants	Epidermis (µm)	Vascular bundle (µm)	Cortex (µm)	Pith (µm)	Sclerenchyma fiber (µm)
Diploid	4.25 ± 0.43[b]	36.27 ± 0.25[b]	34.75 ± 1.98[b]	223.17 ± 1.60[b]	18.25 ± 0.50[a]
Tetraploid	6.35 ± 0.05[a]	46.41 ± 0.72[a]	41.92 ± 0.28[a]	268.67 ± 1.04 [a]	17.92 ± 0.72[b]
t-test	**	**	**	**	*
%C.V.	5.80	1.31	3.70	0.55	3.43

** Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

* Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.05

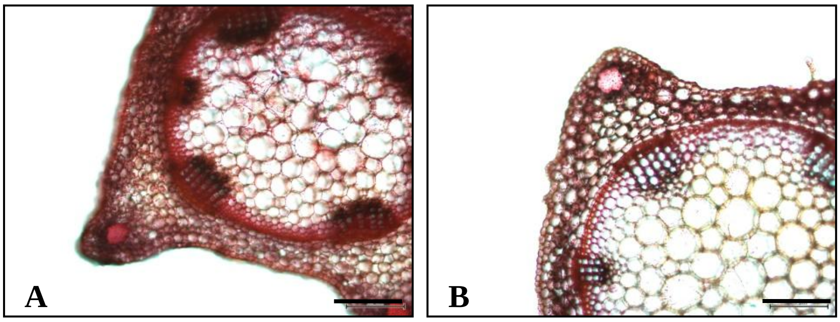


Figure 23 Comparison of anatomical of stem between *T. bentamiana* and *T. asiatica* 90 d. after cutting (Bar = 50 µm)

Table 25 Mean size of Epidermis, Vascular bundle, Cortex and Pith between *T. asiatica* and *T. pierriana* 90 d. after cutting.

Plants	Epidermis (µm)	Vascular bundle (µm)	Cortex (µm)	Pith (µm)	Sclerenchyma fiber (µm)
Diploid	4.60 ± 0.01[b]	31.39 ± 0.72[b]	29.50 ± 0.66[b]	225.33 ± 1.53[b]	19.00 ± 0.25[b]
Tetraploid	6.61 ± 0.11[a]	47.33 ± 0.52[a]	39.17 ± 0.57[a]	294.67 ± 5.00[a]	20.33 ± 0.63[a]
t-test	**	**	**	**	*
%C.V.	1.43	1.60	1.81	1.42	1.56

** Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

* Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.05

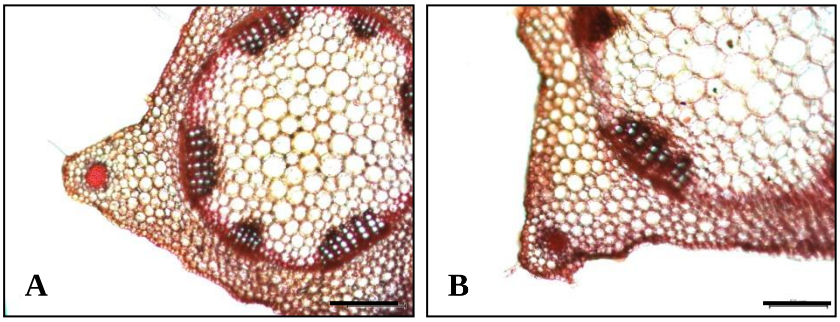


Figure 24 Comparison of anatomical of stem between *T. asiatica* and *T. pierriana* 90 d. after cutting (Bar = 50 µm)

Table 26 Mean size of Epidermis, Vascular bundle, Cortex and Pith between *T. fournieri* and *T. asiatica* 90 d. after cutting**.**

Plants	Epidermis (μm)	Vascular bundle (μm)	Cortex (μm)	Pith (μm)	Sclerenchyma fiber (μm)
Diploid	4.98 ± 1.47[b]	34.96 ± 0.50[b]	24.20 ± 0.06[b]	213.67 ± 4.65[b]	17.58 ± 1.01[b]
Tetraploid	6.05 ± 0.04[a]	49.15 ± 1.49[a]	38.42 ± 1.44[a]	345.83 ± 0.76[a]	20.13 ± 0.33[a]
t-test	**	**	**	**	*
%C.V.	1.95	2.07	3.26	1.19	3.98

** Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

* Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.05

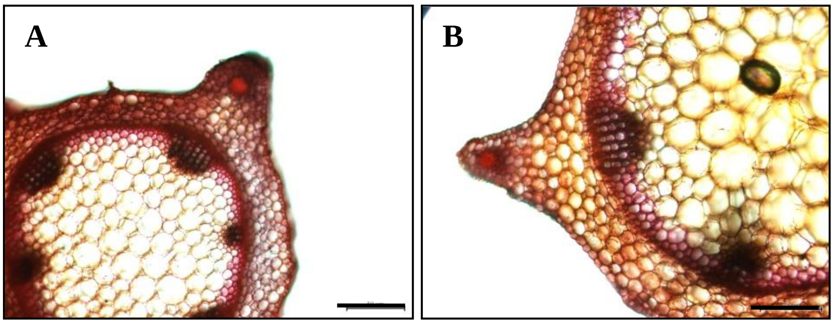


Figure 25 Comparison of anatomical of stem between *T. fournieri* and *T. asiatica* 90 d. after cutting (Bar = 50 μm)

However, at 60 days there was a difference in growth rate between the diploid plants and tetraploid plants; In *T. asiatica* x *T. ranongensis*, differences in plant height, internode length, crown spread, number of branches, number of flowers, and flower size were not statistically significant (Table 27). In *T. bentamiana* x *T. asiatica*, plant height, crown spread, number of branches, and size of flower were greater in diploids than in the tetraploids, but the difference in number of flowers was not statistically significant (Table 28). In *T. asiatica* x *T. pierriana*, crown spread was greater in diploids than tetraploids, but differences in plant height, internode length, number of brances, number of flowers, size of pollen were not to a statistically significant (Table 29). In *T. fournieri* x *T. asiatica*, plant height, internode length, crown spread, number of branches, and size of flower were greater in diploids than in tetraploids (Table 30). However, all hybrids from tetraploid plants had greater survival rate of pollen, greater leaf size and more intense green leaf color, as well as larger stomata than diploid plants, but size of pollen is not different (Figures 34-53).

Comparison of the chromosome number of diploids versus tetraploids: *T. asiatica* x *T. ranongensis* diploid (2n=2x=34) and tetraploid (2n=4x=68) (Figure 26), *T. bentamiana* x *T. asiatica* diploid (2n=2x=34) (A) and tetraploid (2n=4x=68) (Figure 27), *T. asiatica* x *T. pierriana* diploid (2n=2x=34) (A) and tetraploid (2n=4x=68) (Figure 28), *T. fournieri* x *T. asiatica* (2n=2x=26) (A) and an induced tetraploid (2n=4x=52) (Figure 29)

Based on these results, plants were selected for ploidy analysis by flow cytometry based on having mean stomata guard cell length that was greater than control. Flow cytometry results could identify possible tetraploids from having a fluorescence peak that was different than control (Figure 30-33).

Table 27 Mean plant height, internode length, spread, number of branches, number of flowers, flower size, pollen survival rate and size of pollen *T. asiatica* and *T. ranongensis* 60 d after cutting stem.

Plants	Plant height (cm)	Internode length (cm)	Crown spread (cm)	Number of branches	Size of leaves (cm)	Number of flowers	Size of flowers (cm)	Survival of pollen (%)	Size of pollen (µm)	Color of leaf (spad unit)	Size of stomata (µm)
Diploid	54.67 ± 3.77	3.67 ± 0.41	38.75 ± 3.45	11.67 ± 1.86	2.37 ± 1.44	2.5 ± 1.51	11.50 ± 9.98	2.63 ± 0.90^{b}	55.33± 5.88	34.25 ± 1.80^{b}	21.83 ± 1.47^{b}
Tetrapioid	53.66 ± 2.73	3.40 ± 0.32	32.08 ± 1.68	9.83 ± 1.47	2.13 ± 0.19	1.60 ± 0.81	11.50 ± 0.94	44.42 ± 1.85^{a}	62.00 ± 4.19	48.32 ± 5.94^{a}	27.00 ± 1.47^{a}
t-test	ns	ns	ns	ns	ns	ns	ns	**	ns	**	**
% C.V.	6.08	10.48	9.63	15.61	33.48	38.46	8.01	6.19	8.71	10.64	5.9

** Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

ns = non significant

Table 28 Mean plant height, internode length, spread, number of branches, number of flowers, flower size, pollen survival rate and size of pollen *T. bentamiana* and *T. asiatica* 60 d after cutting stem.

Plants	Plant height (cm)	Internode length (cm)	Crown spread (cm)	Number of branches	Size of leaves (cm)	Number of flowers	Size of flowers (mm)	Survival of pollen (%)	Size of pollen (µm)	Color of leaf (spad unit)	Size of stomata (µm)
Diploid	61.33 ± 4.71^{a}	3.08 ± 0.04	20.08 ± 0.80^{b}	10.50 ± 1.04^{a}	1.93 ± 0.11^{b}	18.90 ± 1.41	10.87 ± 0.10^{a}	16.17 ± 2.99^{b}	36.00 ± 3.57^{b}	47.63 ± 6.14^{b}	19.33 ± 1.21^{b}
Tetrapioid	45.83 ± 5.26^{b}	2.95 ± 0.15	18.50 ± 0.70^{a}	8.16 ± 0.75^{b}	2.80 ± 0.21^{a}	15.83 ± 0.98	10.18 ± 0.14^{b}	41.95 ± 1.56^{a}	36.67 ± 3.01	56.40 ± 2.31^{a}	29.17 ± 0.75^{a}
t-test	**	ns	**	**	**	ns	**	**	ns	**	**
% C.V.	9.33	4.23	3.91	9.79	7.18	7.19	12.56	8.22	9.10	8.93	4.16

** Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

ns = non significant

Table 29 Mean plant height, internode length, spread, number of branches, number of flowers, flower size, pollen survival rate and size of pollen *T. asiatica* and *T. pierriana* 60 d after cutting stem.

Plants	Plant height (cm)	Internode length (cm)	Crown spread (cm)	Number of branches	Size of leaves (cm)	Number of flowers	Size of flowers (mm)	Survival of pollen (%)	Size of pollen (µm)	Color of leaf (spad unit)	Size of stomata (µm)
Diploid	56.83 ± 3.31	2.98 ± 0.16	33.75 ± 2.31^{a}	11.5 ± 1.51	1.75 ± 0.03^{b}	11.50 ± 1.51	9.00 ± 0.81^{b}	5.08 ± 0.73^{b}	24.67 ± 3.01	25.85 ± 1.83^{b}	18.5 ± 3.56^{b}
Tetrapioid	52.54 ± 1.87	2.82 ± 0.11	28.83 ± 1.32^{b}	9.67 ± 2.73	1.97 ± 0.11^{a}	9.33 ± 2.33	14.18 ± 1.05^{a}	44.90 ± 1.36^{a}	26.16 ± 2.00	51.80 ± 1.16^{a}	29.87 ± 1.86^{a}
t-test	ns	ns	**	ns	**	ns	**	**	ns	**	**
% C.V.	4.90	4.83	6.03	20.83	5.92	18.92	8.38	4.38	10.12	3.95	11.80

** Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

ns = non significant

Table 30 Mean plant height, internode length, spread, number of branches, number of flowers, flower size, pollen survival rate and size of pollen *T. fournieri* and *T. asiatica* 60 d after cutting stem.

Plants	Plant height (cm)	Internode length (cm)	Crown spread (cm)	Number of branches	Size of leaves (cm)	Number of flowers	Size of flowers (mm)	Survival of pollen (%)	Size of pollen (µm)	Color of leaf (spad unit)	Size of stomata (µm)
Diploid	40.59 ± 2.73^{a}	2.78 ± 0.23^{a}	29.16 ± 1.66^{a}	14.50 ± 1.87^{a}	1.82 ± 0.06^{b}	24.85 ± 2.85	10.69 ± 0.88^{b}	3.66 ± 1.35^{b}	22.33 ± 3.67^{b}	33.73 ± 1.04^{b}	22.67 ± 2.06^{b}
Tetrapioid	30.60 ± 2.73^{b}	2.35 ± 0.22^{b}	25.41 ± 6.73^{b}	9.80 ± 1.47^{b}	2.50 ± 0.15^{a}	24.83 ± 1.47	14.70 ± 0.52^{a}	47.00 ± 4.12^{a}	26.67 ± 2.00^{a}	40.00 ± 4.12^{a}	29.83 ± 1.32^{a}
t-test	**	**	**	**	**	ns	**	**	ns	**	**
% C.V.	7.69	8.91	4.71	13.84	5.34	9.13	5.86	9.08	12.15	8.16	6.62

** Means within the same column followed by different superscripts are significantly different using DMRT, P≤0.01

ns = non significant

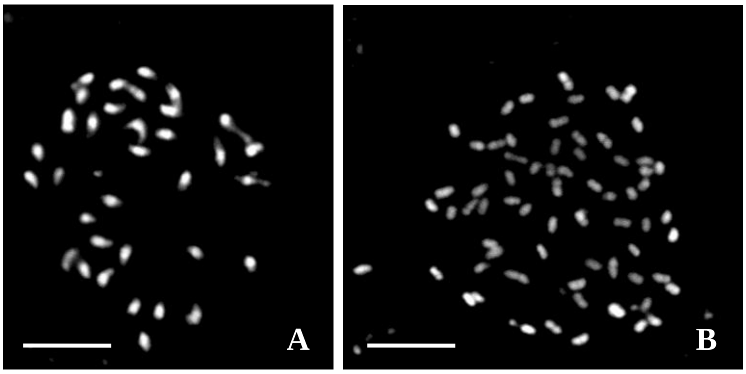


Figure 26 Comparison of the chromosome number of diploid between *T. asiatica* and *T. ranongensis* (2n=2x=34) (A) and an induced tetraploid between *T. asiatica* and *T. ranongensis* (2n=4x=68) (B) (Bar = 10 µm)

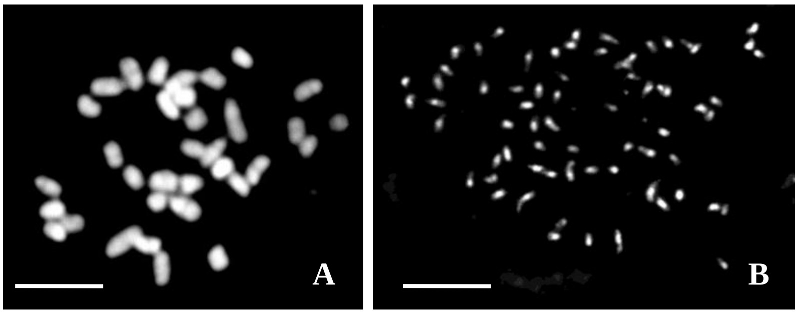


Figure 27 Comparison of the chromosome number of diploid between *T. bentamiana* and *T. asiatica* (2n=2x=34) (A) and an induced tetraploid between between *T. bentamiana* and *T. asiatica* (2n=4x=68) (B) (Bar = 10 µm)

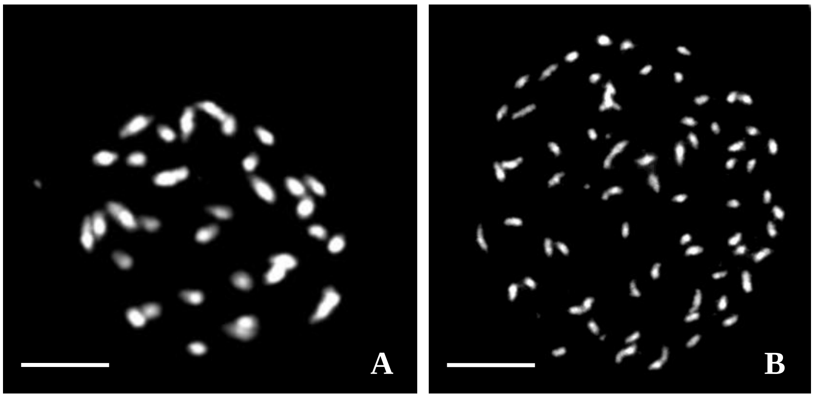


Figure 28 Comparison of the chromosome number of diploid between *T. asiatica* and *T. pierriana* (2n=2x=34) (A) and an induced tetraploid between *T. asiatica* and *T. pierriana* (2n=4x=68) (B)

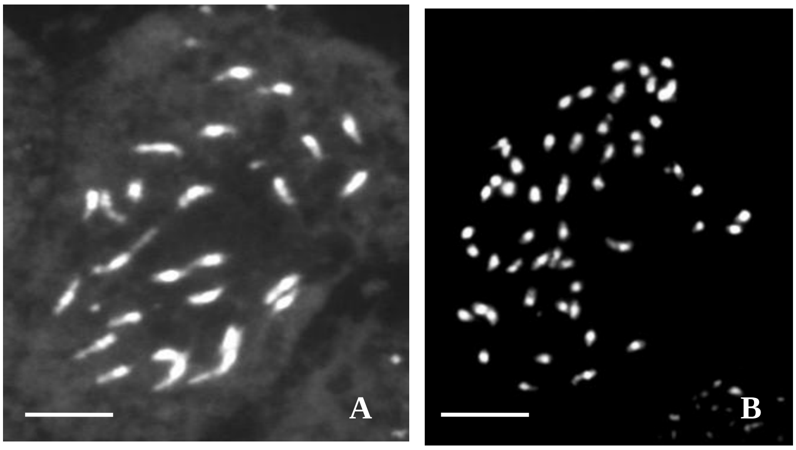


Figure 29 Comparison of the chromosome number of diploid between *T. fournieri* and *T. asiatica* (2n=2x=26) (A) and an induced tetraploid between *T. fournieri* and *T. asiatica* (2n=4x=52) (B)

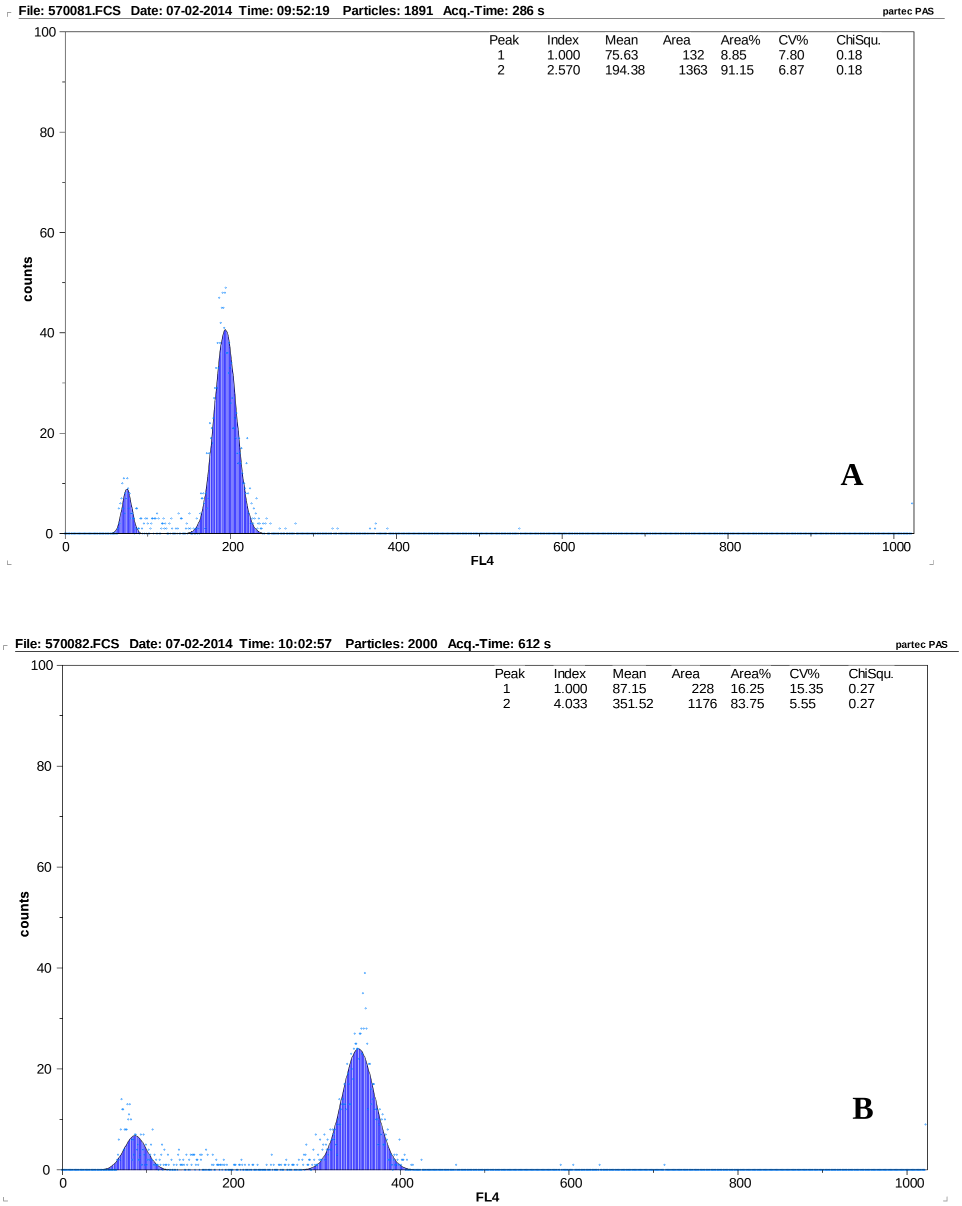


Figure 30 Flow cytometry histograms showing the DNA content of diploid (A) and tetraploid (B) *T. asiatica* × *T. ranongensis*

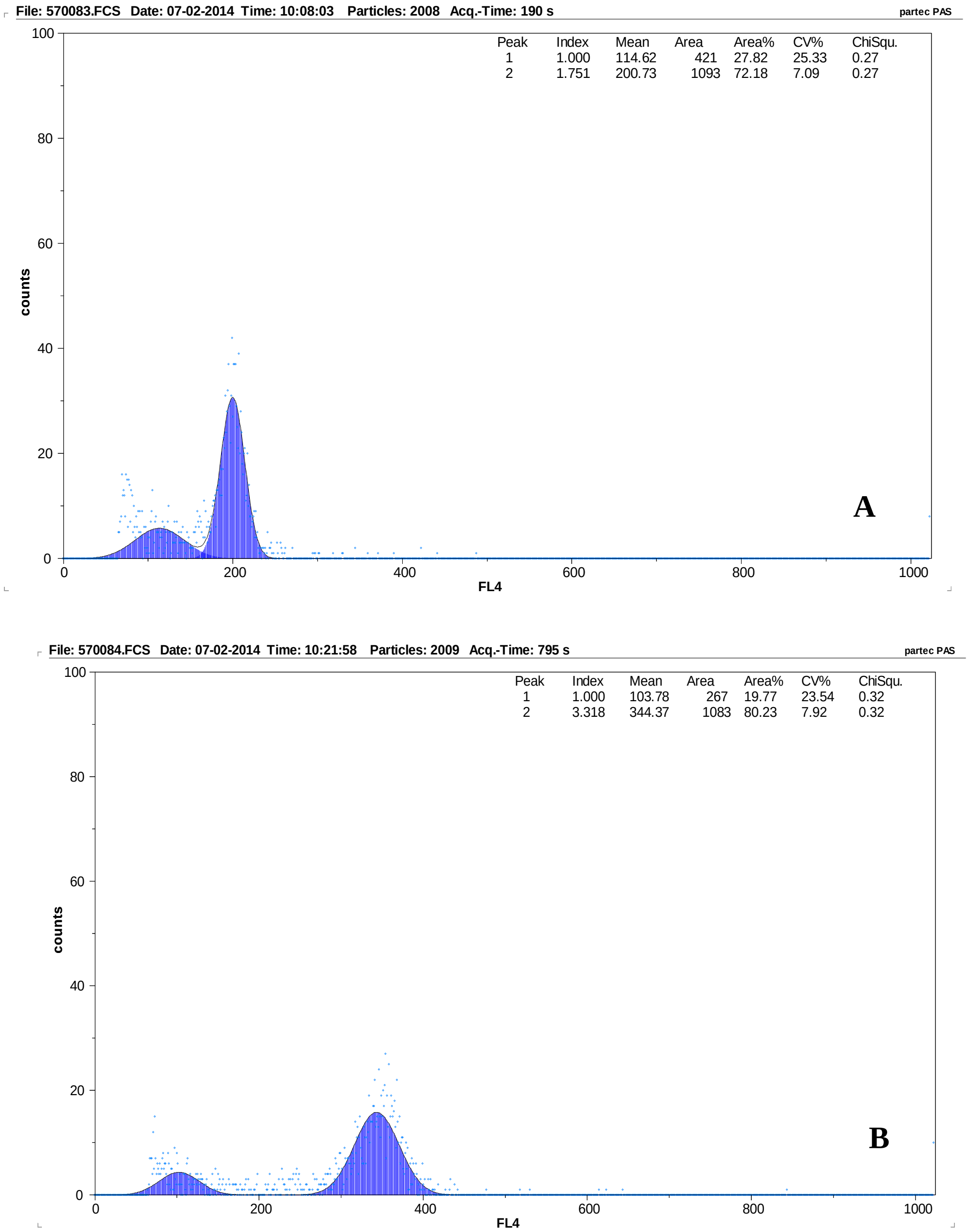


Figure 31 Flow cytometry histograms showing the DNA content of diploid (A) and tetraploid (B) *T. bentamiana* × *T. asiatica*

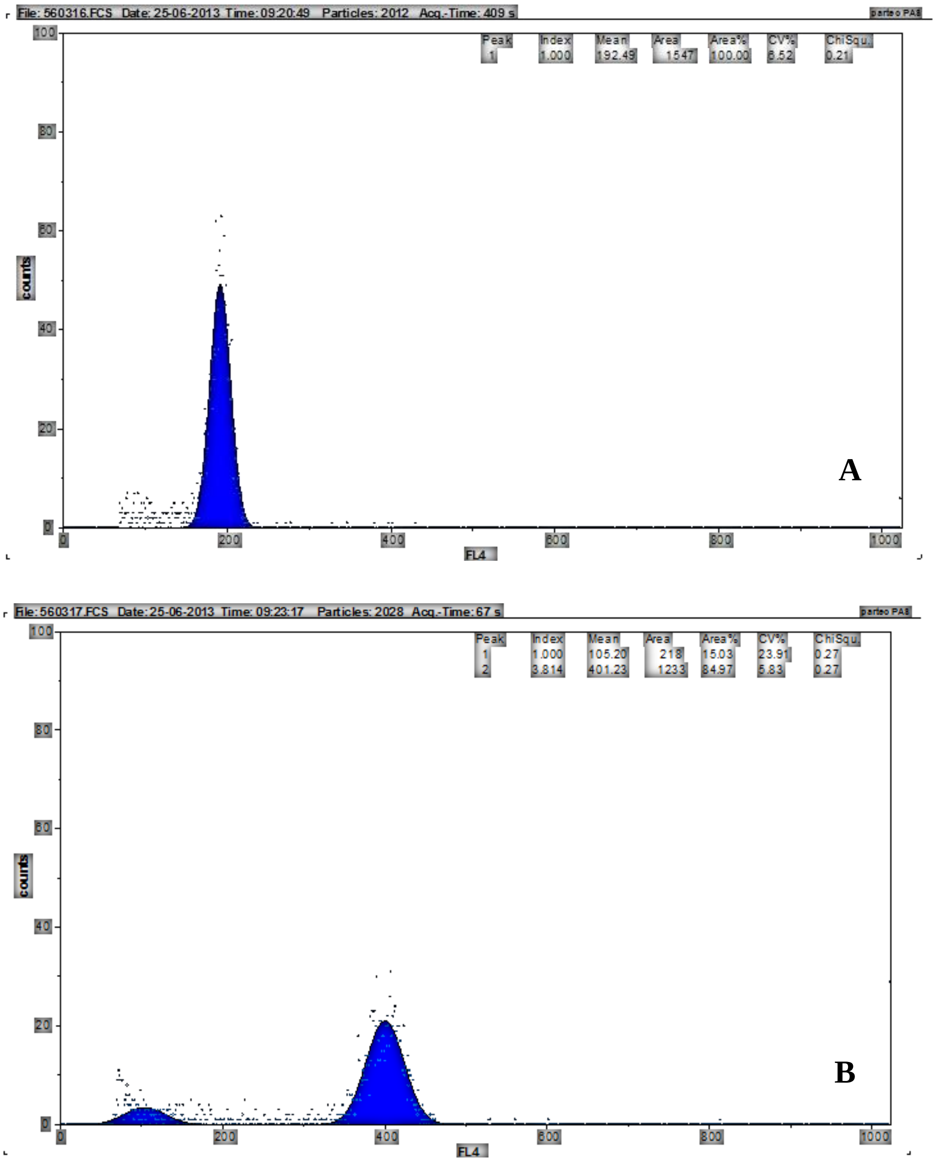
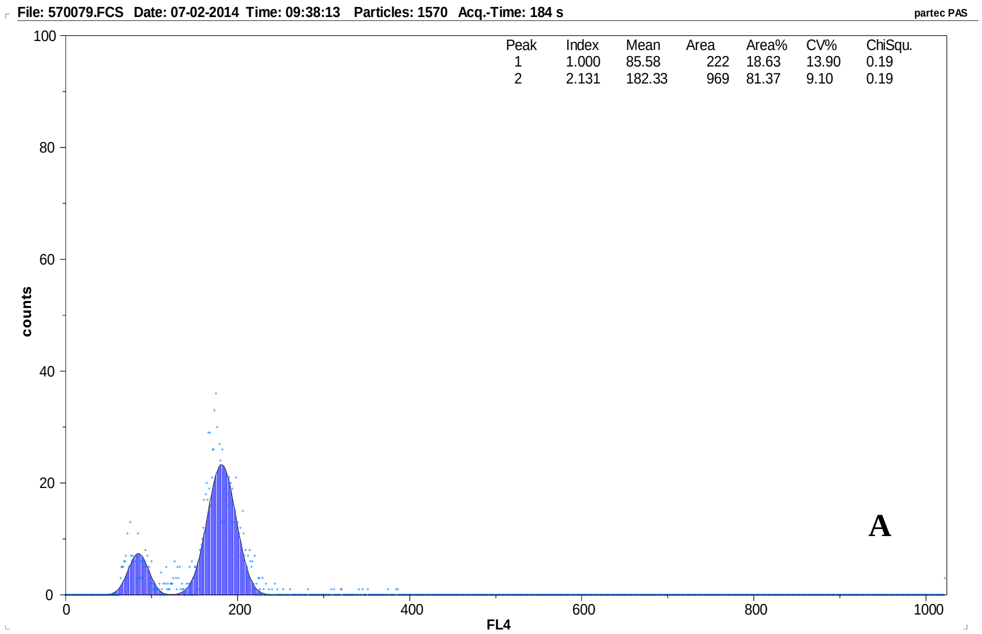


Figure 32 Flow cytometry histograms showing the DNA content of diploid (A) and tetraploid (B) *T. asiatica* x *T. pierriana*



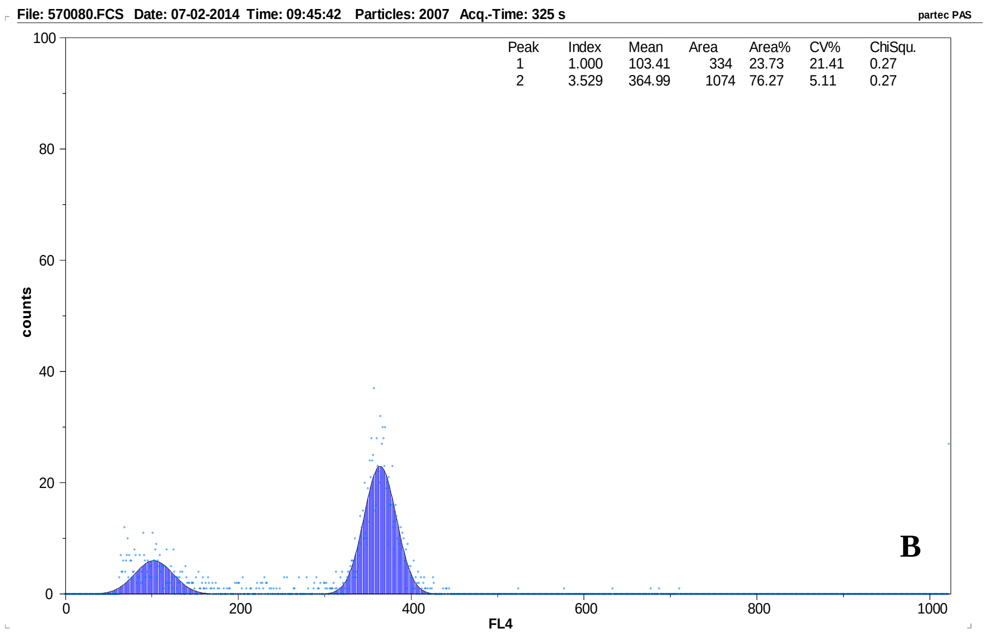


Figure 33 Flow cytometry histograms showing the DNA content of diploid (A) and tetraploid (B) hybrids of *T. fournieri* x *T. asiatica*

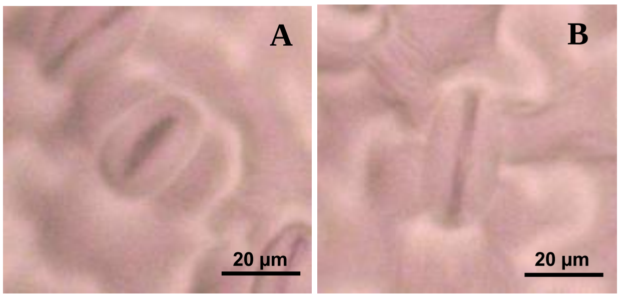


Figure 34 Comparison of stomata of diploid (A) and tetraploid (B) *T. asiatica* × *T. ranongensis*

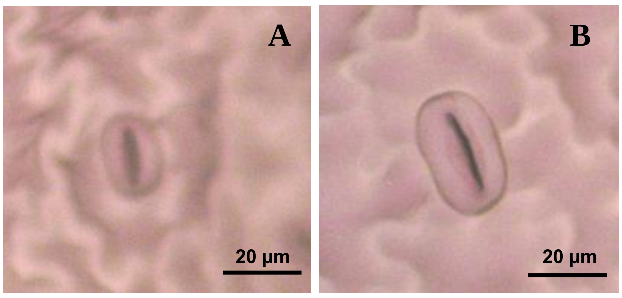


Figure 35 Comparison of stomata of diploid (A) and tetraploid (B) *T. bentamiana* × *T. asiatica*

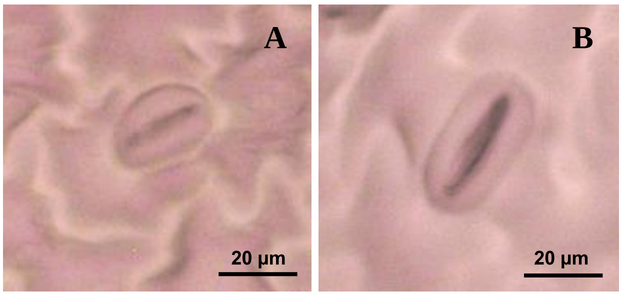


Figure 36 Comparison of stomata of diploid (A) and tetraploid (B) *T. asiatica* × *T. pierriana*

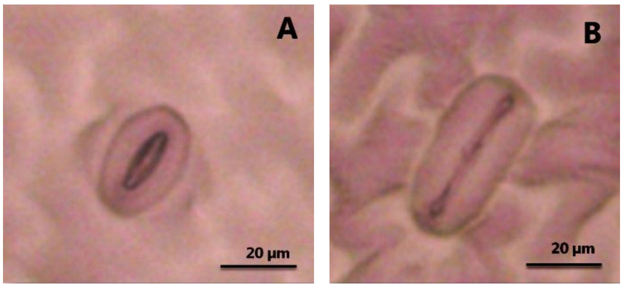


Figure 37 Comparison of stomata of diploid (A) and tetraploid (B) *T. fournieri* × *T. asiatica*

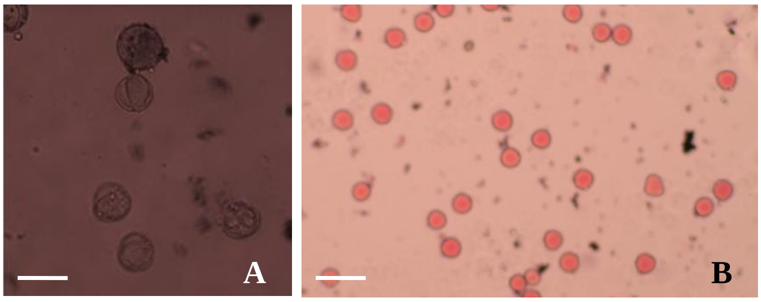


Figure 38 Comparison of pollen diploid (A) and tetraploid (B) *T. asiatica* × *T. ranongensis* (Bar = 40 µm)

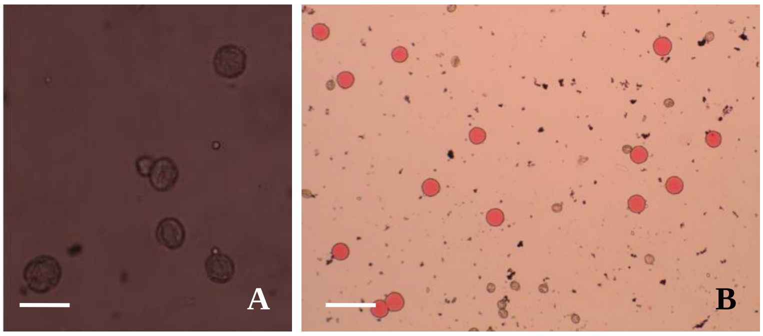


Figure 39 Comparison of pollen diploid (A) and tetraploid (B) *T. bentamiana* × *T. asiatica* (Bar = 40 µm)

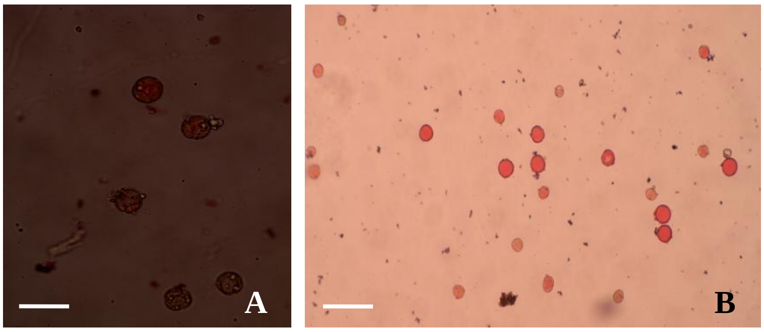


Figure 40 Comparison of pollen diploid (A) and tetraploid (B) *T. asiatica* × *T. pierriana* (Bar = 40 µm)

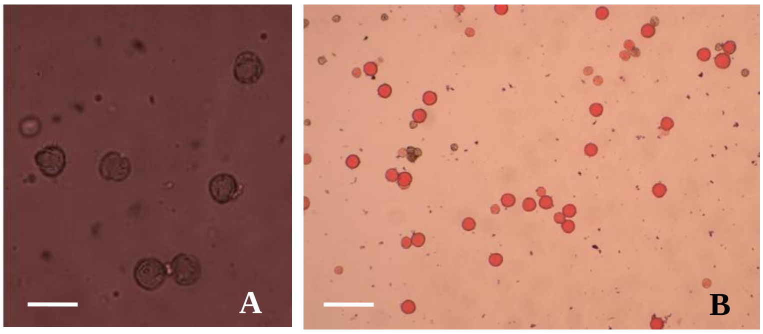


Figure 41 Comparison of pollen diploid (A) and tetraploid (B) *T. fournieri* × *T. asiatica* (Bar = 40 µm)

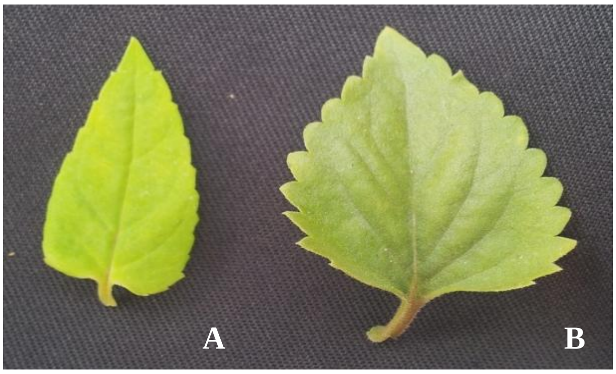


Figure 42 Comparison of leaves diploid (A) and tetraploid (B) *T. asiatica* × *T. ranongensis*

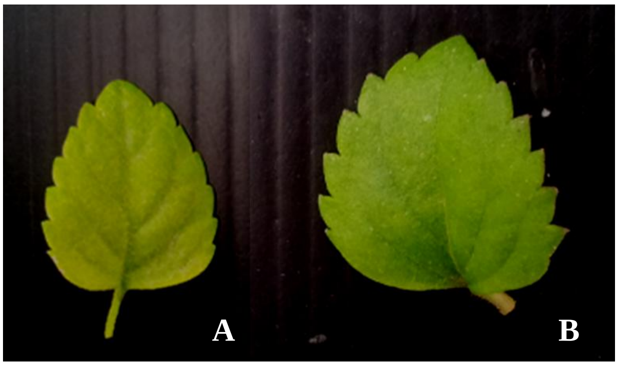


Figure 43 Comparison of leaves diploid (A) and tetraploid (B) *T. bentamiana* × *T. asiatica*

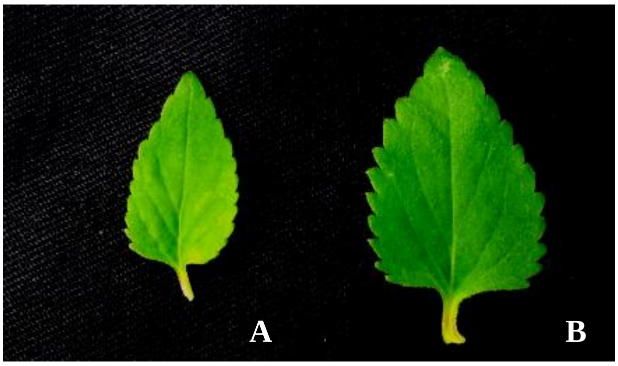


Figure 44 Comparison of leaves diploid (A) and tetraploid (B) *T. asiatica* × *T. pierriana*

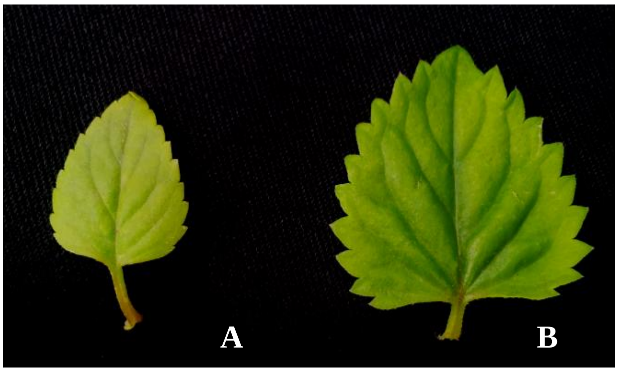


Figure 45 Comparison of leaves diploid (A) and tetraploid (B) *T. fournieri* × *T. asiatica*

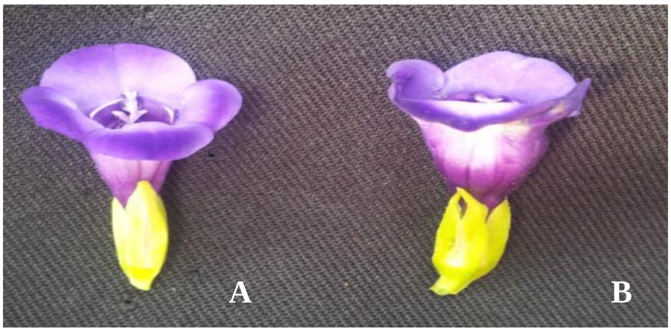


Figure 46 Comparison of flowers diploid (A) and tetraploid (B) *T. asiatica* × *T. ranongensis*

Figure 47 Comparison of flowers diploid (A) and tetraploid (B) *T. bentamiana* × *T. asiatica*

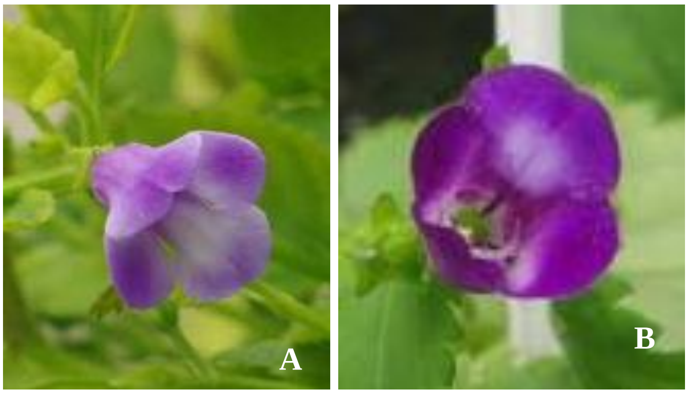


Figure 48 Comparison of flowers diploid (A) and tetraploid (B) *T. asiatica* × *T. pierriana*

Figure 49 Comparison of flowers diploid (A) and tetraploid (B) *T. fournieri* × *T. asiatica*

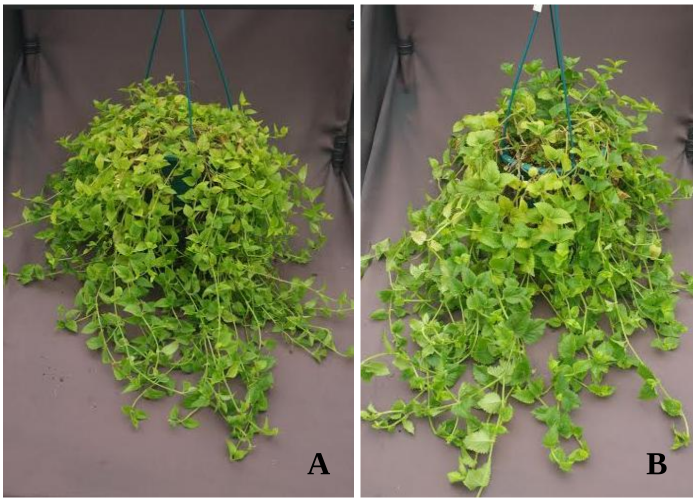


Figure 50 Comparison of morphology of diploid (A) and tetraploid (B) *T. asiatica* × *T. ranongensis*

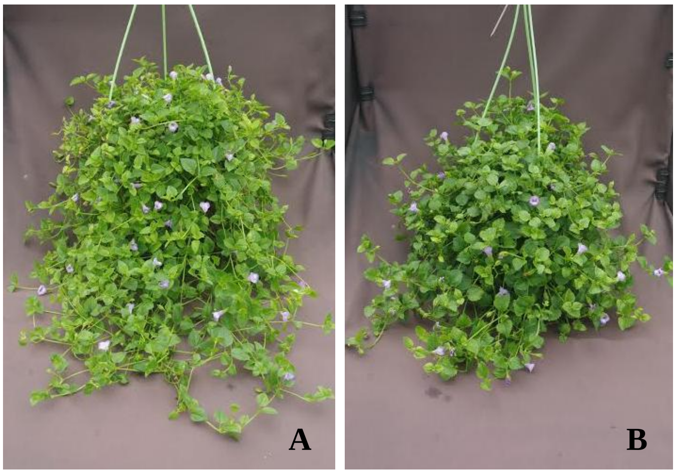


Figure 51 Comparison of morphology of diploid (A) and tetraploid (B) *T. bentamiana* ×*T. asiatica*

Figure 52 Comparison of morphology of diploid (A) and tetraploid (B) *T. asiatica* × *T. pierriana*

Figure 53 Comparison of morphology of diploid (A) and tetraploid (B) *T. fournieri* × *T. asiatica*

In this study, the colchicine tablet for gout drug treatments succeeded in inducing chromosome doubling in 4 hybrids of *Torenia.* By comparision, Boonbongkarn (2013) succeeded in producing tetraploids of *Torenia fournieri* using

15 ppm colchicines, which with exposure time of 3 days could induce polyploidy in 6.67 % of the lines.

Takamura and Miyajima (1996) used colchicine on Cyclamen and reported that lower concentrations and shorter duration treatments were not effective, but they succeeded in producing tetraploid Cyclamen using a concentration of 25 mM colchicine with dose time of 4 days.

Rose (2000) reported a concentration of 2.5 mM colchicine for 72 h was the most effective for inducing polyploidy in Buddleia. In the same way, 0.25 mM colchicine with 48 h exposure time and 1.25 mM colchicine for 48 h could induce polyploidy.

Working with Scoparia, Escandon *et al*. (2005) used concentrations of as low as 0.00125 mM colchicine for 24 h and .025 mM colchicines for 24 and 48 h to induce polyploidy.

In work by Cohen and Yao (1996) on Zantedeschia, 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.

Kanno (2013) Observation of meiotic chromosome pairing revealed appearances of univalent, trivalent and quadrivalents of *T. fournieri* x *T. baillonii*. The result indicates that chromosome rearrangements were accumulated in each. Lines of *T. fournieri*, conceivably, chromosome number variation of x = 8 and x = 9 might be derived from such chromosome arrangement

CONCLUSION

The objectives of this research were to induce tetraploids and mutants by biotechnological techniques in *Torenia* that could be of benefit for horticultural commerce, to create a tetraploid line of *Torenia hybrida* and create new color of *Torenia fournieri* and to compare the effectiveness of colchicine in changing the anatomical characteristics of *Torenia hybrida* for use in breeding programs. The research resulted in achieving the objective of inducing tetraploidy in *Torenia hibrida*, 3 clones of *T. asiatica* x *T. ranongensis*, 15 clones of *T. bentamiana* x *T. asiatica*, 24 clones of *T. asiatica* x *T. pierriana* and 18 clones of *T. fournieri* x *T. asiatica*.

For the results of the tissue culture portion of the research, the *Torenia* leaf explants cultured on MS medium with Picloram added at every concentration tested formed soft, loosely aggregated callus tissue. Callus tissue was induced from leaves of diploid *Torenia* to the greatest degree (95 % callus formation at 3 weeks). For polyploid *Torenia*, the highest percentage of callus formation (92.5 %) was observed on leaves cultured on MS medium containing Picloram at the rates of 1.0 and 1.5 mg^{-l} for 3 weeks in the dark. Following transfer of the embryogenic callus tissue to hormone-free MS medium under light conditions (16-h photoperiod), the greatest rate of somatic embryo formation was observed in the callus derived from the 1.5 mg^{-l} Picloram treatment, in the case of both diploid and polyploid *Toreni*a accessions.

The results of the preliminary gamma ray mutation research, in which axillary buds (1 node cuttings) of new Thai native *Torenia fournieri* Lind. were exposed to acute (Cs-137) gamma ray irradiation at 0, 20, 40, 60, 80 and 100 grays, demonstrated that the LD_{50} was 63.65 grays in diploid plants and 72.00 grays in polyploid plants from tissue culture.

Subsequently, axillary buds from the selected mutated plants from the first generation were irradiated again at 0, 60, 65, and 70 grays. Morphological screening for mutations revealed 3 mutated phenotypes (pink, dark and wavy shaped flowers). Genetic variation was analyzed by HAT-RAPD technique. Polymorphisms revealed by the D30 and F29 random primers could be used to differentiate between the samples, but chromosome numbers (2n=2x=18) were not changed when the chromosome were observed by fluorescence *in situ* hybridization (FISH) analysis. The pollen of some mutants (wavy flower shape) was sterile. The plants with pink, dark and wavy petals were selected for possible development of new cultivars.

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APPENDICES

Appendix A

MS medium

Murashige and Skoog (1962) Medium

Mineral	Volume (mg/l)
Macronutrients	
$NHNO_3$	1,650.000
KNO_3	1,900.000
$CaCl_2 \cdot 2H_2O$	440.000
$MgSO_4 \cdot 7H_2O$	370.000
KH_2PO_4	170.000
Micronutrients	
KI	0.830
H_3BO_3	6.200
$MnSO_4 \cdot 7H_2O$	6.900
$ZnSO_4 \cdot 7H_2O$	6.140
$Na_2MoO_4 \cdot 2H_2O$	0.250
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Fe-EDTA solution	
$FeSO_4 \cdot 7H_2O$	27.850
$Na_2EDTA \cdot 2H_2O$	37.250
Organic compounds	
Myo-inositol	100.000
Glycine	2.000
Nicotinic acid	0.500
Pyridoxine-HCl	0.500
Thiamine-HCl	0.500
Others	
Sucrose	30,000.000
pH	5.7-5.8

Appendix B

Primers for HAT-RAPD

Appendix Table B1 Set of primers for HAT-RAPD (A-2 and B-2)

Set	Names	Seq. (5´→3´)	Set	Names	Seq. (5´→3´)
A-2	A 21	AGAATTGGACCA	B-2	B 21	AAGCCTATACCA
	A 22	GCCTGCCTCACG		B 22	GGTGACTGGTGG
	A 23	ACTGACCTAGTT		B 23	GGTGCCGGAGCA
	A 24	CTCCTGCTGTTG		B 24	CACACTACTTAT
	A 25	CTCAGCGATACG		B 25	AGCACTGAATCT
	A 26	ACTGAGAAAATA		B 26	ATGAGAAAGGAA
	A 27	ATCGCGGAATA		B 27	GGCGGTTATGAA
	A 28	ATTTGGATAGGG		B 28	GTCATTAAAGCT
	A 29	GGTTCGGGAATG		B 29	GCCATCGAAAAA
	A 30	GACCTGCGATCT		B 30	CTTAGGTTACGT
	A 31	AAGGCGCGAACG		B 31	CACAAGGAACAT
	A 32	TTGCCGGGACCA		B 32	ATCGCGGCTTAT

Appendix Table B2 Set of primers for HAT-RAPD (C-2 and D-2)

Set	Names	Seq. (5´⟶3´)	Set	Names	Seq. (5´⟶3´)
C-2	C 21	GGAGAGCGGACG	D-2	D 21	GGCGATTCTGCA
	C 22	GGTCACCGATCC		D 22	TGCCCACTACGG
	C 23	CCGTCTTTTCTG		D 23	ACCATCAAACGG
	C 24	CCTTGGCATCGG		D 24	GTGCAATTTGGC
	C 25	AGATTCTTACTG		D 25	GTTTTGTCACCG
	C 26	GCGTTCGAACGA		D 26	GATGAGCTAAAA
	C 27	GCATTGCAATCG		D 27	AGAATGTCCGTA
	C 28	GTCGACGCATCA		D 28	ACTGAGGGGGGA
	C 29	GTCGCCTTACCA		D 29	ATCAAGTATCCA
	C 30	TATTGGGATTGG		D 30	GAGACTACCGAA
	C 31	TCTGCTGACCGG		D 31	GGAGGTCGACCA
	C 32	TCTACACGAAGT		D 32	AAGCTGGGGGGA

Appendix Table B3 Set of primers for HAT-RAPD (E-2 and F-2)

Set	Names	Seq. (5´→3´)	Set	Names	Seq. (5´→3´)
E-2	E 21	TGCTTCGTATTA	F-2	F 21	AACCTTTAGGGC
	E 22	GGAATGGAACCG		F 22	AAGAGGGTTGAC
	E 23	AGGTACGCCGCA		F 23	CCATCCGCACGA
	E 24	CCGGAGTGGATG		F 24	ACTGTTATAACG
	E 25	ATCGTTACAGTA		F 25	CCAGATCCGAAT
	E 26	CTGCCTGTACCA		F 26	CTCAGCATTGAT
	E 27	CCATTGTCGGTA		F 27	CAGGTGGGAGTA
	E 28	CGCCCTGCAGTA		F 28	CCAAGATCCATT
	E 29	GTTATGCAAGGG		F 29	GCCGCTAATATG
	E 30	TACCTGGTTGAT		F 30	ACTTTCGCCGAA
	E 31	GAGGACAGCAA		F 31	ATCGTGACGCCG
	E 32	CAGGAACAGCAA		F 32	TTCAACATCGAC

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Chanchula, N., T. Taychasipitak, A. Jala, T. Thanananta and S. Kikuchi. 2015. Induction of Somatic Embryogenesis in *Torenia fournieri* Lind. Int. Trans. J. Eng. Mag. Sci. Tech. 6: 165-171.

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INTERNATIONAL CONFERENCES

Chanchula, N., T. Taychasipitak, A. Jala, T. Thananantha and S. Kikuchi. Application of Detached-Leaf Technique and Gout Drug Treatment to Induce Tetraploidy in *Torenia hybrida* (*T. fournieri* x *T. asiatica*). The 5th Asian Chromosome Colloquium, Bangkok, Thailand, April 29-May 1, 2015.