THE IDENTIFICATION OF A SUITABLE IRRADIATION DOSAGE FOR MUTATION INDUCTION IN *ZAMIOCULCAS ZAMIIFOLIA* (LODD.) AND THE POLYPLOIDIZATION OF *Z. ZAMIIFOLIA* AND *MARSDENIA FLORIBUNDA*

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ABSTRACT

Radiation mutation has been successfully used to create a great variety of ornamental crop cultivars by supplementing existing germplasm and improving existing cultivars, and chemical mutagens such as colchicine and oryzalin have been used to create new plant cultivars by doubling the chromosome number of the treated plant material to produce tetraploids. The main objective of this thesis research was to develop protocols to create tetraploid plants of *Zamioculcas zamiifolia* (Lodd.) Engl., an important foliage plant, and *Marsdenia floribunda* (Brongn.), an important lei flower plant, and to determine the LD_{50} of ZZ leaflets. ZZ leaflets and *M. floribunda* seeds were treated with colchicine at various concentrations and durations in order to induce ploidy changes and regenerate polyploids. Five ZZ tetraploids and one *M. floribunda* tetraploid were produced using colchicine. A tissue culture protocol was also developed for the oryzalin treatment of ZZ callus for the *in vitro* polyploidy induction of ZZ. The LD_{50} of ZZ leaflets irradiated with x-rays was calculated as 20±1 Gy. A ZZ germplasm collection was also initiated to provide ZZ plant material for use in future breeding studies.

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2,4-D	2,4-dichlorophenoxyacetic acid
2-ip	6-γ-γ-dimethylaminopurine
ADC	analog-to-digital convertors
BA	6-benzyladenine
CV	coefficients of variation
DI	DNA index
DMSO	dimethyl sulfoxide
Gy	Gray
HCl	hydrochloric acid
FCM	flow cytometry
IAA	indole-3-acetic acid
LD_x	lethal dosage
MS	Murashige and Skoog basal salts
MS_o	Murashige and Skoog basal salts with no plant growth regulators
PGRs	plant growth regulators
PI	propidium iodide
ZZ	*Zamioculcas zamiifolia*

LITERATURE REVIEW

The 2005 wholesale value of floriculture crops in the US is estimated at $5.4 billion, with Hawaii ranking fourth in both the foliage and cut flower/cut greens commodities (USDA, 2006). The introduction of new ornamentals for production can help increase the value of Hawaii's ornamental plant industry. For example, Florida is consistently the greatest producer of foliage plants year after year, accounting for over 55% of the nation's wholesale value. Florida's success has been partially attributed to the continuous introductions of new cultivars and genera into the Florida foliage market (Chen, Henny, and McConnell, 2002).

The main objective of my Master's research program is to develop protocols and systems to create new cultivars of *Zamioculcas zamiifolia* (Lodd.) Engl., an important foliage plant, and *Marsdenia floribunda* (Brongn.), an important lei flower plant formerly known as *Stephanotis floribunda*. Both chemical mutagens and radiation mutation were employed in order to achieve the objective.

New plant cultivars contribute significantly to the ornamental plant industry. Among the various ways to bring about improvements in crops, genetic improvements are more desirable than non-genetic crop improvements such as the use of growth retardants. For example, growth retardants have been shown to reduce vegetative growth in trees and other ornamental crops to produce a more compact plant growth (Manriquez, 2005; Feng, 2004; Criley 2000). The changes induced, however, are not permanent and require repeated inputs. Genetic improvements, on the other hand, are passed on to progeny or to new clones produced by vegetative propagation. It is for this reason that

the use of chemical and physical mutagens was elected as the method of choice for the production of new ZZ and *M. floribunda* cultivars, rather than using other methods that induce a non-permanent change on morphological growth.

Radiation mutation has been successfully used to create a great variety of ornamental crop cultivars by supplementing existing germplasm and improving cultivars (Yamaguchi, 1988; Kaicker and Dhyani, 1986). In ornamental plants, irradiation has produced improved attributes such as earlier flowering, compact growth habits, the production of larger flowers, changes in flower color (Micke *et al.*, 1987), changes in leaf color, and changes in leaf shape (Matsubara, 1982).

The energy radiated from physical mutagens induces chemical reactions within the plant cell resulting in a structural change of the DNA. If these changes are then maintained during DNA replication, they are manifested as mutations. Mutations in somatic tissues are usually induced by treating leaf buds with mutagens or adventitious buds are induced immediately following the mutagenic treatment (Lapins, 1983). The vegetative propagation of ZZ via leaflet cuttings lends itself to irradiation via the latter method. The advantage of this method is that adventitious buds arising from leaves and petioles are thought to arise from a single cell, so that the formation of a chimera is minimized and the irradiation results almost exclusively in solid, non-chimeric mutants and normal, unmutated plants (Broertjes, Haccius, and Weidlich, 1968; Broertjes and Keen, 1980). Though some scientists dispute the origins of adventitious meristems (single vs. multiple cells), Zalewska (2001) concluded that irrespective of their formation, adventitious meristems may give rise to plants with stable genetic changes.

Chemical mutagens such as colchicine (N-acetyltrimethylcolchinic acid), an alkaloid, and oryzalin [(4-(Dipropylamino)-3, 5-dinitrobenzene-sulfonamide], a dinitroaniline herbicide, have been used to create new plant cultivars by doubling the chromosome number of the treated plant material. Colchicine, for example, has proven to be a useful tool in many breeding programs aimed at regaining fertility, preventing hybrid sterility (Kamemoto *et al.*, 1997; Kamemoto, 1985), producing superior cultivars (Vainola, 2000; Tambong and Garton 1998), and producing sterile triploids by breeding induced tetraploids with diploids (Blakesley *et al.,* 2002; St. Marseille and Grant 1997). Similarly, oryzalin has been shown to double the chromosome numbers in *Nepeta* (Mitrofanova *et al.*, 2003), *Alocasia* (Thao *et al.*, 2003), *Rhododendron* hybrids (Vainola, 2000), and Lilium (Tuyl, Meijer, and Van Dien, 1990).

Research has shown that colchicine and oryzalin have a similar mode of action at the molecular level: both chemicals were found to inhibit elongation, produce swelling in the elongation zone, depolarize cell enlargement, and disrupt cell differentiation and polyploidization in maize seedling roots (Upadhyaya and Nooden, 1976). Both agents bind to plant microtubules, which are subcellular structures that are mainly composed of the protein tubulin, and are involved in chromosome migration. Colchicine is thought to depolymerize the microtubules (Morejohn *et al.*, 1987), while oryzalin binds to free tubulin in the plant cell, forming a dinitroaniline-tubulin complex that is incapable of polymerizing into plant microtubules. Consequently, both agents disrupt mitosis by inhibiting spindle fiber formation at metaphase (Strachan and Hess, 1983), resulting in an increase in the chromosome number of the daughter cells. Chemical mutagens are most effective when applied to cells that are in a high state of cell division (Eigsti and Dustin,

1955); therefore, callus tissue, seeds, and freshly cut leaflets were utilized in this research project in order to create polyploids.

Researchers have discovered that oryzalin can disrupt mitosis at a lower concentration than colchicine due to its stronger binding affinity to plant tubulins. Ramulu, Verhoeven, and Dijkhula (1991) showed that in potato, oryzalin was more efficient for chromosome doubling than colchicine. Tuyl *et al.*, (1990) and Thao *et al.*, (2003), reported similar results in *Lilium* and *Alocasia* respectively. Contrastingly, colchicine has been found to be more effective in chromosome doubling for plants such as *Miscanthus sinensis*, a perennial grass (Petersen, Hagberg and Kristiansen, 2003) and two leguminous tree species of *Acacia* (Blakesley, *et al.*, 2002). The variation in the mutagenic effectiveness of both agents indicates that the efficiency of each chemical to double the chromosome number is plant specific, and should be determined on a plant by plant basis. Accordingly, both chemicals were used in attempts to induce chromosome doubling in the subject plants of this research project.

In the past, screening for ploidy level changes was limited to measurement of stomatal guard cells of the treated plant material or by root tip chromosome counts of identified suspects. Measuring stomatal guard cells requires producing an imprint of the abaxial side of the leaf of interest. The imprint is then viewed under the microscope and the size of the guard cells is measured. The theory behind this method of screening for changes in ploidy levels is simple. The cell volume of a plant is directly proportional to the amount of DNA present in the cell, so that doubling the amount of DNA, which occurs when diploids are converted to tetraploids, causes the cell to double its volume. Doubling of the volume allows an increase in the size of the cell in any one dimension by

1.25, and comparisons of guard cells among a batch of chemically treated plants allows for the identification of possible polyploids (Russell, 2004). Typically, the ploidy level of the suspected plants identified using guard cell measurements is then verified by performing root tip chromosome counts, a laborious and time consuming process.

Today, more researchers rely on flow cytometry (FCM) for the early screening of treated plant material and ploidy level confirmation. FCM allows for the quantification of plant nuclear DNA, subsequently providing the user with the ploidy level of the samples screened. A typical flow cytometer contains several components: a light source, a flow chamber and optical assembly, photodetectors and processors to convert light signals into analog electrical impulses, analog-to-digital convertors (ADCs) and a computer system for the analysis and storage of digitized data. Essentially, the plant nuclei is extracted from plant tissue using an extraction buffer and labeled with a fluorescent dye. The sample is then loaded into the cytometer and illuminated, causing the dye to absorb the illuminating light and fluoresce. The emitted light is then converted to electric current pulses, which are fed to amplifiers, digitized using the ADCs, and stored in the computer in the form of a histogram. Computer software may then be used to analyze the data output and study correlations among the parameters (Dolezel, 1991), for example, a tetraploid cell will fluoresce twice as much as a diploid cell. Accordingly, FCM is a valuable, rapid and precise tool to detect converted polyploids (Eeckhaut *et al.*, 2004; Takamura, Lim, and Van Tuyl, 2002; Toska *et al.*, 1995; Vainola 2000; Vainola and Repo, 2001) and was employed as a screening tool in this research project.

SECTION 1

ZAMIOCULCAS ZAMIIFOLIA

Introduction

Zamioculcas zamiifolia (Lodd.), which will be referred to hereafter as ZZ, is a member of the Araceae family and is also commonly known as African coontie, aroid palm, arum fern, cardboard palm, and emerald frond. ZZ is native to tropical east and subtropical southeast Africa, with its native habitat ranging from Kenya to northeastern South Africa. It grows in dry grassland and often stony ground and has several fleshy stalks bearing alternate pinnate leaflets. The leaflets have the capacity to sprout new plants and form tiny rhizomes at their base (Bown, 2000). ZZ is a seasonally dormant or evergreen herb with a short, very thick rhizome and a diploid chromosome number of 2n = 34 (Jones, 1957).

Not only has it been described as a plant that is becoming or will become an important player in the foliage plant industry (Chen, Henny, and McConnell, 2002), but it was also listed among the *Florida Plants of the Year* in 2002 (Chen and Henny 2003). The ability of ZZ to grow under low light conditions, its tolerance to drought stress, its unique appearance, its low maintenance requirements and limited pest problems are characteristics that contribute significantly to its ornamental and interior plantscaping value (Chen and Henny, 2003). Furthermore, there are no reports available on the polyploidization of ZZ, so that success achieved in this research project will contribute significantly to this area of research in ZZ cultivar development. With only one species in the genus of this ornamental, the creation of new clones may allow breeding and variety development to advance more quickly.

Two approaches were taken in an attempt to induce mutations through chromosome doubling in ZZ, namely: (i) colchicine treatment of ZZ leaflets *in vivo* and (ii) oryzalin treatment of ZZ callus *in vitro*. The irradiation of ZZ leaflets was another approach taken in an attempt to create a new ZZ cultivar. For this research, the response of ZZ leaflets to irradiation was limited to the identification of a suitable x-ray dosage for mutation induction, but did not result in a new cultivar. Section 1 has been divided into four chapters, three of which describe the experiments performed using the approaches mentioned above. Chapter 4 provides a brief description of the ZZ germplasm collection at the University of Hawaii.

CHAPTER 1: COLCHICINE TREATMENT OF ZZ LEAFLETS *IN VIVO*

Background Information

ZZ may be propagated vegetatively by leaflet or petiole cuttings. Leaflets are removed from the stock plant and stuck vertically in a highly organic medium such as coir or peat moss. After about 3-4 weeks, a small rhizome begins to appear and roots emerge. Buds begin to form on the rhizome giving rise to new leaves and eventually a whole new plantlet (Fig. 1.1). The described method of propagation proved to be a convenient method for the colchicine treatment of ZZ leaflets *in vivo*. The objective of this experiment was to regenerate tetraploid ZZ plants using varying concentrations of colchicine. The induction of tetraploids using this method has proven to be simple and cheap.

Materials and Methods

ZZ leaflets were treated with colchicine in two ways: immediately after harvest (before rhizome development) or after a small rhizome had been produced. Both methods are described below.

Before rhizome development

Colchicine (PhytoTechnology Laboratories; Shawnee Mission, KS) was dissolved in distilled water to make 0.05%, 0.2 %, and 0.4% solutions. ZZ leaflets were harvested from the tip to the base of the leaf and treated the same day in the colchicine solutions, with 18 leaflets per treatment and water used as the control. The cut portions of the

leaflets (the exposed surface of the petiole) were soaked for 24 hours in 50ml of the treatment solution. After treatment, the leaflets were rinsed with distilled water and transplanted into a 40cm x 55cm metal flat containing 1:1 (v/v) peat:vermiculite. The flat was placed under mist in the Foliage Greenhouse at the Magoon Research Facility. About 3.5 months later the remaining leaflets were transplanted into individual 2 inch pots containing moistened Pro-Mix 'BX' (Premier Horticulture Ltd.; Dorval, Canada).

After rhizome development

ZZ leaflets were harvested from along the entire length to the leaf and stuck in a 40cm x 55cm metal flat containing 1:1(v/v) peat:vermiculite. One month later, after all leaflets had developed a small rhizome, the leaflets were removed from the propagation medium and rinsed with distilled water. The leaflets were then graded according to rhizome size and distributed evenly into 4 treatments containing 18 leaflets each: 0.05%, 0.2 %, and 0.4% colchicine, with water used as a control. The leaflets were soaked for 24 hours in a 50 ml solution, which allowed the rhizome to be completely submerged. After treatment, the leaflets were returned to the mist bench and were transplanted 2.5 months later into individual 2" pots containing moist Pro-Mix 'BX'. The first shoots began to appear 7 months after treatment.

Guard Cell Measurements

After visual inspection of the regenerated plantlets, suspected tetraploids were selected for further screening using stomatal guard cell measurements. Clear nail polish (Markwins Beauty Products Inc.; City of Industry, CA) was thinly applied to the abaxial

side of the leaf. After the polish had dried (approximately 60 seconds), a strip of Scotch 3M Transparent Tape with Gloss Finish (3M Products; St. Paul, MN) was mounted on top of the dried polish. The tape, which now held the abaxial imprint, was removed from the leaflet and mounted onto a coverslip for viewing at 400X magnification under a Leitz Wetzlar light microscope. Fifteen guard cells were randomly selected from each leaf sample and measured (length x width) using a unitless graticule.

Chromosome Counts

Two methods were employed for chromosome counting in ZZ. The first method used is a modified version of the technique used by Shi (2003). Actively growing root tips (Fig. 1.2) were excised, rinsed in distilled water, and pre-treated with 300ppm cycloheximide for 12 hours. Next, the root tips were fixed in Carnoy's fixative (3:1 95% ethanol and acetic acid) for at least 30 minutes followed by a rinse in distilled water. Root tips were then softened in a solution of 1:1 concentrated hydrochloric acid and 95% ethanol for five minutes, rinsed with tap water, and placed on a clean microscope slide that contained a drop of carbolfuchsin stain. The root tip was then squashed using a small scalpel, a cover slip placed on top of the squashed root tip, and the slide viewed under the microscope for counting.

The second method used for chromosome counting was based on the work done by Sharma and Mookerjea (1955). Actively growing root tips were excised, rinsed in distilled water, and pre-treated in a saturated solution of paradichlorobenzene for 24 hours at 10-12°C. The saturated solution was prepared by dissolving 10g of the solid in 500ml distilled water and holding the solution at 60°C overnight. The solution was used

at room temperature. After pretreatment, the root was transferred to an acid-dye fixation solution (2% aceto-orcein and 1N HCl at a 9:1 ratio) and the mixture heated over a flame for 3-4 seconds. The contents were then poured onto a watch glass and allowed to cool for at least 5 minutes. A drop of 1% aceto-orcein solution was placed on a dry slide and the root was transferred to the drop of stain. The intensely colored portion of the tip was retained, while the remainder of the root was discarded. The root tip was then squashed using a small scalpel and a cover slip placed on top of the root. The root tip was further squashed by applying even pressure on the cover slip. A Leitz Wetzlar light microscope was used to observe the prepared slides at 1000X magnification and pictures were taken using a Nikon Coolpix 4500 digital camera.

Flow Cytometry

CyStain PI Absolute P DNA Staining Kit for Plant Genome Size (Partec; Munster, Germany) was used for nuclei extraction and DNA staining of nuclear DNA from ZZ leaflets used for flow cytometric measurement. Approximately 1 cm^2 of each leaflet was chopped for 30 – 60 seconds in 500µl ice cold nuclei extraction buffer with a sharp doubled edged razor blade in a 55mm plastic Petri dish. The slurry was then filtered through a 50µm Cell Trics filter (Partec; Munster, Germany) and the suspension of released nuclei was stained in a solution composed of staining buffer, propidium iodide, and RNAse for a final volume of 2ml. Leaflet samples and all reagents used were kept on ice throughout the entire process from leaflet harvest to sample preparation. All samples were prepared for flow cytometric measurement within 1-2 hours of harvest. Due to the limited availability of plant material, only one leaflet was sampled per leaf in

most cases, with usually one leaf sampled per plant. For those regenerated plants that showed variation in leaf morphology within the same plant, one leaflet was sampled per leaf in order to compare differences in ploidy level throughout the plant. Figure 1.3 shows the process from sample preparation to sample analysis.

The relative fluorescence of total DNA of single nuclei was analyzed using a Beckman-Coulter (Miami, Florida) Altra flow cytometer (www.soest.hawaii.edu/sfcf) using the 488 nm line of a Coherent I90C argon ion laser set at 200 mW. Control diploid plants were used as external standards, and these standards were run intercalated between samples. The linear, log and peak fluorescence signals of the propidium iodide-stained nuclei were collected (610 BP filter), along with forward and side scatter signals. Plots of peak vs. linear propidium iodide fluorescence were used to eliminate doublets (two nuclei stuck together as they pass the laser/particle sensing point). The resulting data was analyzed using Flow Jo (v. 6.3.4, Treestar Inc., www.flowjo.com). Means and coefficient of variance percentages of the resulting peaks were calculated and histograms of linear DNA fluorescence, which allowed for visual analyses of the data, were produced. An Olympus BX51 Epifluorescence Microscope was used at a 480nm excitation frequency in order to view propidium iodide-stained nuclei and detect cellular debris in some of the leaflet samples before flow cytometric analysis.

Results and Discussion

Visual inspection of the regenerated plants resulted in the identification of 26 suspected tetraploids. The leaflets of these plants were rounder (Fig. 1.4), thicker, and the overall plant height was smaller than that of the controls. The growth rate

(emergence of new leaves and roots) of the suspects seemed to be slower than that of the controls. Guard cell measurements of the selected plants support showed that several of the regenerated plants are indeed polyploids (Table 1.1). Doubling the chromosome number in a plant cell leads to an increase in stomatal guard cell size by a factor of 1.25 (Russell, 2004). The results obtained in Table 1.1 show that all the plants regenerated from leaflets treated before rhizome development that were visually identified polyploids were confirmed as such by guard cell measurements. Likewise, Table 1.2 shows that 15 of the 19 visually identified polyploid plants regenerated from leaflets treated after rhizome development were shown to be polyploids via guard cell measurements. Note that for three of the plants screened, the guard cell area decreased in comparison to the control. This decrease does not imply a chromosomal change in the plant, but should be attributed to the inherent variation in guard cell sizes from plant to plant, i.e., the variation that exists between plants of the same ploidy level. The factor of increase in the guard cell area of the plantlets produced before and after rhizome production ranged from 1.27 to 2.13. However, it was not clear what level of polyploidization the new plants had obtained, or whether the plantlets regenerated were solid tetraploids or mixoploids.

In order to avoid misinterpretation of the results obtained, the term *mixoploid*, when used hereafter, applies to the ploidy status of the entire plant (entire stalk including all leaflets, see Fig. 1.3d) or a single leaflet, and will be specified as such. The term *mixoploid* generally applies to a plant structure that has originated from meristematic tissue comprised of cells with varying ploidy levels. This variation results from the polyploidization of some cells, while other cells within the same meristematic region are unaffected and remain diploid. The meristematic tissue composed of cells with mixed

ploidy levels, in turn, gives rise to a structure that varies in ploidy level, e.g., individual leaflets with both diploid and tetraploid cells, where the leaflet may also be described as a chimera (Thao *et al*, 2003).

Further verification of the ploidy state of the regenerated plants using root tip chromosome counts, however, was not possible. ZZ chromosomes are very large and long, so that chromosome overlapping was common. Subsequently, the only chromosome counts obtained were those of the controls (Fig. 1.5), with 2n = 34, supporting the finding of Jones (1954). Even though the length of time allotted for the pre-treatment of the root tips was repeatedly increased in order to allow for further constriction of the chromosomes, no cells were identified in which all the chromosomes could be individually counted. At least 60 - 75 slides were prepared for chromosome counting with no success. Such difficulties in counting chromosomes have also been expressed by other researchers, who have in turn, used flow cytometry for ploidy level determination (Barker *et al.*, 1998; Meng and Finn, 1999). Hence, the results obtained from the flow cytometric analysis were used in order to verify ploidy levels of the suspected polyploids.

Figures 1.6 – 1.9 are representative examples of the different types of histograms that were obtained from analysis of the flow cytometry data with Flow Jo. For all the histograms shown, the number of nuclei is represented on the Y axis and the DNA fluorescence intensity is represented on the X axis. Figure 1.6 shows a histogram that is typical of a diploid sample, with one major peak showing nuclei with 2C DNA content and a smaller peak showing nuclei with 4C DNA content. The first peak is representative of those nuclei that are in the G1 mitotic phase, and the smaller peak represents those

nuclei that are in the G2 mitotic phase of the cell cycle. Figure 1.7 shows a comparison between the diploid control sample and that of an identified tetraploid sample. The fluorescence intensity of the tetraploid (mean channel 90) is almost double that of the control (mean channel number 44), which identifies the sample as a tetraploid. Figure 1.8 is representative of a mixoploid sample that contains both diploid and tetraploid nuclei, which is shown graphically by the two peaks. Unlike other plant species in which 10,000 – 20,000 nuclei are readily available for sample analysis, flow cytometric data was collected from 2000 to 5000 nuclei per ZZ sample. Perennial herbaceous monocotyledons (such as ZZ) tend to have long-lived leaves (Bharathan, *et al.*, 1994), and most of the ZZ polyploid suspects only had older leaves available for sampling. The cells of these older leaves were probably not in an actively dividing state, which accounts for the lower number of nuclei extracted from the leaflets. Nonetheless, the amount of nuclei extracted and analyzed was sufficient for accurate comparisons and data analysis.

Table 1.3 shows the number of ZZ diploids, tetraploids, mixoploids, and DNA aneuploids that were identified via flow cytometry. Plants that showed differences in leaflet morphology between the leaves of the same plant (i.e., different leaves originating from the same rhizome) were sampled more than once, with one leaflet from each leaf stalk being analyzed. For some plants, the leaflets had the same ploidy level, confirming the plants as being diploids, tetraploids, or DNA aneuploids. In others, the leaflets taken from separate leaves of the same plant had different ploidy levels. Such differences in ploidy levels within the same plant may stem from the lack of complete conversion of the colchicine treated cells to a higher ploidy level, so that some of the cells that led to the development of one leaf were of one ploidy level, while other cells that lead to the

development of an adjacent leaf were of another ploidy level. Leaflets with both diploid and tetraploid cells within the same sample were also identified. The results obtained indicate that this lack of complete conversion to tetraploidy holds true for leaflets treated before and after rhizome development, since mixoploids were identified in both cases.

The ZZ mixoploids identified in which one leaf possessed a certain ploidy level while another leaf possessed another ploidy level may have been formed in one of two ways: (i) *before rhizome development*: only some of the leaflet basal cells that were exposed to the colchicine were converted to tetraploids, so that a mixture of diploid and tetraploid basal cells gave rise to the new rhizome. Consequently, some meristematic regions within the rhizome were comprised of diploid cells, while other regions were comprised of tetraploid cells, giving rise to adventitious buds that led to the development of one plant with leaves of different ploidy levels; and (ii) *after rhizome development*: not all of the treated rhizome cells developed 4x daughter cells; subsequently, the meristematic regions that eventually gave rise to new leaves were either of a diploid or tetraploid nature. Though some researchers have reported that adventitious buds arise from a single cell (Broertjes, Haccius, and Weidlich, 1968; Broertjes and Keen, 1980), the mixoploids obtained from the colchicine treatment of leaflets do not support these findings. The leaflet mixoploids indicate that more than one cell was involved in adventitious bud formation, with both diploid and tetraploid cells giving rise to the *de novo* buds that eventually developed into ZZ leaves with mixoploid leaflets.

The mixoploid ZZs that have been regenerated and shown to possess tetraploid leaflets will not be discarded since they may prove to be an additional source for the production of more tetraploid plants. The identified tetraploid leaves may be harvested

and used as propagules from which new plants can be propagated. If all the cells of the leaflet were of tetraploid origin, the rhizome produced should give rise to solid tetraploid plants.

Leaflet samples that produced a shift between 8% - 90% in the mean channel number of the $G_o + G_1$ nuclei from that of the diploid control in the DNA histogram were characterized as *DNA aneuploids* (Fig. 1.9). The term is synonymous with the characterization of a leaf sample as having an abnormal DNA content, but it should not be confused with the cytological term for "true" aneuploidy derived from karyotypic evaluation (chromosome composition involving number variations other than complete genomes). Moreover, because flow cytometry analyzes nuclear DNA content and not the number of chromosomes, the Committee on nomenclature of the Society of Analytical Cytology suggested that the flow cytometry results of ploidy analysis be considered separately from those obtained from cytogenetic analysis (Hiddemann *et al.*, 1984).

The degree of DNA aberration in the DNA aneuploids is expressed by the DNA index (DI), which is the ratio of the mode (or mean, as was used in this experiment) of the relative DNA fluorescence of the $G_{0/1}$ cells of the sample (suspected polyploids) divided by the mode (or mean) of the relative DNA fluorescence of the diploid $G_{0/1}$ reference cells (Hiddemann, *et al.*, 1984). Cytometric analysis of the plants regenerated from colchicine treated leaflets before and after rhizome production resulted in the identification of 13 DNA aneuploids, with the DI index ranging from 1.2 – 3 (data not shown). Though a review of literature has not provided a concrete explanation or mechanism for the production of DNA aneuploids via colchicine treatment, further microscopic investigation of the DNA aneuploids suggests that there are two contributing

factors for identification of DNA aneuploids in this project: (i) error in sample analysis due to the production of cellular debris during sample preparation (tissue chopping) which, in turn, affects the fluorescence intensity of the sample and (ii) error in sample analysis due to unexpected sample browning, which may also affect light scatter after sample illumination, and also affect the fluorescence intensity of that sample.

Due to the slow growing nature of ZZ, only older, mature leaves were available for sampling. These thick, older leaves exude various substances during chopping of the tissue, and though samples are filtered through a 50μm nylon mesh, inspection of some of the prepared samples with an epifluorescence microscope showed debris in the samples (Fig. 1.10). In addition, some samples showed slight browning after being incubated on ice for 1 hour. The sample browning or the presence of sample debris may produce slightly skewed results when the sample is analyzed, producing histograms that may show DNA aneuploidy. Yanpaisan *et al.* (1999) found that both cellular debris and sample browning can contribute to higher peak CVs. Subsequently, sample resolution is decreased, which may lead to the characterization of a sample as a DNA aneuploid.

Brown *et al.*, (1991) provide various troubleshooting solutions for samples that produce broad, unstable, and irreproducible histograms. She reports that the use of a surfactant (Triton X-100) reduces adhesion of cellular debris, the addition of citrate condenses DNA in the samples, and the routine addition of β-mercaptoethanol minimizes sample browning. Likewise, Ulrich and Ulrich (1991) were able to produce high-resolution histograms with coefficients of variation of 1-1.5% for various plant species by pre-treatment with citric acid and Tween 20. These solutions must be considered for further analysis of the DNA aneuploids identified in this study.

The identified tetraploids will continue to be monitored for changes in leaf shape and overall growth morphology. Once the identified tetraploids have flowered, it will also be necessary to confirm the ploidy status of the germinal cell line through chromosome counts of the pollen produced. It has been shown that after colchicine treatment, the ploidy level of leaves or other tissue may not necessarily be representative of the ploidy level of the germinal cell line (Brown *et al.*, 1991). A complete characterization of our regenerated tetraploids will contribute significantly to future breeding studies utilizing these plants.

Tables and Figures

Table 1.1. Guard cell area measurements of ZZ leaflets treated with colchicine before rhizome development and visually identified as possible polyploids. Area values shown were measured using a unitless graticule and are the averages obtained from the measurement of 15 guard cells, with one leaflet sampled per plant. Values in column 4 represent the factor of increase in area as compared to the control (treated plant guard cell area/control guard cell area). Factor values above 1.2 are classified as polyploids.

Colchicine Concentration	Plant Identification	Guard Cell Area (length x width)	Factor of inc./dec. compared to control
0%	1	127.80	-
0.2%	1	250.80	1.96
0.2%	2	257.47	2.01
0.2%	3	272.53	2.13
0.4%	1	237.87	1.86
0.4%	2	234.60	1.84
0.4%	3	248.27	1.94
0.4%	4	271.73	2.13
0.4%	5	243.87	1.91
0.4%	6	224.40	1.76
0.4%	7	212.07	1.66
0.4%	8	200.93	1.57

Table 1.2. Guard cell area measurements of ZZ leaflets treated with colchicine after rhizome development and visually identified as possible polyploids. Area values shown were measured using a unitless graticule and are the averages obtained from 15 guard cells, with one leaflet sampled per plant. Values in column 4 represent the factor of increase or decrease in area as compared to the control (treated plant guard cell area/control guard cell area). Factor values above 1.2 are classified as polyploids.

Colchicine Concentration	Plant Identification	Guard Cell Area (length x width)	Factor of inc./dec. compared to control
0%	1	142.8	-
0.05%	1	231.20	1.62
0.05%	2	227.93	1.60
0.05%	3	137.07	0.96
0.05%	4	216.93	1.52
0.05%	5	218.27	1.53
0.05%	6	262.73	1.84
0.05%	7	260.60	1.82
0.05%	8	127.07	0.89
0.05%	9	244.93	1.72
0.2%	1	236.07	1.65
0.2%	2	189.73	1.33
0.2%	3	238.73	1.67
0.2%	4	214.13	1.50
0.4%	1	118.93	0.83
0.4%	2	236.93	1.66
0.4%	3	140.27	0.98
0.4%	4	227.60	1.59
0.4%	5	181.40	1.27
0.4%	6	245.33	1.72

Table 1.3. Number of tetraploids, mixoploids, and DNA aneuploids identified via flow cytometry. Polyploidy was induced by the *in vivo* colchicine treatment of ZZ leaflets before and after rhizome development.

Colchicine Concentration	Tetraploid	Mixoploid	DNA Aneuploid
Before rhizome development			
0.05 %	0	0	0
0.2 %	0	0	3
0.4 %	1	4	3
After rhizome development			
0.05%	3	3	2
0.2%	1	0	3
0.4%	0	1	2

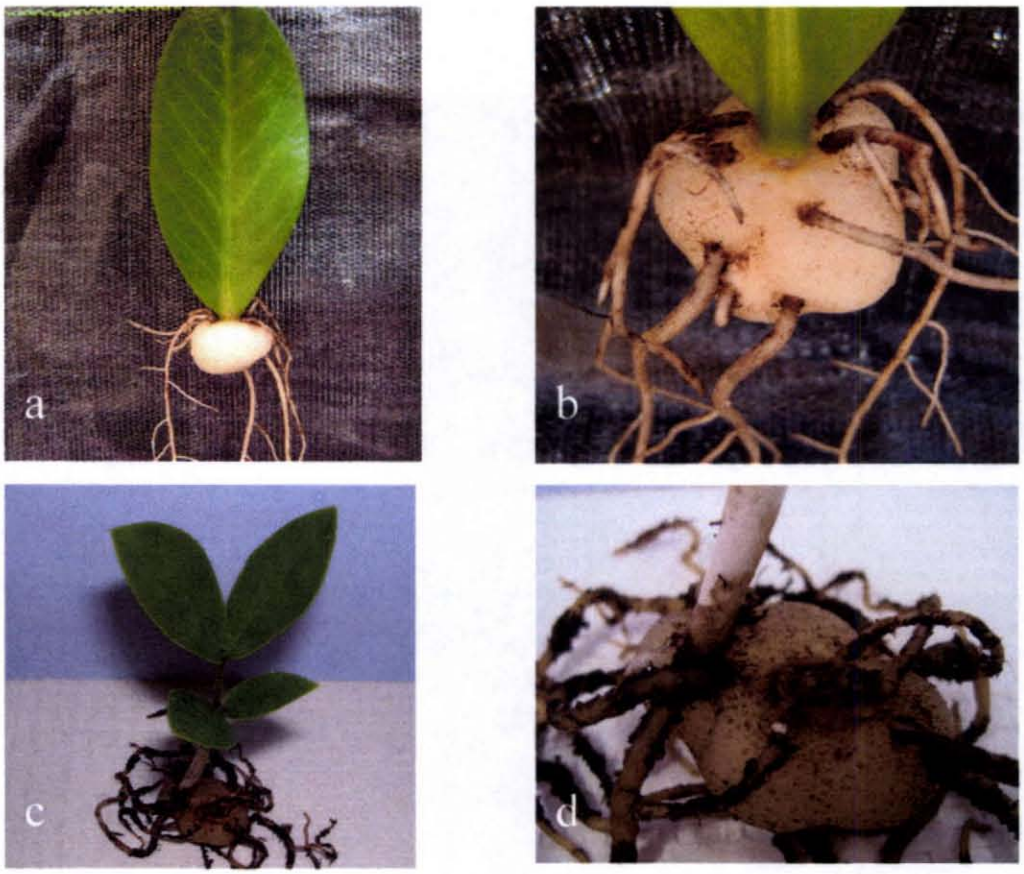

Figure 1.1. Vegetative propagation of ZZ via leaflet cuttings. (a) A ZZ leaflet that has produced a rhizome at its base after leaflet was harvested and stuck in an organic medium. (b) Close-up view of rhizome showing rooting. (c) New ZZ plantlet that was regenerated after adventitious buds produced on rhizome had elongated. (d) Close-up showing where new leaf emerges on the rhizome from an adventitious bud.

Figure 1.2. Actively growing ZZ root tips harvested for chromosome counts.

Figure 1.3. Sample preparation for flow cytometric analysis. (a) Reagents and plant tissue kept on ice throughout the entire process. (b) Sample size varies from 0.5 – 1cm^2. (c) and (d) Samples are chopped in the extraction buffer for 30-60 seconds with a double edged razor (e) suspension of nuclei is filtered, after which staining buffer containing RNAse and propidium iodide is added to the filtrate (f) samples are analyzed using a Beckman-Coulter (Miami, Florida) Altra flow cytometer.

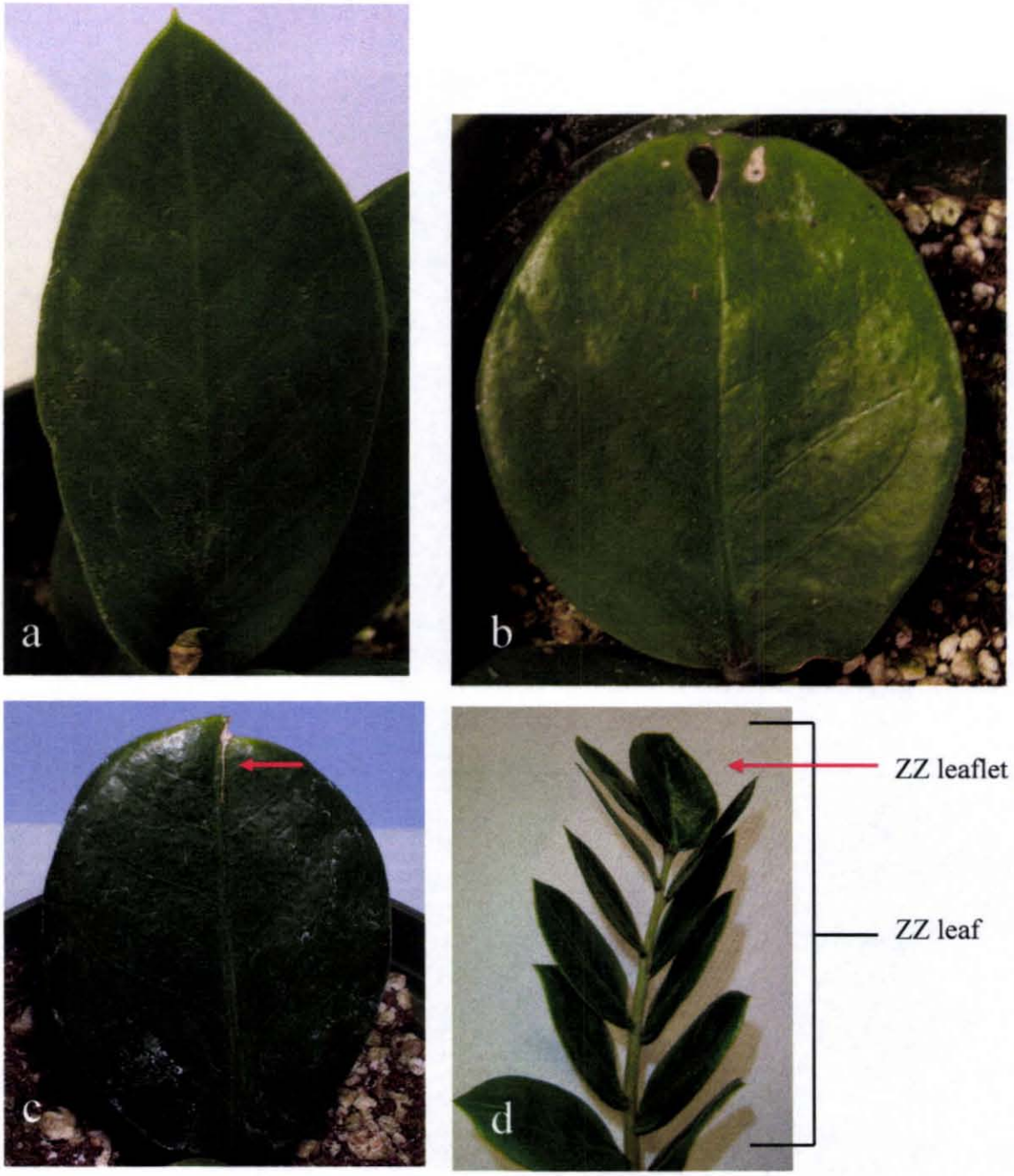

Figure 1.4. (a) ZZ leaflet showing the typical diploid leaf morphology. (b) Leaflet of suspected ZZ tetraploid showing a much rounder shape. (c) Leaflet of another suspected ZZ tetraploid. Notice, the asymmetry of the leaflet: the left side is larger than that of the right, indicating the possibility that the leaflet may in fact be a chimera. (d) ZZ leaf structure

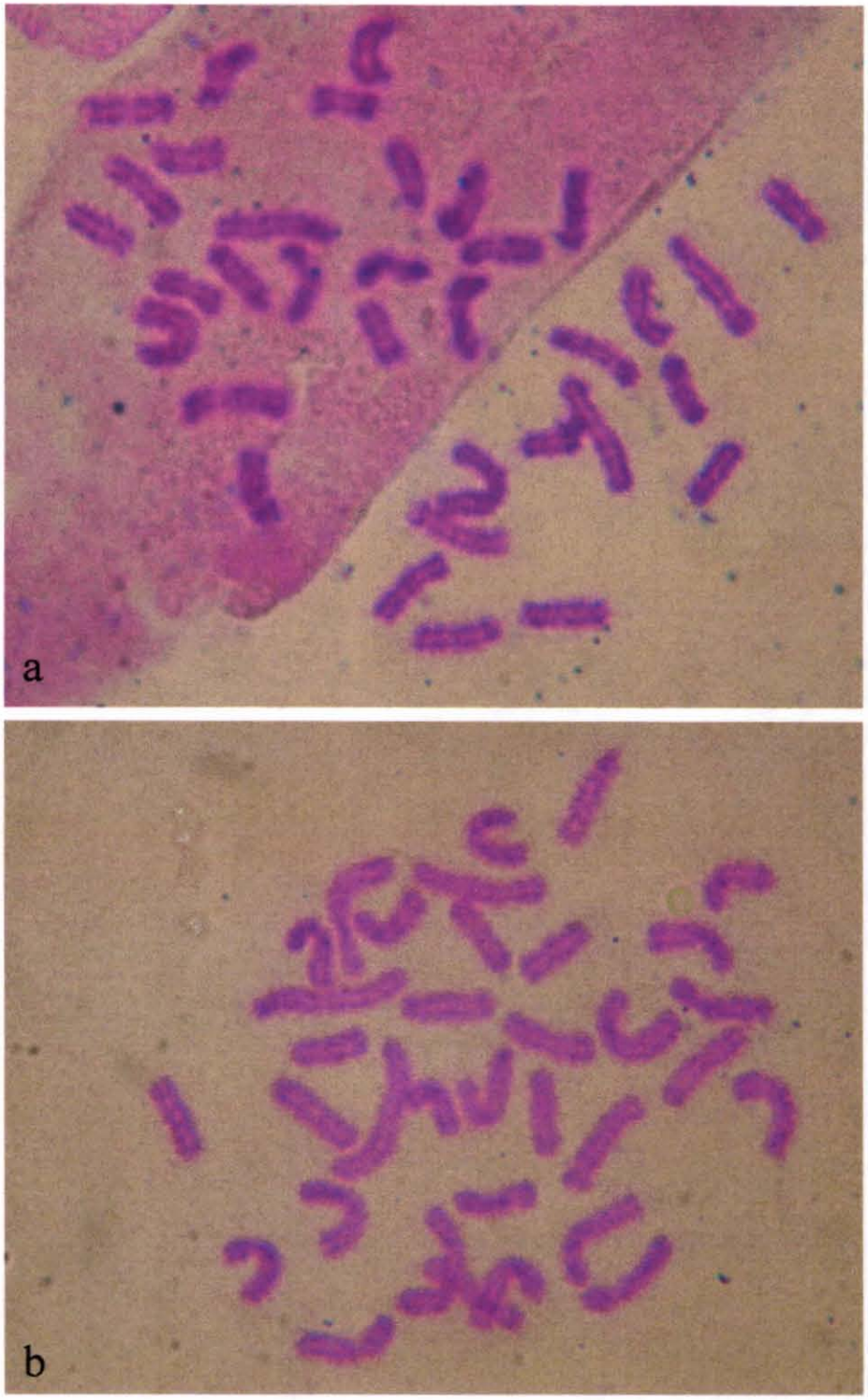

Figure 1.5. Stained ZZ chromosomes isolated from single cells. (a) and (b) Chromosomes from two different cells that were stained using the modified Shi (2003) method. Cells were isolated from actively growing root tips of a regenerated control plant (0% colchicine) and show 2n = 34.

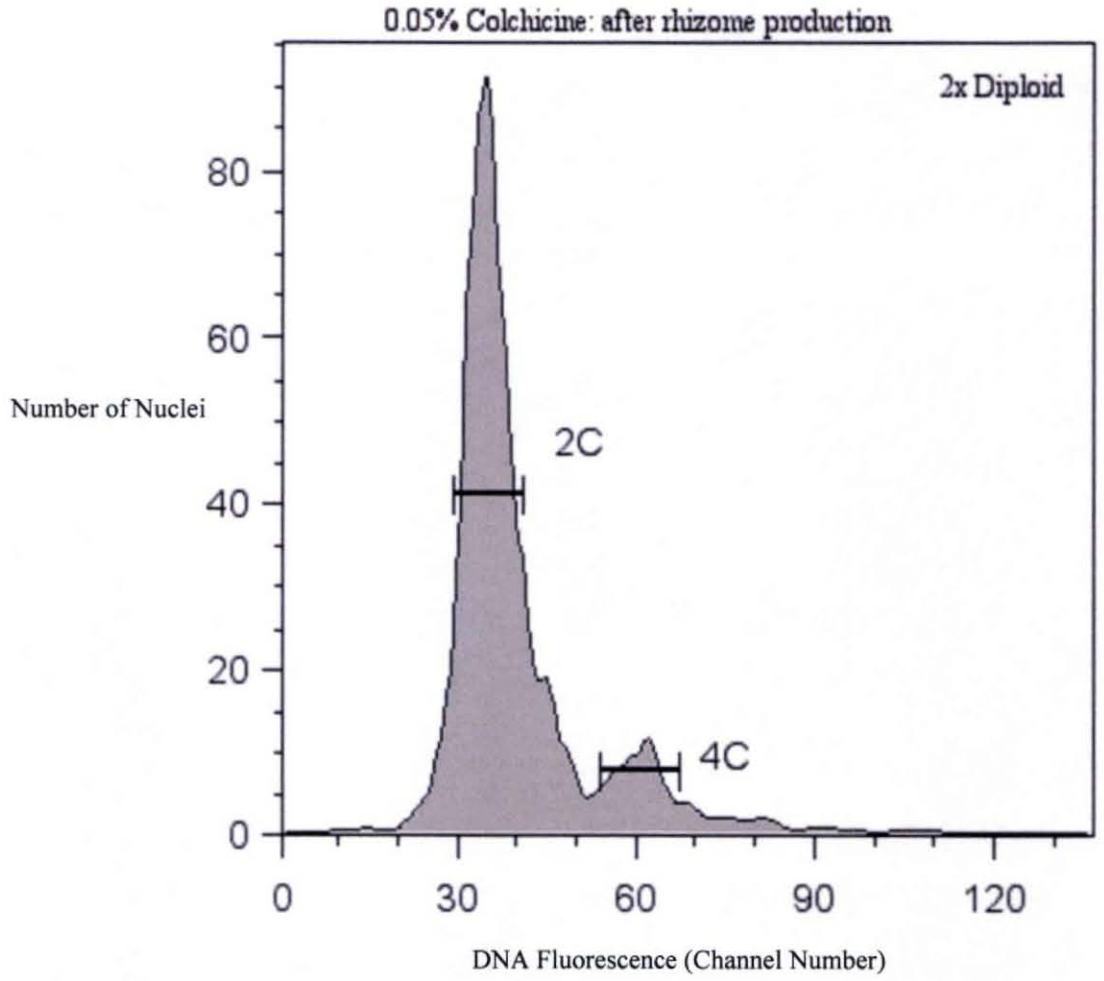

Figure 1.6. Distribution of DNA content of a suspected ZZ polyploid that resulted in being a diploid. Colchicine treatment of the parent leaflet after rhizome production did not result in the regeneration of a tetraploid plant. At least 2000 nuclei were analyzed and both 2C (G1 mitotic phase) and 4C (G2 mitotic phase) peaks have a CV<10%. Cell nuclei were isolated from leaflet tissue and stained with propidium iodide prior to analysis.

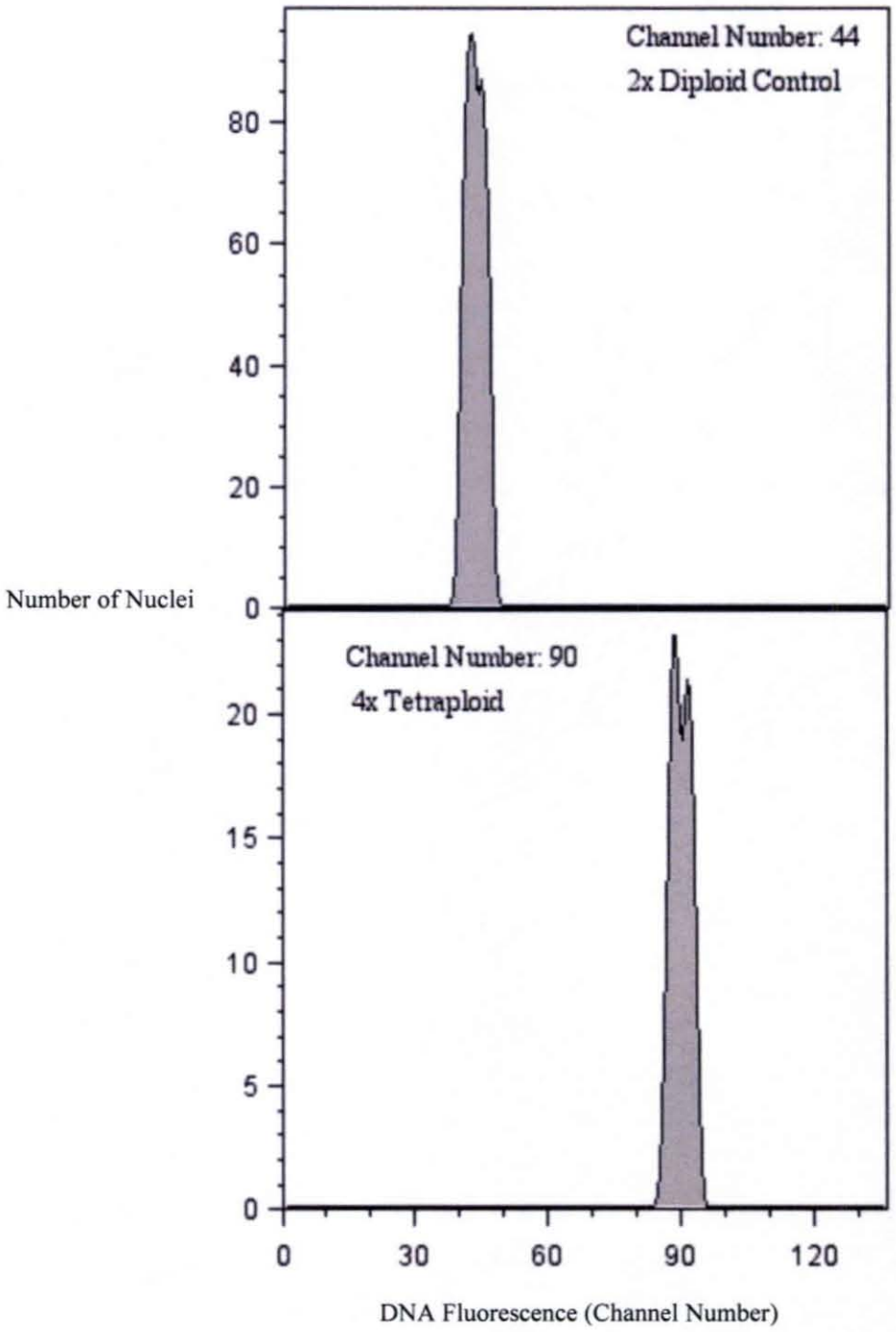

Figure 1.7. Distribution of DNA content of diploid and tetraploid ZZ samples. The DNA fluorescence of the tetraploid sample is almost double that of the diploid control. The tetraploid was produced by soaking a ZZ leaflet (with a small rhizome) in a 0.05% solution of colchicine. At least 2000 nuclei were analyzed and both the diploid and tetraploid peaks have a CV<10%. Cell nuclei were isolated from leaflet tissue and stained with propidium iodide prior to analysis.

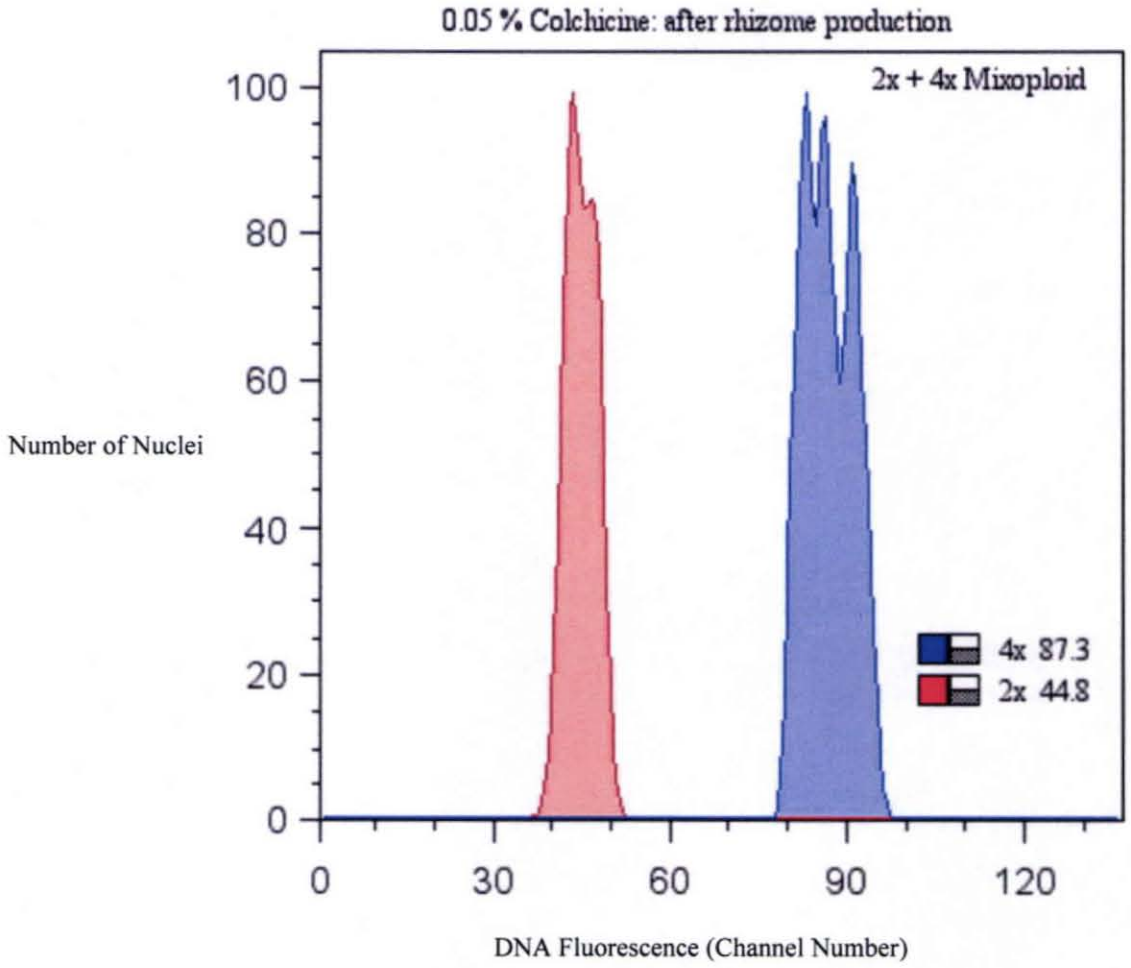

Figure 1.8. Distribution of DNA content of a ZZ mixoploid sample showing both 2x (diploid) and 4x (tetraploid) nuclei. The mixoploid was produced by soaking a ZZ leaflet (with a small rhizome) in a 0.05% solution of colchicine. At least 2000 nuclei were analyzed and both the diploid and tetraploid peaks have a CV<10%. Cell nuclei were isolated from leaflet tissue and stained with propidium iodide prior to analysis.

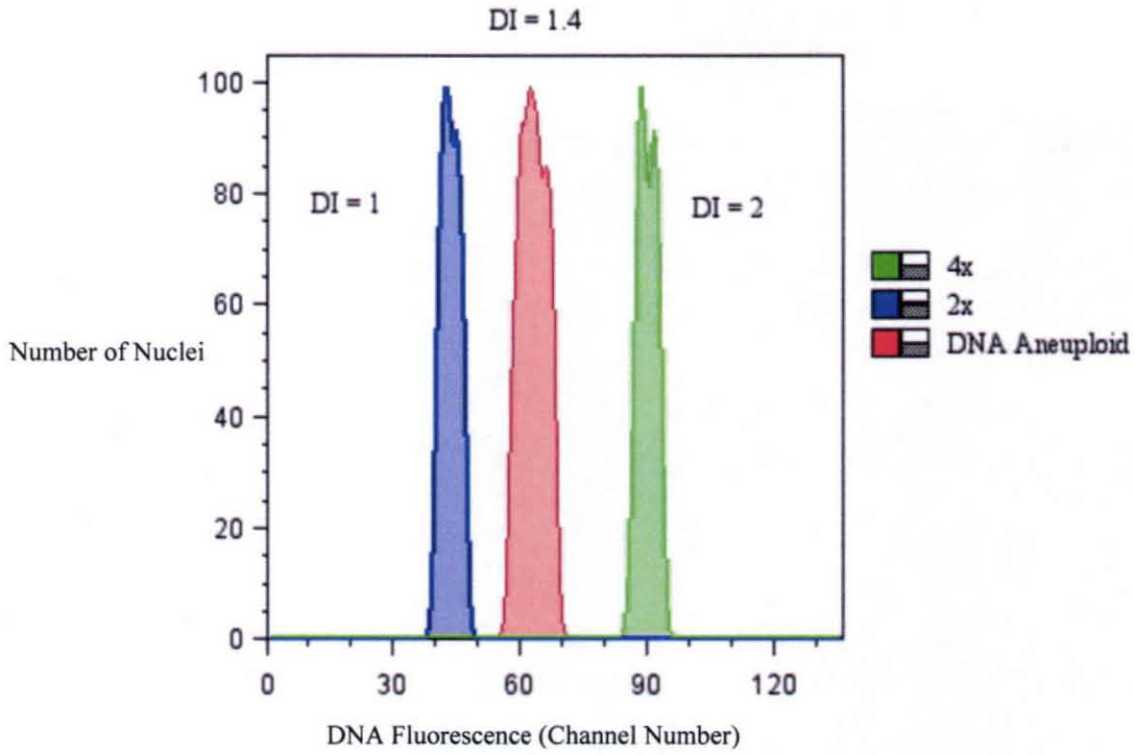

Figure 1.9. Distribution of DNA content of a DNA aneuploid. The diploid peak has been assigned a DI of 1, the tetraploid peak a DI of 2, and the DNA aneuploid was calculated as having a DI of 1.4. At least 2000 nuclei were analyzed and all 3 peaks have a CV<10%. Cell nuclei were isolated from leaflet tissue and stained with propidium iodide prior to analysis.

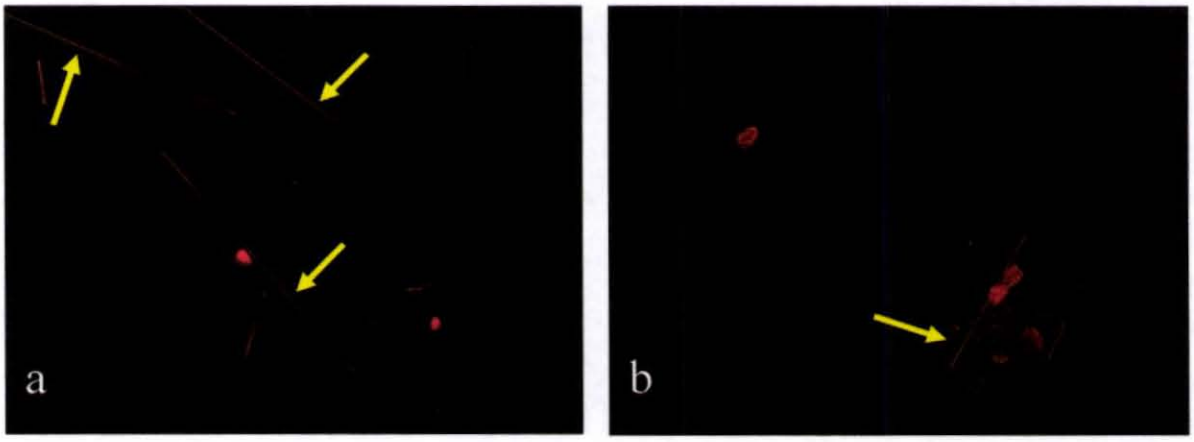

Figure 1.10. Photographs (a) and (b) were taken using an Olympus BX51 Epifluorescence Microscope with 480nm excitation frequency in order to view propidium iodide-stained nuclei (red dots) and detect cellular debris. Yellow arrows indicate debris.

CHAPTER 2: POLYPLOIDIZATION OF ZZ VIA ORYZALIN TREATMENT OF CALLUS

Background Information

Though a simple method was employed for the production of ZZ tetraploids in Chapter1, tissue culture has also been shown to be an effective tool in polyploidy induction. Tissue culture increases the efficiency of mutagenic treatments for variation induction, handling of large populations, use of ready selection methods, and rapid cloning of selected variants (Predieri, 2001). The use of tissue culture in the successful production of tetraploids has been shown in various plants including: *Spathiphyllum* (Eeckhaut *et. al*, 2004), *Alocasia* (Thao *et. al* 2003), *Cocoyam* (Tambong, Sapra, and Garton, 1998), *Miscanthus* (Petersen, 2003) and *Rhododendrons* (Vainola and Repo, 2001; Vainola, 2000). The objective of this experiment was to produce a ZZ tetraploid by the oryzalin treatment of ZZ callus.

In the design of a protocol for the *in vitro* production of ZZ tetraploids, callus tissue was treated with oryzalin. The advantage of treating callus is twofold: (i) callus tissue is typically in a high state of cell division, which has been shown to be most responsive to chemical mutagenesis and (ii) plantlets regenerated from callus tissue may express some genetic variability, which would contribute to the potential for obtaining a new ZZ variety. To date, HePing and Peng (2003 and 2005) are the only researchers to report an *in vitro* micropropagation protocol for ZZ. The 2003 publication, however, describes a protocol for direct shoot organogenesis from ZZ leaf explants with no

intervening callus stage. Similarly, the 2005 publication describes a protocol for tuber induction on leaflet explants. In this research project, a tissue culture protocol was developed for the callus induction and plantlet regeneration of ZZ for the oryzalin treatment of ZZ.

Materials and Methods

Callus Induction and Plantlet Regeneration

A newly emerging ZZ leaf (petiole stalk with leaflets) was harvested from a juvenile-like stock plant for callus induction. The plant material was prepared for disinfestation by soaking it in running water overnight and scrubbing it with liquid antibiotic soap the following day. After rinsing with distilled water, explants (ZZ leaflets and petioles) were harvested and wiped down with 95% ethanol then trimmed at the edges. Explants were soaked in 0.65% sodium hypochlorite solution (10% v/v Clorox Regular Bleach; The Clorox Company; Oakland, CA) for 10 minutes while shaking on a rotator at 140rpm, transferred to 0.13% sodium hypochlorite solution (2% v/v Clorox) for more trimming, and then soaked for an additional 15 minutes in a 0.33% sodium hypochlorite solution (5% v/v Clorox). Leaflet explants were trimmed down to a 1 x 1 cm square (containing the major vein) while petioles were trimmed to about 2.5cm in length, then rinsed in sterilized distilled water before being transferred to 25mm test tubes containing 10 ml solid callus inducing medium. Leaflet explants were stuck vertically in the medium so that half of the explant was submerged into the medium, while the other half was exposed. Similarly, the basal portion of the petiole was submerged in the

medium while the distal portion remained exposed. The callus inducing media is composed of half strength MS macro- and micronutrients (Murashige and Skoog, 1962), half strength MS vitamins, 100mg l^{-1} myo-inositol, 0.2mg l^{-1} BA, 4mg l^{-1} 2,4-D, 20g l^{-1} sucrose, and 3g l^{-1} gellan gum (Miguel, 2004, see pg iii). All cultures were stored in the dark at about 78-80°F. Once callus production had been initiated, cultures were transferred to fresh callus inducing medium approximately every 2-4 weeks for callus multiplication.

For shoot regeneration, callus cultures were removed from the dark, transferred to shoot induction media, and placed on metal racks in the culture room under Gro-Lux lights with a timer set for 18 hour days. The shoot inducing medium used is composed of half strength MS macro- and micronutrients, half strength MS vitamins, 100mg l^{-1} myo-inositol, 1mg l^{-1} BA, 40g l^{-1} sucrose, and 3g l^{-1} gellan gum. Once the adventitious buds had elongated and the leaf sheath was about 2.5cm, they were transferred to ½ MS with no plant growth regulators for further development. Rooted plantlets, about 5cm in height, where then transferred to community pots containing moist Pro-Mix 'BX' medium (Premier Horticulture Ltd.; Dorval, Canada) in the greenhouse at Pope Laboratory. Pots were covered with plastic wrap for about 4 weeks to allow for acclimatization. Figure 2.1 shows the developmental stages observed, and provides an overall view of the protocol that was developed for callus induction and plantlet regeneration of ZZ.

Oryzalin Treatment of ZZ Callus Derived from Leaflet and Petiole Tissue

Callus cultures derived from leaflets were kept separately from those derived from petiole tissue, and the experimental set up described below applies to both leaflet and petiole callus. A completely randomized experimental design with a 2 x 3 factorial treatment design with three oryzalin concentrations (0%, 0.005%, and 0.01%) and two durations (24 and 48 hours) was used for the oryzalin treatment of ZZ callus. Oryzalin (PhytoTechnology Laboratories; Shawnee Mission, KS) was dissolved in DMSO, and added to liquid callus inducing medium to give a final concentration of 1% DMSO. Each treatment consisted of two reps with 10 experimental units ($1cm^2$) per rep for a total of 20 explants per treatment. Due to limited explant material, however, the controls were comprised of one rep with 5 experimental units per rep. A total of 90 callus pieces were used.

Seven month old callus was cut into $1cm^2$ units and transferred to a 125ml flask containing 50ml of the filter sterilized oryzalin solution, with 10 callus pieces per flask. A 1% DMSO solution of callus inducing medium was used as the control. Flasks were placed on a rotary shaker at 75 rpm for 24 or 48 hours. After treatment with oryzalin, the calli were rinsed in sterile distilled water, and each callus piece was transferred to a 25mm test tube containing 10ml solid callus inducing medium, with the callus piece submerged in the medium. Cultures were kept in the dark and transferred to fresh medium every two weeks, for a total of 6 transfers.

After 12 weeks on callus inducing medium, the cultures were transferred to Petri dishes containing 20ml shoot inducing medium and cultures were placed under Gro-Lux lights with 18 hour days. Cultures were transferred to fresh medium every 2 weeks.

Twenty-one weeks after transfer from callus inducting medium to shoot inducing medium, cultures were transferred to Petri dishes containing 20ml MS shoot multiplication medium A (Sigma; St. Louis, MO) in order to speed up the process of adventitious bud development. Medium A contains full strength macro- and micronutrients, vitamins, sucrose, IAA, sodium phosphate, adenine, and 2-ip as described by Huang and Murashige (1976). Once adventitious buds were produced, they were excised from the callus and transferred to Magenta boxes containing 50ml bud elongation medium [transfer to MS with no plant growth regulators resulted in bud death]. The calli were then returned to MS Medium A for further development of adventitious buds. Bud elongation medium is composed of half strength MS macro- and micronutrients, half strength MS vitamins, 100mg l^{-1} myo-inositol, 100mg l^{-1} glutamine, 2mg l^{-1} BA, 40g l^{-1} sucrose, and 3g l^{-1} gellan gum. Cultures were kept on this medium until plantlets had been regenerated, and leaflets were analyzed for changes in ploidy via flow cytometry. Figure 2.2 shows the developmental stages observed and provides an overall view of the protocol that was developed and used for the *in vitro* polyploidization of ZZ using oryzalin.

Flow Cytometry

Refer to the *Materials and Methods* section of Chapter 1 (pages 12-14) for a full description of the procedures performed for flow cytometric analysis of tissue cultured leaflets.

Results and Discussion

Callus Induction and Plantlet Regeneration

Callus was observed on the explants about 4½ weeks after the cultures were initiated, with the callus developing along the submerged edges of the leaflet and petiole explants. About 5 weeks after transfer to shoot induction medium and illuminated conditions, the callus tissue produced tiny hair-like structures that gave some of the calli surfaces a "fuzzy" appearance. Six weeks later, adventitious buds were clearly visible on the callus pieces. Small plantlets were regenerated after 10 weeks on ½ MS with no plant growth regulators. All plantlets transferred to the greenhouse survived.

Oryzalin Treatment of ZZ Callus Derived from Leaflet and Petiole Tissue

To date, two plantlets with the capacity to be analyzed by flow cytometry have been regenerated (Figure 2.2). These plantlets were regenerated from callus pieces that were treated at 0.01% colchicine for 24 hours. Preliminary results obtained from flow cytometric analysis (data not shown) show that both plants have higher ploidy levels, though the fluorescence produced from the extracted nuclei was not exactly twice as much as that of the diploid controls. Subsequently, they are described as DNA aneuploids. It was also shown that the peaks obtained had high coefficients of variation (up to 17%), which had not been previously observed. Though leaflets were also used in the *in vivo* colchicine experiments (Chapter 1), the nature of the plant material used in this experiment was different. It is probable that adjustments such as the addition of citric acid and Tween 20 (Ulrich and Ulrich, 1991), the addition of β-mercaptoethanol,

citrate, and the use of a surfactant (Brown *et al.*, 1991) may need to be applied to the procedure used for flow cytometric analysis of tissue cultured ZZ leaflets. More adventitious buds will need to develop into plantlets in order for further tests to be run.

The length of time required for adventitious bud development to occur on the callus tissue once the cultures had been transferred to shoot induction media varied between the original tissue culture protocol developed for callus in induction in ZZ and in the second procedure in which the calli were treated with oryzalin. In the callus induction protocol, it took 11 weeks for adventitious buds to begin to form, while it took over twice that long and a modification of the medium for all the petiole and leaflet callus cultures (including controls) to develop adventitious buds after oryzalin treatment (26 weeks). It is suspected that DMSO, and not oryzalin, may have affected the regeneration capacity of the callus tissue. Unfortunately, it cannot be confirmed because a control without DMSO was not included in the experiment.

The inability of the callus tissue to produce adventitious buds, however, was overcome with the use of MS Multiplication Medium A. The medium contains key components that helped activate the callus tissues: adenine sulfate promotes cell division, while sodium phosphate provides the cells with energy. Also, the high cytokinin (2-ip at 30 mg l^{-1}): low auxin (IAA at 0.3 mg l^{-1}) ratio helped induce adventitious bud development. Within 5 weeks of callus transfer to Medium A, adventitious buds had developed and callus tissue continued to multiply. Bud elongation to form a new plantlet, however, did not occur upon transfer of the buds to MS medium with no PGRs (MS_o). Within one to two weeks of transfer to MS_o, browning occurred that led to eventual bud death. It was suspected that the buds were affected by the high MS salt concentration in

medium, hence, a bud elongation media was devised that contained ½ the salt concentration and BA was used as the cytokinin to promote elongation (modeled after the original plantlet regeneration protocol devised). The use of this medium proved successful and most buds are now elongating and developing into plantlets.

Though a tetraploid ZZ plantlet has not been identified, there are several emerging buds that must still be analyzed. More importantly, however, is that this project has led to the development of a novel tissue culture protocol for callus induction and plantlet regeneration in *Zamioculcas zamiifolia* (Fig. 2.1). Leaflets or petioles are harvested from a mature mother stock plant, disinfested, and trimmed to an appropriate size. The explant is then stuck into callus inducing medium and cultures are kept in the dark. After sufficient callus has been produced, the callus tissue is transferred to shoot induction medium and cultures are exposed to illuminated conditions. Callus pieces with adventitious buds are then transferred to a medium with no plant growth regulators to allow for rooting and plantlet development. The tissue culture protocol developed may be used for further *in vitro* mutation induction studies in ZZ such as the irradiation of callus tissue or adventitious buds. The protocol developed may also be adopted for the commercial production of ZZ, which may allow for more uniform ZZ propagation.

Figures

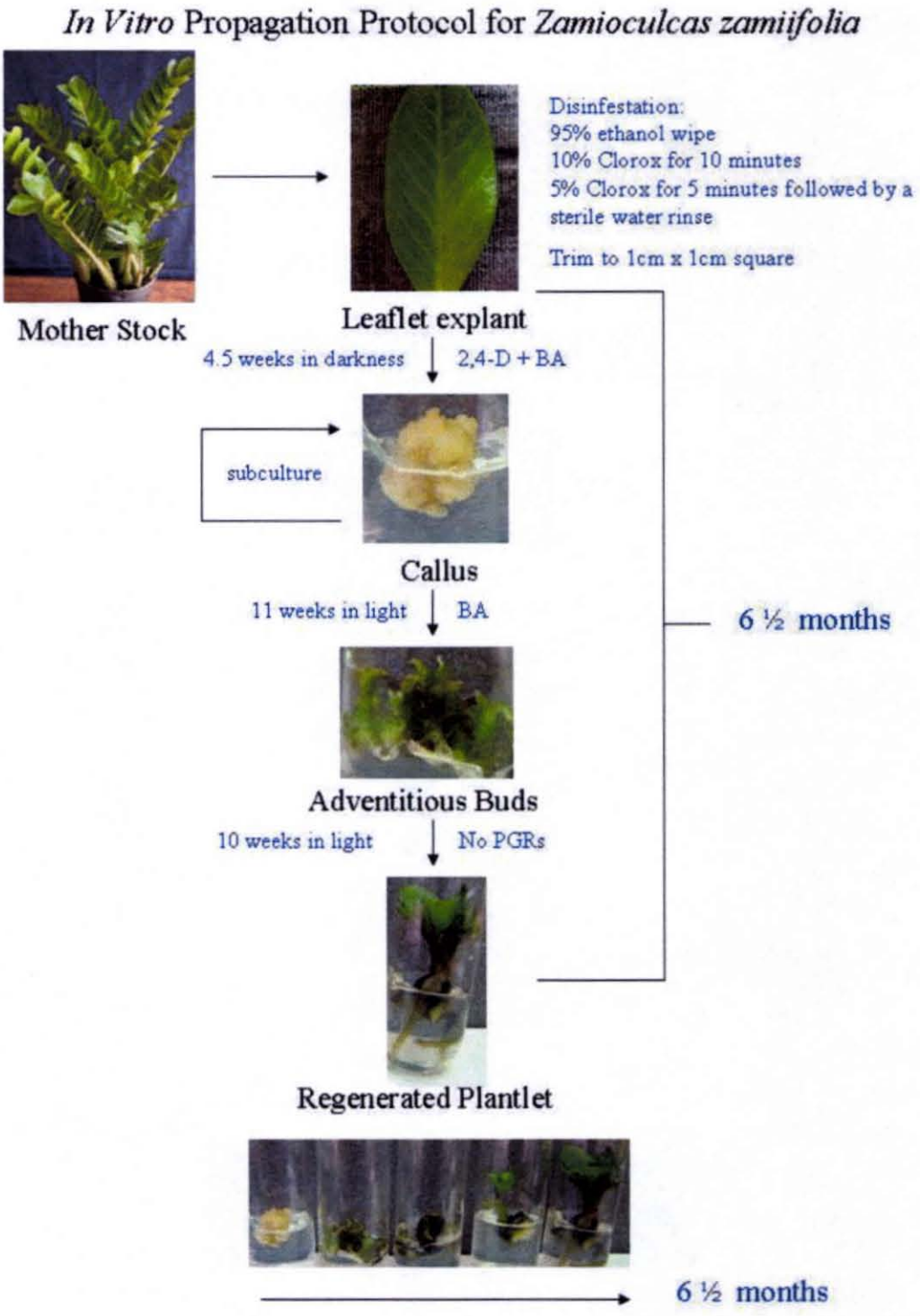

Figure 2.1. Tissue culture protocol developed for callus induction followed by plantlet regeneration of *Zamioculcas zamiifolia*.

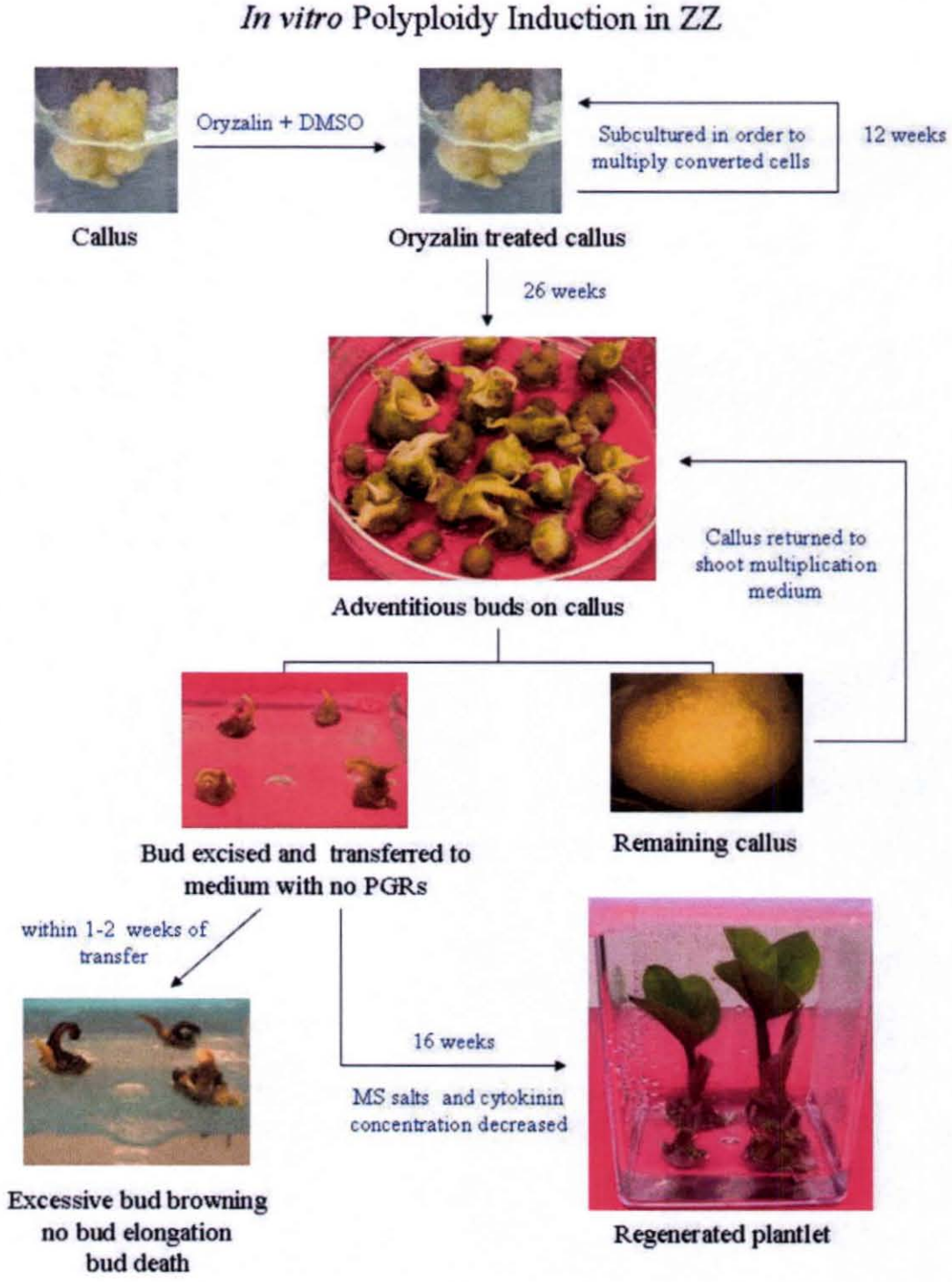

Figure 2.2. Protocol developed for *in vitro* polyploidy induction of *Zamioculcas zamiifolia*. To date, only two fully developed plantlets have been regenerated as shown above. All other adventitious buds are still in the elongation phase.

CHAPTER 3: IDENTIFICATION OF A SUITABLE IRRADIATION DOSAGE FOR *ZAMIOCULCAS ZAMIIFOLIA*

Background Information

Mutation breeding aims to improve an otherwise good cultivar by the modification of one easily recognizable character, leaving the rest of the genotype untouched (Broertjes and Van Harten, 1988; Micke *et al.*, 1987; Lapins, 1983). It is also used to generate more genetic variability for selection and cross breeding (Micke *et al.*, 1987), making it a useful breeding tool for plant species that are to be incorporated into a breeding program. It is for both of these reasons that *ZZ* is an ideal candidate for mutation breeding.

One of the first steps in the establishment of a mutation breeding program is the determination of a suitable irradiation dosage, typically identified as the lethal dosage or LD_x. The LD_x is calculated as the exposure required to produce x percent lethality of the irradiated material, expressed as a percentage of the control (Sparrow *et al.*, 1968). Many breeders use LD_{50} as the desirable irradiation dosage to induce mutations, unless the observed results show otherwise (Predieri, 2001; Cruz T. and Rubi A, 1995; Bhandari, 1993; Yamaguchi, 1988; Lapins, 1983). The objective of this work was to determine the LD_{50} for the irradiation of *ZZ* leaflets using x-rays.

Materials and Methods

Leaflets used for irradiation were harvested from mature (capable of flowering), ZZ stock plants with fully expanded leaves. Harvested leaflets were graded according to size and distributed evenly throughout all treatments, after which leaflets were stacked in sets of (i) 10 leaflets (10 or 30 leaflets irradiated per treatment) or (ii) 11 leaflets (33 leaflets irradiated per treatment). Each set of leaflets was wrapped in a moist paper towel and placed in the irradiator with the leaflets centered within the irradiation zone with the petioles facing inward. After the desired irradiation dosage had been achieved, a stack was randomly removed from the irradiator and placed in a labeled plastic bag until all leaflets had received the appropriate irradiation dosage. Leaflets were then stuck in a 40cm x 55cm metal flat containing moistened Pro-Mix 'BX' (Premier Horticulture Ltd.; Dorval, Canada) and placed under 70% shade in a greenhouse at Pope Laboratory (Figure 3.1). SAS statistical package version 9.1 (SAS Institute Inc., Cary, N. C.) and Microsoft Excel were used to perform probit analysis and regression analyses on the data collected.

Irradiation of ZZ leaflets was performed on three separate occasions using a Hewlett Packard 43804N X-Ray System Faxitron Series. Leaflets were irradiated at a rate of 1.21 Grays per minute. Due to the limited availability of plant materials, only a small number of leaflets were irradiated during the first irradiation. The second and third irradiations were performed based on the preliminary results obtained from the preceding irradiation (Table 3.1). The first irradiation was performed in March 2005, the second in June 2005, and the third in August 2005. Final leaflet survival data was collected in August 2006, and leaflet survival was scored as a ZZ leaflet that regenerated a new plant.

Results and Discussion

Irradiation 1

LD_{100}, the lowest exposure to produce 100% leaflet mortality, was found to be 40Gy, and all leaflets irradiated above this dosage died from exposure to the X-rays (Table 3.2). Within 2 – 3 months, leaflet yellowing was observed, and within 4 months the leaflets had developed a brown, "burnt" appearance (Fig. 3.2). Probit approximation was the model used to determine the LD_{50} from the results shown in Table 3.2. Pearson Chi-Square and L. R. Chi-Square were used as goodness of fit tests, and both models showed that there is no significant difference ($p= 0.2142$ and $p=0.0686$, respectively) between the observed data and the expected data, hence the data were normally distributed. Type III Analysis of Effects showed that irradiation dosage had a highly significant ($p = 0.0026$) effect on leaflet death, and the LD_{50} was identified as 19Gy. The complete SAS output is provided in the Appendix (pages 77 – 80).

Irradiation 2

The second set of irradiations was designed after preliminary observations from the first irradiation showed $LD_{100} = 40$Gy (Table 3.3). It is inappropriate, however, to apply probit analysis to the second data set in order to determine LD_{50} because the data obtained did not fit the normal distribution; hence, regression analysis was used to estimate LD_{50}. Irradiation dosage vs. percent death was plotted and a linear regression equation was calculated as follows: $Y = 0.0261X - 0.0545$, where $Y = LD_{50}$, and $X =$ equivalent dosage, $r = -0.545$, and $R^2 = 0.9348$. Using the calculated regression line, LD_{50} was determined to be 21Gy (Fig. 3.3).

It should also be noted that of the 33 leaflets irradiated at 20Gy, one leaflet regenerated a plant that shows a possible mutation (Fig. 3.4). One of the leaves produced by the regenerated plant shows differences in leaflet size, with half of the leaflets showing a reduction in size as compared to a typical ZZ leaflet. The distance between the successive leaflets also appears to be shorter than is typically observed. Because the plant belongs to the M_1 generation (mutagen-treated plant), further observations of the M_2 and M_3 generations (offspring of the mutagen treated plants) are necessary in order to confirm the differences observed as a mutation. Nonetheless, the results obtained are promising.

Irradiation 3

The data collected from the third irradiation could not be used to calculate the LD_{50}, since the highest percentage of death recorded was 36%, which was obtained at 25Gy, the highest irradiation dosage used in the third irradiation (Table 3.4). The discrepancy between the results for LD_{50} in irradiation 3 and those of irradiations 1 and 2 may have resulted from various reasons. According to Sparrow *et al.*, (1968), several biological, radiological, and environmental factors may contribute to variations in the radiobiological responses of plants. For example, the effectiveness of the prescribed dosage varies according to the moisture content of the irradiated plant material. Similarly, the stage of the plant growth cycle affects plant sensitivity to irradiation, where actively growing plants are more sensitive to irradiation than plants in their dormant stages. In this experiment, the first two irradiations were performed in March and June, while the third irradiation was performed in August. It is plausible that the mother stock

plants used in the third experiment were in a physiologically different stage (growth cycle and vegetative or floral stage of differentiation) than those that were used in the first two irradiations, hence the variation in the calculated lethal dosages. In conclusion, the results obtained have provided useful information that contributes significantly to the establishment of a mutation breeding program for ZZ. Large number of leaflets may now be irradiated at the calculated LD_{50} of 20 ± 1Gy in an attempt to produce ZZ mutants.

Tables and Figures

Table 3.1. Table shows the number of leaflets irradiated and the x-ray dosages used in order to determine the LD_{100} and LD_{50} for ZZ leaflet cuttings. The first irradiation set was performed in March 2006, the second set in June 2006, and the third in August 2006.

Irradiation Set	X-Ray Dosage (Gy)	Number of Leaflets Irradiated
1	0	10
1	10	10
1	20	10
1	30	10
1	40	10
1	50	10
1	60	10
1	70	10
1	80	10
1	90	10
1	100	10
2	0	10
2	10	33
2	20	33
2	30	33
2	40	33
3	0	5
3	15	30
3	20	30
3	25	30

Table 3.2. Table shows the x-ray dosages used and percent death of ZZ leaflets irradiated in March 2006 (irradiation 1). Probit analysis was used on the data shown below to calculate the LD_{50} for ZZ, which was determined to be 19 Gy. A total of 10 leaflets were irradiated per dosage.

Dosage (Gy)	Number of dead Leaflets	Leaflet Total	Percent Death
0	0	10	0
10	5	10	50
20	9	10	90
30	9	10	90
40	10	10	100
50	10	10	100
60	10	10	100
70	10	10	100
80	10	10	100
90	10	10	100
100	10	10	100

Table 3.3. Table shows the x-ray dosages used and the percent death of ZZ leaflets irradiated in June 2006 (irradiation 2). Regression analysis was used to calculate the LD_{50} for ZZ from the data shown below, and was determined to be 21Gy. A total of 10 leaflets were irradiated for the controls and 33 leaflets for each of the other treatments.

Dosage	Number of dead Leaflets	Leaflet Total	Percent Death
0	0	10	0
10	2	33	6
20	20	33	60
30	22	33	66
40	33	33	100

Table 3.4. Table shows the results obtained from irradiation 3, which was performed in August 2006. The data collected could not be used to calculate the LD_{50} of ZZ, since none of the x-ray dosages used produced 50% lethality.

Dosage	Number of dead Leaflets	Leaflet Total	Percent Death
0	0	5	0
15	6	30	20
20	11	30	36
25	11	30	36

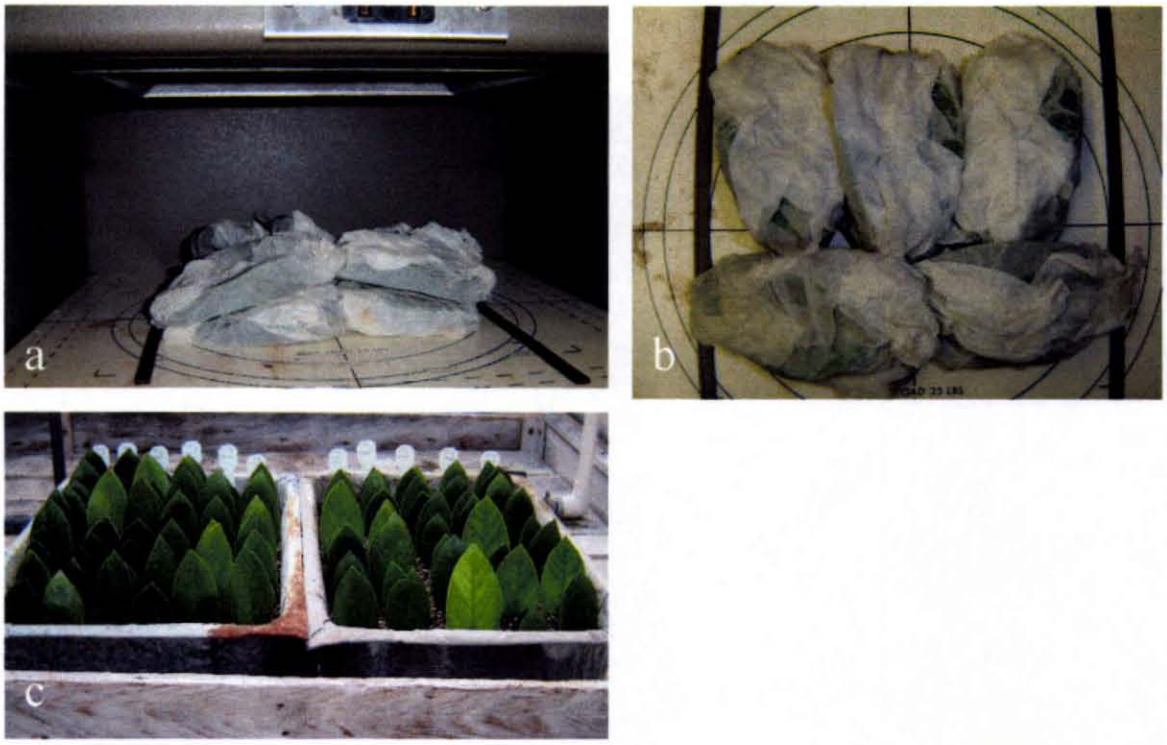

Figure 3.1. (a) Sets of leaflets wrapped in moist paper towels and ready to be irradiated. (b) Leaflets are kept within the irradiation zone on the shelf (c) irradiated leaflets stuck in moist Pro-mix 'BX' and kept under 70% shade.

Figure 3.2. (a) Leaflet on the right was irradiated at 100Gy, and exhibits heavy browning and has a "burnt" look when compared to the control on the left. The picture was taken approximately 4 months after exposure to the x-rays. (b) From left to right: leaflet irradiated at 80Gy, 60Gy, and 0Gy. The picture was taken approximately 3 months after exposure to the x-rays.

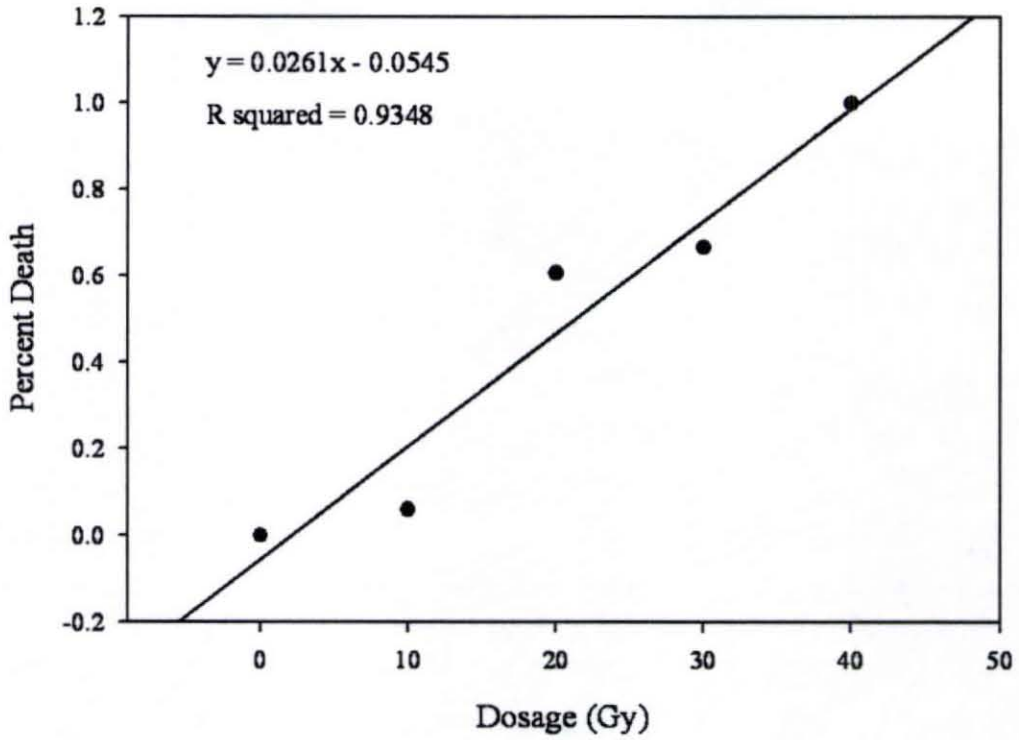

Figure 3.3. Relationship between dosage and percent death of x-ray irradiated ZZ leaflets. Using the calculated regression equations, LD_{50} is estimated at 21Gy.

Figure 3.4. ZZ plant regenerated from leaflet irradiated at 20Gy. Red arrows point to the leaflets that are smaller in comparison to those that are typical in size.

CHAPTER 4: GERMPLASM COLLECTION OF *ZAMIOCULCAS ZAMIIFOLIA*

Our *Zamioculcas zamiifolia* germplasm collection originally began with just a few plants, and has now grown to include plants from six different sources. Though several of the inventoried plants seem to be of the same clone, we have kept records on the date of receipt and source of collection for each of the different plants. Plants from these six different sources have been propagated by division or leaf cuttings to increase our stock numbers. A brief description of the collected plants is given below, followed by photographs of several plants in the collection.

Hawaiian Sunshine Nursery, Oahu

Cultivated variety obtained from Hawaiian Sunshine Nursery in Waimanalo, Oahu, on several occasions. These are the mother stock plants used in my thesis research and are also the variety of ZZ that is commercially grown.

National Botanic Garden of Belgium

Two accessions were purchased from the National Botanic Garden of Belgium:

(i) *Zamioculcas zamiifolia* (Lodd.) Engl.
Accession: 19074359 cultivated material

(ii) *Zamioculcas zamiifolia* (Lodd.) Engl. 'Variegata'
Accession: 19726043 cultivated material

Both accessions were received in February 2005.

Contact person: Frieda Billiet: Botanist, Living Collections, in charge of greenhouses
National Botanic Garden of Belgium
B-1860 MEISE

Tel: +32(0)2.260.09.90
Fax: +32(0)2.260.09.55
email: frieda.billiet@br.fgov.be

Thailand

Collected in Thailand by Dr. Ken Leonhardt in Spring of 2004.

Nong Nooch Tropical Botanical Garden

Plants were donated by Mr. Anders Lindstrom on August 24th, 2005. They were originally collected in Mozambique by Mr. Lindstrom and have an accession number: NNTBG 16090.

Harold L. Lyon Arboretum

Plants were collected from Lyon Arboretum in the Spring of 2005 with the permission of Mr. Ray Baker. He states:

"Our *Zamioculcas zamiifolia* was received in January 1977 from Foster Gardens #9130, and planted in its present location in June 1977. We got 25 plants (probably in pots) originally from Foster Gardens and later more from Elsie Horikawa. Since we only planted 4 where they are now, Elsie's probably died. Foster may have more detailed collection information. You could call Winnie Singeo at 522-7060 for their accession information."

Naomi Hoffman, the botanist at the Honolulu Botanical Garden, provided the following information on November 17th, 2006 about the ZZ plants at the Foster Botanical Garden: Accession # 9130: collected in Zanzibar, 1949, by David Barry.

Contact Person: Naomi Hoffman
nhoffman@honolulu.gov
808-522-7066

Waimea Valley Audubon Center

Plants were donated on June 30th, 2005 by Mr. David Orr.

(i) Accession: 75p2154 received from Tagami & Hart, Kahaluu Oahu.

(ii) Accession: 79p331 received from Conrad Flemming, St. Croix, Virgin Islands; possibly a plant at Fairchild Tropical Gardens Florida

Important Note: Both of these plants are planted in the exact same area at Waimea

Valley Audubon Center, so that the plant material we received can be either of the above.

Contact Person: Mr. David Orr
dorr@audubon.org
808-638-9199

Figures

Figure 4.1. *Zamioculcas zamiifolia* (Lodd.) Engl. acquired from Hawaiian Sunshine Nursery on Oahu. Commercial variety with thick, solid gray/green petioles, and green leaflets.

Figure 4.2. *Zamioculcas zamiifolia* (Lodd.) Engl. acquired from the National Botanic Garden of Belgium. Solid green leaflets are slightly thinner, but broader and larger than those of the commercial variety.

Figure 4.3. *Zamioculcas zamiifolia* (Lodd.) Engl. 'Variegata'. Acquired from the National Botanic Garden of Belgium. Variegation is unstable and may or may not be retained during propagation.

Figure 4.4 *Zamioculcas zamiifolia* (Lodd.) Engl. 'Variegata' acquired from the National Botanic Garden of Belgium. Different forms of ZZ variegation.

Figure 4.5. *Zamioculcas zamiifolia* (Lodd.) Engl. 'Variegata' acquired from the National Botanic Garden of Belgium. Different forms of ZZ variegation.

Figure 4.6. *Zamioculcas zamiifolia* (Lodd.) Engl. 'Variegata' acquired from the National Botanic Garden of Belgium. Different forms of ZZ variegation.

Figure 4.7. *Zamioculcas zamiifolia* (Lodd.) Engl. acquired from Waimea Valley Audubon Center. Solid green leaflets with occasional mottling at the base of the petiole.

Figure 4.8. *Zamioculcas zamiifolia* (Lodd.) Engl. acquired from Harold L. Lyon Arboretum. Closely resembles ZZ from Waimea Valley Audubon Center.

Figure 4.9. *Zamioculcas zamiifolia* (Lodd.) Engl. acquired from Nong Nooch Tropical Botanical Garden. Leaflets have serrated edges and petioles are mottled and brown.

Figure 4.10. *Zamioculcas zamiifolia* (Lodd.) Engl. acquired from Nong Nooch Tropical Botanical Garden. Leaflets possess longer leaflet bases, giving newly emerging leaves a drooped appearance.

SECTION 2

MARSDENIA FLORIBUNDA

CHAPTER 5: POLYPLOIDIZATION OF *MARSDENIA FLORIBUNDA*

Background Information

The botanist Paul Forster (1990) merged the well-known genus *Stephanotis* with the larger genus *Marsdenia*, resulting in the name change of the commonly known stephanotis plant to *Marsdenia floribunda* (Brongn.). Of the 125 species that comprise the genus *Marsdenia*, *M. floribunda* is the only one of horticultural value. It is an evergreen climber native to Madagascar that is cultivated in tropical and subtropical regions (including Hawaii) for its white fragrant flowers. In Hawaii, *M. floribunda* flowers are either strung as lei lengthwise, requiring about 25 flowers, or they are pierced through the side of the corolla, utilizing about 700 flowers (Staples and Herbst, 2005). It has been suggested that winter production of *M. floribunda* flowers under artificial light (Kofranek and Kubota, 1982; Wikesjo,1982; Kofranek and Criley, 1983) would be an economically feasible commercial venture for local growers, since there is a year-round market for the flowers on the mainland (Criley, 1992).

The production of a cultivar possessing larger flowers with thicker, sturdier petals would be beneficial to the lei industry, since fewer flowers would be required to produce the lei. Criley (1992) reported the shelf life of *M. floribunda* flowers as 10 – 14 days when held in a plastic bag. The development of a cultivar with sturdier petals could possibly extend the shelf life of the flower, allowing for even longer storage periods. Such a cultivar could be produced by doubling the chromosome number of *M. floribunda* (2n = 22); hence, the objective of this experiment was to create a tetraploid *M. floribunda*

by treating seeds with colchicine at various concentrations and durations. The resulting tetraploid should theoretically possess larger flowers (Vainola, 2000; Kamemoto, 1985) and could eventually be introduced into the Hawaii floriculture industry as an improved variety.

Colchicine has been used for chromosome doubling in various plants (Kamemoto *et al.*, 1997; Kamemoto, 1985; Vainola, 2000; Tambong and Garton 1998). The flowers produced by polyploid plants have been shown to be larger and the overall plant growth more compact than their diploid forms. New leaves and stems growing from treated sectors are also usually wrinkled, thicker, darker green, and have a coarser texture when compared to untreated plants (Eigsti and Dustin, 1955). Such characteristics were used in the preliminary screening for identification of tetraploid *M. floribunda*.

Materials and Methods

Treatments

Colchicine (PhytoTechnology Laboratories; Shawnee Mission, KS) was dissolved in distilled water to make 0.05, 0.1, 0.2, and 0.4% colchicine solutions. Seeds were treated in 25ml of each solution for 24 and 36 hours, with 44 seeds per treatment for a total of 10 treatments. Distilled water was used in the 24 and 36 hour controls. Fifty milliliter flasks containing the seed treatments were placed on a rotator at 100rpm for the duration of each treatment, after which the seeds were rinsed with water and sown in moist Pro-Mix 'BX' media (Premier Horticulture Ltd.; Dorval, Canada).

Guard Cell Measurements

After visual inspection of the regenerated seedlings, suspected tetraploids were selected for further screening using stomatal guard cell measurements. All guard cell measurements were taken at the same time of day in order to decrease any variation in guard cell size due to natural daytime effects on stomatal opening. Clear nail polish (Markwins Beauty Products Inc.; City of Industry, California) was thinly applied to the abaxial side of the leaf. After the polish had dried (approximately 60 seconds), a strip of Scotch 3M Transparent Tape with Gloss Finish (3M Products; St. Paul, MN) was mounted on top of the dried polish. The tape, which now held the abaxial imprint, was removed from the leaflet and mounted onto a coverslip for viewing at 400X magnification under a Leitz Wetzlar light microscope. Fifteen guard cells were randomly selected from each leaf sample and measured (length x width) using a unitless graticule.

Chromosome Counts

The method employed for chromosome counting of *M. floribunda* was a modified version of the technique used by Sharma and Mookerjea (1955). Actively growing root tips were excised, rinsed in distilled water, and pre-treated in a saturated solution of paradichlorobenzene for 4 hours at 10-12°C. The saturated solution was prepared by dissolving 10g of the solid in 500ml distilled water and holding the solution at 60°C overnight. The solution was used at room temperature. After pretreatment, the root was transferred to an acid-dye fixation solution (2% aceto-orcein and 1N HCl at a 9:1 ratio) and the mixture heated over a flame for 3-4 seconds. The contents were then poured onto a watch glass and allowed to cool for at least 5 minutes. A drop of 1% aceto-orcein

solution was placed on a dry slide and the root was transferred to the drop of stain. The intensely colored portion of the tip was retained, while the remainder of the root was discarded. The root tip was then squashed using a small scalpel and a cover slip placed on top of the root. The root tip was further squashed by applying even pressure on the cover slip. A Leitz Wetzlar light microscope was used to observe the prepared slides at 1000X magnification and pictures were taken using a Nikon Coolpix 4500 digital camera.

Flow Cytometry

CyStain PI Absolute P DNA Staining Kit for Plant Genome Size (Partec; Munster, Germany) was used for nuclei extraction and DNA staining of nuclear DNA from *M. floribunda* leaves used for flow cytometric measurement. Approximately 0.5cm^2 of each leaf was chopped for 30 – 60 seconds in 500µl ice cold nuclei extraction buffer with a sharp doubled edged razor blade in a 55mm plastic Petri dish. The slurry was then filtered through a 50µm Cell Trics filter (Partec; Munster, Germany) and the suspension of released nuclei was stained in a solution composed of staining buffer, RNAse, and Hoechst 33342 (1:40 dye: staining solution volume), which was substituted for the PI provided by nuclei extraction kit. Leaf samples and all reagents used were kept on ice throughout the entire process from leaflet harvest to sample preparation and all samples were prepared for flow cytometric measurement within 1-2 hours of harvest. Figure 1.3 shows the process from sample preparation to sample analysis.

The relative fluorescence of total DNA of single nuclei was analyzed using a Beckman-Coulter (Miami, Florida) Altra flow cytometer (www.soest.hawaii.edu/sfcf)

using the UV excitation of a Coherent I90C argon ion laser set at 200mW. Control diploid plants were used as external standards, and these standards were intercalated between samples. The linear, log and peak fluorescence signals of the Hoechst-stained nuclei were collected (610 BP filter), along with forward and side scatter signals. Plots of peak vs. linear Hoechst fluorescence were used to eliminate doublets (two nuclei stuck together as they pass the laser/particle sensing point). The resulting data was analyzed using Flow Jo (v. 6.3.4, Treestar Inc., www.flowjo.com). Means and coefficients of variance percentages of the resulting peaks were calculated and histograms of linear DNA fluorescence, which allowed for visual analyses of the data, were produced.

Results and Discussion

Five weeks after treatment with colchicine, the number of seedlings that had emerged from each treatment was noted (Fig. 5.1). Though a statistical analysis of the effect of increasing colchicine concentration and duration on seedling emergence of *M. floribunda* could not be performed (due to the low number of reps - one rep per treatment), Figure 5.2 shows an apparent trend: as colchicine concentration increases, percent seedling emergence decreases, with the fewest number of seedlings emerging in the 36 hour 0.4% colchicine treatment. Approximately 4 months after treatment, the number of seedlings per treatment and seedling height (stem base to apical meristem) was recorded (Table 5.1). Those seedlings that did not display polyploid-like qualities (thicker, rounder, greener leaves) were discarded.

Guard cell measurements were performed on the remaining plants approximately 11 months after seed treatment. Of the 33 plants screened by guard cell measurements, 4

were identified as possible polyploids (Figure 5.3 and Table 5.2). Factors of increase in cell area (as compared to the diploid controls) ranged from 1.5 to 1.95. Further analysis of ploidy status via chromosome counts of root tips was unsuccessful. The nucleus of the root tip cells is so small that individual chromosomes could not be identified.

Consequently, flow cytometric analysis was used to confirm the ploidy status of the 4 suspects and of those plants that were identified as non converted diploids. The two plants that showed guard cell areas of 1.59 and 1.66 were identified as mixoploids, the plant with a guard cell area of 1.95 was identified as a diploid, and the plant with a guard cell area of 1.50 was identified as the only tetraploid (Figures 5.4, 5.5, and 5.6,). Also, the ploidy level of the plants that had been shown to be non converted diploids by guard cell measurements was confirmed. The results obtained show that though guard cell measurements may not allow one to distinguish between mixoploids and full tetraploids, it is a useful tool for the screening of suspected polyploids. It cannot be used, however, to confirm ploidy status; flow cytometry or chromosome counts are the most reliable methods available for ploidy confirmation. If financial constraints are not a concern, however, it is advisable to screen for ploidy changes via flow cytometry, since the samples may be analyzed within a few hours as opposed to several days or weeks when compared to chromosome counts.

The identified tetraploid and mixoploids will continue to be monitored, with closer attention given upon flowering (no plants have flowered to date). Further comparisons between the controls and the tetraploid can be made concerning number of flowers produced, floral vase life, petal thickness, fragrance, and flower size. Verification of the ploidy status of the germinal cell line will also be performed through

chromosome counts of the pollen produced, as researchers have found that after colchicine treatment, the ploidy level of leaves or other tissue may not necessarily be representative of the ploidy level of the germinal cell line (Brown *et al.*, 1991). The introduction of the *M. floribunda* tetraploid into the market as an improved variety may be a significant new plant for lei flower producers.

Tables and Figures

Table 5.1. Number of remaining seedlings and average seedling height 4 months after seed treatment of *M. floribunda* with increasing colchicine concentrations and durations, with 44 seeds per treatment. The results show a decrease in the number germinated seedlings as the colchicine concentration and treatment durations increased. Increasing concentration also affected seedling height, with a decrease in height as colchicine concentration and duration increased. Treatment differences could not be statistically confirmed, however, due to the lack of treatment reps.

Treatment		Number of Seedlings Remaining	Average Height (cm)
Duration (Hours)	Concentration (%)		
24	0	42	10.36
24	0.05	39	10.3
24	0.1	16	8.81
24	0.2	7	6.64
24	0.4	15	5.83
36	0	42	9.44
36	0.05	32	9.59
36	0.1	23	7.11
36	0.2	5	6.3
36	0.4	1	4.5

Table 5.2. Table shows guard cell area measurements of those *M. floribunda* seedlings that were visually identified as possible polyploids after seed treatment with colchicine. Guard cell area values shown were measured with a unitless graticule and are the averages obtained from 15 guard cells measured per leaf, with one leaf sampled per plant. Values in column 5 represent the factor of increase or decrease in area as compared to the control (treated plant guard cell area/control guard cell area). Factor values above 1.2 are classified as polyploids and are highlighted in blue.

Colchicine Concentration	Length of Seed Treatment (days)	Plant Identification	Guard Cell Area (length x width)	Factor of inc./dec. compared to control
0%	1	1, 2, 3 (mean)	85.34	-
0%	3	1,2,3 (mean)	87.4	-
0.05%	1	1	100.20	1.17
0.05%	1	2	86.80	1.02
0.05%	1	3	80.91	0.95
0.05%	3	1	71.04	0.81
0.05%	3	2	97.67	1.12
0.05%	3	3	86.76	0.99
0.05%	3	4	93.87	1.07
0.05%	3	5	90.16	1.03
0.05%	3	6	87.63	1.00
0.05%	3	7	93.30	1.07
0.05%	3	8	96.65	1.11
0.1%	1	1	93.45	1.10
0.1%	1	2	101.67	1.19
0.1%	1	3	141.33	1.66
0.1%	3	1	93.87	1.07
0.1%	3	2	85.51	0.98
0.1%	3	3	98.40	1.13
0.1%	3	4	84.43	0.97
0.1%	3	5	74.11	0.85
0.2%	1	1	81.98	0.96
0.2%	1	2	99.76	1.17
0.2%	1	3	89.28	1.05
0.2%	1	4	72.00	0.84
0.2%	1	5	87.63	1.03
0.2%	3	1	73.47	0.84
0.2%	3	2	84.43	0.97
0.2%	1	3	138.92	1.59
0.4%	1	1	127.60	1.50
0.4%	1	2	94.26	1.10
0.4%	1	3	85.87	1.01
0.4%	1	4	86.92	1.02
0.4%	1	5	83.91	0.98
0.4%	3	1	170.52	1.95

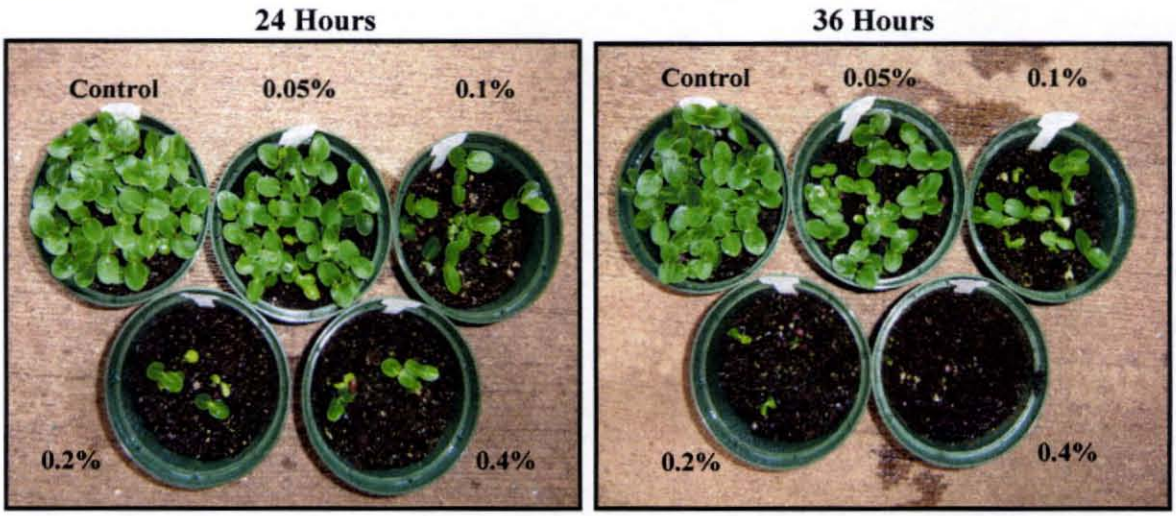

Figure 5.1. Emerging 5 week old seedlings of *M. floribunda* after seed treatment with increasing concentrations and durations of colchicine.

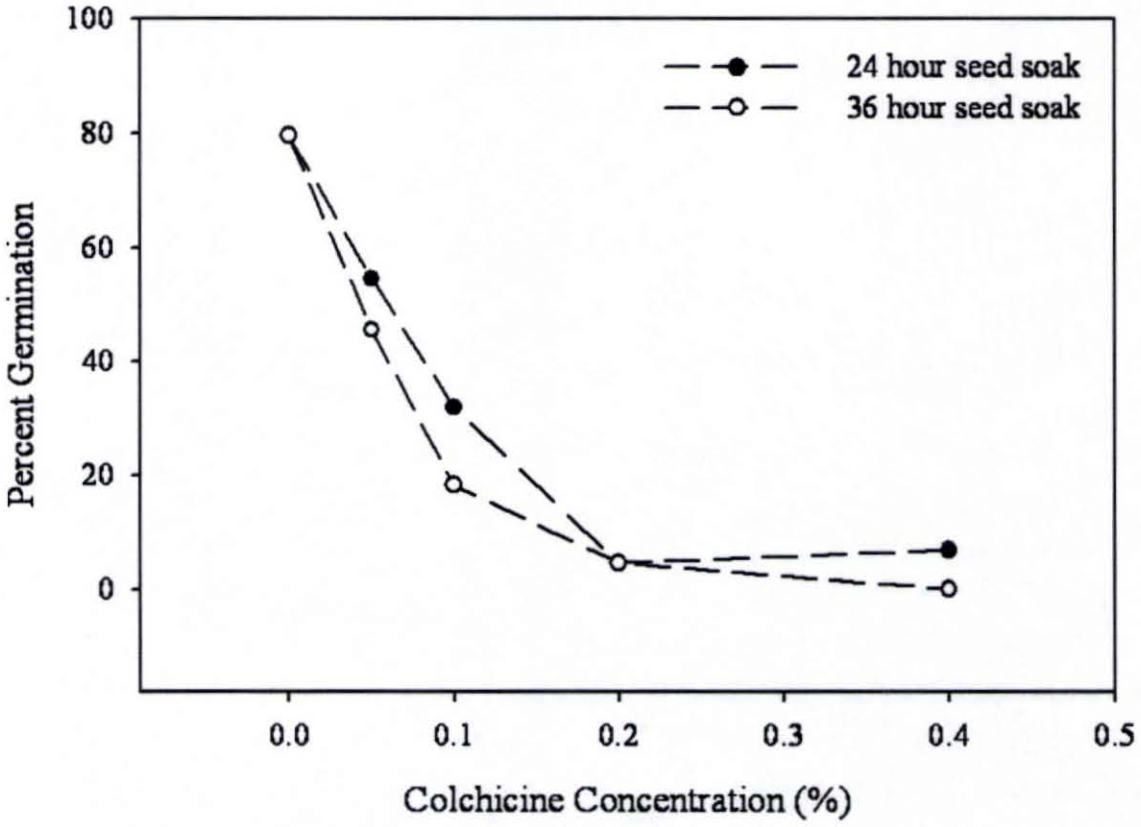

Figure 5.2. Percentage of seedling emergence of *M. floribunda* seeds treated at various concentrations of

colchicine for 24 and 36 hours, with 44 seeds sown per treatment.

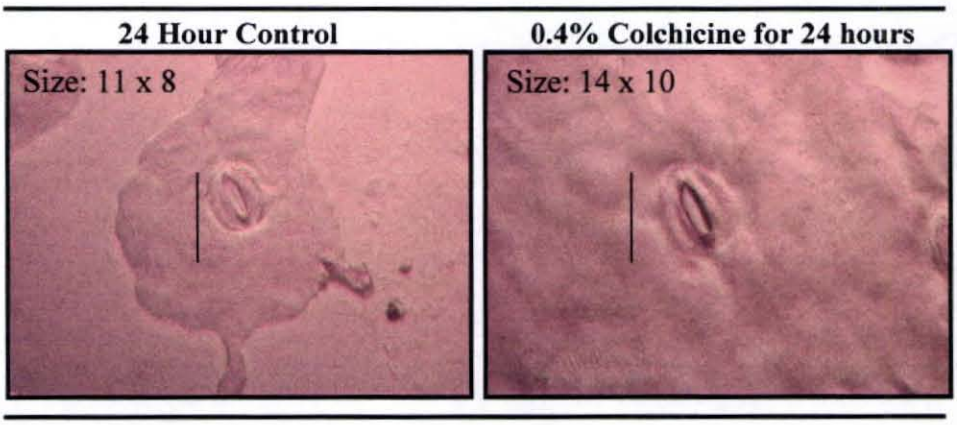

Figure 5.3. Guard cell measurements of *M. floribunda*: 24 hour control measuring 11 x 8 (left) and suspected polyploid measuring 14 x 10 (right). The line visible in both photographs is 1.1cm and was superimposed on the photographs to allow for a visual comparison in size between both guard cells. The remaining three samples identified as possible polyploids by guard cell measurements are not shown.

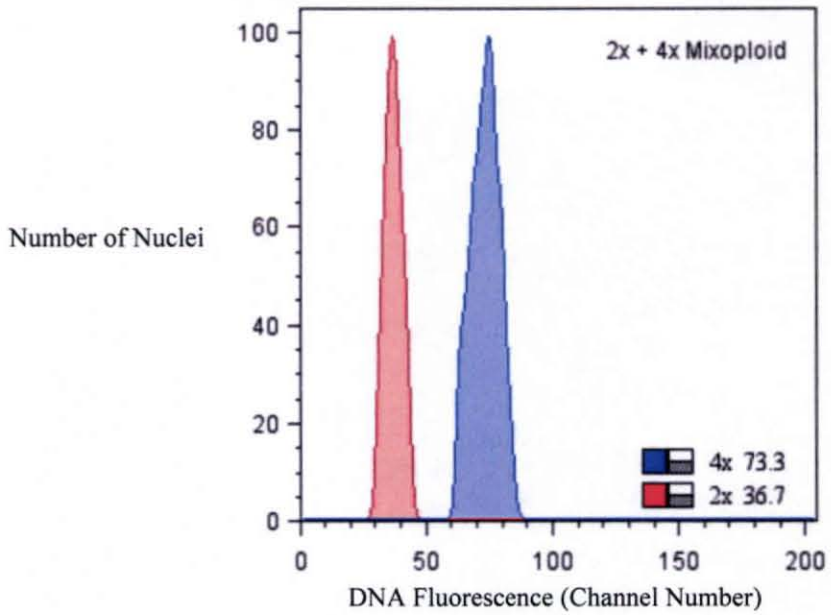

Figure 5.4. Distribution of DNA content of a mixoploid sample showing both 2x (diploid) and 4x (tetraploid) nuclei. The mixoploid was produced by soaking *M. floribunda* seeds in a 0.1% solution of colchicine for 1 day. At least 5000 nuclei were analyzed and both the diploid and tetraploid peaks have a CV<10%. Cell nuclei were isolated from leaf tissue and stained with Hoechst 33342 prior to analysis.

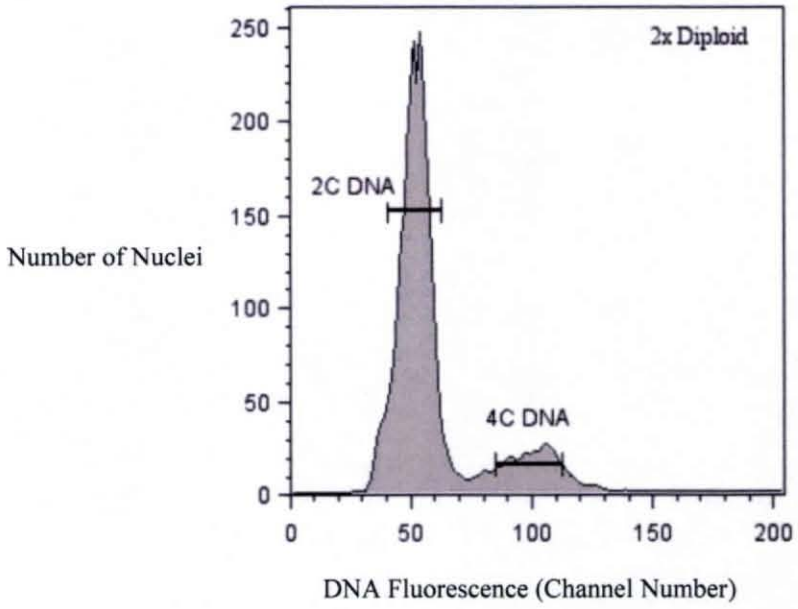

Figure 5.5. Distribution of DNA content of a suspected *M. floribunda* polyploid that showed a guard cell area of 1.95. Flow cytometric analysis showed that the plant that emerged from the colchicine treated seed was in fact a diploid, and had not been polyploidized. At least 5000 nuclei were analyzed and both 2C (G1 mitotic phase) and 4C (G2 mitotic phase) peaks have a CV<10%. Cell nuclei were isolated from leaf tissue and stained with Hoechst 33342 prior to analysis.

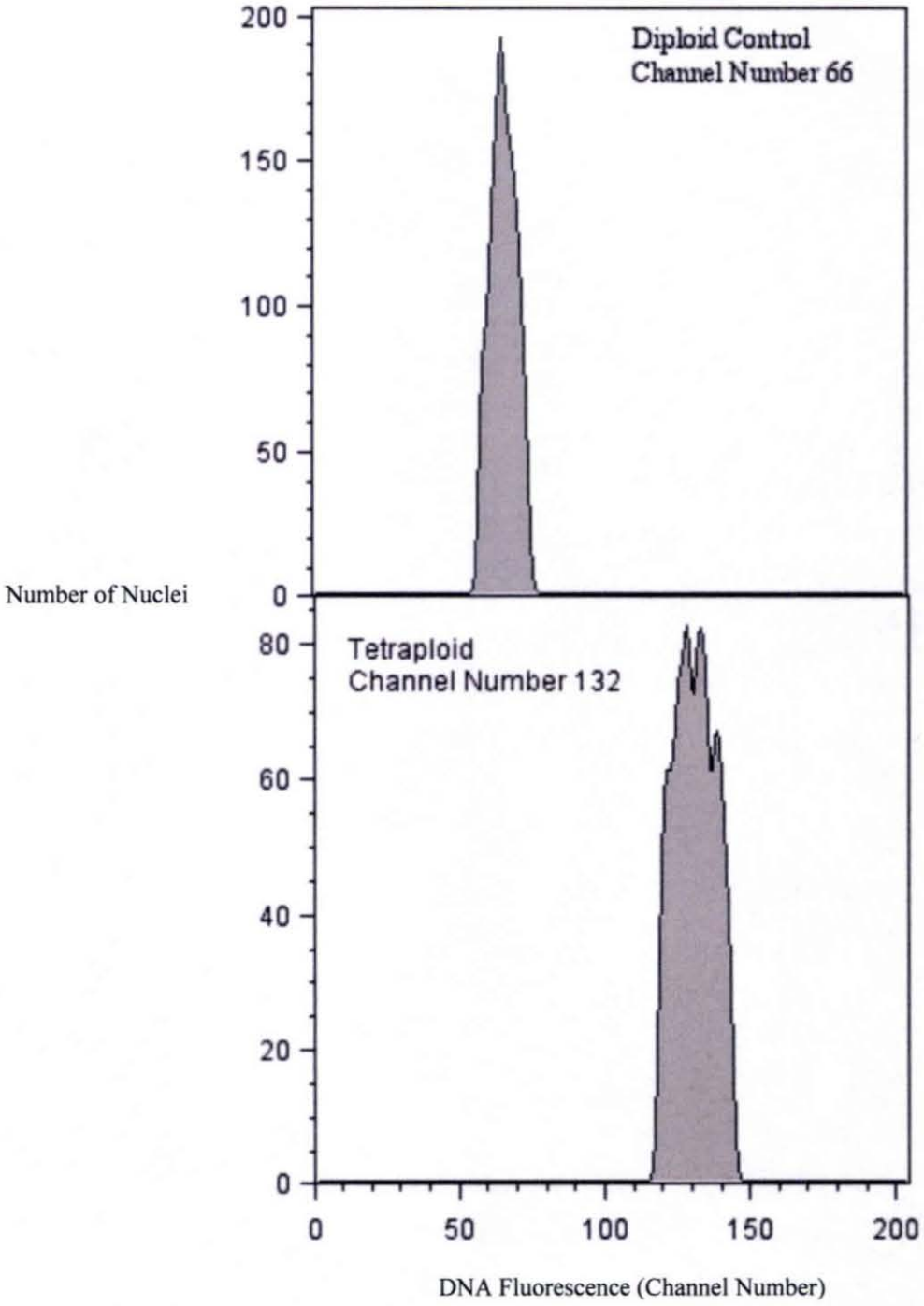

Figure 5.6. Distribution of DNA content of diploid and tetraploid *M. floribunda* samples. The DNA fluorescence of the tetraploid sample is double that of the diploid control. The tetraploid was produced by soaking *M. floribunda* seeds in 0.4% colchicine solution for 1 day. At least 5000 nuclei were analyzed and both the diploid and tetraploid peaks have a CV<10%. Cell nuclei were isolated from leaf tissue and stained with Hoechst 33342 prior to analysis.

APPENDIX

SASS data output for probit analysis of ZZ leaflets irradiated in March 2006. Irradiation dosages ranged from 0 – 100Gy. LD_{50} for ZZ was determined to be 19 Gy. A total of 10 leaflets were irradiated per dosage. Leaflet survival was scored as ZZ leaflet that was capable of regenerating a new plant.

OUTPUT FROM IRRADIATION PROBIT
16:49 Friday, October 20, 2006

Probit Procedure

Iteration History for Parameter Estimates

Iter	Ridge	Loglikelihood	Intercept	Dose
0	0	-50.321437	0	0
1	0	-23.563754	-0.985865807	0.0635619952
2	0	-21.74554	-1.693320317	0.0929560018
3	0	-21.421257	-2.11965983	0.112605381
4	0	-21.392417	-2.301251828	0.1203470989
5	0	-21.391893	-2.330811856	0.1215073582
6	0	-21.391892	-2.33151289	0.1215337199
7	0	-21.391892	-2.33151289	0.1215337199

Model Information

Data Set	WORK.DEAD
Events Variable	dead
Trials Variable	total
Number of Observations	11
Number of Events	93
Number of Trials	110
Name of Distribution	Normal
Log Likelihood	-21.39189244

Number of Observations Read	11
Number of Observations Used	11
Number of Events	93
Number of Trials	110

Parameter Information

Parameter	Effect
Intercept	Intercept

dose dose

Last Evaluation of the Negative of the Gradient

Intercept	Dose (Gy)
4.6665591E-7	8.6813482E-7

Last Evaluation of the Negative of the Hessian

	Intercept	Dose (Gy)
Intercept	5.0790135051	104.82034014
Dose	104.82034014	2776.1596451

Algorithm converged.

Goodness-of-Fit Tests for data collected on ZZ leaflets irradiation in March 2006. There is no significant difference between the observed and expected data; hence the data is normally distributed.

Statistic	Value	DF	Pr > ChiSq
Pearson Chi-Square	11.9843	9	0.2142
L.R. Chi-Square	15.9175	9	0.0686

Response-Covariate Profile

Response Levels	2
Number of Covariate Values	11

Since the chi-square is small ($p > 0.1000$), fiducial limits were calculated using a t value of 1.96.

Type III Analysis of Effects. Irradiation dosage is shown to have a highly significant effect on leaflet death.

Effect	DF	Wald Chi-Square	Pr > ChiSq
dose	1	9.0526	0.0026

Analysis of Parameter Estimates

Parameter	DF	Estimate	Standard Error	95% Confidence Limits		Chi-Square	Pr > ChiSq
Intercept	1	-2.3315	0.9444	-4.1824	-0.4806	6.10	0.0136
dose	1	0.1215	0.0404	0.0424	0.2007	9.05	0.0026
_C_	0	0.5000	0.0000	0.5000	0.5000		

Probit Model in Terms of Tolerance Distribution. LD_{50} is calculated as 19Gy.

MU	SIGMA
19.1840823	8.22816911

Estimated Covariance Matrix for Tolerance Parameters

	MU	SIGMA
MU	13.563394	-1.321450
SIGMA	-1.321450	7.478839

Probit Analysis on Dose. Table shows the calculated lethal dosages and their fiducial limits base on the lethal dosage probabilities . LD_{50} is calculated as 19.184 Gy. LD_{99} is shown to be 38Gy, which is close to the value observed for LD_{100} (40Gy) in Table 3.2 (page 46).

Probability (LD)	Dose (Gy)	95% Fiducial Limits	
0.01	0.042	-40.282	10.003
0.02	2.285	-34.053	11.566
0.03	3.709	-30.125	12.582
0.04	4.779	-27.185	13.362
0.05	5.650	-24.805	14.008
0.06	6.391	-22.789	14.567
0.07	7.041	-21.029	15.065
0.08	7.623	-19.461	15.518
0.09	8.152	-18.040	15.936
0.10	8.639	-16.738	16.327
0.15	10.656	-11.418	18.013
0.20	12.259	-7.291	19.456
0.25	13.634	-3.848	20.791
0.30	14.869	-0.854	22.087
0.35	16.014	1.819	23.391
0.40	17.099	4.245	24.737
0.45	18.150	6.473	26.159
0.50	**19.184**	**8.538**	**27.686**
0.55	20.218	10.465	29.352
0.60	21.269	12.275	31.192
0.65	22.355	13.990	33.250
0.70	23.499	15.636	35.580
0.75	24.734	17.244	38.262
0.80	26.109	18.860	41.424
0.85	27.712	20.556	45.298
0.90	29.729	22.474	50.387
0.91	30.216	22.909	51.645
0.92	30.745	23.371	53.021
0.93	31.327	23.868	54.545
0.94	31.977	24.411	56.261
0.95	32.718	25.015	58.232
0.96	33.589	25.708	60.564
0.97	34.660	26.537	63.455
0.98	36.083	27.608	67.328
0.99	**38.326**	**29.238**	**73.491**

NOTE: The above quantiles and fiducial limits refer to effects due to the independent variable (x-ray dosage) and do not include any effect due to the natural threshold.

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