ABSTRACT

Title of Thesis: *IN VITRO* INDUCTION OF POLYPLOIDY IN *CERCIS YUNNANENSIS* HU ET CHENG

Name of Degree Candidate: Joshua Daniel Nadler

Degree and Year: Masters of Science, 2009

Thesis Directed By: Gary D. Coleman PhD,
Associate Professor,
Department of Plant Science and Landscape Architecture

Methods for *in vitro* induction of polyploid *Cercis yunnanensis* Hu et Cheng using oryzalin were developed and evaluated. Methods included treating either shoot or callus tissue for different exposure durations with either an aqueous solution of 150 μM oryzalin or the addition of oryzalin directly to solid media. Polyploid nuclei were determined by flow cytometry for all oryzalin treatments. Although the results indicate that most tissues measured were likely chimeras with respect to DNA content. Results indicate that treating shoot tissue with an aqueous solution of oryzalin for 12 to 96 hours produced tetraploid plants irrespective of the type of shoot explant treated. An unstable octaploid was formed from the treatment of a pre-cultured lateral shoot in an aqueous solution of oryzalin for 96 hours. In contrast shoots cultured on the solidified media failed to produce polyploid plants and there were no statistical differences between callus treatments regarding polyploid induction.

IN VITRO INDUCTION OF POLYPLOIDY IN *CERCIS YUNNANENSIS* Hu et Cheng

by

Joshua Daniel Nadler

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Masters of Science
2009

Advisory Committee:

Dr. Gary D. Coleman, Advisor/ Chairman
Dr. Margaret Pooler
Dr. Richard Olsen
Dr. Joseph Sullivan

© Copyright by
Joshua Daniel Nadler
2009

Dedication

This thesis is dedicated to my dear friend Earl Cully. Who would have thought that a fieldtrip through a community college could have such a profound impact on an individual? That one day fieldtrip lead to an internship which has lead to so much more. Thank you for your mentoring and patience; you have positively affected my life in so many ways. From writing patents and submitting trademarks, to traveling the globe, your friendship is a major inspiration and a driving force. Thank you for opening the doors to my future. The journey has just begun.

Acknowledgements

Thank you to all… Without friends and family it is difficult for any one person to make progress or achievements. I have had the best team of people standing behind and beside me throughout this journey.

A cordial thanks to:

Dr. Gary D. Coleman – Your assistance throughout the entire process has been invaluable. Thank you for all of your aid and guidance throughout the entire project.

Dr. Margaret Pooler – Your kind words of support were always welcomed. Thank you for the invitation into this project.

Dr. Richard Olsen – Your knowledge of the ornamental tree field is expansive and well articulated. Thank you for all of the discussions on flow cytometry and ornamental trees.

Dr. Joseph Sullivan – Your ecological knowledge is unsurpassed. The courses you taught helped me to better understand and aided me in understanding the ecological attributes sterile plants offer over the invasiveness of many currently planted species.

Table of Contents

Dedication ii
Acknowledgements iii
Table of Contents iv
List of Tables 1
List of Figures 4
List of Abbreviations 6
1.1Nature of Polyploids 8
1.2 Induction of Polyploids 10
1.3 Use of Polyploids 15
2.1 Plant Materials 17
2.2 Preliminary Experiments 17
2.2.1 Root Induction 18
2.2.2 Callus Induction 18
2.3 Polyploid Induction 19
2.3.1 Shoot Treatments 19
2.3.2 Callus Treatments 22
2.4 Flow Cytometry 23
2.5 Statistical Analysis 24
2.5.1 Experiments 24
2.5.2 Polyploid Induction 24
Chapter 3: Results 25
3.1 Experiments 25
3.1.1 Root Induction 25
3.1.2 Callus Induction 25
3.2 Ploidy Induction 29
3.2.1 Aqueous Shoot Treatment 29
3.2.2 Solidified Shoot Media Treatments 41
3.3 Callus Treatments 46
3.3.1 Callus Aqueous Treatment 46
3.3.2 Callus Solidified Media Treatment 49
3.3.3 Callus Treatment Overview 49
4.1 Experiments 56
4.1.1 Rooting Induction 56
4.1.2 Callus Induction 56
4.2 Ploidy Manipulation Treatments 57
4.2.1 Shoot Treatment 57
4.2.2 Callus Media Treatment and Callus Aqueous Treatment 59
4.3 Conclusion 60

List of Tables

Table 1 - Auxin and cytokinin concentrations used in the callus induction experiment……...………………………………………………………………18

Table 2 - Polyploid induction factorial design………..…………………………….20

Table 3 - Percentage (%) of explants that produced roots after six weeks when cultured on two different media (0.5x LS with no supplements or 1x LS media supplemented with 1 μM IBA), either in the dark or in continuous light……………26

Table 4 - Chi-square (α=0.05) analysis of explant rooting on four independent comparisons of 0.5x LS media with no supplements and 1x LS media with 1 μM IBA cultured in light or dark conditions….………………..27

Table 5 - Mean callus weight of internodal sections of 129EC after 12 weeks of treatment……………………………………………….……………………….28

Table 6 - ANOVA of the effect of aqueous oryzalin treatment on shoot leaf growth and development………………………………………………………………..31

Table 7 - Least significant difference (LSD) of mean number of leaves per tissue culture vessel after treatment with 150 μM oryzalin.………………………………..32

Table 8 - ANOVA of the effect of aqueous shoot treatment with 150 μM oryzalin on root development of explants eight weeks after treatment………………………….34

Table 9 - ANOVA analysis of the diploid, tetraploid and octaploid peaks…..……..38

Table 10 - Ploidy distribution of individual plants treated with oryzalin 26 weeks after the initial treatment…...…………………………………………………………...42

Table 11 - ANOVA analysis of the effect of solidified shoot media treatment on the nuclei DNA content of pooled explants …………………………………….…….44

Table 12 - ANOVA analysis of callus viability after treatment with an aqueous solution of 150 μM oryzalin ……………………………………………………...50

Table 13 - ANOVA analysis of the diploid, tetraploid and octaploid peaks of aqueous callus treatments…………………………………………………………………...51

Table 14 - Effect of oryzalin treatment and duration of treatment on callus viability……………………………………………………………………………52

Table 15 - ANOVA analysis of the diploid, tetraploid and octaploid ploidy levels after treatment on solidified callus media………………………………………….53

Table 16 - ANOVA analysis of the diploid, tetraploid and octaploid callus nuclei DNA content after aqueous treatment or solidified media treatment………..……....54

List of Figures

Figure 1 - Mean number of leaves per tissue culture vessel five weeks after treatment with 150 μM oryzalin…………………………………………………………..30

Figure 2 - Examples of flow cytometry histograms for nuclei from pooled-leaf samples of aqueous shoot treatments measured 16 weeks after treatment………..….35

Figure 3 - Ploidy ratio of pooled-leaf nuclei DNA content from shoots ……………37

Figure 4 - Polyploid ratio of pooled-nuclei DNA content isolated from explants after aqueous shoot treatment measured 16 weeks after treatment began………………...39

Figure 5 - Percentage (%) of each ploidy type in the pooled nuclei samples isolated from explants after the aqueous shoot treatment 16 weeks after treatment…….……40

Figure 6 - Tetraploid to diploid ratio of pooled-nuclei DNA isolated from explants after treatment in solidified shoot media …….………...……………………………43

Figure 7 - Ploidy ratio of pooled-nuclei DNA isolated from explants after treatment in solidified shoot media ………...…………………………..…………………………45

Figure 8 - Tetraploid to diploid ratio of pooled-nuclei DNA content isolated from leaves of explants treated with either aqueous shoot treatment or solidified shoot media…...……………………………………………………………………………47

Figure 9 - Polyploid to diploid ratio of pooled-nuclei DNA content isolated from leaf of explants treated with either aqueous shoot treatment or solidified shoot media.....48

List of Abbreviations

129EC - United States National Arboretum selection of *Cercis yunnanensis* Hu et Cheng

2,4-D - 2,4-Dichlorophenoxyacetic acid

2iP - isopentenyl adenine

a.i. - active ingredient

ANOVA - analysis of variance

BA - 6-benzladenine

CIM - callus inducing media

DAPI - 4',6-diamidino-2-phenylindole

DF - degree(s) of freedom

DNA - deoxyribonucleic acid

EPA - Environmental Protection Agency

GLM - General Linear Model

GMO - genetically modified organism

IBA - indole-3-butyric acid

LD-50 - lethal dose that is needed to kill 50% of test subjects

LS - Linsmaier and Skoog media with macro and micronutrients

ns - not significant

MS - mean squared

NAA - 1-naphthaleneacetic acid

PAR - photosynthiclly active radiation

® - registered trademark

SAS® - Statistical Analysis Software, Cary, NC

TO - tubulin - oryzalin complex

RPM - revolutions per minute

w/v - weight per volume

Chapter 1: Literature Review

1.1Nature of Polyploids

Polyploid organisms contain more than two complete sets of chromosomes in the nucleus and occur frequently in three of the four eukaryotic kingdoms (Storchova and Pellman, 2004). In the five kingdom classification system, polyploids have naturally risen in three kingdoms: plantae, protista and animalia (Baatout, 1999). In the plant kingdom polyploids are common in angiosperms and ferns, (Schuettpelz et al., 2008) but are rare in gymnosperms (Briggs and Walters, 1997). Furthermore, genome duplication or polyploidy is believed to have occurred during the evolution of 30 – 70 percent of angiosperms (Masterson, 1994). Although polyploids occur in animals they are rare among mammals but more common in other classes. Algae and bryophytes are not clearly defined in the five kingdoms system; some of these species are polyploids (Briggs and Walters, 1997).

Ploidy is usually represented by the notation n*x,* where a nucleus with two complete sets of chromosomes is referred to as a diploid (2*x*), three complete sets of chromosomes is a triploid (3*x*), four complete sets a tetraploid (4*x*), five complete sets a pentaploid (5*x*) and so on. The highest level of ploidy currently known in angiosperms is an 80-ploid (80*x*) stonecrop (*Sedum suaveolens* Kimnach) (Otto and Whitton, 2000). The wide range of chromosome number observed in plants suggests a role in plant evolution (Leitch and Bennett, 1997). A ploidy series can exist within a species (ex. *Fraxinus americana* Linnaeus) ploidy changes can exhibit morphological, genetic or geographical differences but they do not warrant taxonomic reclassification (Wright, 1944).

Polyploids can have multiple origins that are related to the nature of chromosomal duplication. For example, an autopolyploid is formed through the replication of chromosomes within a species (genome denoted e.g., AA → AAAA). In contrast to an autopolyploid, an allopolyploid arises from the duplication of chromosomes contributed from different species (genome denoted e.g., AABB). Autoallopolyploids contain duplicated chromosomes from one species and at least one set from another species (genome denoted e.g., AAAABB) (Stebbins, 1971); (Grant, 1981).

Polyploids frequently occur within plants used in the fruit industry. Polyploid fruit crops include sour cherries (*Prunus cerasus* L.) (Tavaud et al., 2004), strawberries (*Fragaria* spp. L.) (Ahokas, 1999), kiwi (*Actinidia deliciosa* Liang and Ferguson) (Udall and Wendel, 2006), blueberries (*Vaccinium* spp. L.) (Decroocq, 2004), European plums (*Prunus domestica* L.) (Decroocq, 2004), persimmons (*Diospyros kaki* Thunb.) (Tao et al., 2009) and watermelons (*Citrullus lanatus* Thunberg) (Love et al., 1986). Agronomic crops such as some selections of wheat (*Triticum aestivum* L.) (Udall and Wendel, 2006), potato (*Solanum tuberosum* L.) (McGregor et al., 2000), cotton (*Gossypium* spp. L.) (Udall, 2006), alfalfa (*Medicago sativa* L.) (Udall and Wendel, 2006) and sugar cane (*Saccharum* spp. L.) (Cordeiro et al., 2000) may also be polyploids. Polyploids often have improved horticultural or agronomic traits such as larger fruit, thicker leaves and robust stems (Kehr, 1996), so plant breeders sometimes favor polyploids in breeding programs. Polyploid plants are also found in the ornamental horticulture industry and they may have thicker flower petals that last longer than their diploid counter-parts (Kehr, 1996). Common

polyploid bedding plants include dahlia (*Dahlia* spp. Cav.), pansies (*Viola* spp. L.) and chrysanthemum (*Chrysanthemum* spp. L.).

Naturally occurring polyploids are less common in mammals. The red Viscacha rat (*Tympanoctomys barrerae* Lawrence) is an aneuploid that represents the first known naturally occurring mammal with tetraploid chromosomes for all but the sex-chromosome (Gallardo et al., 1999). This species exhibits a single XY sex-chromosome system [XX (♀) or XY (♂)] which allows diploid like behavior of the sex chromosome while the remaining chromosomes are tetraploid (Gallardo et al., 1999). Other animals such as goldfish, trout, salmon (Seddon, 1997), many orders of insects (Suomalainen, 1962), many amphibians (Bogart and Tandy, 1976), and some reptiles have been reported to be polyploids (Corey, 2000). Some polyploid animals can reproduce because the species completely lack sex chromosomes or certain categories of animals have sex chromosomes that are not well differentiated (McCarthy, 2008).

1.2 Induction of Polyploids

Polyploids can arise spontaneously within plants during somatic cell division (mitosis) which can result in an autopolyploid shoot often noticeable by its enlarged "gigas" condition (Stebbins, 1971). Autopolyploids can also arise during the meiotic process through the union of unreduced gametes. Allopolyploids are much more common than autopolyploids and are the function of the hybridization of separate species that contain separate sets of non homologous chromosomes (Stebbins, 1971; Soltis and Soltis, 2000). Polyploids can also be experimentally induced in both animals and plants. For example, the fish, grass carp (*Ctenopharyngodon idella*

Valenciennes), naturally occurs as a diploid but the ploidy level can be increased to triploid through hydrostatic pressure treatment after egg fertilization (Cassani and Caton, 1986). Other methods of inducing polyploids include treatments with mitotic inhibiting chemicals such as colchicine (Derman, 1940), oryzalin (Alberts et al., 1994), trifluralin (Eeckhaut et al., 2004), amiprophos- methyl (Hansen et al., 2000) and N_2O (Kitamura et al., 2009).

Colchicine is an alkaloid extracted from seeds or corms of *Colchicum autumnale* L. (autumn crocus or meadow saffron) and was first isolated in 1820 by the French chemists P.S. Pelletier and J. Caventon (Pelletier and Caventon, 1820). Colchicine blocks inflammation caused by uric acid crystals and is used to treat acute gouty arthritis in humans (Eustice and Eustice, 2007). Medicinal colchicine is used as a 0.5 mg and 0.6 mg oral tablets, or as an intravenous injection, but the treatments have a high risk of serious toxicity (Eustice and Eustice, 2007). Fatalities have been reported after ingestion of 7 mg to 12 mg of colchicine in adult humans (Stapczynski et al., 1981). The lethal dose of 50% (LD 50) for oral ingestion in rats was reported at 6 mg/kg (Extension Toxicology Network, 1993). The Environmental Protection Agency (EPA) mandates a “toxic” label on all containers containing colchicine because of this toxicity. Ploidy levels have been manipulated in animals and plants (Derman, 1940) by submerging the specimen in a solution of colchicine. The most effective range of treatment ranges from micro to millimolar concentration of colchicine with the optimal concentration for a species needing to be determined empirically.

Oryzalin [3,5-dinitro-N4, N4-dipropylsulfanilamide] is the active ingredient (a.i.) of the pre-emergence herbicide Surflan® (Sourthern, 1998) and is also used to induce polyploids in plants. Pre-emergent herbicides are applied to the surface of the soil, inhibiting the germination and growth of weed seeds. The main labeled use of oryzalin is to control annual grasses and broadleaf weeds in a wide array of growing situations. Oryzalin is available as a dry flow-able, an aqueous suspension, or a wet-able powder (Extension Toxicology Network, 1993). The established oral LD 50 in rats is >5000 mg/kg (Extension Toxicology Network, 1993) making it significantly less toxic than colchicine. The EPA classifies oryzalin in a toxicity class IV: slightly to practically nontoxic, and products containing the compound must display a "caution" label.

Oryzalin binds to plant tubulin heterodimers only during metaphase through a pH-dependent interaction forming a rapid and reversible tubulin – oryzalin (TO) complex (Hugdahl and Morejohn, 1993). Further research concluded that the oryzalin binding site is under the N loop of the α-tubulin and consistently docks to Arg2, Glu3, Val4, Trp21, Phe24, His28, Ile42, Asp47, Arg64, Cys65, Thr239, Arg243 and Phe244 sites (Morrissette et al., 2004). It is suggested that the oryzalin or the TO complex binds to the microtublin positive (+) end leading to the disruption of the polymerization of microtublin (Hugdahl and Morejohn, 1993). The spindle fibers, which are composed of microtubules, function to pull the sister chromatids to opposite poles of the cell and without their action the mitotic process is disrupted (Alberts et al., 1994). This disruption can result in DNA replication without cell division when oryzalin is used at low concentrations (Bartels and Hilton, 1973). The

cytoskeleton of plant cells is also composed of microtubules which when treated with oryzalin can lead to abnormalities of cytoskeleton function such as maintaining cell shape, maintaining cell protection and cell motion. Although a LD 50 has been established in animals, oryzalin does not disrupt animal microtubules due to the absence of an oryzalin – binding site on mammalian tubulin (Hugdahl and Morejohn, 1993), therefore, chromosome numbers in mammals are not altered (Bartels and Hilton, 1973).

The ploidy level of several plant species have been altered using oryzalin. This includes *Miscanthus sinensis* Anderson (Petersen et al., 2002), *Pyrus* L. (Bouvie, 2002), *Solanum* L. (Chauvin et al., 2003), *Rosa* L. (Kermani et al., 2003), *Lilium* L. (Van Tuyl et al., 1992) and *Tulipa* L. (Chauvin et al., 2005). Research with *M. sinensis* determined that treating shoot apices in 15 μM oryzalin solution for a period of 96 hours was the most effective treatment for inducing polyploids (Petersen et al., 2002). Petersen et al. (2002) also found that 60 μM oryzalin prevented callus initiation of immature inflorescences of *M. sinensis* that were cultured *in vitro*. Bouvie et al. (2002) found that 200 μM – 300 μM concentrations of oryzalin were required to induce polyploidy in *Pyrus* L. In *Solanum* L. the most effective treatment for producing tetraploids was a 24 hour treatment with 28.8 μM oryzalin solution applied to apical buds (Chauvin et al., 2003). From prior research it is evident that the optimal oryzalin concentration and treatment duration for polyploid induction varies among species and must be determined empirically.

Trifluralin [2, 6-dinitro-N, N-din-propyl-4-trifluomethyl aniline] has also been successfully used to manipulate ploidy levels. Approximately 5% of the anther

filaments of *Spathiphyllum wallisii* Regal 'Speedy' treated with 10 μM trifluralin or oryzalin became polyploids (Eeckhaut et al., 2004). The Eechhaut et al. study suggested that both of these chemicals, (both structurally similar dinitroanilines) could effectively replace colchicine as an anti-mitotic agent, thus removing the risk of colchicine exposure in laboratory procedures (Eeckhaut et al., 2004). Amiprophos-methyl [APM; O-methyl-O-(4-methyl-6-nitrophenyl)-N-isopropyl phosphorothioam-idate] has also been used to induce chromosome doubling (Hansen et al., 2000). Diploid sugar beets (*Beta ulgaris* L.) treated *in vitro* with amiprophos-methyl yielded 10% - 25% tetraploids (Hansen et al., 2000). Exposure to N_2O gas has also been reported to alter the ploidy level although the mode of action, in plants, is not known (Kitamura et al., 2009). N_2O has been used on *Zea mays* L. (Kato and Birchler, 2006) and *Triticum dicoccum* Khapli (Kihair and Tsunewak, 1960).

For all the reports described above, flow cytometry was used to quantify nuclei DNA content. A flow cytometer can evaluate a large population of single cells or nuclei by quantifying the amount of nuclear DNA that is present. This is done by aligning cells or nuclei, via hydrodynamic forcing, and passing them by a single wavelength light source (Greve et al., 2004). When a DNA fluorescent stain such as DAPI is included the light excites the bound DAPI and emits fluorescence in proportional to the DNA/ DAPI binding ratio. This emission is in the form of an electrical signal that is translated into a numerical data set that is compiled in real time (Rahman, 2006).

1.3 Use of Polyploids

Polyploid plants may have a wide variety of uses including overcoming hybridization barriers (due to differences in ploidy), improved stress tolerance, improved pest resistance, and restoring fertility in wide hybrids (Levin, 1983). Polyploids can have larger flowers and large thick leaves with shorter internodes (Pryor and Frazier, 1968). In addition triploids can be advantageous because they typically fail to reproduce sexually due to unbalanced chromosome numbers.

Many nursery crops are not native to the areas where they are planted, and they have the potential to escape the managed landscape and become invasive. For example, *Pyrus calleryana* Decaisne 'Bradford' (Bradford Pear) (Dirr, 1998) and *Berberis thunbergii* DC var. *atropurpurea* (Japanese barberry) (Lehrer and Lubell, 2008) are two examples of nursery crops that are commonly planted in the ornamental landscape that are now considered invasive. One possible approach to reduce the invasive nature of some non-native crops is to develop triploid selections with reduced fertility. Reproductive sterility can also be utilized in genetically modified organisms (GMO) as a mechanism to eliminate or reduce gene introgression into native species.

Ploidy manipulation that results in seedless fruit can increase the quality and quantity of the fruits. Triploid watermelons (*Citrullus* spp. Forsskal) are commonly grown to produce seedless watermelon. Additional seedless horticulturally important crops include grapes (*Vitis* spp. L.), apples (*Malus* spp. Tournefort) plantains and bananas (*Musa* spp. L.) (Lehrer and Lubell, 2008). Tetraploid apples may produce fruit almost double the size of diploid fruit. However, a disadvantage of increasing

the fruit size is that the larger fruit maybe watery and misshapen. Triploid fruit crops, produced through breeding, can have traits found in tetraploids and diploid parents while reducing the number of seeds within the fruit (Ranney, 2007). There are a few varieties of apples on the market that are triploids including Crispin, Holstein and Jupiter that retain the flavor of the diploid parent yet have the increased size found in tetraploid fruit.

The goal of this research was to manipulate the ploidy of the diploid *Cercis yunnanensis* Hu et Cheng (Yunnan redbud native to Yunnan, China) using *in vitro* treatments with oryzalin to create tetraploid plants. This species was chosen due to its easy of propagation *in vitro* along with its exotic (non-native to the United States) origin. If this species is planted into a landscape there is potential for hybridization to the native *Cercis canadensis* L. or even potential for the plant to become invasive unless precautions are taken. These tetraploid plants (established through this project) can then be used to hybridize with a diploid plant (original plant material) to produce triploid offspring. This research laid the foundation to develop a protocol for *in vitro* polyploidy induction that could be applied other ornamental crops. The next stage of this research will be to produce a sterile triploid *C. yunnanensis.*

Chapter 2: Materials and Methods

2.1 Plant Materials

Cercis yunnanensis Hu et Cheng (United States National Arboretum clone 129EC) shoot cultures were *in vitro* propagated on a shoot proliferation media of full strength (1x) Linsmaier and Skoog (LS) media (Linsmaier and Skoog, 1965) supplemented with 1.8 μM 6-benzyl adenine (BA) and 0.5 μM indole-3-butyric acid (IBA). Media was solidified using 0.15% (w/v) Gelrite™ (Caisson Laboratories Inc., North Logan, UT), and 0.35% (w/v) agar (Caisson Laboratories Inc., North Logan, UT). Shoot cultures were grown in Magenta® GA7 vessels. Nine 129EC plantlets with ~ 4 or 5 internodes were cultured per vessel. Shoot cultures were grown in a controlled environment growth chamber where they received continuous light from cool-white fluorescent light (Ecolux® Technology Plant and Aquarium F40T12 Bulbs) with a PAR of 200 to 300 μmol m^{-2} s^{-1} at 23 ± 1° C. Shoot cultures were transferred to new media at 4 to 5 week intervals.

2.2 Preliminary Experiments

These studies on the induction of root and callus were done to supplement the main studies on polyploidy induction. The root induction study was done to give guidance on which media and lighting situations developed the highest percentage of roots from 129EC. The callus induction study was done to give guidance to which hormone combination induced the most callus from 129EC.

2.2.1 Root Induction

Nodal sections consisting of 4 to 5 nodes of clone 129EC were rooted on two types of media: half strength (0.5x) LS media with no supplements or full strength (1x) LS media supplemented with 1 μM of IBA. A total of four nodal sections were placed in each container. Half the culture vessels were placed in the dark while the remaining were cultured in continuous light. After seven weeks the number of rooted plantlets was recorded.

2.2.2 Callus Induction

Internode sections, 2 to 2.5 cm in length, of clone 129EC were cultured on full strength (1x) LS media, supplemented with various concentrations of 1-naphthaleneacetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-benzladenine (BA) as described in Table 1. 12 internodal sections were cultured

		NAA	NAA	2,4-D	2,4-D
		2.5 μM	5 μM	2.5 μM	5 μM
BA	0.44 μM	X	X	X	X
BA	1.1 μM	X	X	X	X

Table 1. Auxin and cytokinin concentrations used in the callus induction experiment. Combinations of 1-naphthaleneacetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-benzladenine (BA) were used in the callus induction experiment. Eight combinations in total were used.

on the specified media in 100 x 15 mm sterile polystyrene Petri dishes. Each treatment included seven replicate plates. Cultured stem sections were placed in a dark box for 11 weeks. To determine callus induction and growth, each Petri dish was initially weighed aseptically and the callus tissue was then removed and placed in a new Petri dish containing the same media. The old Petri dishes were reweighed

again and the weight of the 12 stem/ callus explant was determined by subtracting the initial weight from the weight of the Petri dish after callus transfer.

2.3 Polyploid Induction

Four different treatment methods used oryzalin at a constant concentration of 150 μM. Two of the four treatments consisted of either nodal sections with lateral buds or nodal sections with the shoot tip that were either treated with aqueous solutions of oryzalin or the oryzalin was incorporated into the culture media. The remaining two treatments consisted of either aqueous or media treatments of oryzalin on callus induced from internodal explants. Table 2 outlines the polyploid induction factorial design.

2.3.1 Shoot Treatments

2.3.1.1 Aqueous Treatments

The aqueous shoot treatment experiments used *in vitro* shoot cultures to evaluate the effect of placing shoot explants into an oryzalin solution. A stock solution (75 *m*M) of oryzalin (Sigma-Aldrich®: St. Louis, MO) was made by dissolving oryzalin in 100% ethanol. Treatments were performed on *in vitro* (a) shoot apices, (b) nodal sections with lateral buds or (c) nodal sections with lateral buds that were first cultured for one week (herein referenced as pre-cultured laterals) on shoot proliferating media. Leaves were removed prior to treatment to ensure full submersion in oryzalin solution.

Treatments were conducted in sterile 125 mL flasks containing 50 mL of sterilized full strength (1x) liquid LS media with 150 μM oryzalin for 0 (dipped into

Shoot Treatments (Section 2.3.1)
Aqueous (Section 2.3.1.1)
Duration (Hours): 0 (Dip), 6, 12, 24, 48 and 96
Explant: Apical, Lateral and Pre-Cultured Lateral
Treatment: Control and Treatment
Total Number of Treated Explant: 216
Media (Section 2.3.1.2)
Duration (Day): 1, 2, 4, 8 and 16
Explant: Apical, Lateral and Pre-Cultured Lateral
Treatment: Control and Treatment
Total Number of Treated Explant: 540
Callus Treatments (Section 2.3.2)
Aqueous (Section 2.3.2.1)
Duration (Day): 0 (Fresh Cut), 5, 10, 15, 20, 25, 30, 35 and 40
Treatment: Control and Treatment
Total Number of Treated Calli: 324
Media (Section 2.3.2.2)
Duration (Day): 5, 10, 15, 20, 25, 30, 35 and 40
Treatment: Control and Treatment
Total Number of Treated Calli: 288

Table 2. Polyploid induction factorial design.

solution), 6, 12, 24, 48 or 96 hours. Flasks containing the explants were agitated at room temperature on an orbital shaker at 100 RPM for the given treatment duration. Control treatments were conducted by using the same procedure with ethanol and lacking oryzalin (ethanol was used to dilute oryzalin therefore it must be used with control treatments). After treatment for the specified time, explants were blotted dry using sterile paper towels to remove excess treatment solution and then transferred to solidified shoot proliferation media. 12 explants were used for each oryzalin treatment for a total of 216 explants. After ten weeks of culture, new growth was removed from the treated tissue and shoot explants were transferred to fresh media. The existing callus, leaves, and terminal apices were removed from the shoot explants when transferred giving a 4 to 5 internodal explant from basal tissue which was then transferred to shoot proliferation media. These plants were then grown for two weeks after which time leaves from the six plants within each culture vessel were collected and pooled-nucleic DNA content determination using flow cytometry. After 14 weeks of culture, the callus and apical portion of the explant was removed and the plants were transferred to a root inducing medium containing half strength (0.5x) LS.

2.3.1.2. Solidified Media Treatment

To determine if polyploidy could be induced in shoot explants cultured on solidified media containing oryzalin, nodal explants with shoot apices, nodal sections with lateral buds and nodal sections with lateral buds containing 4 to 5 internodes that were first cultured for one week on shoot proliferating media were cultured on full strength (1x) LS media supplemented with 150 μM oryzalin. Explants were cultured on the solidified media for 1, 2, 4, 8 or 16 days. 540 explants were treated, with 36

explants per treatment. 270 explants were used as controls; 18 explants per treatment. After treatment, the explants were transferred to fresh shoot proliferation media for ten weeks. Any callus, leaves, and terminal apices were removed to make nodal explants containing 4 to 5 buds at the base of the plantlets (original tissue that was treated) which was then transferred to the shoot proliferation media. These plants were grown for two weeks to allow new leaf production. New leaves from each culture vessel were collected and pooled-nuclei DNA content determination using flow cytometry**.**

2.3.2 Callus Treatments

2.3.2.1 Aqueous Treatment

Internodal sections of clone 129EC were cultured on full strength (1x) LS callus induction media supplemented with 5 μM 2,4-D and 0.44 μM BA for 5, 10, 15, 20, 25, 30, 35 and 40 days. Callus generated after the respective culture times were placed in a sterilized 125 mL flask with 50 mL of full strength (1 x) LS media that contained 150 μM oryzalin (treatment) or equal parts 100% ethanol (control). The flasks were sealed with Identi-Plugs® (Jaece Industries Inc.) and agitated on an orbital shaker for 12 hours at 100 RPM. Following this, the solution was filtered from the callus tissue using a sterile mesh and washed with 10 mL of aqueous full strength (1x) LS. The internodal sections were then placed in sterile 100 x 15 mm polystyrene Petri dishes containing callus induction media. Analysis of nuclear DNA content by flow cytometry was conducted seven weeks after oryzalin treatment began.

2.3.2.2 Solidified Media

Results from the callus induction study indicated that the media that produced the greatest amount of callus was full strength (1 x) LS supplemented with 5 μM 2,4-D and 0.44 μM BA. To this media 150 μM oryzalin (treatment) or equal volume of 100% ethanol (control) were filter sterilized (Fisherbrand® 25mm syringe filter, 22 μM, MCE, sterile) then added to the media. The media was cooled, and poured into sterile 100 x 15 mm polystyrene Petri dishes. 12 internodal sections of clone 129EC were placed on the respective media with three replicate plates per treatment. Internodal sections were cultured on media with oryzalin for either for 5, 10, 15, 20, 25, 30, 35 or 40 days. At the end of given culture time, callus tissue was transferred to callus induction media without oryzalin.

2.4 Flow Cytometry

A Partec PAII flow cytometer, with 100W UV HBO lamp was used to determine nuclear DNA content according to the manufacturer's instructions. For the shoot treatments, the most basal leaves were collected and pooled from each treatment. From this pool 0.5 cm^2 of leaf tissue was placed in a 100 x 15 mm polystyrene Petri dish, 400 μL of nuclei extraction buffer (Partec) was added and a sharp razor blade was used to chop the leaf tissue in the buffer. After one minute incubation, 1.6 mL of staining buffer (Partec) was then added to the plate. The solution was filtered through a Partec 30 μm (Green) CellTrics® disposable filter and collected in a Rohren-Tube (Sarstedt 3.5 mm x 55 mm x 12 mm). Approximately 3 hours later the solution was cycled through a Partec PAII with the gains set at 505 (FL4). Using the analysis function, peaks were assigned to the output data. If peaks

were not assigned by the program then they were determined manually. Peaks assigned represent the quantity of nuclei present in each diploid, tetraploid and octaploid peak. This information was used for statistical analysis.

2.5 Statistical Analysis

2.5.1 Experiments

2.5.1.1 Root Induction

Chi-squared (Proc FREQ, SAS 9.2, Cary, NC) was used to calculate results of the root induction experiment. Continuity adjusted chi-square was used for analysis since only 1 degree of freedom was present for each of the given tests.

2.5.1.2 Callus Induction

Fisher's least significant difference (LSD) (α=0.05) test was performed to test determine that significant differences existed between the mean weights of callus for each treatment group.

2.5.2 Polyploid Induction

The samples were run through the flow cytometer and the visible diploid, tetraploid or octaploid peaks were separated and quantified. The peak means of the sample groups were then tested with ANOVA (α=0.05) to determine differences. All of the statistical analysis was done by importing the data sets into SAS 9.2. Analysis of variance (ANOVA) the Proc MIXED test was used if the variances were not balanced (ex. uneven replicates), while the Proc GLM was used if the variances were balanced. Fisher's least significant difference (LSD) (α=0.05) was performed on shoot treatments for mean separation when the ANOVA showed that the effects of the raw data were significantly different.

Chapter 3: Results

3.1 Experiments

3.1.1 Root Induction

The rooting experiment determined the effect of media composition and light on the presence or absence of root initiation and growth. Table 3 shows the percentage of explants that produced roots after seven weeks of treatment. No difference was observed in rooting among the various treatments six weeks after the treatment (Table 3). Culturing explants on half strength (0.5x) LS media resulted in the rooting of 57.9% of the explants in the light while 55.0% of the explants rooted in the dark. Similar results were obtained when explants were treated with full strength (1x) LS media supplemented with 1 μM IBA where 52.7% of the explants rooted in the light and 56.2% rooted in the dark (Table 3). Four independently run Chi-Square analyses (α=0.05) revealed no statistical difference among the treatments of differing media or in light or dark condtions (Table 4).

3.1.2 Callus Induction

The average callus weight for all 2,4-D treatments was 1.0728 grams while the average callus weight for all NAA treatments was 0.5681 grams after 12 weeks of treatment. On average, treatments with 2,4-D resulted in more callus growth than NAA treatments (Table 5). LSD (α=0.05) revealed no difference in callus growth from treatments supplemented with NAA. Treatment of full strength (1x) LS media supplemented with 5 μM 2,4-D plus 0.44 μM BA was designated “callus inducing media” (CIM) and used in subsequent experiments. The growth of

Growing Media	Light	Dark
0.5x LS Media	(11/19) 57.9%	(11/20) 55.0%
1 x LS Media with 1 μM IBA	(19/36) 52.7%	(20/36) 56.2%

Table 3. Percentage (%) of explants that produced roots after seven weeks when cultured on two different media (0.5x LS with no supplements or 1x LS media supplemented with 1 μM IBA), either in the dark or in continuous light. Numbers within parenthesis indicate actual number out of total explants that produced roots.

Comparisons	Continuity X^2	P	Significant
0.5x LS Media verse 1x Media with 1 μM IBA in the Light	0.0060	0.9381	ns
0.5x LS Media verse 1x Media with 1 μM IBA in the Dark	1.3832	0.2396	ns
Light verse Dark culturing on 0.5x LS Media	0.0000	1.0000	ns
Light verse Dark culturing on 1x LS Media with 1 μM IBA	1.6406	0.2002	ns

Respectively; ns = non-significant

Table 4. Chi-square (α=0.05) analysis of explant rooting on four independent comparisons of 0.5x LS media with no supplements and 1x LS media with 1 μM IBA cultured in light or dark conditions. No significant differences were observed between tested medias or lighting.

Significant Levels (α=0.05)	Mean (gm)	N	Treatment
A	1.2657	7	5μM 2,4-D & 0.44 μM BA
A	1.1164	7	2.5 μM 2,4-D & 1.1 μM BA
AB	1.0594	7	2.5 μM 2,4-D & 0.44 μM BA
BC	0.8497	7	5 μM 2,4-D & 1.1 μM BA
CD	0.6271	7	5 μM NAA & 0.44 μM BA
D	0.5554	7	5 μM NAA & 1.1 μM BA
D	0.5546	7	2.5 μM NAA & 1.1 μM BA
D	0.5353	7	2.5 μM NAA & 0.44 μM BA

Table 5. Callus weight of internodal sections of 129EC after 12 weeks of treatment. Differences between means designated by different letters (A, B, C and D) based on LSD of α=0.05. The mean weights are composed of the weight (gm) of 12 internodal sections per container. The total error degree of freedom for this test is 48. For the LSD test the error mean square is 0.056436 and the least significant difference between significant levels is 0.2553.

callus on CIM was not statistically different from callus growth that occurred for the 2.5 μM 2,4-D plus 1.1 μM BA or 2.5 μM 2,4-D plus 0.44 μM BA treatments.

3.2 Ploidy Induction

3.2.1 Aqueous Shoot Treatment

3.2.1.1 Leaf Growth and Development

Aqueous shoot solution treatment with 150 μM oryzalin reduced the number of new leaves that were produced after five weeks of culture, irrespective of treatment time or explant type (Figure 1). On average, treatment with oryzalin reduced new leaf production by approximately one-half. ANOVA (Proc GLM) analysis (α=0.05) revealed that both treatment with oryzalin and the duration of the treatment had an effect on leaf growth and development (Table 6). The greatest number of leaves, regardless of explant type (apical, lateral or pre-cultured lateral), was observed for treatment of 12 hr with 150 μM oryzalin. Treatment for 96 hours with 150 μM oryzalin produced the least number of leaves. When averaged across treatment durations and explant type, treatment with 150 μM oryzalin produced 14.8 leaves per culture vessel compared to 29.5 leaves for control explants (Table 7). Pre-Cultured laterals produced an average of 24.2 leaves per vessel which is significantly more than the average 21.2 leaves for the apical explants and 21.0 leaves for the lateral explants irrespective of treatment and treatment duration. There was a significant interaction between the type of explant treated and the treatment duration.

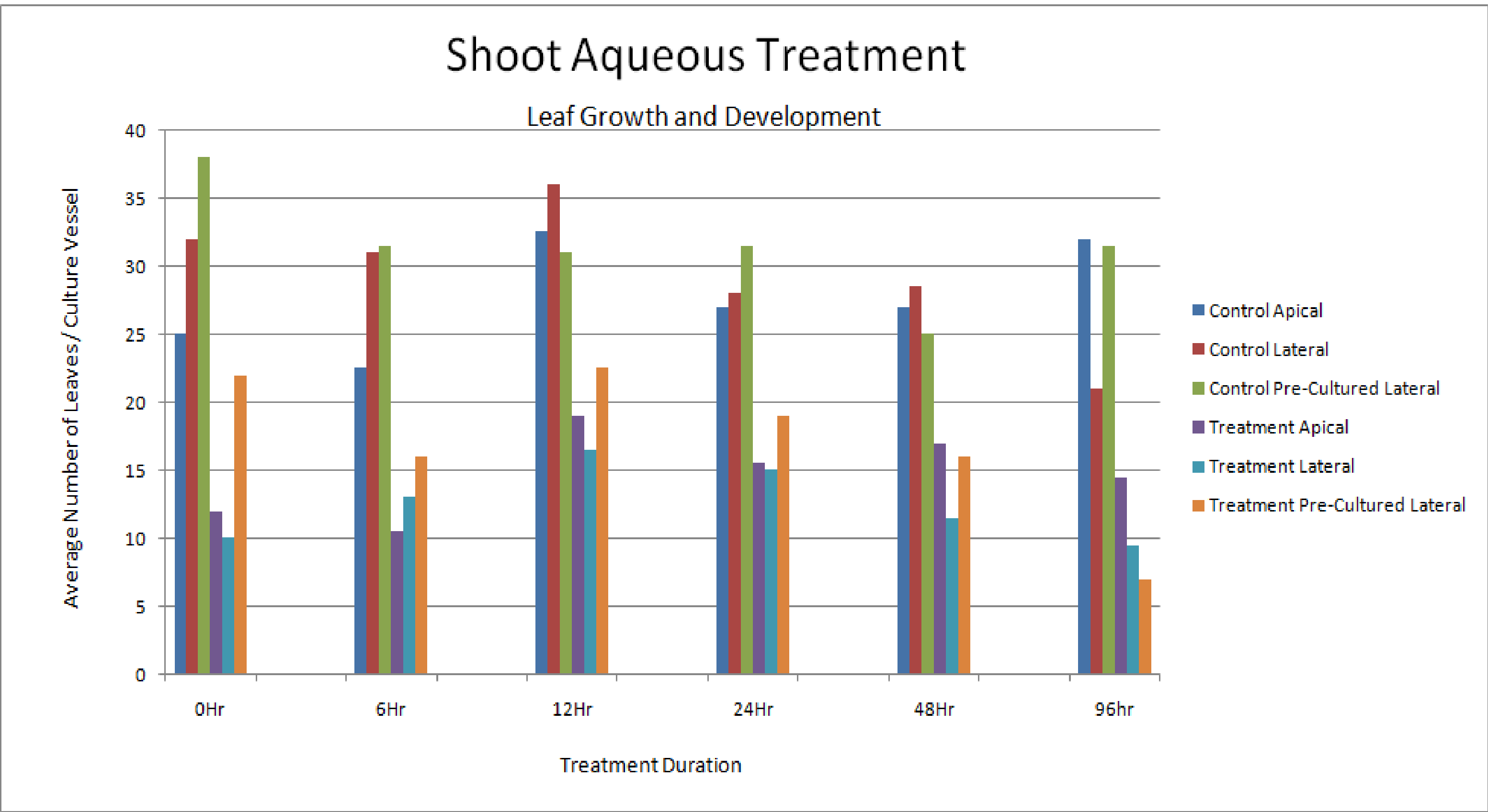

Figure 1. Mean number of leaves per tissue culture vessel five weeks after treatment with 150 μM oryzalin. Each culture vessel contained six shoot explants with two replicate culture vessels.

Analysis of Variance

Source of Variance	d.f	SS	MS	F-value	Pr>F	Signif.
Treatment Duration	5	362.569	72.513	4.71	0.0021	*
Explant	2	158.861	79.431	5.61	0.0107	*
Treatment	1	3886.680	3886.681	252.34	<0.0001	*
Treatment Duration* Explant	10	427.138	42.719	2.77	0.0120	*
Treatment*Treatment Duration	5	86.902	17.381	1.13	0.3631	ns
Explant*Treatment	2	47.194	23.597	1.53	0.2299	ns
Treatment Duration*Treatment* Explant	10	197.472	19.747	1.28	0.2769	ns

* Significant (α =0.05) Respectively; ns = non-significant

Table 6. ANOVA of the effect of aqueous oryzalin treatment on shoot leaf growth and development five weeks after treatment. Shoots were treated with 150 μM oryzalin for durations of 0, 12, 24, 48 and 96 hours. Three different explant types (apical, lateral and pre-cultured lateral) were treated.

Significant Levels	Mean	N	Treatment
A	29.5	36	Ethanol (Control)
B	14.8	36	Oryzalin (Treatment)

Table 7. Least significant difference (LSD) of mean number of leaves per tissue culture vessel after treatment with 150 μM oryzalin. Leaves were counted five weeks after treatment. Treatments were statistically different ($\alpha= 0.05$) the mean is based on 36 culture vessels.

3.2.1.2 Root Development

Aqueous shoot treatment with 150 μM oryzalin reduced the development of roots when measured eight weeks after treatment. An average of 5.68 out of 6 explants in the control vessels developed roots, while an average of 1.11 explants treated with 150 μM oryzalin developed roots. According to ANOVA (SAS MIXED procedure) a significant difference exists between treatments and the interactions of treatments (Table 8) while explant type alone did not significatnly influence rooting. Within the interaction of explant type and oryzalin treatment, the lateral explants treated with 150 μM oryzalin developed the fewest roots with an average of 0.58 explants that developed roots per culture vessel, while control explants consisting of the apical shoot developed the most roots and averaged 5.75 rooted plants per culture vessel. The average for all treated explants (regardless of duration or explant type) was 2.67 rooted plants per culture vessel while the average for all control explants was 4.92 rooted plants per culture vessel. Treatment duration (0, 6, 12, 24, 48 and 96 hours) had no effect on the development of roots. The oryzalin mantained a residual effect on the root development.

3.2.1.3 Polyploid Induction

The DNA content of leaf nuclei was measured using flow cytometry 16 weeks after treatment. Figure 2 illustrates the typical data obtained for diploid (Figure A), tetraploid and diploid (Figure B) and diploid, tetraploid and octaploid (Figure C) nuclei. The data obtained from the flow cytometry analysis of nuclear DNA content was used to evaluate ploidy levels. Since each sample varied in the number of nuclei measured, data was normalized to the ratio of tetraploid nuclei to diploid nuclei. As

Analysis of Variance

Source of Variance	d.f.	F-value	Pr>F	Significant
Treatment Duration	5	0.27	0.9250	ns
Treatment	1	108.80	<0.0001	*
Explant	2	0.82	0.4494	ns
Treatment Duration*Treatment	5	1.91	0.1192	ns
Treatment*Explant	2	5.67	0.0075	*
Treatment Duration*Explant	10	0.46	0.9061	ns
Treatment Duration*Treatment*Explant	10	1.26	0.2902	ns

* Significant (α=0.05);
Respectively; ns = non-significant

Table 8. ANOVA of the effect of aqueous shoot treatment with 150 μM oryzalin on root development of explants eight weeks after treatment. Treatment duration represents 0, 12, 24, 48 and 96 hours of treatment. The explant variable represents the apical, lateral and pre-cultured lateral explant type. The treatment variable represents control verse plants treated 150 μM oryzalin.

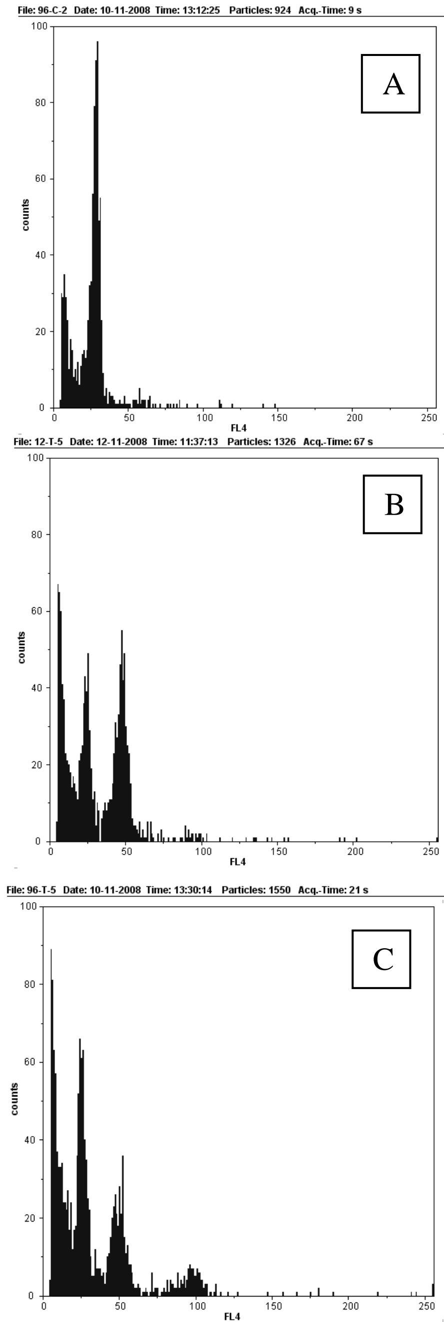

Figure 2. Examples of flow cytometry histograms for nuclei from pooled leaf samples of aqueous shoot treatments measured 16 weeks after treatment. The x-axis is relative fluorescence of DAPI stained nuclei and y-axis is the number of nuclei per fluorescence channel (Gains 505). Figure A shows a pooled diploid sample consisting of six apical stems that were treated with an aqueous solution of ethanol for 96 hours. Figure B shows diploid and tetraploid peaks form a pooled sample of nuclei from six pre-cultured lateral stems that were treated with an aqueous solution of 150 μM oryzalin for 12 hours. Figure C shows a diploid, tetraploid and an octaploid peak from a pooled sample of nuclei from six pre-cultured laterals that were treated with 150 μM aqueous oryzalin for 96 hours.

shown in Figure 3, treatment with an aqueous solution of oryzalin increased the ratio of polyploid nuclei in the pooled nuclei sample irrespective of explant type or treatment duration. The oryzalin treatment and the duration of the oryzalin treatment were significantly different from control treatments (Table 9) and the interaction between treatment and duration was also significant (α=0.05) for both diploid and tetraploid nuclei. Explant type had no effect on tetraploid to diploid ratios (Table 9). In general, treatments of 12 hours or greater resulted in an increased ratio of tetraploid to diploid nuclei. The 12 hour lateral treatment may have an artificial ratio inflation because of a contamination issue that resulted in the culling of half of the treated plants, all other treatments and explants treated have the mean of 12 plants represented. Including octaploid nuclei into the ratio calculations did not change the results (Figure 4 and Table 9). Similar to the tetraploid calculation, the ratio of polyploid nuclei (tetraploid plus octaploid) was significantly affected by oryzalin treatment and the duration of the treatment (Figure 4). In addition, like the tetraploid results, treatment durations of 12 hours or greater produced an increased ratio of polyploid to diploid nuclei. The type of explant had no effect on nuclei DNA content (Figure 4 and Table 9). To further characterize the effect of oryzalin treatment on nuclei DNA content, the percentage of polyploid nuclei (tetraploid plus octaploid) to the total nuclei measured was determined. Since previous analysis showed that the type of explant had no effect on DNA content, all explant types were combined in the calculation. As shown in Figure 5, the percentage of polyploid nuclei increased relative to all nuclei with oryzalin treatment, and polyploid nuclei accounted for 30% - 40% of nuclei after 12 hours of aqueous treatment with 150 μM oryzalin (Figure 5). Based on this pooled analysis, individual plants from

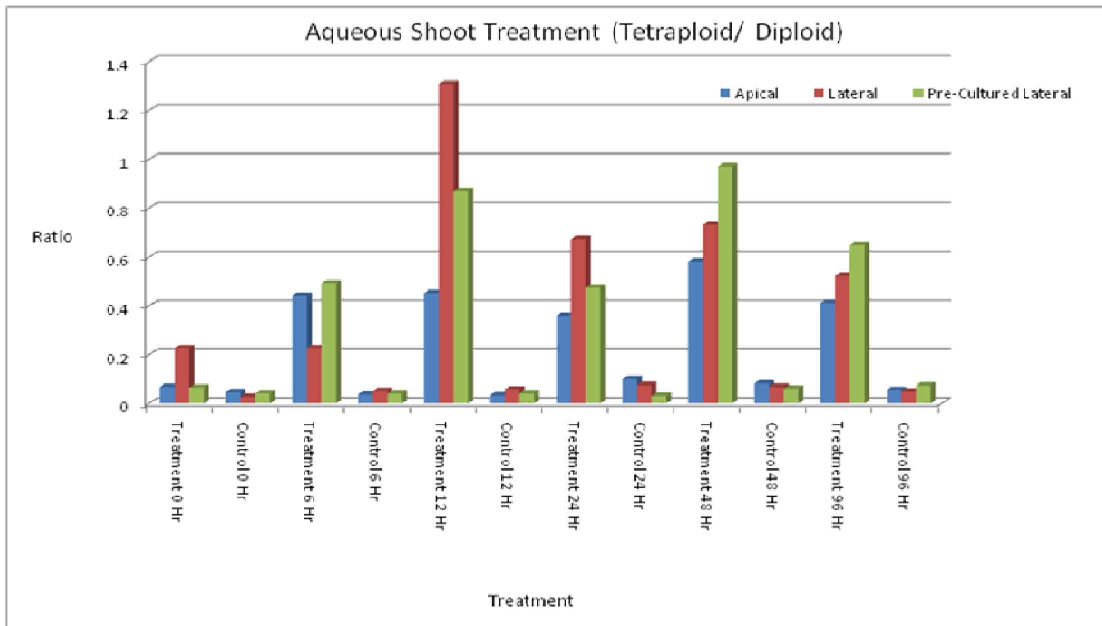

Figure 3. Ploidy ratio of pooled-leaf nuclei DNA content from shoots. Nuclei were isolated from explants consisting of either internodal shoots with apices (Apical), internodal shoots without an apex (Lateral) or internodal explants without an apex that were first pre-cultured on full strength (1x) LS media supplemented with 1.8 μM BA and 0.5 μM IBA for seven days (Pre-Cultured Laterals). DNA content was measured 16 weeks after treatment. Leaves from growing explants were pooled from each tissue culture vessel (replication) and used to measure nuclei DNA content.

Analysis of Variance -

Source of Variance	d.f	F-value	Pr>F	Significant
Diploid peak results of ANOVA				
Treatment Duration	5	4.01	0.0062	*
Treatment	1	104.60	<0.0001	*
Explant	2	0.86	0.4339	ns
Treatment Duration*Treatment	5	2.90	0.0286	*
Treatment*Explant	2	1.31	0.2851	ns
Treatment Duration*Explant	10	0.55	0.8430	ns
Treatment Duration *Treatment*Explant	10	0.45	0.9099	ns
Tetraploid peak results of ANOVA				
Treatment Duration	5	3.34	0.0154	*
Treatment	1	94.57	<0.0001	*
Explant	2	0.87	0.4288	ns
Treatment Duration *Treatment	5	2.66	0.0405	*
Treatment*Explant	2	1.16	0.3261	ns
Treatment Duration *Explant	10	0.47	0.8951	ns
Treatment Duration *Treatment*Explant	10	0.48	0.8930	ns
Octaploid peak results of ANOVA				
Treatment Duration	5	4.00	0.0063	*
Treatment	1	18.88	0.0001	*
Explant	2	0.55	0.5816	ns
Treatment Duration *Treatment	5	3.44	0.0135	*
Treatment*Explant	2	1.18	0.3215	ns
Treatment Duration*Explant	10	2.20	0.0440	*
Treatment Duration *Treatment*Explant	10	1.72	0.1193	ns

* Significant (α =0.05)
Respectively; ns = non-significant

Table 9. ANOVA analysis of the diploid, tetraploid and octaploid peaks. Pooled leaves of aqueous shoot treatments were measured 16 weeks after treatment. This table is composed of three independently run ANOVA tests. The variable tested was the percentage of nuclei (out of 100%) that occurred within each peak (the percent of each of the 3 peaks added together equal 100%).

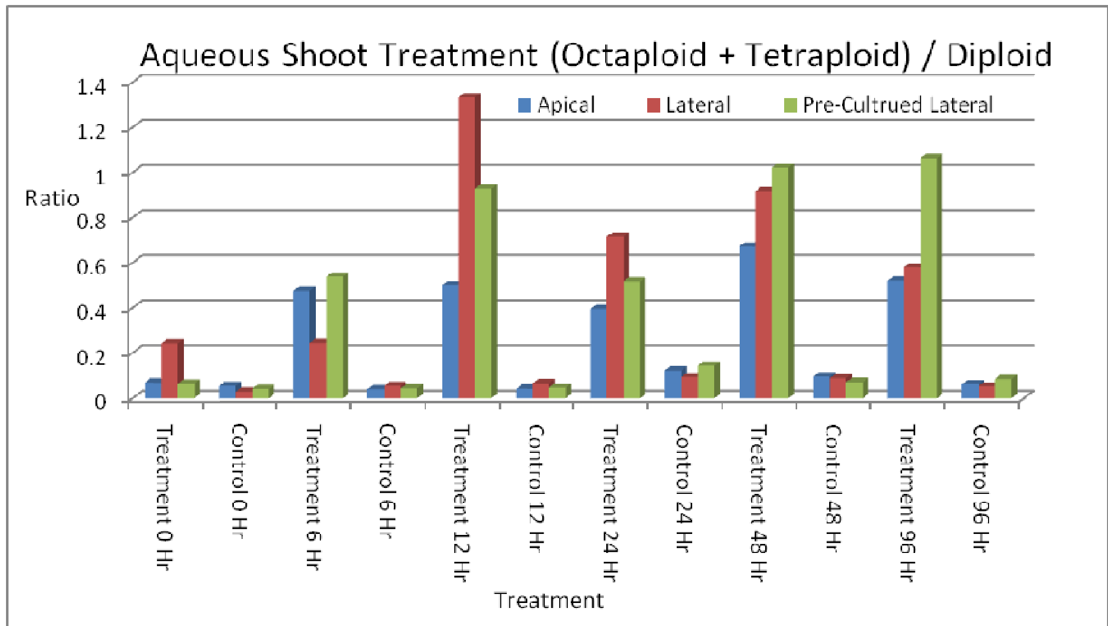

Figure 4. Polyploid ratio of pooled-nuclei DNA content isolated from explants after aqueous shoot treatment. This figure takes into account the octaploid nuclei plus the tetraploid nuclei.

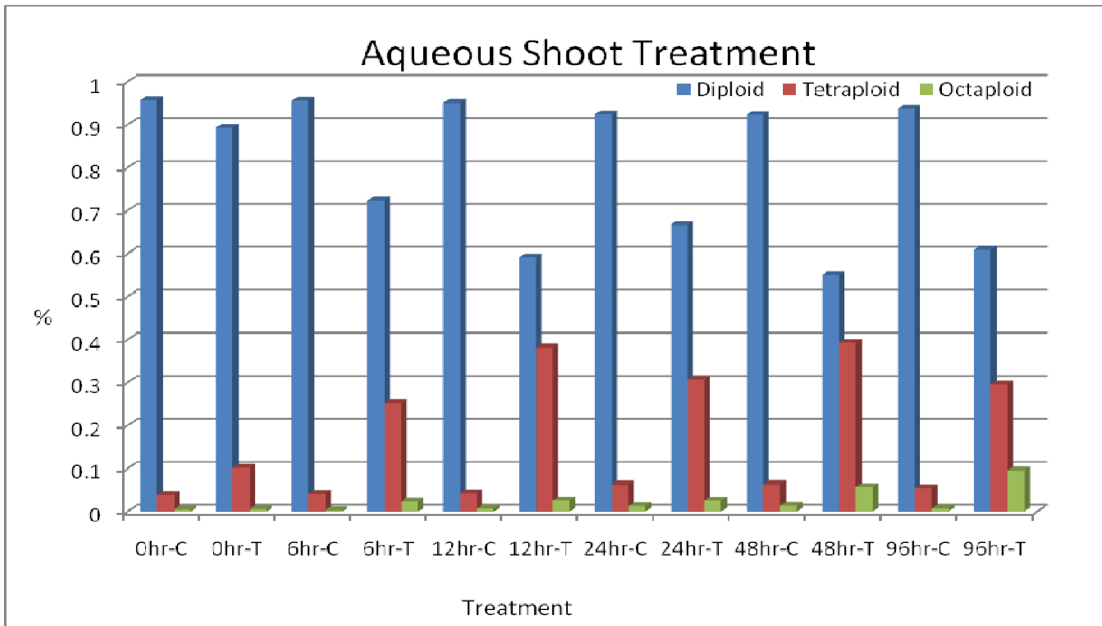

Figure 5. Percentage (%) of each ploidy type in the pooled-nuclei samples isolated from explants after the aqueous shoot treatment 16 weeks after treatment. Sum of diploid, tetraploid and octaploid peaks equal 100%. This data reflects the mean of the apical, lateral and pre-cultured lateral explant treatments.

selected treatments were analyzed for nuclei DNA content. The longer treatment durations (12, 24, 48 and 96 hours) were shown to give the greatest increase of polyploid nuclei, individual plants from these treatments were tested by flow cytometry (Table 10) 26 weeks after initial treatment. In general the greatest percentage of tetraploid plants occurred after 96 hours of treatment and also resulted in the only mixaploid plants consisting of tetraploid and octaploid and the only octaploid plants. At the 26 week mark there were 19 polyploids out the 216 treated plants (8.8% throughout all treatments including dip and 6 hour). At 36 weeks the 19 polyploids were sub-cultured into 65 smaller explants and allowed to grow. 60 weeks after initial treatment, the sub-cultured explants were tested again and nine plants remained stable tetraploids.

3.2.2 Solidified Shoot Media Treatments

This treatment involved placing shoots into media supplemented with 150 μM oryzalin. 12 weeks after the initial treatment, plants were pooled and DNA nuclei content was measured via flow cytometry. Figure 6 shows the ratio of tetraploid nuclei to diploid nuclei as opposed to absolute values since the number of nuclei tested for DNA content varied among samples. These results show very little variation between treatment or explant types. ANOVA confirmed that the only significant treatment effect with oryzalin was on the frequencies of diploid and tetraploid nuclei (Table 11). Although no tetraploid plants were generated from treatment with 150 μM there was a significant ($\alpha=0.05$) increase in the number of tetraploid nuclei and a significant reduction in the number of diploid nuclei. Treatment duration and explant type had no effect on nuclei DNA content. Figure 7

Treatment Duration	Explant	Diploid no. (%)	Mixaploid (Diploid and Tetraploid) no. (%)	Tetraploid no. (%)	Mixaploid (Tetraploid & Octaploid) no. (%)	Octaploid no. (%)
12 Hour	Apical	6 (50)	5 (41.6)	1 (8.3)	0 (0)	0 (0)
12 Hour	Lateral	8 (66.6)	4 (33.3)	0 (0)	0 (0)	0 (0)
12 Hour	Pre-Cultured Laterals	10 (83.3)	0 (0)	2 (16.6)	0 (0)	0 (0)
24 Hour	Apical	7 (58.3)	3 (25)	2 (16.6)	0 (0)	0 (0)
24 Hour	Lateral	5 (41.6)	7 (58.3)	0 (0)	0 (0)	0 (0)
24 Hour	Pre-Cultured Laterals	9 (75)	3 (25)	0 (0)	0 (0)	0 (0)
48 Hour	Apical	7 (58.3)	5 (41.6)	0 (0)	0 (0)	0 (0)
48 Hour	Lateral	6 (50)	3 (25)	2 (16.6)	0 (0)	0 (0)
48 Hour	Pre-Cultured Laterals	4 (33.3)	7 (58.3)	1 (8.3)	0 (0)	0 (0)
96 Hour	Apical	5 (41.6)	6 (50)	1 (8.3)	0 (0)	0 (0)
96 Hour	Lateral	6 (50)	3 (25)	3 (25)	0 (0)	0 (0)
96 Hour	Pre-Cultured Laterals	3 (25)	2 (16.6)	3 (25)	3 (25)	1 (8.3)

Table 10. Ploidy distribution of individual plants treated with oryzalin 26 weeks after the initial treatment. Plants were tested individually and were chosen due to the tetraploid or octaploid peaks of the pooled samples.

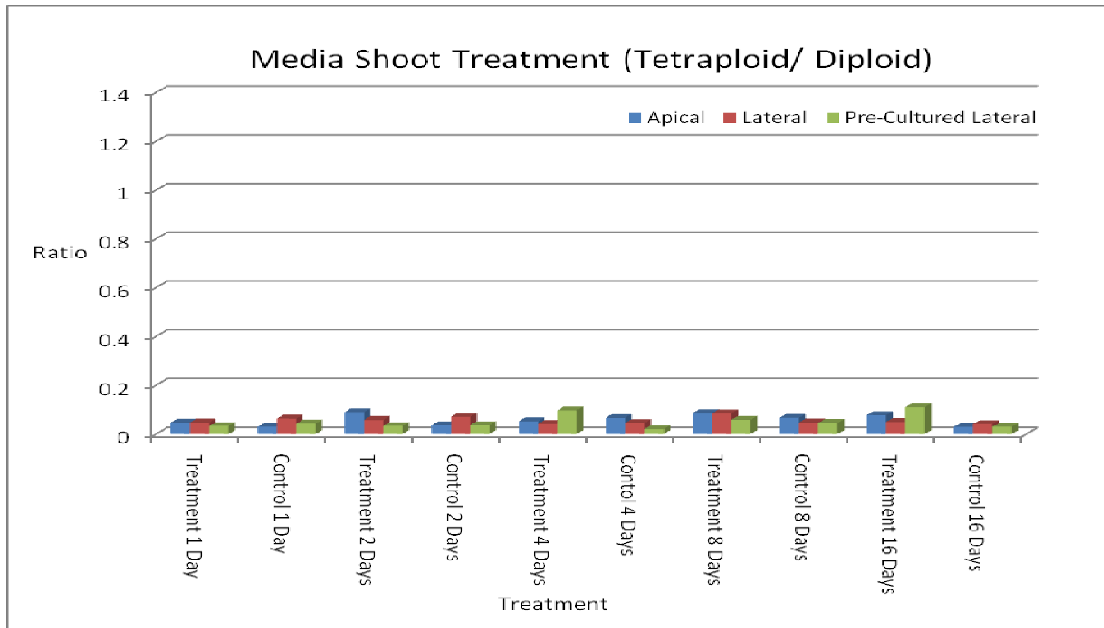

Figure 6. Tetraploid to diploid ratio of pooled-nuclei DNA isolated from explants after treatment in solidified shoot media. Ratio of pooled samples of tetraploid nuclei were divided by diploid nuclei. Explant type and treatment duration are shown. Flow cytometry was used on pooled samples of explants within a treatment. The numbers of nuclei within each peak (tetraploid or diploid) were compared as a ratio since the numbers of nuclei tested differed among samples.

Analysis of Variance

Source of Variance	d.f	F-value	Pr>F	Significant
Diploid peak result of ANOVA				
Treatment Duration	4	0.99	0.4218	ns
Treatment	1	4.71	0.0343	*
Explant	2	0.28	0.7571	ns
Treatment Duration *Treatment	4	0.91	0.4661	ns
Treatment*Explant	2	1.30	0.2807	ns
Treatment Duration *Explant	8	0.76	0.6381	ns
Treatment Duration *Treatment*Explant	8	0.85	0.5648	ns
Tetraploid peak result of ANOVA				
Treatment Duration	4	0.66	0.6227	ns
Treatment	1	5.04	0.0287	*
Explant	2	0.26	0.7717	ns
Treatment Duration *Treatment	4	0.96	0.4367	ns
Treatment*Explant	2	1.04	0.3609	ns
Treatment Duration *Explant	8	0.61	0.7629	ns
Treatment Duration *Treatment*Explant	8	0.91	0.5170	ns
Octaploid peak result of ANOVA				
Treatment Duration	4	1.18	0.3291	ns
Treatment	1	0.73	0.3973	ns
Explant	2	0.10	0.9061	ns
Treatment Duration *Treatment	4	0.65	0.6307	ns
Treatment*Explant	2	0.81	0.4482	ns
Treatment Duration *Explant	8	0.90	0.5195	ns
Treatment Duration*Treatment*Explant	8	0.65	0.7341	ns

* Significant (α=0.05),
Respectively; ns = non-significant

Table 11. ANOVA analysis of the effect of solidified shoot media treatment on the nuclei DNA content of pooled explants. Diploid, tetraploid and octaploid peaks were assigned after flow cytometry analysis. The numbers of nuclei within each peak were compared to the peaks of the other treatments for each pooled sample. This table is composed of three independently run ANOVA test that compared the percentage of nuclei within each peak. Treatment duration represents 1, 2, 4, 8 or 16 days in the solidified media. Treatments represent either treatments of 150 μM oryzalin or controls of ethanol. Explant represents either apical, lateral or pre-cultured lateral internodal sections.

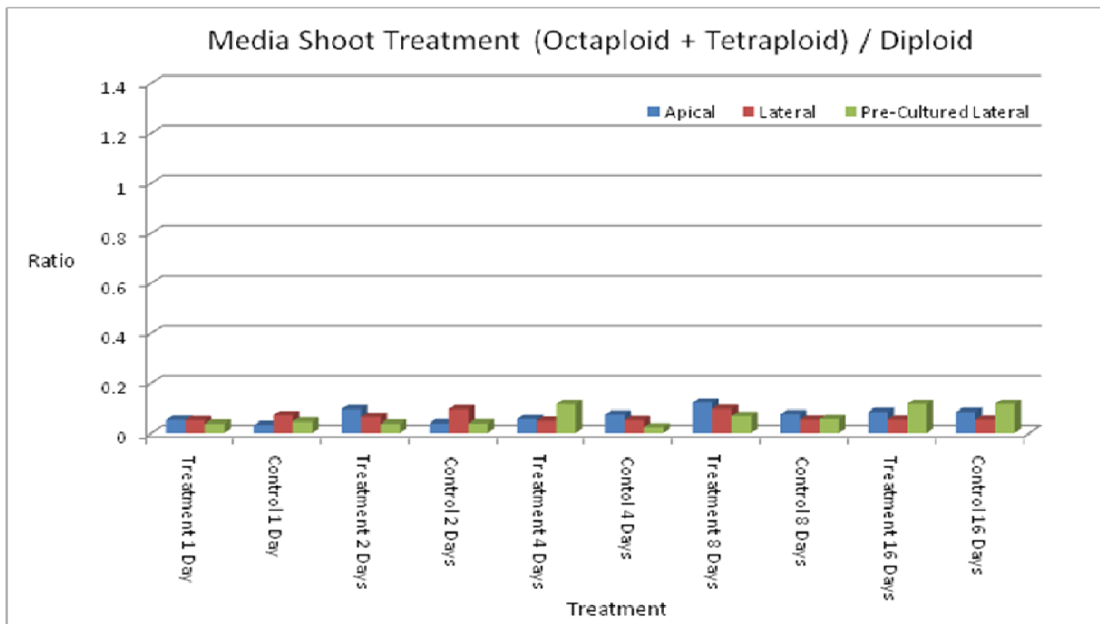

Figure 7. Ploidy ratio of pooled-nuclei DNA isolated from explants after treatment in solidified shoot media. Explant type treatment and treatment duration are represented. Flow cytometry was run on pooled samples of explants. The numbers of nuclei within each peak (octaploid, tetraploid or diploid) were compared as a ratio since the numbers of nuclei tested were never the same.

combines the percentage of total polyploidy (tetraploid plus octaploid) peaks divided by the diploid peak percentage. Little difference was observed among treatment explant type or duration of treatment. This would be expected because the ANOVA (Table 11) shows no significant differences with the octaploid peak. The lack of tetraploid plant production indicates that the solidified shoot media treatment is not an efficient method of polyploid induction because oryzalin is not a systemic and the plant tubulin must be in direct contact with the oryzalin for polyploid induction to occur.

Figure 8 present a side by side comparison of both methods used for shoot treatment (aqueous and solidified media). Figure 8 shows the tetraploid to diploid ratio while Figure 9 shows the polyploid peak to diploid ratio. From this data it is clear that the controls from both methods maintained a consistent level of DNA content. Having both methods of shoot treatment on the same graph with the same scale also shows that the aqueous treatment of 150 μM oryzalin produced a much greater ratio of tetraploid nuclei.

3.3 Callus Treatments

3.3.1 Callus Aqueous Treatment

The aqueous callus treatment consisted of callus that was initiated on CIM for 0, 5, 10, 15, 20, 25, 30, 35 or 40 days and then treated in an aqueous solution of 150 μM for 12 hours on an orbital shaker. 12 weeks after initial treatments began portions of the callus were removed and nuclei DNA content was determined by flow cytometry. Also at that time the number of explants producing callus was also

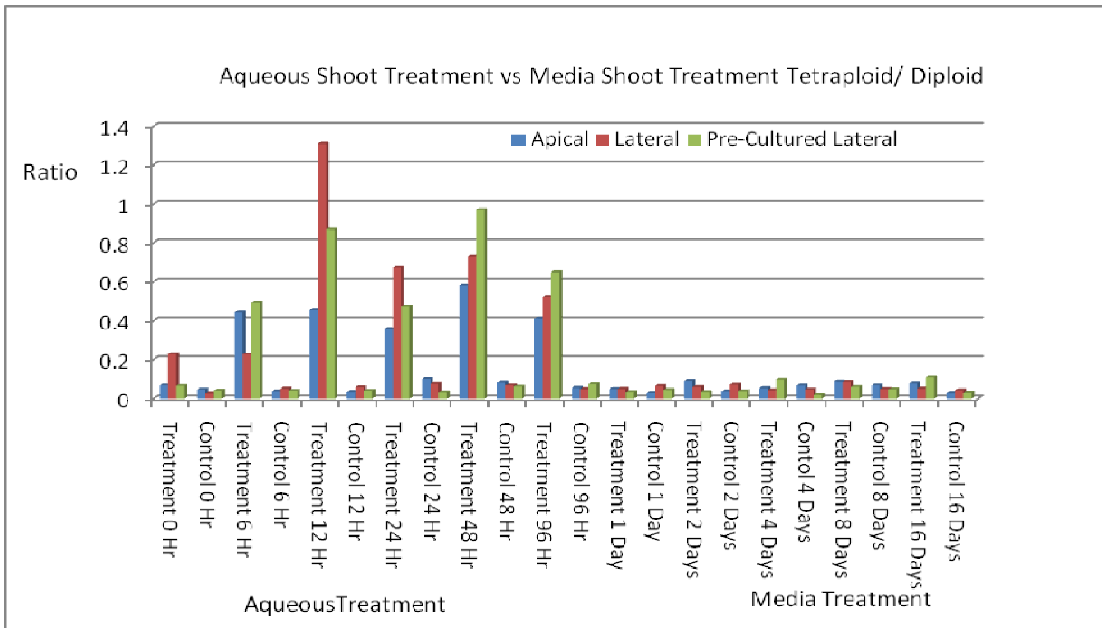

Figure 8. Tetraploid to diploid ratio of pooled-nuclei DNA content isolated from leaves of explants treated with either aqueous shoot treatment or solidified shoot media.

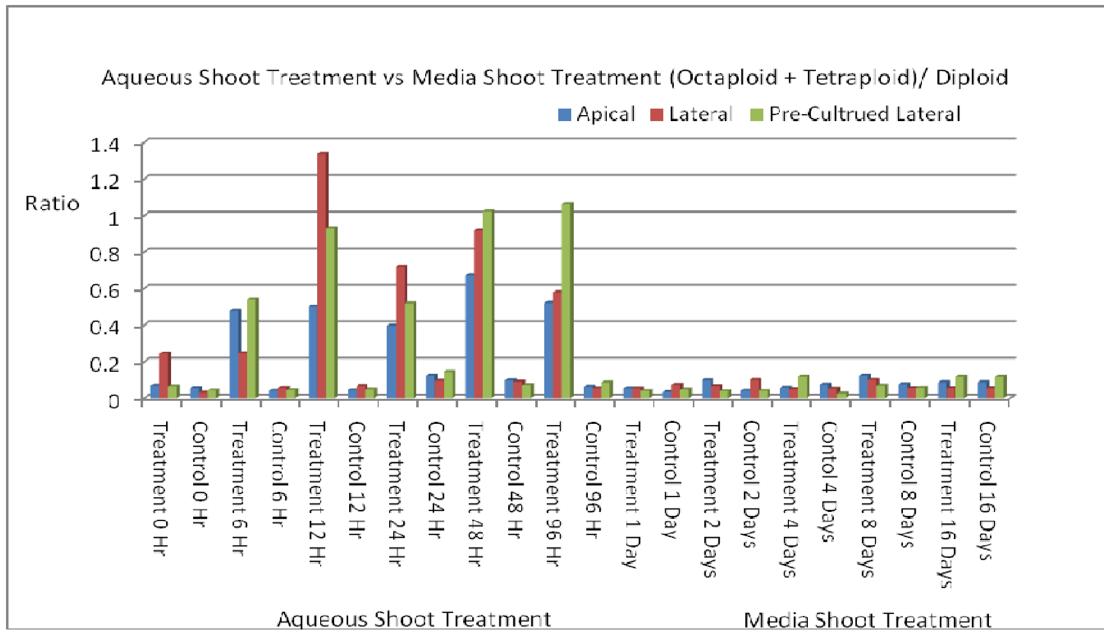

Figure 9. Polyploid to diploid ratio of pooled-nuclei DNA content isolated from leaf of explants treated with either aqueous shoot treatment or solidified shoot media.

determined. ANOVA (Proc GLM) analysis confirms that the number of dead callus was not dependent on the treatment duration while treatment, with oryzalin did significantly affect callus viability (Table 12). Table 13 is composed of three independently run ANOVA tests. The analysis revealed similar results in all three tests in that the treatment duration has no significant effect on nuclei DNA content. The only significant factor different from the control was treatment with 150 μM oryzalin which had an effect on diploid, tetraploid and octaploid peaks.

3.3.2 Callus Solidified Media Treatment

Callus was initiated on solidified media that contained 150 μM oryzalin for time durations of 5, 10, 15, 20, 25, 30, 35 and 40 days. 12 weeks after the callus initiation began, portions of the callus were removed and nuclei DNA content was evaluated via flow cytometry. After 12 weeks viable callus was also counted and compared to dead callus. ANOVA analysis (α=0.05) presented in Table 14 reveals that neither treatment with oryzalin or length of treatment affected callus viability. Although callus viability was not altered by oryzalin treatment, the diploid, tetraploid and octaploid nuclei DNA content was significantly higher after the treatment on the media containing oryzalin (Table 15). Treatment duration had no significant effect on ploidy levels.

3.3.3 Callus Treatment Overview

Table 16 shows the ANOVA results of overall treatments with 150 μM oryzalin or ethanol (control), and the treatment method of either growing callus on a CIM followed by oryzalin or inducing callus growth on solidified media containing 150 μM oryzalin on the change of ploid. The ANOVA analysis (α= 0.05) revealed

Analysis of Variance

Source of Variance	d.f	F-value	Pr>F	Significant
Treatment Duration	8	1.15	0.4242	ns
Treatment	1	66.82	<0.0001	*

* Significant (α =0.05)
Respectively; ns = non-significant

Table 12. ANOVA analysis of callus viability after treatment with an aqueous solution of 150 μM oryzalin.

Analysis of Variance

Source of Variance	d.f	F-value	Pr>F	Significant
Diploid peak result of ANOVA				
Treatment Duration	4	4.94	0.1102	ns
Treatment	1	83.41	0.0028	*
Treatment*Treatment Duration	3	4.70	0.1181	ns
Tetraploid peak result of ANOVA				
Treatment Duration	4	4.10	0.1381	ns
Treatment	1	62.01	0.0043	*
Treatment*Treatment Duration	3	3.86	0.1482	ns
Octaploid peak result of ANOVA				
Treatment Duration	4	6.04	0.0856	ns
Treatment	1	91.22	0.0024	*
Treatment*Treatment Duration	3	9.07	0.0515	ns

* Significant (α =0.05)
Respectively; ns = non-significant

Table 13. ANOVA analysis of the diploid, tetraploid and octaploid peaks of aqueous callus treatments. Three independently run ANOVA tests are present.

Analysis of Variance

Source of Variance	d.f	F-value	Pr>F	Significant
Treatment Duration	7	2.68	0.1084	ns
Treatment	1	2.17	0.1841	ns

* Significant (α =0.05)
Respectively; ns = non-significant

Table 14. Effect of oryzalin treatment and duration of treatment on callus viability. Treatment durations consisted of 5, 15, 20, 25, 30, 35 and 40 days on the solidified media containing 150 μM oryzalin.

Analysis of Variance -

Source of Variance	d.f	F-value	Pr>F	Significant
Diploid peak result of ANOVA				
Treatment Duration	7	1.39	0.2335	ns
Treatment	1	176.43	<0.0001	*
Treatment*Treatment Duration	7	1.25	0.2973	ns
Tetraploid peak result of ANOVA				
Treatment Duration	7	1.65	0.1477	ns
Treatment	1	145.10	<0.0001	*
Treatment*Treatment Duration	7	1.69	0.1374	ns
Octaploid peak result of ANOVA				
Treatment Duration	7	1.05	0.4103	ns
Treatment	1	123.70	<0.0001	*
Treatment*Treatment Duration	7	1.23	0.3072	ns

* Significant (α=0.05)
Respectively; ns = non-significant

Table 15. ANOVA analysis of the diploid, tetraploid and octaploid ploidy levels after treatment on solidified callus media. This table is composed of three independently run ANOVA tests.

Analysis of Variance -

Source of Variance	d.f	F-value	Pr>F	Significant
Diploid peak result of ANOVA				
Treatment	1	83.78	<0.0001	*
Treatment Method	1	2.55	0.1148	ns
Treatment*Treatment Method	1	0.79	0.3765	ns
Tetraploid peak result of ANOVA				
Treatment	1	65.41	<0.0001	*
Treatment Method	1	1.94	0.1683	ns
Treatment*Treatment Method	1	0.35	0.5542	ns
Octaploid peak result of ANOVA				
Treatment	1	56.26	<0.0001	*
Treatment Method	1	1.85	0.1789	ns
Treatment*Treatment Method	1	1.50	0.2253	ns

* Significant (α =0.05)
Respectively; ns = non-significant

Table 16. ANOVA analysis of the diploid, tetraploid and octaploid callus nuclei DNA content after aqueous treatment or solidified media treatment. This table is composed of three independently run ANOVA tests.

the 150 μM oryzalin a significant increase in polyploid nuclei compared to ethanol controls. The treatment method, when controls and treated callus were pooled together (aqueous and solidified media), had no effect on the number of tetraploid and octaploid nuclei. The source of variance treatment: pooled together the treatment method and views if more tetraploid and octaploid nuclei existed between the oryzalin treatment or the control.

Overall treatments with 150 μM regardless of the method of treatment resulted in 51.9% (18.7 dead of 36 calli) of the calli dying while only 10.5% (3.8 of 36 calli) of the calli were dead in control treatments. Aqueous treatment also reduced viability since overall 37.7% (13.6 of 36 calli) of aqueous calli died while only 24% of the overall calli died from treatment with solidified media. Statistically, the aqueous treatment killed more calli than the solidified media treatment and both methods were successful at producing polyploid nuclei.

Chapter 4: Discussion

4.1 Experiments

4.1.1 Rooting Induction

The root induction study found no statistically significant results when using these media and light combinations on selection 129EC of *Cercis yunnanensis* Hu et Cheng. It did not make a significant difference if the plants were rooted in half strength (0.5x) LS media with no supplements or full (1x) LS media supplemented with 1 μM IBA. In addition, the presence or absence of light did not affect the number of roots produced. These results are similar to findings of a previous study (Cheong and Pooler, 2003) that tested the type and concentration of auxin on *in vitro* root development of 129EC. Therefore the results of this study are consistent with previous studies. The previous study (Cheong and Pooler, 2003) showed that dark treatment affected adventitious shoot induction from leaf tissue and that increased duration of dark treatment resulted in significantly (α=0.05) more roots on media supplemented with TDZ (thidiazuron). Although plant tissue (leaf versus shoot), media supplements (TDZ versus IBA) and time duration (0 to 8 weeks versus 0 to 7 weeks) were different, the findings of this rooting induction experiment cannot be directly compared to previous findings.

4.1.2 Callus Induction

Callus was grown on full strength (1x) LS media with four different combinations of 2,4-D and BA and four different combinations of NAA and BA. Callus initiated in the presence of 2,4-D with BA produced approximately twice the

mean weight of callus compared to the combination of NAA with BA. This study was important to find which treatment combination induced the greatest callus growth for use in the callus ploidy induction experiment. The greatest callus growth was induced by full strength (1x) LS media supplemented with 5μM 2,4-D and 0.44 μM BA. The findings of this callus culture protocol were incorporated into the polyploid induction of callus.

4.2 Ploidy Manipulation Treatments

4.2.1 Shoot Treatment

4.2.1.1 Aqueous

Polyploid plants, including tetraploids and unstable octaploids, were produced with the aqueous shoot treatment. Furthermore, the aqueous shoot treatment method resulted in a 100% survival of all treated explants. The type of explant (apical, lateral or pre-cultured lateral) had an effect on the number of leaves produced following the aqueous shoot treatment. Explant type affected ploidy through the production of unstable octaploids. This study found that when pre-cultured laterals were treated for 96 hours with 150 μM oryzalin, unstable octaploids were produced. The 96 hour treatment with oryzalin was the only treatment to produce octaploids. Perhaps a longer treatment of oryzalin could ultimately produce stable octaploids or higher levels of ploidy. For octaploid production, the nuclei must go through two mitotic cycles; first the diploid nuclei must double its nucleic DNA content, then the new tetraploid nuclei must double its content. This may explain why the shorter time duration of treatments did not yield octaploid nuclei. The instability of the octaploid nuclei may be a function of slower cell division for the polyploids compared to the

diploids (Baatout, 1999). The duration of treatment with 150 μM oryzalin had a significant effect. Tetraploids were produced from 12, 24, 48 and 96 hour treatments for the aqueous oryzalin treatment. These durations might have been successful because the nuclei went through a complete mitotic cycle in the presence of oryzalin. Plants that had altered ploidy levels were sub-cultured into six plants (depending upon size at time of division). The plants were allowed to grow and apical or basal leaves were harvested and evaluated via flow cytometry. Results indicated that all of the octaploid plants reverted to a lower level of ploidy (tetraploid). This may be a result of the tetraploid nuclei outcompeting or out growing the octaploid nuclei.

Chromosomes have been doubled in *Solanum* spp. L. (potato) (Chauvin et al., 2003) *in vitro* with an apical dipping application of 28.8 μM oryzalin for 24 hour period produced tetraploid explants. This is consistent with the findings of this study. Other published results (Petersen et al., 2002) indicate that colchicine treatments with *Miscanthus sinensis* A. on unrooted *in vitro* shoots produced higher percentages of chromosome doubled shoots compared to the rooted shoots, and single shoots produced more chromosome doubled shoots than treatment of clustered shoots. Their findings indicate that the toxic levels to the roots were reached before chromosome doubling occurred. Although their findings were with colchicine and not with oryzalin, assumptions could be made that oryzalin will have the same effect on shoot cultures.

4.2.1.2 Solidified

No polyploid plants were recovered from the solidified shoot media treatments. Plants treated by this method were tested by pooling basal leaves of treated plants. Through flow cytometry pooling efforts this method was proven to

significantly increase the amount of polyploidy nuclei. Although polyploid nuclei were detected, it appeared that not enough nuclei were transformed to justify sampling individual plants via flow cytometry. As stated by Ascough et al. in 2008 "perhaps a method in which the entire explant is submerged (like in a liquid-shake culture) would improve penetration" which is supported by the results of this study. If oryzalin acted systemically, it may be possible for the nuclei throughout the plant to be affected by solidified media treatments. However, oryzalin does not act systemically and therefore the mitotically active nuclei (cell) must be placed in direct contact for tubulin binding to occur resulting in ploidy manipulation.

4.2.1.3 Aqueous Shoot Treatment Verse Solidified Shoot Media Treatments

In comparing the aqueous shoot treatment to the solidified shoot media treatment, the aqueous treatment produced more polyploid nuclei. The aqueous shoot treatment produced tetraploid plants while the solidified shoot media treatment did not. Results published by Chauvin et al. (2003) also agree with these findings. The oryzalin that they placed in the media did not perform effectively in doubling chromosomes, compared to the aqueous agitated oryzalin solution which was the most efficient tetraploid producing method among oryzalin treatments.

4.2.2 Callus Media Treatment and Callus Aqueous Treatment

Statistically, more callus died from the aqueous treatment than the media treatment and the significant factor contributing the amount of callus death was treatment with oryzalin verses the ethanol control. Although callus death was an issue, the formation of callus was not a hindrance. In other studies, no callus was formed on immature inflorescence of *Miscanthus sinensis* A. when exposed to 60 μM oryzalin (Petersen, 2002). When comparing the amount diploid to polyploid nuclei

produced it was clear that the method (solidified media or aqueous) of treatment was not significant since both treatments produced statistically equal numbers of polyploid nuclei. Both methods of oryzalin treatment did produce more polyploid nuclei than the controls. In an evaluation of the aqueous treatment it did not make a difference whether the callus was dipped in the 150 μM oryzalin solution or if the callus was left on the media supplemented with 150 μM oryzalin for 40 days; the ploidy manipulation and death rate were not significantly different. Results were similar with the solidified media callus treatment in that it did not make a difference whether the callus was treated on the media for five days or 40 days. A hurdle exists within treating callus tissue; a protocol of forming the callus and then generating shoots and roots from the treated callus tissue must be established. Once the protocol is established, callus treatments many serve as a practical method of ploidy manipulation.

4.3 Conclusion

In conclusion the aqueous shoot treatment produced the greatest number of polyploid plants compared to any other tested method conducted in this study. This study found that significant difference did exist with the explant treated; all of the unstable octaploid that arose from this study originated with pre-cultured lateral explants. Time duration was also significant; plants submerged in an aqueous solution of 150 μM oryzalin for 12 to 96 hours produced polyploids while explants dipped in the solution and 6 hour treatment produced no polyploid plants. 60 weeks after initial treatment there are 9 stable tetraploids.

The solidified shoot media produced significantly more tetraploid nuclei than controls but no full tetraploids arose from that treatment. Aqueous callus treatment and solidified media callus treatment produced many chimeric calli but was unsuccessful at producing a full tetraploid plant. The aqueous callus treatment had a significant negative effect on the viability of the callus while the solidified media treatment did not affect callus viability.

These finding advance the scientific field by aiding to the foundation of polyploid induction through *in vitro* methods. With a method established for polyploid induction a protocol can be pioneered for the hybridization of the tetraploid and diploid plants. The outcome of this hybrid can yield sterile triploids that alleviate the threat of invasive plants around the globe.

References

Ahokas, H. 1999. Spontaneous tetraploidy in strawberry (*Fragaria* spp.) *Nordic Journal of Botany*. 19: 227–234.

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts and D. Watson. 1994. *Molecular Biology of the Cell*. 3rd edition. Garland Publishing. New York, NY. 925-927.

Ascough, G.D., J.V. Standen and J.E. Erwin. 2008. Effectiveness of colchicine and oryzalin at inducing polyploidy in *Watsonia lepida* N.E. Brown. *HortScience.* 43(7):2248-2251.

Baatout, S. 1999. Molecular basis to understand polyploidy. *Hematology and Cell Therapy*. 41:169–170.

Bartels, P.G. and J.L. Hilton. 1973. Comparison of trifluralin, oryzalin, pronamide, propham, and colchicine treatments on microtubules. *Pesticide Biochemistry* and *Physiology*. 3:462-472.

Bogart, J.P. and M. Tandy. 1976. Polyploid amphibians: three more diploid - tetraploid cryptic species of frogs. *Science.* 193:334–335.

Bouvier, L.P., P. Guerif, M. Djulbic, C. Durel, E. Chevreau and Y. Lespinasse. 2002. Chromosome doubling of pear haploid plants and homozygosity assessment using isozyme and microsatellite markers. *Euphytica.* 123:255-262.

Briggs, D. and S.M. Walters. 1997. *Plant variation and evolution*. 3rd edition. Cambridge University Press. Cambridge, United Kingdom. 312.

Brown, W.P. 1972. *Textbook of Cyotgenetics*. The C.V. Mosby Company, Saint Louis.

Cassani, J. R. and W.E. Caton. 1986. Efficient production of triploid grass carp (*Ctenopharyngodon idella*) utilizing hydrostatic pressure. *Aquaculture.* 56:43-50.

Chauvin, J.E., C. Souchet, J.P. Dantec and D. Ellisseche. 2003. Chromosome doubling of 2*x Solanum* species by oryzalin: method development and comparison with spontaneous chromosome doubling *in vitro*. *Plant Cell, Tissue and Organ Culture.* 73:65-73(9).

Chauvin, J.E., A. Label and M.P. Kermarrec. 2005. *In vitro* chromosome - doubling in tulip (*Tulipa gesneriana* L.). *HortScience Biotechnol.* 80:693–698.

Cheong, E. and M.R. Pooler. 2003. Micro propagation of Chinese redbud (*Cercis yunnanensis*) through lateral bud breeding and induction of adventitious shoots from leaf pieces. *In Vitro Cellular and Developmental Biology - Plant*. 39:455–458.

Cordeiro, G.M., G.O. Taylor and R.J. Henry. 2000. Characterisation of microsatellite markers from Sugarcane (*Saccharum* sp.) a highly polyploid species. *Plant Science*. 155(2) 161-168.

Corey, M.A. 2000. *Evolution and the problem of natural evil*. New York: University Press of America.

Decroocq, V., L. Hagen, M. Fave, J. Eyquard and A. Pierronnet. 2004. Microsatellite markers in the hexaploid *Prunus domestica* species and parentage lineage of three European plum cultivars using nuclear and chloroplast simple-sequence repeat. *Molecular Breeding*. 13(2): 135-142.

Derman, H. 1940. Colchicine polyploidy technique. *Botanical Reviews*. 6:599-635.

Dirr, M.A. 1998. *Manual of woody landscape plants*: Their identification, ornamental characteristics, culture, propagation and uses. 5th edition. Stipes Publishing Co. Champaign, Illinois.

Dunn, B. L. and J.T. Lindstrom. 2007. Oryzalin-induced chromosome doubling in *Buddleja* to facilitate inter-specific hybridization. *HortScience.* 42(6):1326-1328.

Eeckhaut, T.G., S.P. Werbrouck, L.W. Leus, E.J. Van Bockstaele and P.C. Debergh. 2004. Chemically induced polyploidization in *Spathiphyllum wallisii* Regel through somatic embryogenesis. *Plant Cell Tissue Organ Culture.* 78:241–246.

Eustice, C. and R. Eustice. 2007. Colchicine: 10 things you should know. *About.com*.

Extension Toxicology Network, 2006. 24 December 2007. <http://extoxnet.orst.edu/pips/oryzalin.htm>.

Flow Cytometry. 2009. *Mosby's Medical Dictionary*. 8th edition. <http://medicaldictionary.thefreedictionary.com/flow +cytometry>.

Gallardo, M. H., W.J. Bircham, L.R. Honeycutt, R.A. Odeja and N. Kohler. 1999. Discovery of tetraploidy in a mammal. *Nature.* 401:341.

Grant, V. 1981. *Plant Speciation*. Columbia University Press, New York.

Greve, B G. Valet, A. Humpe, T. Tonn and U. Cassens. 2004. Flow cytometry in transfusion medicine: development, strategies and applications. T*ransfusion Medicine and Hemotherapy.* 31:152–161.

Hansen, A.L., A. Gertz, M. Joersbo and S.B. Andersen. 1998. Antimicrotubule herbicides for *in vitro* chromosome doubling in *Beta vulgaris* L. ovule culture, *Euphytica.* 101:231–237.

Hugdahl, J.D., and L.C. Morejohn. 1993. Rapid and reversible high-affinity binding of the dinitroaniline herbicide oryzalin to tubulin of *Zea mays* L. *Plant Physiology*. 102:725–740.

Kato, A. and J.A. Birchler. 2006. Induction of tetraploid derivatives of maize inbred lines by nitrous oxide gas treatment. *Journal of Heredity.* 97:39–44.

Kermani, M.J., V. Sarasan, A.V. Roberts, A. Yokoya, J. Wentworth and V.K. Sieber. 2003. Oryzalin-induced chromosome doubling in *Rosa* and its effects on plant morphology and pollen viability. *Theory of Applied Genetics*. 107:1195–1200.

Kehr, A.E. 1996. Woody plant polyploidy. *American Nurseryman*. 183:38-47.

Kihara, H. and K. Tsunewaki. 1960. Production of polyploid wheat by nitrous oxide. *Proceedings Japanese Academy Science*. 36:658–663.

Kitamura, S., M. Akutsu and K. Okazaki. 2009. Mechanism of action of nitrous oxide gas applied to as a polyploidizing agent during meiosis in lilies. *Sex Plant Reproduction.* 22:9-14.

Lehrer, J. D., M. Band and J. Lubell. 2008. Induction of tetraploidy in meristematically active seeds of Japanese barberry (*Berberis thunbergii* var. *atropurpurea*) through exposure to colchicine and oryzalin. *HortScience.* 119:67-71.

Leitch, I.J. and M.D. Bennett. 1997. Polyploidy in angiosperms. *Trends In Plant Science.* 2:470–476.

Levin, D.A. 1983. Polyploidy and novelty in flowering plants. *American Naturalist*. 122: 1-25.

Linsmaier, E.M. and F. Skoog. 1965. Organic growth factor requirements of tobacco tissue cultures. *Physiol Plant* 18: 100-127.

Love, S. L., B. B. Rhodes and P. E. Nugent, 1986. Controlled pollination transfer of a nuclear male sterile gene from a diploid to a tetraploid watermelon line. *Euphytica.* 35, 633–635.

Masterson, J. 1994. Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. *Science*. 264: 421-423.

McCarthy, E.M. 2008. Macroevolution – The origin of new life forms. Appendix E: H. J. Muller and Polyploidy. <http://www.macroevolution.net/muller.html>.

McGregor, C.E., C.A. Lambert, M.M. Greyling, J.H. Louw and L. Warnich, 2000. A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) in tetraploid potato (*Solanum tuberosum* L) germplasm. *Euphytica*. 113: 135–144.

Morrissette, N. S., A. Mitra, D. Sept and L.D. Sibley. 2004. Dinitroanilines bind alpha-tubulin to disrupt microtubules. *Molecular Biology Cell.* 15: 1960-1968.

Olsen, R. 2006. Utilizing polyploidy for developing improved nursery crops: restoring fertility in wide hybrids, limiting fertility of invasive species, embryo culture of triploid, pest resistance, and inheritance of ornamental traits., (*PhD dissertation* North Carolina State University).

Otto, S.P. and J.Whitton. 2000. Polyploid incidence and evolution. *Annual Review of Genetics*. 34:401–437.

Pelletier, P.S. and J.Caventon. 1820. *Chim. Phys*. 14:69.

Petersen, K.K., P. Hagberg and K. Kristiansen. 2002. Plant *in vitro* doubling of *Miscanthus sinensis* A. *Plant Breeding.* 121:445-450.

"-ploid." *Merriam-Webster Online Dictionary*. 2009. Merriam-Webster Online. 1 July 2009 <http://www.merriam-webster.com/dictionary/-ploid>.

Poehlman, J.M. and D.A.Sleper. 1995. *Breeding Field Crops*, Iowa State University Press/Ames, 4th edition.

Pryor, R.L. and L.C. Frazier. 1968. Colchicine induced tetraploid azaleas. *HortScience.* 3:282-286.

Rahman, M. 2006. Introduction to flow cytometry. Serotec Ltd. Endeavour House. Oxford, UK.

Ranney, T.G. 2007. Polyploidy: from evolution to landscape plant improvement. <http://www.ces.ncsu.edu/fletcher/programs/nursery/metria/metria11/ranney/polyploidy.htm>.

Ranney, T.G. and J.R. Jones. 2008. Understanding Polyploidy: Insight into the evolution and breeding of azaleas. *The Azalean.* 81-84.

Sanders, L, J.J. Hoover and K.J. Killgore. 1991. Triploid grass carp as a biological control of aquatic vegetation. Aquatic plant control research program A-91-92. *U.S. Army Corps of Engineers Waterways Experiment Station*.

Schuettpelz, E., A.L. Grusz, M.D. Windham and K.M. Pryer. 2008. The utility of nuclear gap Cp in resolving polyploidy fern origins. *Systematic Botany.* 33:621-629.

Seddon, W.L. 1997.Mechanisms of temperature acclimation in the channel catfish *Ictalurus punctatus*: Isozymes and quantitative changes. *Comparative Physiology and Biochemistry.* 118a:813–820.
Soltis, P.S. and D. E. Soltis. 2000. The role of genetic and genomic attributes in the success of polyploids. ONAS. 97: 7051-7057.

Southern Agriculture Insecticides Inc.1998. Surflan® A.S. *Pre-emergent Herbicide Label*. Palmetto, Fl.

Stapczynski, J.S., R.J. Rothstein , W.A. Gaye and J.T. Niemann. 1981. Colchicine overdose: report of two cases and review of the literature. *Annuals Emergency Medicine*. 10(7): 364-369.

Stebbins, L. 1971. *Chromosomal evolution in higher plants*. London: Edward Arnold Ltd Publisher.

Storchova, Z. and D. Pellman. 2004. From polyploidy to aneuploidy, genome instability and cancer. *Nature Reviews Molecular Cell Biology*. 5:45–54.

Suomalainen, E. 1962. Significance of parthenogenesis in the evolution of insects. *Annual Review Entomology*. 1:349–366.

Tao, R., M. Gao, T. Esumi, Y. Kitamura and A. Yamada. 2009. High frequency ploidy variation observed in seedlings of a hexaploid persimmon cultivar 'Fujiwaragosho'. *Acta Horticulture*. 833:131-134.

Tavaud, M., A. Zanetto, J.L. David, F. Laigret, and E. Dirlewanger. 2004. Genetic relationships between diploid and allotetraploid cherry species (*Prunus avium*, *Prunus* × *gondouinii* and *Prunus cerasus*). *Heredity.* 93:631-638.

Udall, J and J. Wendel. 2006. Polyploid and crop improvement. *The Plant Genome: Review and Interpretation* (supplement to *Crop Science*)*.* 1:S3–S14.

Van Tuyl, J., M. B. Meijer and M .P. van Din. 1992. The use of oryzalin as an alternative for colchicine in *in vitro* chromosome doubling of *Lilium* and *Nerine*. *Acta Horticulture.* 325: 625-630.

Wright, J. W. 1944. Genotypic variation in white ash. *Journal of Forestry.* 42:489-495.