ABSTRACT

OLSEN, RICHARD THOMAS. Utilizing Polyploidy for Developing Improved Nursery Crops: Restoring Fertility in Wide Hybrids, Limiting Fertility of Invasive Species, Embryo Culture of Triploids, Pest Resistance, and Inheritance of Ornamental Traits. (Under the direction of Thomas G. Ranney.)

Multiple projects were conducted to investigate the potential for developing a breeding program utilizing species of *Catalpa* Scop. and *Chilopsis* D. Don. The efficacy of oryzalin was evaluated for inducing polyploidy and restoring fertility in the sterile, intergeneric hybrid ×*Chitalpa tashkentensis* Elias & Wisura [*Catalpa bignonioides* Walt. x *Chilopsis linearis* (Cav.) Sweet] 'Pink Dawn'. Submerging meristems in 150 μM oryzalin for up to 24 hours was effective at inducing tetraploids and cytochimeras. Pollen from the diploid cultivar was non-viable, but pollen from the polyploid stained and germinated as well as pollen from progenitor taxa. Polyploid ×*Chitalpa* were self-compatible yielding tetraploids when self pollinated and triploids when crossed with *C. bignonioides*, but reciprocal crosses with *Chilopsis* taxa failed. To increase recovery of triploids, we investigated germination of ovules and embryos at various harvest dates on Schenk and Hildebrandt (SH) basal salts supplemented with various medium components. Germination of triploid [(polyploid ×*Chitalpa*) x *C. bignonioides*] and tetraploid (selfed polyploid ×*Chitalpa*) embryos was greatest at 7 weeks after pollination on SH with 20 g·L^{-1} sucrose and ≥ 1 μM gibberellic acid (GA$_3$). Triploids [*Chilopsis linearis* x (polyploid ×*Chitalpa*)] germinated < 5%. Additional studies were conducted to screen diverse taxa for resistance to powdery mildew (PM), *Erysiphe elevata* (Burr.) U. Braun & S. Takam, and catalpa sphinx larvae (CSL), *Ceratomia catalpae* (Boisduval). Twenty-four taxa from *Catalpa* (section *Catalpa* Paclt and *Macrocatalpa* Grisebach),

Chilopsis, and ×*Chitalpa* were screened in 2004-05 for susceptibility to PM. Disease incidence and severity were recorded to calculate area under the disease progress curves (AUDPC) for each year. North American *Catalpa* spp. in sect. *Catalpa*, *Chilopsis,* and ×*Chitalpa* taxa were all moderate to highly susceptible to PM. Chinese *Catalpa* spp. in sect. *Catalpa* and West Indian sect. *Macrocatalpa* were resistant to PM. Hybrids among North American and Chinese *Catalpa* spp. in sect. *Catalpa* varied in susceptibility, indicating inheritance of partial resistance to PM. A no-choice feeding study conducted with CSL in 2005 found no differences in survival or growth of larvae reared on taxa from both sections of *Catalpa, Chilopsis,* and ×*Chitalpa.* Future breeding of ×*Chitalpa* can utilize the identified sources of resistance for PM; however, a source of resistance to CSL was not found. Triploids are generally infertile and may be deployed by breeders to limit invasive potential of introduced ornamentals. However, inheritance of ornamental traits can be complex at higher ploidy levels. Inheritance of two mutant foliage types, variegated and purple, was investigated for diploid, triploid and tetraploid tutsan (*Hypericum androsaemum* L.). Fertility of progeny was evaluated with pollen viability tests, percent fruit set, and germinative capacity of seed from specific crosses. Segregation ratios were determined for diploids in reciprocal di-hybrid F_1, F_2, BC_{1P1}, and BC_{1P2} families and selfed F_2s with the parental phenotypes and triploid and tetraploid F_2s. Diploid di-hybrid crosses fit the expected 9:3:3:1 ratio for a single, simple recessive gene for both traits, with no evidence of linkage. A novel phenotype representing a combination of parental phenotypes was recovered. Data from backcrosses and selfing also supported the recessive model. Both traits behaved as expected at the triploid level; however, at the tetraploid level the number of variegated progeny increased, with

segregation ratios between random chromosome and random chromatid assortment models. We propose the gene symbol *var* (*variegated*) and *pl* (*purple leaf*) for the variegated and purple alleles, respectively. Triploid pollen stained moderately well, but pollen germination was low. Triploid plants demonstrated extremely low male fertility and no measurable female fertility (no viable seed production). Research presented herein demonstrates the feasibility of manipulating ploidy levels for breeding desirable ornamental traits including non-invasiveness.

UTILIZING POLYPLOIDY FOR DEVELOPING IMPROVED NURSERY CROPS:
RESTORING FERTILITY IN WIDE HYBRIDS, LIMITING FERTILITY OF INVASIVE
SPECIES, EMBRYO CULTURE OF TRIPLOIDS, PEST RESISTANCE, AND
INHERITANCE OF ORNAMENTAL TRAITS

by

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A dissertation submitted to the Graduate Faculty of
North Carolina State University
In partial fulfillment of the
Requirements for the degree of
Doctor of Philosophy

HORTICULTURAL SCIENCE

Raleigh, NC

2006

APPROVED BY:

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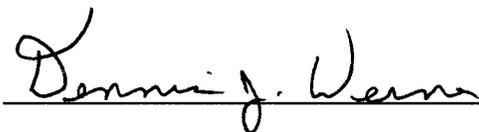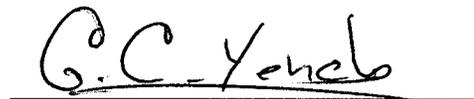
Dr. Dennis J. Werner   Dr. G. Craig Yencho

Dr. David A. Danehower

DEDICATION

J.C.

Thank you for your erudition, encouragement, and exemplary dedication to public

horticulture. Generations of horticulturists, gardeners, and an unknowing public are in your

debt. May your spirit live on in those blessed to have known you and who are still

"planning and planting for a better world."

Dr. James "J.C." Chester Raulston, 1940 -1996

professor, advisor, mentor

Director, NCSU Arboretum

BIOGRAPHY

Richard Thomas Olsen was born eight minutes after his bigger, stronger, and more intelligent fraternal twin brother, Michael Andrew, on April 21, 1975 in Milwaukee, Wisconsin. After five years in the frigid north, the Olsen family (father, Theodore; mother, Janice; eldest son, Scott; eldest twin, Michael; younger twin, Richard; and daughter, Jill) moved to Raleigh, North Carolina. Besides a brief move to Sanford, N.C., Raleigh would be home for the remaining formative years. Sports and camping were favorite activities as a youth, with most summers spent out of doors exploring, hiking, backpacking, and camping, manifesting ultimately in the rank of Eagle Scout in Boy Scouts of America in 1992.

Richard met Erin Kath his sophomore year of high school, and finally asked her out a year later; their first date was his junior prom. Both were excellent soccer players, who happened to both be goalies, and wear #15. A brief stint of studying international relations and playing soccer at American University gave Richard a taste for Washington, D.C. and travel, but not for politics. Richard transferred to NC State University in August of 1994 intent on enrolling at the School of Design in Landscape architecture, a career that would combine his love of outdoors and art. However, after his first plant identification class, he realized his passion lay with ornamental plants and public horticulture, and he remained in Horticultural Science, graduating magna cum laude in 1998 with a Bachelor of Science degree in Horticultural Science and a minor in Botany. Seminal events during his undergraduate career were employment at the then NCSU Arboretum with Dr. J.C. Raulston (1995-97) and an internship at Heronswood Nursery in Kingston, Washington with Dan Hinkley (1997).

A year was spent working on his own as a landscape designer and gardener after graduation, enabling him to travel for two months in Europe with college friends and Erin. A two-week trip visiting gardens and arboreta in Scotland and England with his good friend and plantsman Todd Lasseigne was squeezed in just before going back to school.

In August of 1999, Richard enrolled at the University of Georgia to pursue a Master of Science degree in Horticulture under the guidance of Dr. John Ruter, and indirectly, Dr. Michael A. Dirr. The research was focused on plant stress physiology, specifically, photosynthetic responses of container-grown *Illicium* species to varying light levels and nitrogen nutrition. Erin followed him to Athens, Georgia, and in 2000 the high school sweethearts married, after nine years of "courtship." After turning in his thesis in 2001, Erin and Richard took a delayed honeymoon to Peru to visit Macchu Picchu, a month before he would begin his doctoral research.

Richard returned to NC State University to pursue his doctoral degree in plant breeding under the tutelage of Dr. Thomas G. Ranney at the Mountain Horticultural Crops Research and Extension Center, Fletcher, N.C. Richard split his time between classes and teaching assignments in Raleigh with research in the mountains. Four years and countless classes, meetings, miles, and presentations later, Richard is set to earn his doctorate at the very institution where his passion for horticulture, plants, and research was ignited. Upon completion of his Ph.D., Richard will begin his career with the United States Department of Agriculture-Agricultural Research Service, as research geneticist in charge of the urban tree breeding program at the U.S. National Arboretum in Washington, D.C.

ACKNOWLEDGMENTS

student into their home without any expectations. Your compassion and self-sacrifice are both saintly and heroic. You made the last two summers bearable for Erin and me, and we are forever in your debt.

And back to Raleigh and the main campus… Returning to NC State after my stop at Georgia reunited me with individuals in the department who had significant impact on my career as an undergraduate and showed renewed interest and support during my doctoral training. Without Bryce Lane's intervention or Will Hooker's mentoring, I would very likely not be in horticulture, let alone pursuing a doctorate. Tracy Traer, now retired, was responsible for capturing my personality in one word that brought my life into focus: *cavalier*. It sounds silly how powerful one word can be when said from someone you admire. Thank you. To Denny Werner, you have had a model career and at a time when you could be retiring you have moved on to a new challenge as Director of the J.C. Raulston Arboretum, a fitting position for one so dedicated to horticulture and the well being of our society; the arboretum could not be in better hands and I anticipate great things. To Dr. Yencho and Danehower, thank you for serving on my committee, although I feel I have failed to utilize fully the breadth of your knowledge and brilliance. To Rachel McLaughlin, the one person who I had to see everyday or it was not complete; your pragmatism brought sanity and normalcy to all around you at your own expense, reminding me always to be thoughtful and human! I have not forgotten the other professors, faculty, and staff and again to all, I say thank you.

To past and fellow graduate students. To those before me, thank you for showing me the way: Todd Lasseigne, your enthusiasm and passion for plants was inspiring, your friendship honored; Jeff Adkins, you beat me by two years, both your grit and commitment

were commendable; Andy Bell, your honor and integrity was heartening, your continued

friendship a blessing. To those of you in the hunt, you are almost there! Jim Owen, I'll be

seeing and collaborating with you, you are my twin spirit of the nursery substrate world. To

Ryan Contreras, watching you mature and come into your own makes me want to mentor

graduate students. You are a friend and colleague who will one day surpass me in breeding

and genetic accomplishments and I look forward to it. To the rest of the graduate students I

wish you all the best and bid *adieu* and *adios* (Brian you inherit my bookshelf).

I feel like I am hitting my stride for the homestretch. If it is not apparent by now, I

have been influenced and inspired by a number of individuals in my career, and through

serendipity, luck, fate, or destiny, I have managed to be in the right place at the right time.

Mark Starrett, professor at the University of Vermont, in 1994 taught my first plant

identification class when he was a doctoral student, but taught as if a seasoned professor.

From that point forward he has cheered, often behind the scenes and unbeknownst by me,

and championed for my continued success. I value your advice both professional and

personal and you are a dear friend.

It was no secret that I had told my friends that I would not return to NC State for my

doctorate unless I could work with Tom Ranney, for my early schooling and interests lay

with plant stress physiology, and he is one of the best. One phone call and conversation

later, I was packing my bags and returning to NC State and went from screening plants for

stress tolerance, to breeding them. From the beginning Tom called me a colleague, which

when I let you down meant I could not look you in the eye, which was all too often.

However, on the occasions I have met or exceeded expectations I was truly lifted in spirit.

Thank you for stimulating scientific discourse and expert mentoring. You welcomed me

both into your career and family, and for that I have been deeply touched. Cool summer evenings drinking beer in the company of friends at the Ranney house will not be long forgotten.

Professional and personal families seem to blur in horticulture, but my wife's family and my immediate family deserve the final praise and acknowledgements for my accomplishments. Moving back to Raleigh meant being near our families, and during the tumultuous path of a doctoral degree, a luxury. We lived briefly with, and vicariously through, Sarah and Brendan Jones, Erin's sister and brother-in-law. Thank you for being a safety valve for Erin and me to vent, and support for Erin while I was away each summer. It will be a shock when Erin and I can't stop by on a whim simply to say hi to you or the dogs or now, our niece. For the past three years we have lived in a 480 square foot loft/guesthouse/tree house on Erin's parent's property. A thirty year old living with his in-laws was the epitome of yin and yang; full of compromises and sacrifice on both sides, and I am forever in debited to Dann and Donna Kath for their undying support and self-sacrifice to ensure their daughter and her husband had some semblance of a normal life during this degree. To Dann, you put two and two together a long time ago, and suggested I think about landscape architecture, a decision that lead me back to NC State and where I am now.

My family has always valued education, between my parents and siblings there are ten college degrees. My mother's parents, Margaret and Allan Beaumont, provided encouragement and inspiration, and the "Beaumont scholarship" for the grandkids during our undergraduate degrees. Getting a monthly check in college from your grandparents was not as important as their words of advice or love and support. Thank you for all you have done since 1975. To Margie, my father's sister, thank you for taking a post-doc at UNC

where our family visited when I was a young boy, allowing me a first glimpse of academia and the nature of science! You don't get to this point in your education and soon to career, and not wonder why or how you made the journey. Camping with my brothers and father fostered a deep and profound love of the outdoors, the environment, and an ecological conscience. I know for a fact that I am at this junction because of my father's interest and participation in my life, and words cannot relay my love and appreciation. What do you say to the woman who brought you into the world? Memories of planting rhubarb in Wisconsin and tomatoes in North Carolina before I knew Latin or photosynthesis form the foundation for all that I have learned. I love you and will miss using gardening as an excuse for a son to spend time with his mother. To my brothers, Scott and Michael, thank you for being the brilliant big brothers that I looked up to, and Jill, thanks for the being the little sister I looked out for.

I am now five pages into my acknowledgements, following an allegorical progression that has lead me to the penultimate acknowledgement. Erin, my wife, you were and are never last. You are what remain when my protective shells of professionalism, congeniality, and practicality are removed. You are, to paraphrase Ralph Waldo Emerson, part and participle of my soul and being. You fell in love with a long haired, carefree and cavalier high school soccer player who morphed into a balding, Latin spewing horticulturist with a passion for plants and making the world a better place. I know at times you have questioned, perhaps, what it is I love most. You are unequivocally loved above all else. You are the reason I strive to excel at all that I do. You have sacrificed and compromised your interests and goals for these past seven years, and from this point forward it is your, and of course our future family's, time… I love you.

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General Introduction

Polyploidy, the condition when an organism contains more than two complete sets of chromosomes, plays a major role in plant evolution and speciation (Leitch and Bennett, 1997; Masterson, 1994; Sparrow and Nauman, 1976; Wendel, 2000). Although rare in gymnosperms and cycads, polyploidy, or genomic duplication, occurs in nearly all extant species of ferns and flowering plants (Soltis et al., 2003). In nature, the fusion of unreduced gametes between different species (i.e., homoeologous chromosomes) can give rise to an allopolyploid with a full set of chromosomes from each parent, representing two different genomes. The fusion of unreduced gametes within a single plant species (i.e., homologous chromosomes) or spontaneous doubling of somatic cells in a meristem results in an autopolyploid, or a duplication of one genome. Both allopolyploids and autopolyploids may be reproductively isolated from their progenitor diploid species, which can result in abrupt speciation (Briggs and Walters, 1997).

Utilization of available germplasm pools as sources for novel traits and resistances for biotic and abiotic stresses often involves crossing distantly related species. Diploid wide hybrids are often sterile due to chromosomal and genic imbalances during meiosis and gamete production. However, induced allotetraploids exhibit varying levels of fertility due to the restoration of chromosome pairing and viable gamete production. During normal meiosis, synapsis of homologous chromosomes occurs during zygotene of prophase I, followed by condensation of chromatids (pachytene) and chiasmata formation during diplotene. By metaphase I, the bivalents are aligned on the spindle plate, with a balanced number of centromeres per pole. The bivalents separate and are pulled towards the poles during anaphase I. The production of chromosomally balanced haploid gametes in meiosis

II depends ultimately, upon proper pairing during prophase and metaphase I.   In wide

hybrids, chromosomal divergence between the two parental genomes may result in

incomplete pairing between chromosomes, or homoeologous pairing, rather than strict

pairing between homologues. If the parent genomes lack adequate homology, asynapsis

occurs, and chromosomes do not pair. However, it is likely that a combination of the

possibilities occurs, resulting in a mixture of univalents, bivalents, and multivalents at

metaphase I (Sybenga, 1992). Non-random distribution of univalents, and separation of

centromeres from multivalents, leads to uneven segregation at the first meiotic division,

chromosomal imbalance in the gametes, and abnormal or abortive tetrad development.

Induced polyploidy is a viable method for improving meiotic pairing and restoring fertility

in wide crosses (Hadley and Openshaw, 1980; Sybenga, 1992). By doubling the

chromosomes from a wide cross at the diploid level, each chromosome pairs with its

duplicate, complete synapsis followed by regular segregation occurs, and fertility can be

restored. In woody ornamental plants, induced tetraploids from diploid hybrids have

successfully been generated in *Bougainvillea* (Zadoo et al., 1975), *Camellia* (Ackerman and

Dermen, 1972), *Rhododendron* (Pryor and Frazier, 1968), *Rosa* (Byrne et al., 1996), and

Syringa (Rose et al., 2000).

Since the discovery of mitotic inhibition by colchicine, ploidy levels have been

manipulated, with varying levels of success, in a large number of agricultural and

horticultural crops (Hancock, 1997), often in an attempt to restore fertility in interspecific

hybrids or to induce ploidy as a genetic bridge for crossing between species of different

ploidy levels. Although colchicine is still used in tissue culture and some breeding programs

to induce polyploidy it has mostly been superseded by chemicals which are less toxic to

humans. By the 1970's, the mitotic inhibiting effects of several classes of herbicides were well established (Bartels and Hilton, 1973; Upadhyaya and Noodèn, 1977; Upadhyaya and Noodèn, 1980). In particular, dinitroaniline herbicides were found to interfere with the synthesis of microtubular proteins and the assembly of microtubules during mitosis (Bartels and Hilton, 1973). Oryzalin, [4(dipropylamino)-3,5-dinitrobenzenesufonamide], is a dinitroaniline herbicide used for pre-emergent control of annual grasses and broadleaf weeds. Oryzalin binds to tubulin heterodimers preventing polymerization of both mitotic spindles and cortical microtubules, leading to multi-nucleate and isodiametric cells (Vaughn and Lehnen, 1991). Brief exposure of meristematic cells to oryzalin results in a temporary loss of cell division, which is recovered upon removal from the chemical of the plant tissue.

Although induction of polyploids using mitotic inhibitors in vitro has been shown to be efficient and rapid, it is also costly and time consuming, requiring that protocols for tissue culturing the plants be first worked out. A system whereby vegetative meristems of asexually propagated plants or seedlings can be targeted directly, without the need for tissue culture would be highly desirable. The advent of flow cytometry, a rapid and efficient screen for identifying polyploids, has facilitated renewed interest in integrating ploidy manipulation into plant breeding programs. In this manner, the time necessary to produce fertile autopolyploids and allopolyploids can be shortened and new avenues of breeding may be elucidated.

In the United States, approximately 85% of invasive plant species were introduced for ornamental use (Reichard and White, 2001). This is alarming when one considers that horticulturists are often selecting for traits that risk assessment models consider intrinsic to invasive plants (White and Schwarz, 1998). These traits include rapid growth,

environmental fitness, early and abundant flowering and fruiting, and ease of propagation (White and Schwarz, 1998). Furthermore, many plants already considered invasive, or having the potential to be invasive, are currently being sold by the nursery industry.

The development of tetraploid plants would facilitate backcrossing to their diploid progenitors and may result in infertile triploid plants. In horticulture, the principle of sterile triploid plants has been used in the development of seedless fruit crops such as watermelons and bananas. In woody ornamental plants, there are limited examples where plant breeders have integrated sterile triploids into their programs. A notable exception is the development of triploid rose-of-sharon (*Hibiscus syriacus* L.) cultivars including 'Diana', 'Helene', 'Minerva', and 'Aphrodite' (Egolf, 1970; Egolf, 1981; Egolf, 1986; Egolf, 1988).

There are many opportunities for developing and utilizing polyploids in ornamental plant breeding programs. Significant opportunities include restoring fertility in wide hybrids that have proven themselves in the landscape, but that would benefit from further breeding to refine ornamental traits or introduce novel traits and pest resistance. Likewise, plant breeders can manipulate ploidy to develop highly infertile triploid cultivars to limit the invasive potential of ornamental plants in the landscape. This dissertation is presents the results of applying ploidy manipulation to address the above two issues (sterile hybrids and invasiveness) using two different model woody ornamental plants: ×*Chitalpa tashkentensis* Elias & Wisura and *Hypericum androsaemum* L. We also address the issues concomitant with increasing ploidy: crossability between ploidy levels and the presence of interploid blocks, rescue of triploid embryos using in vitro embryo culture, inheritance of desirable phenotypic traits across ploidy levels, and the fecundity of triploid progeny. In addition, we

began screening germplasm of *Catalpa* Scop*., Chilopsis* D. Don*.,* and ×*Chitalpa* for

resistance to two common pests of ×*Chitalpa*, powdery mildew and Catalpa sphinx moth

larvae, in order to initiate an ×*Chitalpa* improvement program.

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Chapter 1

Reproductive Behavior of Induced Allotetraploid ×*Chitalpa* and In Vitro Embryo Culture of

Polyploid Progeny

(In the format appropriate for submission to the

Journal of the American Society for Horticultural Science)

Reproductive Behavior of Induced Allotetraploid ×*Chitalpa* and In Vitro Embryo Culture of Polyploid Progeny

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Subject Category: Genetics and Breeding

Reproductive Behavior of Induced Allotetraploid ×*Chitalpa* and In Vitro Embryo Culture of Polyploid Progeny

Additional index words. *Catalpa bignonioides*, *Chilopsis linearis*, cytochimera, embryo rescue, flow cytometry, pollen viability, triploid block

Abstract. ×*Chitalpa tashkentensis* Elias & Wisura is a sterile intergeneric hybrid [*Catalpa bignonioides* Walt. × *Chilopsis linearis* (Cav.) Sweet]. To restore fertility in ×*Chitalpa* the following were evaluated: (1) oryzalin as a polyploidization agent, (2) fertility of induced polyploids, and (3) in vitro culture methods for embryo rescue of interploid crosses. Meristems of ×*Chitalpa* 'Pink Dawn' were submerged in aqueous solution of 150 μM oryzalin for 0, 6, 12, or 24 hours and ploidy analyzed via flow cytometry. As treatment duration increased, recovery of diploids decreased as mixoploids and shoot mortality increased. Two tetraploid shoots occurred in the 24-hour treatment. Four tetraploids and two cytochimeras were stabilized in total. Tetraploids flowered sparsely; however, cytochimeras flowered profusely and these were used to study fertility at the tetraploid level. Diploid ×*Chitalpa* 'Pink Dawn' pollen was essentially nonviable, but cytochimera pollen stained and germinated equal to or greater than pollen of *Catalpa bignonioides* and *Chilopsis linearis* 'Bubba'. Cytochimera ×*Chitalpa* were selfed yielding tetraploid seedlings, crossed with *C. bignonioides* to yield triploids, but failed in reciprocal crosses

with *Chilopsis* 'Bubba' and 'Burgundy Lace'. To increase recovery of triploids,

germination of triploid and tetraploid embryos was investigated, as either intact ovules or

excised embryos, on Schenk and Hildebrandt (SH) basal salts supplemented with sucrose at

20, 40, and 80 g·L^{-1}, presence or absence of 2% coconutwater, and gibberellic acid (GA$_3$) at

0, 1, 2, or 4 μM, and harvested weekly beginning 2 weeks after pollination (WAP).

Germination of triploids (cytochimera ×*Chitalpa* x diploid *C. bignonioides*) and tetraploids

(selfed cytochimera ×*Chitalpa*) were greatest with excised embryos at 7 WAP on SH

supplemented with sucrose at 20 g·L^{-1} and ≥ 1 μM GA$_3$. Germination of triploids (diploid

Chilopsis linearis x cytochimera ×*Chitalpa*) was < 5% at 4, 5, or 6 WAP on the same

medium as above. Oryzalin effectively induced polyploidy and restored fertility in

×*Chitalpa* 'Pink Dawn'. Successful crosses between hybrid and parental taxa of different

ploidy levels, coupled with embryo culture will facilitate a ×*Chitalpa* breeding program.

Chemical names used: 4(dipropylamino)-3,5-dinitrobenzenesufonamide (oryzalin).

In the early 1960s, F. N. Rusanov, of the Uzbek Academy of Sciences Botanical Garden, Tashkent, Uzbekistan (formerly the Republic of Uzbekistan, U.S.S.R) made a series of intergeneric crosses between species of *Catalpa* Scop. and C*hilopsis* D. Don. (Rusanov, 1964). *Chilopsis* is a monotypic genus, sister to *Catalpa* within the tribe Tecomeae Endl. of the Bignoniaceae Juss. Both genera share a base chromosome number, $n = 20$ (Goldblatt and Gentry, 1979) and are morphologically similar (Henrickson, 1985) including their pollen type (Gentry and Tomb, 1979) and self-incompatible breeding behavior (Petersen et al., 1982; Stephenson and Thomas, 1977). Reciprocal crosses were made between several species of *Catalpa* and the monotypic *Chilopsis*, resulting, ultimately, in describing and naming of one new hybrid species: ×*Chitalpa tashkentensis*, an intergeneric hybrid between *Catalpa bignonioides* and *Chilopsis linearis* (Elias and Wisura, 1991). According to Elias and Wisura, who translated from Rusanov (1971), the hybrids were sterile with pollen grains developing abnormally into pentads and hexads which contain uninuclear pollen and normally degenerate.

Sterility in wide hybrids is often a result of abnormal meiotic pairing between the divergent parental chromosomes, leading to both chromosomal and genic imbalances during chromosome segregation (Hadley and Openshaw, 1980; Rieseberg and Carney, 1998) and ultimately, abnormal or abortive gamete development. Inducing polyploidy is a viable method for improving meiotic pairing and restoring fertility in wide crosses (Hadley and Openshaw, 1980; Sanford, 1983). Mitotic inhibiting chemicals such as colchicine and the dinitroaniline herbicide oryzalin inhibit formation of the spindle apparatus during mitosis, allowing replication of DNA but preventing cell division (Bajer and Molè-Bajer, 1986; Bartels and Hilton, 1973; Vaughn and Lehnen, 1991). Several in vitro studies involving

diverse plant genera have shown that oryzalin is more efficient than colchicine at inducing

polyploidy (Ramulu et al., 1991; Tosca et al., 1995; Väinölä, 2000). Oryzalin uptake in

plant tissues is a rapid, nonactive process; its extremely lipophilic nature facilitates passive

absorption and diffusion into lipid components of cellular membranes with little

translocation (Upadhyaya and Noodèn, 1977; Upadhyaya and Noodèn, 1980). In actively

dividing cells, oryzalin binds to tubulin heterodimers, preventing polymerization of both

mitotic spindles and cortical microtubules, thus leading to polyploid cells (Morejohn et al.,

1987; Vaughn and Lehnen, 1991).

Induction of polyploidy in interspecific or intergeneric hybrids results in duplication

of the two genomes present in the hybrid and formation of an allopolyploid. The degree of

fertility in the ensuing allopolyploid is related to the amount of divergence between the

genomes of the parental taxa and the degree of homologous versus homoeologous pairing

(Gottschalk, 1978; Levin, 2002). Meiotic pairing may or may not be fully restored in

allopolyploids; however, they normally exhibit improved fertility over the original wide

hybrid. This allows for continued introgression of desired genes into the cultivated gene

pool. The technique has been used for the overall genetic improvement of a number of

important agronomic crops, such as cotton, *Gossypium* L. (Brubaker et al., 1999),

sunflowers, *Helianthus* L. (Jan and Chandler, 1989), various small fruit crops (Sanford,

1983), but only a few ornamental nursery crops, a notable exception being roses, *Rosa* L.

(Ma et al., 1997). For cultivated crops where germplasm encompasses several ploidy levels,

such as potato, *Solanum tuberosum L.* (Jackson and Hanneman, 1999), cotton (Brubaker et

al., 1999), and roses (Cole and Melton, 1986; Ma et al., 1997), inducing polyploids not only

restores fertility, but facilitates hybridization with other taxa at the same ploidy level

(intraploid). For predominantly diploid crops, the increase in ploidy may represent a hindrance as backcrosses to desirable parental taxa involve crossing between ploidy levels (interploid). The success of interploid crosses depends, in part, on the maternal:paternal genomic ratio in the developing endosperm, with crosses that violate a 2:1 maternal:paternal ratio often resulting in abnormal or abortive embryos according to the endosperm balance number theory (EBN) (Carputo et al., 1999; Johnston et al., 1980; Sanford, 1983).

Although fertile interspecific hybrids have been reported in *Catalpa* (Jones and Filley, 1920; Sargent, 1889; Smith, 1941), the intergeneric hybrid between *Chilopsis* and *Catalpa*, *×Chitalpa tashkentensis*, is sterile (Elias and Wisura, 1991). Sterility of the bi-generic cross prevents use of the hybrid in breeding programs to further combine desirable traits such as improved disease resistance, greater cold hardiness, and novel flower and foliage characteristics. Therefore, to initiate a breeding program for *×Chitalpa* our objectives were to 1) evaluate the efficacy of oryzalin to induce polyploidy in vegetative meristems of *×Chitalpa*, 2) evaluate fertility and crossability of induced allotetraploids, and 3) develop in vitro methods for recovering progeny from interploid crosses between *×Chitalpa, Catalpa,* and *Chilopsis*.

Materials and Methods

Inducing polyploidy. Thirty-two plants of *×Chitalpa tashkentensis* 'Pink Dawn' growing in 1-L containers were selected from stock material growing at the Mountain Horticultural Crops Research Station (MHCREC), Fletcher, N.C. in June 2002. Plants were grown in a substrate of 3 pine bark : 1 peat (by volume) amended with 2.8 kg·m^{-3} dolomitic

limestone and 0.5 kg·m^{-3} Micromax micronutrients (The Scotts Co., Marysville, Ohio).

Plants were transported to the laboratory and each plant was pruned to three actively

growing shoots and terminal leaves removed to expose apical meristems. Apical meristems

were submerged in beakers containing a saturated aqueous solution of 150 μM oryzalin

[0.004% v/v Surflan® (Dow AgroSciences, Indianapolis)] for one of four treatment

durations: 0, 6, 12 or 24 h. After treatment, plants were moved to the greenhouse, re-potted

into 7.6-L containers with the same substrate and top-dressed with 20 g/container 17N-7.4P-

14.1K Multicote controlled-release fertilizer (Vicksburg Chem. Co., Vicksburg, Mo.).

After growth resumed, DNA content and ploidy levels were determined via flow

cytometry (de Laat et al., 1987; Doležel, 1991; Galbraith et al., 1983). Nuclei isolation and

staining followed the protocols provided by Partec (Partec GmbH, Münster, Germany).

Approximately 0.5 cm^2 of newly expanded leaf tissue was chopped with a double-sided

razor blade in a petri dish containing 400 μL of extraction buffer (CyStain UV Precise P,

Partec). The suspension was filtered through 50-μm nylon mesh and nuclei were stained

using 1.6 mL staining buffer containing 4',6-diamidino-2-phenylindole (DAPI) (CyStain

UV Precise P, Partec). The suspension was analyzed using a flow cytometer with

fluorescence excitation provided by a mercury arc lamp (PA-I Ploidy Analyzer, Partec).

The mean fluorescence of each sample was compared with diploid ×*Chitalpa tashkentensis*

'Pink Dawn' and an internal standard of known genome size [*Pisum sativum* L. 'Ctirad', 2C

= 9.09 pg; (Doležel et al., 1998)]. A minimum of 4,500 nuclei were analyzed to calculate

the ratio of sample peak to the internal standard for determining genome size [2C pg =

(sample peak/internal standard peak) × 9.09 pg]. Sample ploidy levels were calculated by

dividing sample genome size by the diploid ×*Chitalpa tashkentensis* 'Pink Dawn' genome

size [ploidy = sample 2C pg/ 2C ×*Chitalpa tashkentensis* 'Pink Dawn' pg]. Peaks used for

calculating genome size and ploidy levels had coefficients of variation (cvs) ≤ 8%.

The experimental design was completely randomized with eight single plant

replicates and three sub-samples (shoots) per plant. Oryzalin efficacy was analyzed using

nonlinear and linear regression (PROC NLIN and PROC GLM; SAS version 8.02, SAS

Inst., Cary, N.C.). After initial treatments and testing, mixoploids and tetraploids were cut

back to the point of treatment, forcing new shoots from axillary buds. Ploidy levels for new

shoots were determined to chart the stability of the original mixoploids or tetraploids. This

cycle was repeated. Shoots were considered stabilized when every leaf on a stem and

subsequent shoots that arose from the axils exhibited the same ploidy level. Stem cuttings

from stabilized shoots were rooted and grown in a greenhouse under the same conditions as

the original plants.

Pollen viability. Diploid (2*x*), cytochimera (2*x* + 4*x*) and tetraploid (4*x*) ×*Chitalpa*

tashkentensis 'Pink Dawn', identified from oryzalin treatments, were grown along with

Chilopsis linearis 'Bubba' to flowering in greenhouses at MHCREC. All pollen for

viability tests were collected at anthesis from greenhouse plants with the exception of

Catalpa bignonioides, which was collected from naturalized trees growing near the research

station. Pollen viability was quantified using pollen staining and pollen germination tests

conducted during 2003 and repeated in 2004. Pollen grains were treated using Müntzing's

stain, 1 aceto-carmine (1%) : 1 glycerol, for 3 h. Stained pollen grains were scored as

viable. Pollen germination was quantified utilizing spot tests with Brewbaker-Kwack media

(Brewbaker and Kwack, 1963) supplemented with sucrose. In 2003, consistent pollen

germination occurred at 10% sucrose. In 2004, pollen germination was sporadic at 10%, so

sucrose concentration was increased to 15%. Pollen grains with pollen tubes greater than

one-half the diameter of the pollen grain after 8 h were scored as germinated. Pollen

staining and germination were observed using a compound light microscope (Micromaster,

Fisher Scientific, Pittsburgh) under ×100 and ×400 magnifications. Each pollen replicate

contained eight subsamples, with $\geq$ 100 pollen grains. Each test was a completely

randomized design with 2 replicates (years) × 4 treatments (taxa) × 8 subsamples. Data

were analyzed using PROC GLM (SAS) and means compared using Tukey's Honestly

Significantly Difference test with the experimentwise Type I error rate controlled at α =

0.05.

  Crossability studies. Female fertility was assessed using the same plant material as

for the pollen viability tests, with the addition of *Chilopsis linearis* 'Burgundy Lace'. For all

crosses, flowers were emasculated prior to anthesis. Stamens were collected, dried

overnight at 5 °C using indicator drierite (Drierite, Xenia, Ohio) and stored at 5 °C for use in

subsequent crosses. Stigmas were receptive to pollen when stigma lobes separated,

generally the afternoon after emasculation. Pollen was applied to stigmas using fine

brushes. *Catalpa* and *Chilopsis* stigmas are haptonastic, and close permanently when

pollinated with viable pollen grains (Newcombe, 1922; Petersen et al., 1982); ×*Chitalpa*

stigmas responded in a similar manner (personal observation). The number of pollinations

varied depending on the availability of flowers on female parents. Percent fruit set, average

seed per fruit, and seed germination were recorded for each cross. All seed were counted at

maturity, even seeds that appeared to contain aborted embryos, so as to elucidate triploid blocks. Seeds were surface-sown on a substrate of 1 peat : 1 vermiculite (by vol.) in flats and misted regularly until germination occurred in 1 to 4 weeks. Crosses were conducted in the greenhouses during each growing season from 2003-05.

In vitro ovule and embryo rescue. During summers 2004 and 2005, a series of experiments were designed using the above crosses to investigate optimal timing and medium components for ovule and embryo culture of ×*Chitalpa* hybrids to facilitate recovery of triploids (3*x*). Fruit collection and sterilization were the same for each experiment. Fruit were removed and brought to the laboratory, washed in dish-soap and water, stirring frequently for 5 to 10 min. Fruit were rinsed with running water and transferred to 70% ethanol for 2 min, rinsed with sterile distilled water, and further sterilized by submersion in a solution of 1.05% sodium hypochlorite with 2 to 3 drops of Tween 20 (polyethylene-20-sorbitan monolaurate, Fisher Scientific, Pittsburgh) for 30 min. Fruit were rinsed three times with sterile distilled water and ovules and embryos excised under aseptic conditions. Fruit collection and sterilization were the same for each experiment. The basic medium for each experiment included Schenk and Hildebrandt (SH) salts (S6765, Sigma-Aldrich Co., St. Louis) and vitamins (Beyl, 2000), pH adjusted to 5.7 prior to autoclaving, and solidified with agar at 7 g·L^{-1} (A6686, Sigma-Aldrich). Twenty-five millimeter petri dishes were used with ≈15 mL of medium added to each dish. Cultures were maintained at 25 °C under a 16 h photoperiod of 75 μmol·s^{-1}·m^{-2} photosynthetic active radiation (*PAR*) provided by cool-white fluorescent lamps.

Expt. 1. To test the effects of harvest date and the medium supplements coconut-water and sucrose on germination of triploid ($3x$) and tetraploid ($4x$) embryos, fruit were collected at 2, 3, 4, 5, or 6 weeks after pollination (WAP) from ($\times$*Chitalpa tashkentensis* $2x$ + $4x$) x *C. bignonioides* $2x$ and selfed $\times$*Chitalpa tashkentensis* $2x$ + $4x$, respectively. Intact ovules were excised and plated in petri dishes containing SH medium, with or without coconutwater (2%) (C-5915, Sigma-Aldrich), and one of three sucrose concentrations (20, 40 or 80 g·L^{-1}). Experimental design was a split plot with 2 (ploidy) $\times$ 2 (coconut-water) $\times$ 3 (sucrose) factorial main plot with WAP as the subplot, and five replicates (petri dishes) with six subsamples (ovules) per replicate and treatment combination. Germination percentage for each treatment combination was recorded 6 weeks after plating.

Expt. 2. At 6 WAP, expanding embryos were apparent with the naked eye, so a new study was initiated to investigate the effect of coconutwater and sucrose concentration on excised embryos at this single harvest date. Embryos were harvested from the same crosses and factorial treatment combinations as in Expt. 1, but with four replicates. Embryo germination was recorded 6 weeks after plating.

Expt. 3. The effect of gibberellic acid (GA$_3$) in promoting germination of embryos was tested using embryos from both crosses at 7 WAP. Embryos were excised and plated in SH medium supplemented with sucrose at 20 g·L^{-1} and 0, 1, 2, or 4 μM GA$_3$. The GA$_3$ treatment consisted of six replicates (petri dishes) with six subsamples (embryos) per dish. Embryo germination was recorded 4 weeks after plating.

Expt. 4. To test the effect of harvest date on in vitro germination of $3x$ embryos from $2x \times (2x + 4x)$ cross, fruit were collected at 3, 4, 5, 6, or 7 WAP from *Chilopsis linearis*

'Bubba' 2*x* and 'Burgundy Lace' as 2*x* female parents and cytochimeric ×*Chitalpa*

tashkentensis (2*x* + 4*x*) as the male parent. Ovules were excised, torn in half longitudinally

to expose the developing embryo and plated on SH medium supplemented with sucrose at

20 g·L^{-1} and 1 μM GA$_3$. For each harvest date there were six replicates (petri dishes) with

eight subsamples (embryos) per replicate. Percentages of embryos producing callus and

germinating were recorded 6 weeks after plating. Callus production was recorded in this

experiment to note which treatments to pursue in future studies of somatic embryogenesis or

shoot organogenesis from 3*x* embryonic callus. Data for each ovule and embryo culture

experiment were analyzed separately according to individual experimental designs using

PROC ANOVA (SAS) with the appropriate error term for F tests.

Results

Inducing polyploidy. Duration of oryzalin treatment had a significant effect on the

percentage of diploids, mixoploids, and mortality of shoot meristems (Fig. 1). As treatment

duration increased, the percentage of diploids decreased exponentially. Reduction in diploid

shoots was concurrent with an increase in both mixoploids and shoot mortality. The

percentage of mixoploids demonstrated a quadratic response, increasing up to the 12-h

treatment. In the 24-h treatment, decreases in percent diploid and mixoploid shoots were the

result of increases in shoot mortality and induction of two tetraploid shoots. The absence of

tetraploid shoots in treatments other than the 24-h duration precluded regression analysis

and *t* tests for comparing this treatment with all other durations.

Flow cytometry provided a rapid and reliable screen for determining ploidy levels of treated shoots (Fig. 2A-B and D). DNA content for diploid ($2n = 2x = 20$) and tetraploid ($2n = 4x = 40$) ×*Chitalpa tashkentensis* were 2.27 ± 0.2 pg and 4.59 ± 0.2 pg, respectively. Of the original 96 shoots treated, 34% (33 of 96) were mixoploids and 2% (2 of 96) were tetraploids. These shoots were cut back to recover additional tetraploids from mixoploids or to ensure stability of non-chimeric tetraploids. After removing terminal shoots, new axillary shoots were screened for ploidy level. Of the original mixoploids, 45% (15 of 33) reverted back to diploids, 45% (15 of 33) remained mixoploid, and 9% (3 of 33) stabilized to tetraploids (data not shown). Of the two shoots identified as non-chimeric tetraploids from the original 24-h treatment, one reverted to the diploid state. The remaining mixoploids and tetraploids were cut-back a second time, again, in an effort to further isolate stable tetraploid shoots. After the second cut, 40% (6 of 15) of the mixoploids reverted to diploid shoots. However, a similar percent of shoots stabilized to tetraploids as in the first cut, 13% (2 of 15). One of the three tetraploids from the first cut-back reverted to a mixoploid. Ultimately, four shoots of the original treated meristems stabilized to tetraploid shoots: three stabilized from mixoploids and one induced directly from a diploid to a tetraploid (Fig. 2D). Of the remaining mixoploids, two stabilized as cytochimeras ($2x + 4x$) (Fig. 2B). Flow cytometry of stamens and petals of the cytochimeras indicated these tissues maintained the cytochimeric condition of the shoots ($2x + 4x$). Likewise, dehiscing anthers contained copious, bright yellow pollen, suggesting the L-II histogenic layer was tetraploid in these cytochimeras.

Pollen viability. Induced tetraploid plants were reluctant to flower, so the stable cytochimeras, which flowered regularly, were used for pollen viability tests. There was a

significant interaction between year and pollen germination (F value = 2.51; *P* = 0.052) as a result of moderate increases in pollen germination for all taxa except 2*x* ×*Chitalpa* in 2004 with 15% sucrose (data not shown). The random effect of year is not repeatable, and since rankings of germination percentages between taxa did not change, the data for the 2 years was combined for analysis of variance (ANOVA) [PROC GLM, TEST h = taxa e = year (treatment)]. There was a significant effect due to taxa on pollen staining (F value = 16503; *P* < 0.0001) and pollen germination (F value = 43.8; *P* = 0.0016). Pollen of diploid ×*Chitalpa tashkentensis* 'Pink Dawn' was essentially nonviable, with almost zero percent pollen staining and germination (Table 1). Pollen of cytochimera ×*Chitalpa tashkentensis* 'Pink Dawn' were highly viable, staining and germinating equal to, or greater than, the representative parental taxa *Catalpa bignonioides* and *Chilopsis linearis* 'Bubba' (Table 1).

Crossability studies. Controlled crosses from 2003-05 and resultant fruit set, average seed per fruit, and percent seed germination are presented in Table 2. Diploid ×*Chitalpa tashkentensis* 'Pink Dawn' failed to initiate fruit when self-pollinated, pollinated with *Catalpa bignonioides* or *Chilopsis linearis* 'Bubba', or when used to pollinate *Chilopsis linearis* 'Bubba'. However, our cytochimera ×*Chitalpa tashkentensis* 'Pink Dawn' was both male and female fertile, with fruit and viable seed developing when selfed or pollinated with *Catalpa bignonioides*. Selfed crosses yielded 50.5 seed per fruit which germinated at 80.7%. Seedlings were tested via flow cytometry and found to be tetraploid. Cytochimeric ×*Chitalpa tashkentensis* 'Pink Dawn' x *Catalpa bignonioides* resulted in 16.5% fruit set, 94.1 seed per fruit, and 19.5% germination. Flow cytometry of these seedlings showed that nearly all were triploids (Fig. 2C), although a few diploids and tetraploids were found (data

not shown), apparently the result of the rare production of a viable haploid ($n = 1x$) egg cells

in the cytochimera (for diploid seedlings) and unreduced pollen ($n = 2x$) from *Catalpa* (for

tetraploid seedlings). No viable seed formed when the cytochimera or tetraploid ×*Chitalpa*

tashkentensis 'Pink Dawn' were pollinated with diploid *Chilopsis linearis* 'Bubba'. The

reciprocal cross, *Chilopsis linearis* 'Bubba' x tetraploid ×*Chitalpa tashkentensis* 'Pink

Dawn', resulted in 21.1% fruit set, 42.7 seed per fruit, but essentially no seed germination

(0.01%) (Table 2). The seedling of the one seed that germinated was found to be diploid

(data not shown). Similar seed germination results occurred when *Chilopsis linearis*

'Bubba' or *C. linearis* 'Burgundy Lace' were pollinated with cytochimera ×*Chitalpa*

tashkentensis 'Pink Dawn': 12.8% fruit set, 28.0 seed per fruit, and 0% germination and

13.7% fruit set, 112.3 seed per fruit, and 0.3% germination (all diploid), respectively.

Chilopsis linearis 'Burgundy Lace' x 'Bubba' crosses resulted in 22.6% fruit set, 35.8 seed

per fruit, and 29.3% seed germination (Table 2).

   In vitro ovule and embryo rescue. Fruit from successful pollinations grew rapidly in

length up to 7 WAP, reaching 25 cm or more, and then leveled off until mature at 14-15

WAP (data not shown). Embryos were first visible with the naked eye at 6 WAP and

continued to grow, as measured transversally from cotyledon tip to tip, until 11 WAP.

Mature embryos from viable crosses were between 4 and 9 mm (data not shown).

   Expt. 1. No ovules germinated for either cross at 2, 3, or 4 WAP or in the sucrose

treatments of 80 g·L^{-1} (data not shown). Coconut treatment was not significant in combined

ANOVA analysis, so this treatment was excluded from the final ANOVA for main plot

factors [PROC GLM, TEST h=ploidy|sucrose e=rep (ploidy × sucrose)]. The main plot

factor ploidy was significant (F value = 48.38, $P < 0.0001$) as well as the interaction

between ploidy × sucrose × WAP (F value = 13.9, $P = 0.0005$) so data are presented for each

ploidy level, sucrose, and WAP (Table 3). Triploid ($3x$) ovules germinated only in the 6

WAP and sucrose treatment of 20 g·L^{-1}. Tetraploid ($4x$) ovules germinated at both 5 and 6

WAP and both levels of sucrose. At 5 WAP, highest germination of $4x$ ovules (30%)

occurred in the sucrose treatment of 40 g·L^{-1}. At 6 WAP, there was a reversal, with greatest

germination of $4x$ ovules (25%) in sucrose at 20 g·L^{-1}. Ovules germinating in the presence

of coconutwater had constricted, thickened roots with little secondary root development.

Expt. 2. Analysis of variance revealed a significant sucrose main effect (F value =

10.07, $P = 0.0003$) and coconutwater × ploidy interaction (F value = 6.39, $P = 0.016$) on

germination of excised embryos at 6 WAP. Sucrose was partitioned into linear and lack of

fit to linear components, with a significant linear effect (F value = 18.21, $P = 0.001$). Within

a ploidy level, percent germination decreased linearly as sucrose concentration increased

(Table 4). For $3x$ embryos, coconutwater had no effect on germination (8.3% vs. 7.0%,

absent versus present, respectively). The presence of coconut-water increased germination

from 62.5% to 83.3% for $4x$ embryos. However, those embryos germinating in

coconutwater had constricted, thickened roots as in Expt. 1.

Expt. 3. At 7 WAP, $3x$ and $4x$ embryos on SH medium with sucrose at 20 g·L^{-1}

germinated across all GA$_3$ concentrations (Table 5). Treatments were partitioned into absent

(0 μM GA$_3$) and present (1, 2, and 4 μM GA$_3$) for ANOVA analysis. The presence of GA$_3$

significantly increased germination across each ploidy level (F value = 2069.34, $P <$

0.0001), though perhaps to a lesser extent in the $4x$ embryos, which resulted in a significant ploidy $\times$ GA$_3$ interaction (F value = 10.65, $P < 0.0001$)].

Expt. 4. Callus production of triploid *Chilopsis* x (×*Chitalpa tashkentensis* $2x + 4x$) embryos was significantly affected by WAP (F value = 7.56, $P = 0.0004$), with 4, 5, or 6 WAP resulting in high callus production (Table 6). Weeks after pollination did not effect germination percent (F value = 0.97, $P = 0.4402$) in part due in part to low germination across three of the harvest dates (4, 5, or 6 WAP). Excised embryos at 7 WAP were visually smaller than those at 6 WAP, and appeared flat and desiccated.

Discussion

Oryzalin treatment of apical meristems of ×*Chitalpa tashkentensis* 'Pink Dawn' was effective at inducing tetraploid cells, albeit in the form of mixoploid or chimeric shoots (Fig. 1). The prevalence of mixoploids over non-chimeric tetraploids in our treatments is a typical response when inducing polyploidy. In *Solanum* spp., treating excised apical meristems of various dihaploid × diploid crosses and parental taxa with 28.8 μM oryzalin in vitro resulted in low tetraploid conversion rates (0 to 12%, taxa dependent) and a preponderance of mixoploid plantlets (Chauvin et al., 2003). Väinölä (2000) exposed microshoots of three different cultivars of *Rhododendron* to either 30 or 150 μM oryzalin for 24 or 48 h. The combined oryzalin treatments, resulted in 37% mixoploids and 8% tetraploids, which is similar to the 34% and 2% for mixoploids and tetraploids, respectively, reported herein. Optimal treatments for inducing polyploid shoots should approach 50% mortality (Sanford, 1983). Our efficacy data demonstrate that as shoot mortality increases

up to the 24-h treatment, the remaining shoots are predominantly mixoploid with a few non-chimeric tetraploids (Fig. 1). Since our shoot mortality in the 24-h treatment was approaching 40%, a treatment > 24 h may lead to increased incidence of tetraploid shoots. The prevalence of mixoploids can be attributed to gradations in mitotic activity within the apical meristem (Francis, 1997) such that some cells are cell-cycling faster than others and thus more susceptible to inhibition by oryzalin. Longer oryzalin treatments would ensure that a greater percentage of cells are exposed during cell-cycling. Furthermore, unless each apical initial cell in the meristem is doubled, the resulting shoot will be chimeric, displaying both diploid and tetraploid sectors (sectorial or mericlinal chimera). The ploidy level of leaves can be used as a surrogate for the ploidy level of axillary buds, since both share a common lineage with meristematic cells in the peripheral zone of the apical meristem (Tian and Marcotrigiano, 1994). Removing apical meristems forces axillary buds to break, and in a sectorial cytochimera, may result in axillary shoots comprised primarily of tetraploid cells. After two rounds of cutting back shoots and ploidy sampling, we were able to stabilize four non-chimeric tetraploids and two cytochimeras. Previous induction studies with rose (Kermani et al., 2003) and potato (Chauvin et al., 2003) have likewise required re-screening and propagation for stabilizing induced tetraploids.

Flow cytometry of leaf tissue of ×*Chitalpa* (Fig. 2A-D) permitted efficient screening and rescreening of ploidy levels. The flexibility of flow cytometry was further demonstrated by identification of the chimeral state of stamens and petal tissues in our cytochimeras. Since angiosperm stamens typically are comprised of two histogenic layers, an L-I epidermis and L-II gamete-producing internal layers (Goldberg et al., 1993), it was assumed

that our stable cytochimeras consisted of a 2*x* L-I epidermis and 4*x* L-II or LII-LIII histogenic layers.

Pollen grains in *Catalpa* and *Chilopsis* are united into tetrads with coarsely reticulate areoles (Gentry and Tomb, 1979). Both parental taxa exhibited high pollen viability and germination, although pollen viability may have been overestimated by the aceto-carmine stain (Table 1). In diploid ×*Chitalpa*, the pollen grains form highly variable polyads, but rarely, if ever tetrads (Elias and Wisura, 1991), indicating a high degree of sterility, which we confirmed using pollen viability and germination tests (Table 1). Cytochimera ×*Chitalpa* produced well-formed tetrads and pollen grains that stained and germinated in greater percentages than diploid ×*Chitalpa* and greater than or equal to the parent taxa, *Catalpa* and *Chilopsis* (Table 1). Because our non-chimeric allotetraploids were reluctant to flower, the cytochimeras became important for crossing studies, since for breeding purposes, they behaved primarily as allotetraploids (Pratt, 1983).

Concomitant with restoration of pollen viability was female fertility in the induced cytochimera and tetraploid ×*Chitalpa tashkentensis*. As suggested previously (Elias and Wisura, 1991), diploid ×*Chitalpa tashkentensis* 'Pink Dawn' is completely female sterile, which was confirmed by selfing and crossing with *Catalpa bignonioides* and *Chilopsis linearis* 'Bubba' (Table 2). Cytochimeric ×*Chitalpa tashkentensis* were selfed successfully and resulted in moderate seed production and germination. For viable crosses, fruit set never exceeded 25% of the flowers pollinated. In hand pollination experiments with *Catalpa speciosa*, successful pollinations decline after the first four flowers are successfully pollinated, with the earliest flowers having the greatest fruit set and later flowers functioning

as males only (Stephenson, 1979). Thus, in *Catalpa speciosa* and perhaps *Chilopsis* and

fertile *×Chitalpa,* fruit set is not limited by successful pollinations but appears to be

regulated by the plant (Stephenson, 1980). Furthermore, Stephenson (1982) found that fruit

set in natural populations of *Catalpa speciosa* over a 2-year period varied from 7% and

20%, which is within our fruit set percentages for controlled crosses in the greenhouse for

the various successful crosses. Both *Catalpa* and *Chilopsis* are self-incompatible (SI)

(Petersen et al., 1982; Stephenson and Thomas, 1977). Late-acting self-incompatibility

(LSI), a type of gametophytic self-incompatibility (GSI), is common in the Bignoniaceae

(Gibbs and Bianchi, 1999), and GSI is often overcome upon induction of polyploidy (Lewis,

1947). Although it is not possible to separate self-sterility from SI reaction in our original

diploid *×Chitalpa tashkentensis*, the appearance of self-fertility in the induced allotetraploid

×Chitalpa raises the question of overcoming SI in tetraploid forms of *Catalpa* and *Chilopsis*.

Cytochimeric *×Chitalpa tashkentensis* were crossed successfully with *Catalpa*

bignonioides, with fruit set similar to that from selfing, but twice as much seed per fruit

(50.5 vs. 94.1 seeds) developed and germination was only 19.5%. The recovery of triploids

in this cross demonstrates lack of a triploid block when a tetraploid female is pollinated by a

diploid male, as predicted by the endosperm balance number theory (EBN) (Johnston et al.,

1980). Pollination of cytochimeric *×Chitalpa tashkentensis* with *Chilopsis linearis* 'Bubba'

resulted in extremely poor fruit set with 60.0 seed per fruit that were not viable. This may

represent a pre-fertilization gametophytic incompatibility (e.g., failure of pollen germination

and pollen tube growth). When *Chilopsis linearis* 'Bubba' or 'Burgundy Lace' were

pollinated with cytochimeric and tetraploid *×Chitalpa tashkentensis*, fruit set ranged from

12.8% to 21.1% and average seed from 28.8 to 112.3 but again, no viable triploid seed was produced, as predicted by EBN. Seedlings recovered from these crosses were all diploid, evidence that triploid embryos triggered fruit set and maturation before aborting. This allowed for the recovery of diploid progeny that were the result of the rare production of viable pollen with a haploid chromosome number from the cytochimera and tetraploid plants (Sanford, 1983). Development of stamens involves contributions of all three histogenic layers (Dermen and Stewart, 1973), but sporogenesis originates and the anther sac is derived from the L-II layer. Chimeric plants are not completely stable, with frequent replacement or displacement of histogenic layers occurring from cells derived from adjacent layers. Our cytochimeric ×*Chitalpa tashkentensis* must have produced rare, viable haploid pollen from displacement of the tetraploid L-II with a diploid cell from L-I or L-III. Diploid pollen produced by the tetraploid L-II fertilized haploid eggs in the *Chilopsis,* forming triploid embryos which trigger fruit maturation, but which later abort, leaving only the rare, diploid embryo to develop. In the original sterile diploid ×*Chitalpa tashkentensis* 'Pink Dawn', viable pollen grains are only occasionally observed (Table 1). The one diploid seedling that resulted from the seed of *Chilopsis linearis* 'Bubba' crossed with our non-chimeric tetraploid again maybe the result of the rare production of haploid pollen grains from the induced tetraploid male parent.

In the present investigation, fruit of ×*Chitalpa tashkentensis* and *Chilopsis* grew rapidly in length the first 7 WAP then leveled off until maturation 14 or 15 WAP. Fruits of *Catalpa speciosa* accumulate dry matter and proteins slowly during the first 6 WAP, then rapidly for the next several weeks (Stephenson, 1980). Rapid cell division and elongation in

×*Chitalpa*, and the transition to dry matter and protein accumulation in *Catalpa speciosa*,
appear to coincide with growth of the embryos, which was first noted 6 WAP. Ovule and
embryo culture experiments one through three utilized selfed cytochimeric ×*Chitalpa*
tashkentensis and crosses with diploid *Catalpa bignonioides,* a cross that was subsequently
shown to be viable and lead to triploid progeny (Fig. 2C).

Previous research has demonstrated suitability of SH basal salts for
micropropagation of *Catalpa bignonioides* (Wysokińska and Światek, 1989) and *C. ovata*
(Lisowska and Wysokinska, 2000). In Expt. 1, tetraploid and triploid ovules harvested prior
to 5 WAP failed to germinate in our SH medium and tissue culture system. In ovule culture
may be inappropriate for heterotrophic embryos embedded in excess ovule tissue which may
impede uptake and translocation of vitamins and nutrients from the medium, although
embryo germination as high as 30% was observed. Five WAP for tetraploids and 6 WAP
for triploids, in ovule embryos may have developed sufficiently on the plant to reach a
developmental stage responsive to lower sucrose concentrations. There was a benefit of
higher sucrose for tetraploid ovules 5 WAP, but not at 6 WAP, and may also reflect
increased development of in ovule embryos, with higher osmolarity beneficial at a younger
developmental stage (Bridgen, 1994; Sharma et al., 1996).

In Expt. 2, embryos were excised 6 WAP. High sucrose concentrations inhibited
germination of excised embryos of ×*Chitalpa* and have been known to prevent precocious
germination of embryos in vitro (Sharma et al., 1996). Although coconutwater had no effect
on intact ovules, for excised embryos, there was a slight increase in germination for

tetraploid embryos but no effect on triploids. Germination in coconutwater or in sucrose at

80 g·L^{-1}, led to constricted, thickened roots, possibly an effect of higher osmolarity.

In Expt. 3, excised embryos at 7 WAP were treated with GA$_3$, a hormone that

promotes germination in tissue culture (Sharma et al., 1996). The presence of GA$_3$ from 1 –

4 µM significantly increased germination for triploids and tetraploids, indicating harvesting

embryos, independent of the ploidy level, at 7 WAP could lead to high germination rates in

vitro. These first three experiments demonstrated that germination of embryos from crosses

of ×*Chitalpa tashkentensis* (2*x* + 4*x*) x *Catalpa bignonioides* (2*x*) are possible as early 5

WAP for tetraploids, and 6 WAP for triploids, with high germination by 7 WAP for both

crosses. Seeds harvested from this cross germinated at 19.5%, indicating a triploid block

does not exist in this direction with these parents. The reciprocal cross, *Catalpa*

bignonioides (2*x*) x ×*Chitalpa tashkentensis* (2*x* + 4*x*), was not performed due to a lack of

flowering *C. bignonioides* at the MHCREC.

Early observations from pollination experiments indicated that a triploid block may

be present, as predicted by EBN theory, when diploid *Chilopsis* is pollinated by tetraploid

×*Chitalpa tashkentensis*. Using the media optimized for germination of viable tetraploid

and triploid embryos, we investigated timing of harvest of *Chilopsis* fruit, with the

hypothesis that our previous optimized SH medium was sufficient to rescue triploid embryos

with poor endosperm development as a result of violating the 2 maternal : 1 paternal genome

ratio in the endosperm (Expt. 4). We were able to recover triploid embryos at 4, 5, and 6

WAP, but germination rate was never > 5%. Abundant callus was produced up to 6 WAP,

facilitating future studies to investigate somatic embryogenesis and shoot organogenesis

from triploid callus as an alternative to rescuing intact triploid embryos. By 7 WAP, the

embryos appeared to be degenerating, with significantly lower callus production and no

germination was induced. Maximum embryo development, and hence optimal timing for

excising embryos, of *Chilopsis* (2*x*) **x** *×Chitalpa tashkentensis* (2*x* + 4*x*) was after 3 WAP

but before 7 WAP. Lack of seed germination from mature fruit of this cross, combined with

observations of degenerate embryos, indicates a triploid block exists in this direction. Late

abortion of embryos (i.e., embryos beyond the torpedo stage) is often the result of nutritional

imbalances or starvation of the embryo from poor endosperm development (Mont et al.,

1993), which itself is a result of violation of EBN (Carputo et al., 1999). Although

coconutwater and sucrose concentrations > 20 g·L^{-1} were not appropriate for in ovule

embryos and excised embryos from viable triploid crosses, they may be appropriate as a

source of unreduced organic nitrogen or increased osmolarity, respectively, for culturing

embryos with poor endosperm development from triploid crosses that violate EBN (Sharma

et al., 1996). Further studies will be needed to optimize our media for increasing

germination of embryos of *×Chitalpa* earlier than 7 WAP in crosses in the direction of the

triploid block.

Results herein demonstrate the feasibility of inducing polyploidy in vegetative

meristems of *×Chitalpa tashkentensis* and isolation and recovery of stable, allotetraploid

breeding clones. Discovery of self-fertility in allotetraploid *×Chitalpa* allows for production

of advanced generations with increased recombination between genomes of *Catalpa* and

Chilopsis. Introgression of desired traits from *Catalpa* is possible due to absence of a

triploid block when crossing with allotetraploid *×Chitalpa*. Failure of crosses between

allotetraploid ×*Chitalpa tashkentensis* and *Chilopsis* in either direction, either due to pollen-pistil interactions or a triploid block is an important note. Embryo rescue of these crosses were successful, though methods for increasing the cross successes are needed to increase the number of embryos for evaluation. Use of *Chilopsis* as a breeding parent is desirable owing to superior flower colors, foliage and plant habit, and tolerances to high temperatures and drought available within germplasm of *Chilopsis*. Integration of these traits can lead to development of novel, improved selections of ×*Chitalpa* that increases the palette of small trees tolerant of wide extremes in growing conditions present in urban environments.

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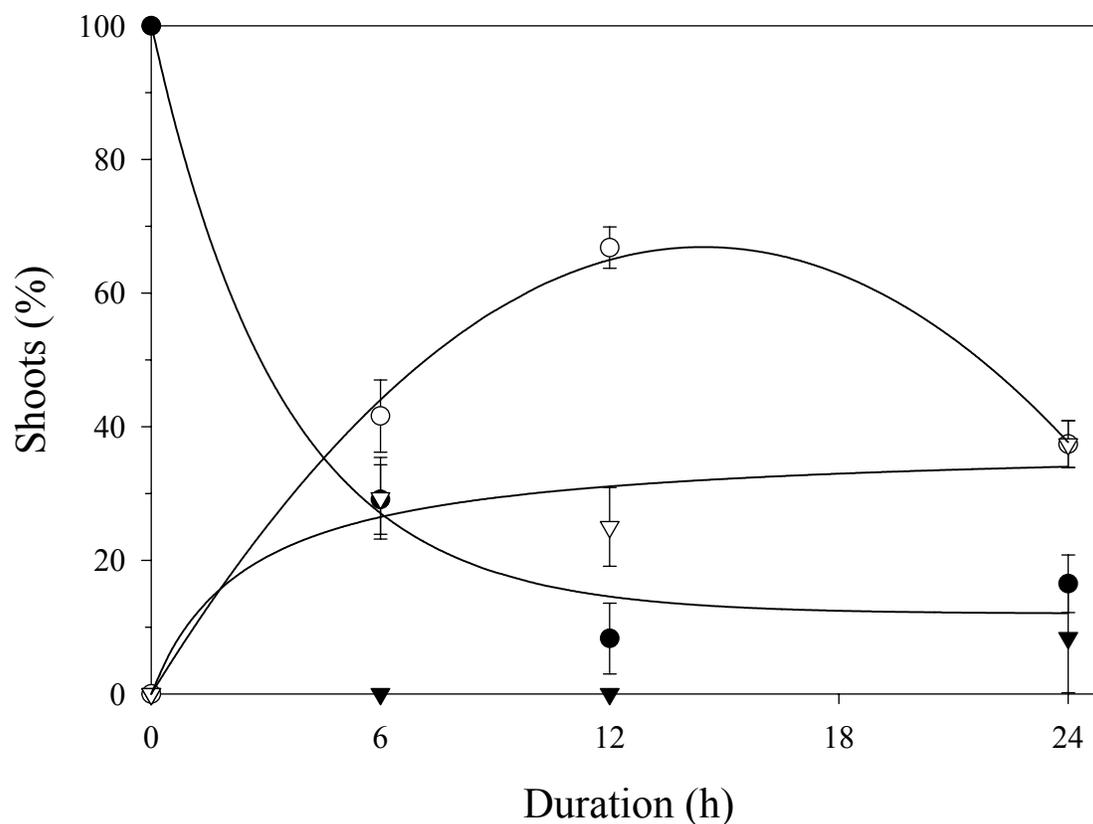
Fig. 1. Efficacy of a 150 μM oryzalin solution for inducing polyploidy in actively growing shoot meristems of ×*Chitalpa tashkentensis* 'Pink Dawn'. Symbols represent mean percentage of diploid (!), mixoploid (") and tetraploid (▼) shoots and shoot mortality (▽) as a function of treatment duration ± 1 SE.   Solid lines represent curves fitted using linear and nonlinear regression analysis: diploids (!) = 12.0 + 88.17exp [-0.29 (duration)], F value = 56.14, $P < 0.0001$; mixoploid (") = 9.26 (duration) – 0.32 (duration)^2, $r^2 = 0.79$; dead shoots (▽) = 37.57 (duration)/ (2.52 + duration), F value = 19.28, $P < 0.0001$. Two solid tetraploids (▼) were found at the 24-h treatment which was not sufficient for proper regression analysis. Each treatment consisted of eight single plant replicates and three subsamples (shoots) per replicate.
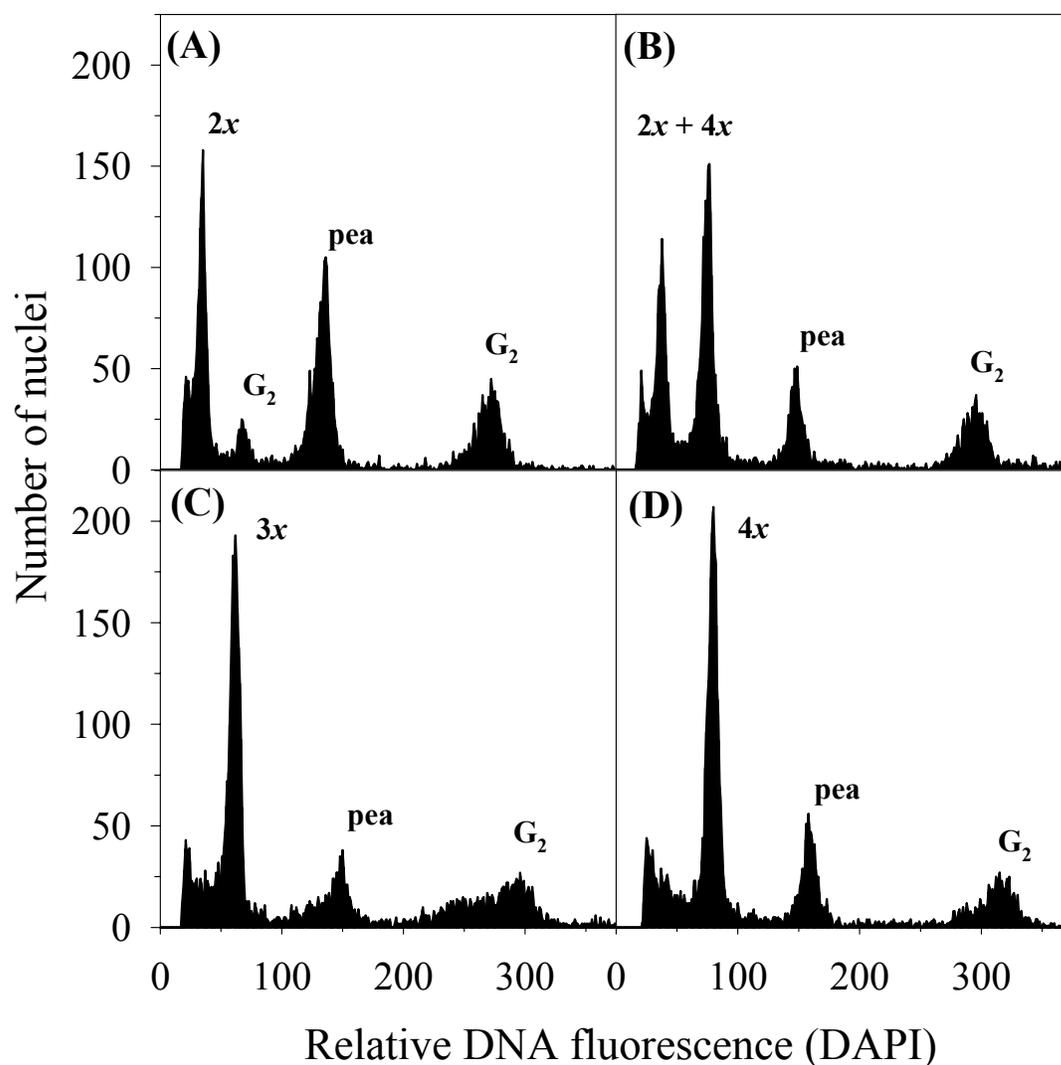
Fig. 2. Flow cytometry histograms of ×*Chitalpa tashkentensis* (A) diploid, (B) cytochimera, (C) triploid, and (D) tetraploid plants. Relative DNA content and ploidy levels were calculated from the ratio of sample peaks to that of the internal standard, pea (*Pisum sativum* 'Ctirad', 2C DNA content = 9.09 pg). Mode of diploid (A) 2*x* peak = 35, G_2 = 67, pea = 136; cytochimera (B) 2*x* peak = 38, 4*x* peak = 77, pea = 149; triploid (C) 3*x* peak = 62, pea = 150; and tetraploid (D) 4*x* peak = 80, pea = 158. Peaks beyond fluorescent channel 200 represent cell cycling (G_2) and sticky nuclei of the internal standard. Extracted nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI).

Table 1. Pollen viability tests for diploid ($2x$) and cytochimeric ($2x + 4x$) ×*Chitalpa tashkentensis* 'Pink Dawn' and representative diploid parental taxa, *Catalpa bignonioides* and *Chilopsis linearis* 'Bubba'.

Taxa	Ploidy	Pollen viability tests[z]	
		Staining (%)	Germination (%)
Catalpa bignonioides	$2x$	98.0 a[y]	62.3 b
Chilopsis linearis 'Bubba'	$2x$	94.9 b	73.8 ab
×*Chitalpa tashkentensis* 'Pink Dawn'	$2x$	0.8 c	0.04 c
×*Chitalpa tashkentensis* 'Pink Dawn'	$2x + 4x$	98.6 a	65.9 a

[z]Means are n = 2 with eight subsamples per replicate and ≥ 100 pollen grains counted per subsample for each test.
yMean separation within columns by Tukey's HSD$_{0.05}$.

Table 2. Fruit set and seed germination for controlled pollinations between diploid (2*x*), cytochimera (2*x* + 4*x*) and tetraploid (4*x*) ×*Chitalpa tashkentensis* 'Pink Dawn' and representative diploid parental taxa *Catalpa* and *Chilopsis*.

Maternal parent	Pollen source	Pollinations (no.)	Fruit set[z] (no.)	Fruit set[z] (%)	Seed Avg. per fruit[y]	Seed Germination (%)
×*Chitalpa tashkentensis* 'Pink Dawn' 2*x*	selfed	459	0	0	---	---
	Catalpa bignonioides 2*x*	619	0	0	---	---
	Chilopsis linearis 'Bubba' 2*x*	129	0	0	---	---
×*Chitalpa tashkentensis* 'Pink Dawn' 2*x* + 4*x*	selfed	581	118	20.3	50.5	80.7
	Catalpa bignonioides 2*x*	1390	229	16.5	94.1	19.5
	Chilopsis linearis 'Bubba' 2*x*	243	2	0.8	60.0	0.0
×*Chitalpa tashkentensis* 'Pink Dawn' 4*x*	*Chilopsis linearis* 'Bubba' 2*x*	43	0	0	---	---
Chilopsis linearis 'Bubba' 2*x*	×*Chitalpa tashkentensis* 'Pink Dawn' 2*x*	103	0	0	---	---
	×*Chitalpa tashkentensis* 'Pink Dawn' 2*x* + 4*x*	329	42	12.8	28.0	0.17[x]
	×*Chitalpa tashkentensis* 'Pink Dawn' 4x	57	12	21.1	42.7	0.01[x]
Chilopsis linearis 'Burgundy Lace' 2*x*	*Chilopsis linearis* 'Bubba' 2*x*	53	12	22.6	35.8	29.3
	×*Chitalpa tashkentensis* 'Pink Dawn' 2*x* + 4*x*	80	11	13.7	112.3	0.32[x]

[z]Includes immature fruit harvested for in vitro ovule and embryo experiments.
[y]Average seed per fruit harvested at maturity (15 weeks after pollination).
[x]Flow cytometry indicated these seedlings were diploid.

Table 3. In vitro germination for triploid (3*x*) and tetraploid (4*x*) ×*Chitalpa* ovules from ×*Chitalpa tashkentensis* 'Pink Dawn' (2*x* + 4*x*) x *Catalpa bignonioides* (2*x*) harvested 5 and 6 weeks after pollination (WAP) and plated at one of two sucrose concentrations.

Embryo ploidy	Sucrose[z] (g·L^{-1})	Germination (%)	
		5 WAP	6 WAP
3*x*	20	0.0	3.3 a[y]
	40	0.0	0.0 b
4*x*	20	5.0 b	25.0 a
	40	30.0 a	13.4 b

[z]Schenk and Hildebrandt basal salts and vitamins supplemented with sucrose as indicated, 0.7% agar and pH adjusted to 5.7.
[y]Within a ploidy level and WAP, mean germination (n = 5) between sucrose treatments are not significantly different according to F test's at *P* < 0.05.

Table 4. Effect of coconutwater and sucrose concentration on germination of ×*Chitalpa* embryos from ×*Chitalpa tashkentensis* 'Pink Dawn' ($2x + 4x$) x *Catalpa bignonioides* ($2x$) crosses harvested 6 weeks after pollination.

Embryo ploidy	Sucrose[z] ($g{\cdot}L^{-1}$)	Germination (%)	
		2% coconutwater	
		Absent	Present
$3x$	20	20.8[y]	16.7
	40	4.2	4.2
	80	0.0	0.0
Mean[x]		8.3 c	7.0 c
$4x$	20	83.3	91.6
	40	62.5	83.3
	80	41.7	75.0
Mean		62.5 b	83.3 a

[z]Schenk and Hildebrandt basal salts and vitamins, supplemented with sucrose as indicated, 0.7% agar, and pH adjusted to 5.7
[y]Significant linear effect for sucrose over each ploidy and coconut level (n = 4), according to partitioned sums of squares (F value = 18.21, $P = 0.0001$).
[x]Means (n = 12), summed over sucrose concentration, within rows or columns followed by the same letter are not significantly different at $P < 0.05$ based on least significant means pairwise comparisons.

Table 5. Effect of GA_3 on germination of
×*Chitalpa* embryos from ×*Chitalpa*
tashkentensis 'Pink Dawn' ($2x + 4x$) x
Catalpa bignonioides ($2x$) crosses harvested
7 weeks after pollination (WAP).

GA_3[z]	Germination (%)		
(μM)	$3x$	$4x$	Mean[x]
0	11.1[y]	30.6	20.8 b
1	100.0	100.0	
2	100.0	100.0	99.1 a
4	97.2	97.2	

[z]Schenk and Hildebrandt basal salts and
vitamins supplemented with GA_3 as indicated,
sucrose at 20 g·L^{-1}, 0.7% agar, and pH
adjusted to 5.7.
[y]Means presented as GA_3 treatment partitioned
as absence (n = 12) or presence (n = 36) of GA_3
(0 vs. 1, 2, or 4 μM, respectively) across ploidy
levels (F value = 2069, $P < 0.0001$).
[x]Means (n = 6) within a ploidy.

Table 6. Callus production and embryo germination for triploid ($3x$) ×*Chitalpa* embryos from *Chilopsis linearis* ($2x$) x ×*Chitalpa tashkentensis* 'Pink Dawn' ($2x + 4x$) crosses harvested 3, 4, 5, 6, or 7 weeks after pollination (WAP).

WAP[z]	Callus (%)	Germination (%)
3	47.9 bc[y]	0.0[x]
4	89.7 a	2.1
5	87.5 a	2.1
6	66.7 ab	4.2
7	25.0 c	0.0

[z]Schenk and Hildebrandt basal salts and vitamins supplemented with sucrose at 20 g·L^{-1}, 0.7% agar, 1 μM GA$_3$, pH adjusted to 5.7, and harvested when indicated.
[y]Means (n = 6) followed by the same letter are not significantly different according to Fisher's Protected least significance difference at $\alpha = 0.05$.
[x]WAP had no significant effect on germination (F value = 0.97, $P = 0.4402$).

Chapter 2

Susceptibility of *Catalpa*, *Chilopsis*, and Hybrids to Powdery Mildew and Catalpa Sphinx

Larvae

(In the format appropriate for submission to HortScience)

Susceptibility of *Catalpa*, *Chilopsis*, and Hybrids to Powdery Mildew and Catalpa Sphinx Larvae

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Research Report: Plant Pathology/Pest Management

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Additional index words. ×*Chitalpa tashkentensis*, *Macrocatalpa,* Tecomeae, Bignoniaceae, *Erysiphe elevata*, *Ceratomia catalpae,* disease resistance

Abstract. A diverse collection of germplasm representing 24 taxa from *Catalpa* sect. *Catalpa* Paclt and sect. *Macrocatalpa* Grisebach, *Chilopsis* D. Don*,* and ×*Chitalpa* Elias & Wisura were screened for susceptibility to powdery mildew (PM), *Erysiphe elevata* (Burr.) U. Braun & S. Takam, and feeding by catalpa sphinx larvae (CSL), *Ceratomia catalpae* (Boisduval). PM screening was conducted in 2004-05, with plants grown in a lathhouse (50% shade) in 2004, and a gravel pad (100% full sun) in 2005. The PM causal organism was identified as *Erysiphe elevata* both years. Disease incidence and severity were recorded at two-week intervals for six weeks and used to calculate area under the disease progress curves (AUDPC) for each year. North American *Catalpa* in sect. *Catalpa*, *Chilopsis,* and ×*Chitalpa* taxa were all moderate to highly susceptible to PM. Chinese *Catalpa* in sect. *Catalpa* and West Indian species in sect. *Macrocatalpa* were resistant to PM. Hybrids between North American and Chinese *Catalpa* in sect. *Catalpa* varied in susceptibility, indicating inheritance of partial resistance to PM. A no-choice feeding study conducted with CSL in 2005 found no differences in survival or growth of larvae reared on taxa from

Chilopsis, ×*Chitalpa,* or either section of *Catalpa.* Future breeding of ×*Chitalpa* can utilize

identified sources of PM resistance, but a source for resistance to CSL was not found.

The genus *Catalpa* Scop. (Bignoniaceae Juss.) is composed of 11 species in two well-defined sections, sect. *Catalpa* and *Macrocatalpa,* differentiated on leaf morphology and seed characteristics as well as geographical distribution (Paclt, 1952). Section *Catalpa* contains six species of deciduous trees with a disjunct distribution between East Asia (four species) and eastern North America (two species). All species from this section are in cultivation except *C. tibetica* Forrest, but only the two North American species are commonly cultivated. Southern catalpa, *C. bignonioides* Walt., is native to the southeastern U.S. (Alabama to western Florida) and northern catalpa, *C. speciosa* (Ward. ex Barn.) Ward ex. Engelm., is native to south central U.S. Both are cultivated, as well as naturalized in many urban areas of the eastern U.S. (Rehder, 1940). Section *Macrocatalpa* is comprised of five species of semi-evergreen trees restricted to the West Indies. These species are poorly represented in cultivation, with the exception of *C. longissima* (Jacq.) Dum.-Cours. (Haitian yokewood) which is cultivated throughout the West Indies as an important landscape and timber species, and in Florida and Hawaii for landscapes (Francis, 1990).

Desertwillow or desertcatalpa, *Chilopsis* D. Don.*,* is a monotypic genus related to *Catalpa*. *Chilopsis linearis* (Cav.) Sweet is a small to medium sized tree, with willow-like leaves and attractive flowers in summer, found in washes and arroyos in desert regions of southwestern U.S., from southern California to Texas, and south-central Mexico (Henrickson, 1985). The species and its cultivars are grown throughout its native range and adjacent regions and are valued for drought tolerance and attractive flowers (Dirr, 1998; Henrickson, 1985; Tipton, 1987).

Catalpa and *Chilopsis* are very similar, but are segregated by number of stamens, two in *Catalpa* versus four in *Chilopsis*, and leaf morphology, large ovate to cordate leaves

in *Catalpa* versus linear to lanceolate leaves in *Chilopsis*. Traditional classifications have placed them in the large, pan-tropical tribe Tecomeae Endl. (Henrickson, 1985). However, this tribe was shown recently to be paraphyletic, suggesting that the tribe be divided with *Catalpa* (both sections) and *Chilopsis* forming a new tribe sister to the Oroxyleae Gentry (Olmstead, unpublished data; Spangler and Olmstead, 1999). Intergeneric hybrids between *Chilopsis linearis* and *Catalpa bignonioides* bred by Rusanov (1964) were introduced into the U.S. in 1977 and formally described by Elias and Wisura (1991) as ×*Chitalpa tashkentensis*. ×*Chitalpa* has performed well in arid climates, but suffers in more humid climates from severe powdery mildew (PM) infections (Dirr, 1998) and in eastern North America from herbivory by catalpa sphinx moth larvae, *Ceratomia catalpae* (Lepidoptera: Sphingidae) (T.G. Ranney, pers. obs.).

Powdery mildews (PM) are obligate fungal parasites (Ascomycetes) characterized by epiphytic white mycelium that often cause distortions of new growth, chlorosis, necrosis and premature leaf fall (Braun, 1987), limiting both growth and aesthetic qualities of infected plants. Seven different species of PM have been identified on *Catalpa* spp., with two host-specific to *Catalpa*: *Erysiphe catalpae* Simonyan and *E. elevata* (syn. *Microsphaera elevata* Burr.) (Ale-Agha et al., 2004; Braun, 1987; Braun et al., 2002; Farr et al., 1989). Of the two *Catalpa*-specific PM's, only *E. elevata* has been reported in N. America, with *E. catalpae* restricted to Europe and Asia currently (Braun, 1987; Sinclair et al., 1987).

Catalpa sphinx moth is distributed throughout eastern U.S from New York to Florida west to Texas and Iowa, overlapping with the native and naturalized range of natural host-plant species *Catalpa bignonioides* and *C. speciosa* (Baerg, 1935). Previous studies

confirmed the suitability of Chinese *Catalpa* species as a food source for CSL (Baerg, 1935; Bowers, 2003). Host plant species significantly affected larval growth (fresh weight), but survival was not affected for larvae reared on *C. bignonioides, C. bungei* C.A. Mey*., C. fargesii* Bureau*, C. ovata* G. Don*,* and *C. speciosa* (Bowers, 2003)*. Ceratomia catalpae* larvae sequester catalpol, converted from catalposide and other iridoid glycosides ingested from *Catalpa* (Bowers, 2003), that serves as a defense against generalist predators (Bowers and Puttick, 1986). It is unknown whether *Chilopsis* and *Catalpa* spp. in section *Macrocatalpa* are susceptible to PM infections or are suitable hosts for CSL.

Both *Catalpa* and ×*Chitalpa* are under-utilized in north temperate zones, owing to a lack of diversity of available germplasm, and knowledge of susceptibility of the common species (*C. bignonioides* and *C. speciosa*) to pathogens and insects, particularly PM and CSL. In order to establish a breeding program targeting improvement of *Catalpa* and ×*Chitalpa* hybrids, sources of resistance to both pest species are needed. Our objective was to assemble and screen a diverse collection of *Catalpa, Chilopsis*, and ×*Chitalpa* for 1) PM susceptibility and 2) hostplant suitability for CSL. A part of the PM study was concerned with identification of the causal organism.

Materials and Methods

Plant Material. Germplasm acquired from various botanical institutions and nurseries accessioned at the Mountain Horticultural Crops Research Station (MHCREC), Fletcher, N.C., between 2002-04, including several breeding lines from the ×*Chitalpa* breeding program at the MHCREC are listed in Table 1. Germplasm was received as un-

rooted softwood or hardwood cuttings, seed, or young plants. Plants were propagated and grown under standard nursery conditions until needed for screening studies.

Powdery Mildew Studies. Two separate screening studies were conducted over successive years, 2004 and 2005. In July 2004, available taxa were potted into 11.2 L containers with a 3 pine bark : 1 peat (by volume) substrate amended with 2.8 kg·m^{-3} dolomitic limestone and 0.5 kg·m^{-3} micronutrients (Micromax, The Scotts Co., Marysville, Ohio) and top-dressed with 35 g per container of 15N-3.9P-10.0K controlled-release fertilizer (15-9-12 Osmocote® Plus 3-4 mo. @70°C, The Scott's Co.). Plants were placed in a lathhouse providing 50% shade in 11 rows of approximately 20 plants, on 0.75 m centers and watered as needed using drip irrigation. The experimental design was completely randomized with 19 treatments (taxa) using single plant replicates. For most taxa, n was $\geq$ 8, except *Catalpa* sp. #2 (n = 3), *C. bungei* var. *heterophylla* (n = 2), and *C. ovata* 'Flavescens' (n = 4).

Catalpa bignonioides stock plants were grown separately in the lathhouse and allowed to develop PM infections naturally. Beginning on 4 Aug. 2004, infected stock plants were placed in and around study plants, and shaken over the study plants to release conidia every other day for one week (total 4×) to supplement natural inoculation. Powdery mildew incidence (I) and severity (S) were recorded for each replicate once every two-weeks, for a total of four observation dates. Incidence (I) represented the percentage of leaves infected per plant and severity (S) the mean percent leaf area covered with mycelium per infected leaf. After six weeks, plants severely infected began abscising leaves; thus the study was terminated.

Selected taxa from the 2004 study and several additional taxa were screened in 2005 for PM. On 23 May, plants were potted into 26.5 L containers using the substrate used in 2004 experiment and controlled-release fertilizer but at 64 g per container.   Plants were placed on a gravel pad in full sun, and watered as needed using drip-irrigation. The experimental design was completely randomized with 17 treatments (taxa) using single plant replicates. For most taxa, n was $\geq$ 6, except *Catalpa* sp. #2 (n = 2), *C. bungei* var. *heterophylla* (n = 2), *C. ovata* 'Flavescens' (n = 4), and *C. punctata* (n = 5). Plants were allowed to develop infections from natural inoculla, no supplemental inoculation was provided. Measurements of I and S were made after the first signs of PM were noted on *Catalpa bignonioides* plants (25 July), and thereafter every two-weeks for six weeks, terminating on 5 Sept. for a total of four observation dates.

Cleistothecia (ascomata) were collected in 2004 and 2005 from infected senescent leaves in late fall, after temperatures decreased and mildew ratings were concluded. Cleistothecia were mounted in 0.01% cotton blue-lactophenol (v/v) on glass slides and viewed under a compound light microscope (Nikon Eclipse E400, Nikon Instruments, Melville, N.Y.) at $\times$400 magnification. Pathogen identification was based on size of cleistothecia, length and branching characteristics of cleistothecial appendages, number of asci per cleistothecium, size and shape of ascospores, and number of ascospores per ascus. Voucher specimens were made and placed in the mycological herbarium, Dept. of Plant Pathology, NCSU.

Area under the disease progress curves (AUDPC) were constructed for each year using the formula of Shaner and Finney (1977) with modification:

$$\sum_{i=1}^{n}[(Y_{i+1}+Y_i)/2][X_{i+1}-X_i]$$

Where $Y_i = I \times S$ at the ith observation, X_i = time (days) at the ith observation, and n = total

number of observations. Multiplying I by S results in a relative disease intensity rating for a

given plant at a given observation date (Seem, 1984). Then, AUDPC represents the

cumulative disease intensity for a given plant over the length of the study (Jeger and

Viljanen-Rollinson, 2001). Data for each year were analyzed separately. Differences

between taxa were compared using analysis of variance (PROC GLM; SAS version 8.02,

SAS Inst., Cary, N.C.) and means separated using Fisher's protected least significant

difference (LSD) at $\alpha = 0.05$.

Catalpa Sphinx Moth Larvae Screening. A no-choice feeding study, with the addition of

Chilopsis linearis 'Regal', was conducted in summer 2005. On 22 June, fourth and fifth

instars were collected from infested trees at the University of Georgia Horticulture Farm,

Athens, Georgia (Ness, 2003). Larvae were transported to the greenhouses at MHCREC

and allowed to pupate in plastic boxes filled with sand and covered with perforated plastic.

On 21 July, the first adult moth emerged, at which time the containers were transferred

along with two stock plants of *Catalpa bignonioides* to a mesh cage ($2 \times 2 \times 2$ m) located in

a lathhouse (50% shade). Adults emerged daily with the first egg masses laid on 23 July.

Eggs were collected from the walls of the flight cage and *Catalpa* leaves and placed in petri

dishes (15 cm), then incubated in the insect rearing room at the MHCREC. Conditions

within the rearing room were maintained at 16 h day (15 μmol·s^{-1}·m^{-2} photosynthetically

active radiation, fluorescent lights), 25°C and 60% relative humidity for the duration of the

study. Waxed paper cups (0.9 L) covered with cheesecloth served as individual rearing

chambers with one cup per single plant replicate. Cups were located randomly on shelves

within the chamber. Recently expanded leaves or small shoots from each taxon were placed

in water picks and inserted into cups. Leaves and water were exchanged daily, or as needed

to maintain adequate leaf turgor and plant material for larvae.

The study began on 28 July. Five first instar larvae (1 d old) were placed in each

cup. The study ended at 15 d when surviving larvae reached the fifth instar and feeding was

negligible. Percent survival data on each taxon was recorded. For surviving larvae, final

mass and headcapsule width (mm) were recorded. Growth of the headcapsule was used to

estimate instar stage (Baerg, 1935). Percentage survival data were arcsin transformed prior

to statistical analysis. The experiment was a completely randomized design, with nine taxa

(treatments) and single plant replicates (n = 8 except *Catalpa longissima,* n = 5, and *C.*

punctata, n = 6) and five subsamples (larvae) per replicate. Data were analyzed using

general linear model (PROC GLM; SAS version 8.02, SAS Inst.).

Results and Discussion

Severe PM epiphytotic developed in the lathhouse in 2004, with moderate but similar

pattern of infections developing in 2005 (Table 2). Infections generally began on recently

expanded leaves, rather than immature growth. Levels of natural inoculum in the proximity

of MHCREC were assumed to be high, with epiphytotic events observed in the years prior

to 2004 on ×*Chitalpa tashkentensis* 'Pink Dawn' growing at MHCREC (pers. obs.). White

superficial mycelium on the leaf surface was well-developed and heavy cleistothecial

development in the fall of 2004 facilitated identification of the causal organism. Length and

branching of cleistothecial appendages are key taxonomic characters for distinguishing

Erysiphe spp. (Ale-Agha et al., 2004; Braun, 1987). Cleistothecial appendages were

hyaline, 4-7× longer than the width of the cleistothecial body, and dichotomously branched

at the terminus, characteristic of *Erysiphe elevata*. *Erysiphe catalpae* has non-branched

appendages, and *E. penicillata* [syn. *Microsphaera penicillata* (Wallr.:Fr.) Lév.], a broadly

adapted PM in N. America, has branched appendages 1-1.5× as long as the width of the

cleistothecial body (Braun, 1987). In North America, *E. elevata* (including synonyms

Microsphaera vaccinii auct. p. p. and *M. alnii* var. *vaccinii* auct. p. p.) is the most commonly

sited PM on *Catalpa* (Braun, 1987; Sinclair et al., 1987), but our collection and positive

identification was the first for North Carolina. In Europe, *E. catalpae* is the common

Catalpa-specific PM (Ale-Agha et al., 2004; Braun, 1987). However, *E. elevata* has been

introduced recently and identified in Hungary (Vajna et al., 2004) and is spreading in

Europe currently (Ale-Agha et al., 2004; Cook et al., 2004). In both study years, a leaf spot

infection occurred on *Catalpa* taxa within both sections, and was identified as *Cristulariella*

moricola (I. Hino) Redhead. This fungus has a wide host range of several plant families.

There were significant differences among taxa in susceptibility to PM as measured

by AUDPC values in 2004 (F value = 183.4, *P* < 0.0001) and 2005 (F value = 51.6, *P* <

0.0001). Taxa with no PM infection (0 AUDPC) were not used in determining differences

among means using Fisher's protected LSD. The natural host species for *E. elevata*, N.

American *C. bignonioides* and *C. speciosa*, had intermediate levels of PM infection in 2004.

All three cultivars of *C. bignonioides*, 'Aurea', 'Koehnei' and 'Nana', likewise were

susceptible, although 'Nana' was more susceptible than the other cultivars and type species

(Table 2). All of the above were dropped from the 2005 study, except *C. bignonioides*, that served as a susceptible control both years.

Chinese *Catalpa, C. bungei* var. *heterophylla, C. ovata,* and *C. ovata* 'Flavescens', were resistant to *E. elevata* in both study years. *Catalpa fargesii* var. *duclouxii* was not available in 2004, but was resistant during 2005. The behavior of *C.* sp. #1, #2, and # 3, received as *C. bungei*, is of special note. *Catalpa bungei* is confused in the nursery trade and herbaria with *C. bignonioides* 'Nana' and *C. ovata* (Bean, 1936; Dirr, 1998; Paclt, 1952). *Catalpa* sp. #1 and #2 were received from Morton Arboretum, Lisle, Ill. as un-rooted cuttings from plants grown from seed received as *C. bungei* from the Uzbek Academy of Sciences Botanical Garden, Tashkent, Uzbekistan (formerly the Republic of Uzbekistan, U.S.S.R.) (Table 1). *Catalpa* and *Chilopsis* are self-incompatible (Petersen et al., 1982; Stephenson and Thomas, 1977); therefore trueness-to-type may be questioned in seed of cultivated origin. These plants do not fit the description of *C. bungei* by Paclt (1952) nor Bean (1936), in that leaves are not glabrous, inflorescences are not corymb-like, and flowers are not rose-colored. These plants appear to be hybrids with *C. bignonioides*, although this cross has not been reported previously. In terms of PM susceptibility, they responded similarly to cultivars of *C.* ×*erubescens* (*C. ovata* x *C. bignonioides*), with moderate to low levels of susceptibility in both 2004 and 2005 (Table 2). *Catalpa* sp. #3 represented nursery collected seed and was nearly identical to *C. ovata* in foliage and flower. It was available only for 2005, and in that year was resistant to *E. elevata,* as were the other taxa of *C. ovata* (Table 2). In our study, *Catalpa bungei* var. *heterophylla* accessioned from the Brooklyn Botanical Garden was the only *C. bungei* taxon that matched its taxonomic description: leaves were dark green and glabrous, and differed from the type species only in it's more

deeply lobed leaves (Paclt, 1952). Although we only had two plants, this taxon remained free of PM infection both years.

Hybrids between Chinese *C. ovata* and N. American *C. bignonioides* (*C. ×erubescens*) and *C. speciosa* (*×galleana*) exhibited a broad range of susceptibility to *E. elevata*. Of the hybrids, *C. ×erubescens* 'J.C. Teas' was most susceptible in both 2004 and 2005 (AUDPC of 914 and 1261, respectively), followed by *C. ×erubescens* 'Purpurea' (24 and 33, respectively) and *C. ×galleana* (4 and 1, respectively). Our clone of *C. ×erubescens* 'J.C. Teas' resembles the *C. bignonioides* parent, whereas the *Catalpa ×erubescens* 'Purpurea' resembles the *C. ovata* parent. In the United States, *C. ×erubescens* was first documented by Sargent (1889) who reported the existence of hybrid seedlings sent to the Arnold Arboretum by a Mr. J.C. Teas of Carthage, Ind. The origin of the cultivar 'J.C. Teas' is unknown, but may represent one of the original F_1 hybrids sent to the Arnold Arboretum or an F_2 segregate derived from the original hybrids. Segregates were known to exist (Jones and Filley, 1920; Paclt, 1952; Sargent, 1889) and the greater susceptibility of 'J.C. Teas' to PM infection may reflect segregation towards susceptibility of *C. bignonioides* to *E. elevata,* and for *C. ×erubescens* 'Purpurea' segregation towards resistance in *C. ovata* (Table 2). *Catalpa ×galleana* had extremely low levels of PM infection in both years, with only a few spots noted on the new growth and no secondary infection or spread (Table 2). Our clone of Galle's hybrid catalpa is one of the original F_1 hybrid seedlings bred by Karl Sax at the Arnold Arboretum in 1940 (Table 1). Morphologically, the hybrid is intermediate between *C. ovata* and *C. speciosa*, but has the largest leaves of the genus (pers. obs.). The lack of complete resistance in *C. ×galleana,* a known F_1, and the range of susceptibilities in

C. ×*erubescens* clones, suggesting inheritance of PM resistance in *Catalpa* is polygenic or quantitative in nature.

Evergreen species of sect. *Macrocatalpa, C. longissima* and *C. punctata*, were resistant to PM infection by *E. elevata*. Section *Macrocatalpa* is restricted to the New World, with five species poorly understood occurring throughout the West Indies (Paclt, 1952). Little cultural information existed for these taxa, with the exception of *C. longissima,* an important agro-forest tree species in Haiti. In seedling nurseries, leaf spots and anthracnose have been reported (Francis, 1990) but PM infections on this species or other West Indian species was lacking in the literature.

Although *Chilopsis linearis* was not available for the 2004 study, in 2005 PM infections developed late on *Chilopsis linearis* 'Bubba' grown in full sun. Thus, low AUDPC values for this taxon (Table 2). Our limited data suggest that some *Chilopsis* taxa are not resistant to PM infection by *E. elevata*, but the degree of susceptibility needs further investigation.

×*Chitalpa* cultivars were amongst the most susceptible taxa in both 2004 and 2005. Infection and spread of mycelia were rapid, as noted by the high AUDPC values for ×*Chitalpa*'s in both years (Table 2). Powdery mildew infections on ×*Chitalpa* have been observed previously in Georgia (Dirr, 1998), Arkansas (J. Lindstrom, pers. comm.) and North Carolina (T. Ranney, pers. obs.), but the causal organism was not identified. In both years, ×*Chitalpa* 'Pink Dawn' was more susceptible than 'Morning Cloud' (Table 2). The induced allotetraploid 'Pink Dawn' was as susceptible to PM infection as the diploid 'Pink Dawn' in 2004, but in 2005 had significantly less PM. Also, progeny derived from the

induced allotetraploid 'Pink Dawn' (MHREC #1 and F_2 $4x$ seedlings) were highly

susceptible to PM (Table 2). These progeny were uniform in appearance and susceptibility,

suggesting that these allopolyploids have fixed heterozygosity (strong intrageneric disomic

meiotic pairing) resulting in little intergenomic recombination and segregation.

There were no significant differences for CSL survival (F value = 1.74, P = 0.113),

final weight (F value = 2.09, P = 0.173) or head carapace width (F value = 0.65, P = 0.723)

in the no-choice feeding study (Table 3). In a no-choice feeding study using deciduous

members of sect. *Catalpa*, Bowers (2003) found a significant effect of hostplant on larval

growth, but no differences in larval survival, even though survivorship ranged from 47% for

C. ovata to 70% for *C. bignonioides*. Bowers (2003) concluded that all deciduous species of

Catalpa are suitable hostplant species for *Ceratomia catalpae* larvae. We included

deciduous representatives of sect. *Catalpa*, evergreens from sect. *Macrocatalpa*, *Chilopsis*,

and ×*Chitalpa* in our no-choice feeding study. Survival of larvae reared on various taxa

ranged from 27.3% for ×*Chitalpa* 'Pink Dawn' to 84.1% for *Catalpa longissima* (Table 3).

Final weight and headcapsule width varied less among taxa than survival (Table 3).

Headcapsule width of mature larvae ranged from 3.4 to 4.1 mm. Baerg (1935) reported that

C. catalpae headcapsule width for fourth and fifth instars typically average 2.20 mm and

4.35 mm, respectively. Although head carapace width of surviving larvae in our study was

slightly less than that reported for fifth instars by Baerg (1935), the majority of larvae

pupated upon completion of the study (data not shown) suggests that they were indeed in the

fifth instar stage. North American *C. bignonioides* and *C. speciosa,* host-plants that have

co-evolved with *Ceratomia catalpae* were expected to be suitable hosts, as was Chinese

Catalpa species in sect. *Catalpa* (Baerg, 1935; Bowers, 2003). This is the first report on suitability of taxa from *Catalpa* sect. *Macrocatalpa*, *Chilopsis,* and ×*Chitalpa* to act as host-plants for CSL. The coriaceous leaves of *Catalpa* spp. in sect. *Macrocatalpa* are known to contain calcium oxalate crystals (Elias and Newcombe, 1979), but were not detrimental to larval survival and growth. Other sources of resistance to CSL may lie in manipulation of iridoid glycoside content in *Catalpa* spp., particularly catalposide, which is thought to act as a larval feeding-stimulant (Nayar and Fraenkel, 1963) and may play a role in acceptance as a host for oviposition.

In conclusion, North American *Catalpa*-specific *E. elevata* was identified as the causal organism of PM epiphytotics on study plants of *Catalpa* sect. *Catalpa* and ×*Chitalpa.* Cultivars and hybrids derived from ×*Chitalpa tashkentensis* were especially susceptible. Taxa of Chinese *Catalpa* in sect. *Catalpa* and West Indian evergreen species in sect. *Macrocatalpa* were resistant. Existing hybrids between susceptible and resistant species (*C. ×erubescens* and ×*galleana*) demonstrated heritability of partial resistance to *E. elevata*. *Catalpa* species from sect. *Macrocatalpa* will be an alternative source of resistance for introgression of PM resistance into novel hybrid *Catalpa*'s and ×*Chitalpa*'s. Also, *Chilopsis linearis* was susceptible to PM infection by *E. elevata*, thus its value to breeding programs will lie in introducing novel flower color, drought tolerance, refined foliage and reduced height to new ×*Chitalpa* cultivars. Unfortunately, no immediate source of resistance to CSL was found in existing germplasm, indicating that while *Ceratomia catalpae* is monophagous on *Catalpa* in the eastern U.S., its sister genus *Chilopsis* in southwestern U.S. is also a suitable host.

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Table 1. Germplasm within the tribe Tecomeae Endl. (Bignoniaceae Juss.) screened for susceptibility to powdery mildew and catalpa sphinx larvae.

Taxa[z]		Source of plant material	Accession no.[y]	Comments[x]
Catalpa Section *Catalpa* Paclt				
Catalpa	*bignonioides* Walt.	MHCREC, Fletcher, NC 28732-9244	na	Southern catalpa. Cuttings from naturalized plant along the French Broad River, Asheville, NC. Native to SE US from Ala. to W. Fla.
	bignonioides 'Aurea'	JCR Arboretum, Raleigh, NC 27695-7609	960617	Yellow new growth, maturing to green, introduced pre-1871.
	bignonioides 'Koehnei'	Sir Harold Hillier Gard. Hampshire SO51 0QA, England, UK	1976.9778	New growth broadly margined with yellow, c.1903. Syn. 'Aureo-marginata'.
	bignonioides 'Nana'	Sir Harold Hillier Gard.	1976.9602	Umbrella catalpa. Heavily branched, dwarf cultivar of French origin, c. 1850. Often confused with *C. bungei*.
C.	sp. clone #1	Morton Arboretum, Lisle, IL 60532-1293	498-80 03-79	Cuttings of a plant raised from seed received from the Bot. Gard. at Uzbek Acad. of Sciences, Tashkent, Uzbekistan as *C. bungei* C.A. Mey. but appears to be of hybrid origin.
	sp. clone #2	Morton Arboretum	498-80 38-39	Cuttings from sister seedling to *C.* sp. clone 1. Also appears to be of hybrid origin.
	sp. clone #3	Heritage Seedlings, Salem, OR 97301	na	Nursery grown seedlings, originally from Lawyer Nursery, Plains, MT 59859. Labled as *C. bungei*, but appeared to be *C. ovata*.
C.	*bungei* var. *heterophylla* C.A. Mey.	Brooklyn Bot. Gard. Brooklyn, NY 11225	340008	Lobed Manchurian catalpa. Unknown provenance. Form grows with the type species in the wild, c. 1907. N. China.
C.	×*erubescens* Carr. 'J.C. Teas'	Cornell Plantings, Cornell University, Ithaca, NY 14853	97-072	Teas' hybrid catalpa (*C. ovata* × *bignonioides*). Spontaneous hybrid occurring before 1869 in France and 1874 in the US.
	×*erubescens* 'Purpurea'	MHCREC	1996-139	Purple-leaf catalpa. New growth dark purple, fades to green, discovered in cultivation c. 1886 in US.
C.	*fargesii* f. *duclouxii* (Dode) Gilm.	Scott Arboretum, Swarthmore, PA 19081	93-661-A	Ducloux's catalpa. Smooth-leaf form with deeper pink flowers than the type species, c.1908. W. China.
C.	×*galleana* Dode	Arnold Arboretum, Jamaica Plain, MA 02130-3500	925-42B	Galle's hybrid catalpa (*C. ovata* × *speciosa*). Spontaneous cross in France c. 1907. This clone is from artificial crosses made at the Arnold Arboretum in 1940 by Karl Sax.

Table 1. (continued)

C.	*ovata* G. Don	Arnold Arboretum	516-87B	Chinese catalpa. Received as wild collected seed from Yunnan Inst. Trop. Bot., China as *C. fargesii* f. *duclouxii.*
	ovata 'Flavescens'	Sir Harold Hillier Gard.	1992-1036	Pale yellow Chinese catalpa. Smaller, and more yellow flowers than the species, c.1863 in Europe.
C.	*speciosa* (Ward. ex Barn.) Ward. ex. Engelm.	Arnold Arboretum	1245-79-B	Northern catalpa. Cuttings of wild collected tree from Mo., US. Native to south central US.

Catalpa Section *Macrocatalpa* Grisebach

Catalpa	*longissima* (Jacq.) Dum.-Cours.	Fairchild Tropical Bot. Gard., Coral Gables FL 33156	X.1-21B	Haitian yokewood. Seedlings of a wild collected tree. Native to Hispaniola, but cultivated in Caribbean, S. Fla. and Hawaii.
C.	*punctata* Griseb.	Fairchild Tropical Bot. Gard.	66396C	Cuban catalpa. Seedlings from a tree collected in the Bahamas. Native to Cuba, rare in cultivation.

Chilopsis D. Don

Chilopsis	*linearis* (Cav.) Sweet 'Bubba'	SFA Mast Arboretum, Nacogdoches, TX 75962-3000	na	Bubba desertwillow. Popular cultivar with deep purple flowers, introduced by Paul Cox of San Antonio Bot. Gard., Texas.
C.	*linearis* 'Regal'	Native Texas Nursery, Austin, TX 78725	na	Regal desertwillow. Lavendar flower with burgundy lower lip. Introduced by Los Lunas Plant Material Center, N.M. in 1989.

×*Chitalpa* (*Chilopsis linearis* × *Catalpa bignonioides*)

×*Chitalpa*	*tashkentensis* Elias & Wis. 'Morning Cloud'	Forestfarm Nursery, Williams, OR 97544-9599	2005-311	Morning Cloud chitalpa. White-flowered cultivar named in 1991. One of two clones introduced from Uzbekistan in 1977.
×*C.*	*tashkentensis* 'Pink Dawn' 2*x*	MHCREC	2005-312	Diploid Pink Dawn chitalpa. Pink-flowered cultivar named in 1991. One of two clones introduced from Uzbekistan in 1977.
×*C.*	*tashkentensis* 'Pink Dawn' 4*x*	MHCREC	na	Oryzalin-induced tetraploid form of 'Pink Dawn' chitalpa.
×*C.*	*tashkentensis* F$_2$ 4*x* seedlings	MHCREC	na	Seedlings from selfed tetraploid 'Pink Dawn' chitalpa.
×*C.*	*tashkentensis* MHCREC#1	MHCREC	H2004-003	Diploid seedling from *Chilopsis* 'Bubba' × 24-2-1 cytochimera 'Pink Dawn' (2*x* + 4*x*).

[z]Author of scientific name follows the first listing of each taxon above the rank of cultivar, unless the taxon is solely represented by a cultivar(s).
[y]Accession number from the original source, if available. na = no accession number.
[x]Accession information, including taxa description, origin, and date of introduction.

Table 2. Mean area under the disease progress curve for powdery mildew infection among a diverse collection of taxa within the tribe Tecomeae Endl. (Bignoniaceae Juss.) grown in containers under nursery conditions during 2004 and 2005.

		AUDPC^z	
Taxa		2004^y	2005^x
×*Chitalpa tashkentensis*	MHREC#1	2416 a^w	---^{v}
	'Pink Dawn' 4x	2300 a	1167 c
	'Pink Dawn' 2x	2290 a	2154 a
	F$_2$ 4x seedlings	2268 a	---
	'Morning Cloud'	1300 b	1655 b
Catalpa bignonioides	'Nana'	1270 b	---
C. speciosa		1134 b	---
C. ×erubescens	'J.C. Teas'	914 c	1261 c
C. bignonioides		694 d	172 d
C. bignonioides	'Koehnei'	637 d	---
C. sp.	#2	622 d	90 d
C. bignonioides	'Aurea'	273 e	---
Chilopsis linearis	'Bubba'	---	48 d
C. ×erubescens	'Purpurea'	24 f	33 d
C. sp.	#1	15 f	0
C. ×galleana		4 f	1 d
C. sp.	#3	---	0
C. bungei	var. *heterophylla*	0	0
C. fargesii	var. *duclouxii*	---	0
C. ovata		0	0
C. ovata	'Flavescens'	0	0
C. longissima		0	0
C. punctata		---	0

^{z}Area under the disease progress curve (AUDPC) calculated using the product of disease incidence (% leaves affected) and severity (% leaf area affected).
^{y}Plants grown under 50% shade with drip irrigation.
^{x}Plants grown in full sun with drip irrigation.
^{w}Means within a year, followed by the same letter are not significantly different based on Fisher's LSD$_{0.05}$.
^{v}Dropped from study in 2005, or in 2004 not available.

Table 3. Survival and final growth measurements for *Ceratomia catalpae*
larvae on a known host (*Catalpa bignonioides*) and non-host members of
the Tecomeae tribe (Bignoniaceae) in a 15 day no-choice feeding study.

Taxa[z]		Survival (%)[y]	Final wt (g)[x]	Head width (mm)[w]
C. bignonioides		59.5	1.4	3.7
C. longissima		84.1	2.0	4.1
C. ovata		47.5	2.2	4.0
C. punctata		70.7	1.5	3.9
Chilopsis linearis	'Bubba'	70.7	1.6	3.6
Chilopsis linearis	'Regal'	78.1	1.1	3.8
×*Chitalpa tashkentensis*	'Morning Cloud'	45.6	1.7	3.9
	'Pink Dawn' 2*x*	27.3	1.3	3.4
	'Pink Dawn' 4*x*	44.0	2.5	3.9

[z]n =8 except *C. longissima* (n = 5) and *C. punctata* (n = 6).
[y]Began with 5 larvae per replicate. Arcsin transformed for data analysis,
untransformed data presented. Nonsignificant (F value = 1.74, $P = 0.113$).
[x]Final weight measured on day 15 prior to pupation. Nonsignificant (F
value = 2.09, $P = 0.173$).
[w]Width measured across head capsule at day 15, (fifth instars ≈ 4.0 mm).
Nonsignificant (F value = 0.65, $P = 0.723$).

Chapter 3

Fertility and Inheritance of Variegated and Purple Foliage across a Polyploid Series in

Hypericum androsaemum

(In the format appropriate for submission to

the Journal of the American Society for Horticultural Science)

Fertility and Inheritance of Variegated and Purple Foliage across a Polyploid Series in
Hypericum androsaemum

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Genetics and Breeding

Fertility and Inheritance of Variegated and Purple Foliage across a Polyploid Series in
Hypericum androsaemum

Additional index words. St. John's wort, tutsan, autotetraploid, oryzalin, invasive plants,
pollen viability, ornamental plant breeding

Abstract. Inheritance of two mutant foliage types, variegated and purple, was investigated
for diploid, triploid and tetraploid tutsan (*Hypericum androsaemum* L.). The fertility of
progeny was evaluated by pollen viability tests and reciprocal crosses with diploids, triploids
and tetraploids and germinative capacity of seed from successful crosses. Segregation ratios
were determined for diploid crosses in reciprocal di-hybrid F_1, F_2, BC_{1P1}, and BC_{1P2} families
and selfed F_2s with the parental phenotypes. F_2 tetraploids were derived from induced
autotetraploid F_1s. Triploid segregation ratios were determined for crosses between
tetraploid F_2s and diploid F_1s. Diploid di-hybrid crosses fit the expected 9:3:3:1 ratio for a
single, simple recessive gene for both traits, with no evidence of linkage. A novel
phenotype representing a combination of parental phenotypes was recovered. Data from
backcrosses and selfing support the recessive model. Both traits behaved as expected at the
triploid level, however, at the tetraploid level the number of variegated progeny increased,
with segregation ratios falling between random chromosome and random chromatid
assortment models. We propose the gene symbol *var* (*variegated*) and *pl* (*purple leaf*) for
the variegated and purple alleles, respectively. Triploid pollen stained moderately well, but

pollen germination was low. Triploid plants were highly infertile demonstrating extremely low male fertility and no measurable female fertility (no viable seed production). The present research demonstrates the feasibility of breeding simultaneously for ornamental traits and non-invasiveness.

The genus *Hypericum* L. is a member of the large family Guttiferae Juss. [(Clusiaceae) subfamily Hypericoideae Engler. tribe Hypericeae Choisy] (Gustafsson et al., 2002) and contains c. 370 species of herbs, shrubs and trees distributed across the temperate zones and high elevations of the tropics (Mabberley, 1997; Robson, 1985). St. John's worts, as they are collectively known, have garnered much attention of late, with research focused on phytochemicals unique to the genus, including hypericins and hyperforins from common St. John's wort (*H. perforatum* L.) (Kirakosyan et al., 2004) and xanthones from tutsan (*H. androsaemum*) (Dias et al., 2000; Valentão et al., 2002). In addition to pharmacological value, the genus has contributed a number of important ornamental species including *H. calcyinum* L.*, H. forrestii* (Chittenden) N. Robson*,* and *H. androsaemum* (Robson, 1985)*.*

Hypericum androsaemum ($2n = 2x = 40$) is native from western Europe, through the Mediterranean and north Africa, east into northern Iran; however, it is now found naturalized throughout Europe and parts of Australia, New Zealand and Chile (Robson, 1985). This semi-evergreen shrub grows less than one meter in height and has abundant, yellow, star-like flowers and fleshy red fruit which mature to a brown capsule. As a landscape plant, *H. androsaemum* and its hybrids with *H. hircinum* L. (*H. ×inodorum* Miller), perform best in the cooler parts of hardiness zones 6-8 (U.S.D.A.). Various cultivars have been selected for the landscape and floriculture trade including yellow, variegated, and purple leaf forms. 'Mrs. Gladis Brabazon' and 'Glacier' are cultivars derived from *H. androsaemum* f. *variegatum* McClintock & Nelson with heavily mottled leaves consisting of white, green, and light green patches (McClintock et al., 1986). 'Albury Purple' has purple blushed foliage and red-veined flowers; the origin of the cultivar is unknown.

Cultivation of *Hypericum androsaemum* outside of its native range has led to naturalization in Europe, Australia, New Zealand and Chile (Robson, 1985) and is a species of concern in the Pacific Northwest, USA (Dan Hinkley, Heronswood Nursery, pers. comm.). It is considered an invasive species in Australia where biological control mechanisms are being studied (McLaren et al., 1997). Increasing awareness and concern about invasive plant species (Reichard and White, 2001) provides an opportunity for plant breeders to address this problem through the development of non-invasive cultivars. Bananas (*Musa* L.) and watermelons [*Citrullus lanatus* (Thunb.) Matsum. & Nakai], are well-known example of triploid plants displaying low fertility and seed development (Kihara, 1951; Ortiz and Vuylsteke, 1995). Few triploid ornamental plants have been developed, though the potential for using this approach to develop non-invasive cultivars is promising (Ranney, 2004). The triploid rose-of-sharon (*Hibiscus syriacus* L.) cultivars released by the U.S. National Arboretum shrub breeding program are notable exceptions (Egolf, 1970; 1981; 1986; 1988).

The objectives of this study were to investigate the mode of inheritance for two important ornamental traits, leaf variegation and purple foliage, across three ploidy levels [diploid ($2x$), triploid ($3x$) and tetraploid ($4x$)] and to determine the fertility of progeny to assess the feasibility of developing non-invasive, triploid cultivars.

Materials and Methods

Inheritance studies. Plants of *H. androsaemum* f. *variegatum* 'Glacier' (G) and 'Albury Purple' (AP) were maintained in the ornamental tree and shrub collection at the Mountain Horticultural Crops Research Station (MHCREC), Fletcher, N.C. Prior to

beginning the study, open-pollinated seedlings from G and AP displayed their parental

phenotypes, indicating that both traits were heritable. Plants for all crosses were grown in

the greenhouse at MHCREC in 18.9 L containers in 3 pine bark : 1 peat (by volume)

substrate amended with 2.8 kg·m^{-3} dolomitic limestone and 0.5 kg·m^{-3} micronutrients

(Micromax, The Scotts Co., Marysville, Ohio) and watered and fertilized as needed.

Reciprocal, di-hybrid F_1 and F_2 families were generated to investigate inheritance (Table 1).

BC_{1P1} and BC_{1P2} families were produced as additional test crosses. $F_{2P1[2x]}$ plants exhibiting

the original parental phenotypes were selfed (S_0) to further verify genotypes. For all

crosses, flowers were emasculated prior to anthesis, generally when the elongating floral

bud was equal to subtending calyx lobes in length. Pollen was collected from recently

dehisced anthers and either used fresh or dried overnight at 5 °C using indicator drierite

(Drierite, Xenia, Ohio) and stored at 5 °C for use in later crosses. Pollen was applied daily

to stigmas using small brushes, until stigmas turned brown after ≈ 10 days. Fruit were

collected when capsules turned from bright red to brown and allowed to dry at room

temperature for 1-3 days. Seeds were surface-sown onto a substrate of 1 peat : 1 vermiculite

(by volume) in seedling flats and misted regularly until germination occurred in 1 to 4

weeks. Seedlings were transplanted into 40-cell trays and phenotypes were scored when

seedlings had > 3 sets of true leaves.

A subset of seedlings from G x AP F_1 ($F_{1P1[2x]}$) were treated with the mitotic-

inhibiting herbicide oryzalin [4(dipropylamino)-3,5-dinitrobenzenesufonamide] to induce

tetraploidy ($F_{1P1[4x]}$). A total of 488 seedlings were selected from seedling flats after the

first set of true leaves had emerged. Substrate was washed from roots and the entire

seedling submerged in 150 μM oryzalin [0.004% Surflan®, Dow AgroSciences,

Indianapolis] for 24 h. Following treatment, seedlings were washed under running water for

30 min and potted into 40-cell trays containing a 1 peat : 1 vermiculite (by volume) substrate

and placed under intermittent mist for three days then grown off under normal greenhouse

conditions. Relative DNA content and associated ploidy levels were determined using a

flow cytometer (PA-I Ploidy Analyzer, Partec GmbH, Münster, Germany). Nuclei isolation

and staining [4',6-diamidino-2-phenylindole (DAPI)] followed the protocols provided by

(Olsen et al., in prep.). Induced tetraploids ($F_{1P1[4x]}$) were grown to flowering and selfed to

investigate segregation of traits in an F_2 tetraploid family ($F_{2[4x]}$) or pollinized with diploid

F_1 ($F_{1P1[2x]}$) to investigate segregation at the triploid level ($F_{2[3x]}$) (Table 1). Samples of

seedlings from all crosses were analyzed using flow cytometry to confirm ploidy level of the

progeny. Flow cytometry was also used to detect apomixis by analyzing ploidy levels of

seed from F_1 crosses. Seed from sexual crosses will have a diploid embryo peak and a

triploid endosperm peak, while seed from apomictic (unreduced embryo sac) origin will

have a diploid embryo peak and a tetraploid endosperm peak (Matzk et al., 2000). Seeds (25

seeds per sample) were chopped using the same buffer and staining procedure as for foliage.

Inheritance of variegated and purple leaf traits was tested on the hypothesis that both

traits were inherited in a simple Mendelian recessive manner. Segregation data were

analyzed for departures from expected ratios using chi-square analysis on segregating

families ($F_{2P1[2x]}$, $F_{2P2[2x]}$, BC_{1P1}, BC_{1P2}, $F_{2[3x]}$, and $F_{2[4x]}$). The chi-square test of

independence for linkage was calculated for diploid F_2's ($F_{2P1[2x]}$ and $F_{2P2[2x]}$). Crosses were

conducted during the summers of 2003-05.

Fertility of progeny. Reciprocal crosses between diploid ($2x$), triploid ($3x$), and tetraploid plants ($4x$) were made in 2005 to investigate male and female fertility as a function of ploidy level. Plants were grown in 18.9 L containers in the greenhouse using the same substrate and conditions described earlier and the same pollination techniques. Six plants per ploidy level were randomly sampled for pollen viability during the study. One flower per plant per ploidy level was harvested and anthers divided into two groups, with one group for pollen staining and the other for pollen germination tests. Pollen grains were stained in 40 μL of 1% aceto-carmine stain on a microscope slide. The slide was heated $3\times$ times over a hot-plate for 5 s, sealed with valap (1 vaseline : 1 lanolin : 1 paraffin by weight) and incubated at room temperature (23 °C) for 5 h. Stained pollen grains were scored as viable. Pollen germination was performed in 5 mL petri dishes containing Brewbaker-Kwack media supplemented with 5% sucrose and solidified with 2% agarose (Marquard, 1992). Pollen grains with pollen tubes greater than one-half the diameter of the pollen grain after 5 h were scored as germinated. Pollen staining and germination were observed using a compound light microscope (Micromaster, Fisher Scientific, Pittsburgh) under $\times 100$ and $\times 400$ magnifications. For each replicate ≥ 100 pollen grains were scored. The experiment was a completely randomized design with three treatments (ploidy levels) and six replicates ($n = 6$).

Crosses among the three ploidy levels resulted in nine, factorial treatment combinations. The experiment was a completely randomized design with five single plant replicates ($n = 5$) per cross with 10 flowers (subsamples) pollinated per plant. Fruit from successful crosses matured $\approx$ 40 d after pollination. Fruit were harvested, dried, and seed

separated and stored in sealed vials at 5 °C until used for germination studies. Total number of fruit per cross, percent fruit set, and average seed per fruit were recorded.

Germinative capacity of seed harvested from successful crosses was determined using in vitro germination studies. Two germination blotters (SDB3.5, Anchor Paper Co., St. Paul, Minn.) soaked with 100 μM gibberellic acid (GA_{4+7}) (21 g·L^{-1} Provide®, Valent Biosciences, Walnut Creek, Calif.) were placed in petri dishes (100 mm × 15 mm). A sample of 50 seed per fruit, or all seed available when < 50, were sown per petri dish. Petri dishes were sealed with parafilm and placed in a growth chamber maintained at 22 °C, 16 h day/ 8 h night period, and 42 μmol·s^{-1}·m^{-2} photosynthetically active radiation (PAR) provided by fluorescent lights. After 8 weeks, seeds with emergent radicles were scored as germinated.

Data from pollen, crossing, and germination studies were analyzed using analysis of variance (PROC GLM; SAS version 8.02, SAS Inst., Cary, N.C.) and means compared using Fisher's protected least significant difference (LSD) at α = 0.05.

Results

Inheritance studies. Reciprocal F_1 crosses yielded progeny that were all green, with the exception of three variegated seedlings ($F_{1P1[2x]}$) and one purple seedling ($F_{1P2[2x]}$), which were attributed to accidental self-pollination (Table 2). No evidence for apomixis was observed from flow cytometry analysis of F_1 seed, thus all progeny appeared to be the result of sexual crosses (data not shown). Segregation ratios for the diploid F_2 families ($F_{2P1[2x]}$ and $F_{2P2[2x]}$) fit the expected 9:3:3:1 ratio, supporting the hypothesis of simple recessive

inheritance for both traits. The chi-square test for independence of linkage ($\Pi^2 = 1.75$, $P = 0.19$ and $\Pi^2 = 0.02$, $P = 0.89$ for $F_{2P1[2x]}$ and $F_{2P2[2x]}$, respectively) suggests no evidence for linkage between the variegated and purple loci. Furthermore, recovery of variegated and purple seedlings in both F_2 populations ($F_{2P1[2x]}$ and $F_{2P2[2x]}$) effectively rules out strict maternal inheritance. Segregation ratios for BC_{1P1} fit the expected 1:1 ratio of green : variegated phenotypes (Table 2). However, in BC_{1P2} a few spontaneous variegated and variegated : purple seedlings were observed which can not be explained by accidental self-pollination or pollen contamination. These seedlings were scored as either green or purple for chi-square analysis; however, due to the presence of more purple seedlings than expected (221 observed versus 199.5 expected) we still had a poor fit. F_2 plants ($F_{2P1[2x]}$) exhibiting the original G or AP phenotype when selfed (S_0) yielded all variegated or all purple progeny, respectively (Table 2).

Induction of autotetraploids ($F_{1P1[4x]}$) from $F_{1P1[2x]}$ seedlings was successful. Of 488 seedlings treated, 16.2% survived (79 of 488) the treatment. Of those that survived, 39.2% remained diploid, 45.6% were mixoploids, and 15.2% were non-chimeric tetraploids. Autotetraploids grew slowly, but otherwise were similar to their diploid counterparts. Triploid ($F_{2[3x]}$) and tetraploid ($F_{2[4x]}$) seedlings from crosses with the autotetraploids grew normally, except tetraploid AP phenotypes which tended to be poor growers.

Triploid F_2 ($F_{2[3x]}$) progeny fit the expected 121:11:11:1 segregation ratio (Table 2). However at the tetraploid level ($F_{2[4x]}$) we observed more than three times as many variegated phenotypes than expected (76 observed versus 21.6 expected) and thus had a poor fit to the expected 1225:35:35:1 segregation ratio for tetrasomic inheritance at the

tetraploid level. We analyzed inheritance for each locus separately for random chromosome and chromatid assortment (at 50% recombination), as well as for selective pairing at the tetraploid level to elucidate possible mechanisms for departure from our expected ratio for the variegated trait. Random chromosome assortment for one recessive gene at the tetraploid level would segregrate 35 wild-type : 1 mutant. For the purple locus, there was no reason to reject the assumption of random chromosome assortment at the tetraploid level (Π^2 = 0.028, P = 0.867) however the variegated locus did not fit (Π^2 = 138.9, $P < 0.001$). For random chromatid assortment at 50% recombination we would expect 20.8 wild-type : 1 mutant, and the variegated locus failed to fit (Π^2 = 46.4, $P < 0.001$). Assuming preferential or selective pairing, we would expect 15 wild-type : 1 mutant, and again, the variegated locus failed to fit (Π^2 = 15.6, $P < 0.001$).

If above the diploid level the variegated trait behaves quantitatively, the frequency of variegated progeny in both triploid and tetraploid crosses would increase, and classes of variegation from weak (duplex) to strong variegation (quadriplex) may be discerned. At the tetraploid level we would then expect 3 variegated : 1 green progeny however, the variegated locus did not fit this either (Π^2 = 100.9, $P < 0.001$).

Fertility of progeny. Ploidy level had a significant effect on pollen staining (*F*-value = 274.9, $P < 0.0001$) and germination (*F*-value = 136.3, $P < 0.0001$). Diploid ($2x$) and tetraploid ($4x$) plants had greater pollen staining (96.8% and 94.1%, respectively) and germination (89.1% and 76.3%, respectively) than triploids ($3x$) (40.6% staining and 5.9% germination).

Crosses between diploids ($2x$) resulted in 64.0% fruit set with an average of 632 seed per fruit and a germination percentage of 26.3% (Table 3). Only two fruit formed in $2x$ x $3x$ crosses, with an average of 138 seed per fruit which germinated at 1.0%. Crosses between $2x$ x $4x$ were not successful. Triploids failed to set fruit and viable seed when pollinated with $2x$ or $3x$ pollen. One fruit was collected from $3x$ x $4x$ crosses, yielding four seeds which failed to germinate. Tetraploids were successfully crossed with $2x$ and $4x$ pollen, resulting in similar percent fruit sets, average seed per fruits and seed germination values. Crosses between $4x$ x $3x$ were unsuccessful. Germination was erratic for diploid ($2x$ x $2x$), triploid ($4x$ x $2x$) and tetraploid ($4x$ x $4x$) seed, as evident in the very broad least significant difference ($LSD_{0.05}$) for seed germination (28.4%).

Discussion

Reciprocal di-hybrid crosses were performed to rule out maternal effects on inheritance of each trait, in particular, variegation. Variegated foliage is often the result of abnormal plastid development due to mutations in chloroplast genes and can result in strict maternal inheritance or non-Mendelian inheritance due to stochastic sorting of aberrant versus normal plastids during cell division and transmission to germ cells (Tilney-Bassett, 1978; Walbot and Coe, 1979). F_1 reciprocal crosses of G x AP resulted in green, or wild-type progeny, indicating that variegation in *H. androsaemum* f. *variegatum* 'Glacier' is not the result of maternally (or paternally) transmitted aberrant plastids. Recovery of variegated progeny in F_2s at the expected 9:3:3:1 segregation ratio and in BC_{1P1} at 1:1 is evidence that variegation is controlled by a monogenic, recessive nuclear gene. We propose the gene symbol *var* (*variegated*) for the recessive allele producing the variegated phenotype of *H.*

androsaemum f. *variegatum* and the cultivars 'Mrs. Gladis Brabazon' and 'Glacier' derived

from this forma. Presence of variegation only in homozygous genotypes, with no aberrant

plastid transmission, suggests the variegated allele is a mutation at a locus that regulates

chloroplast gene expression or encodes a protein necessary for proper functioning of the

chloroplast. Plastid restitution occurs when at least one wild-type allele is present, as in the

immutans mutant of *Arabidopsis thaliana* (L.) Heynh. (Aluru and Rodermel, 2004).

The lack of purple foliage in F_1s and recovery of purple progeny at the expected ratio

in the F_2s supports our hypothesis of simple recessive inheritance for purple foliage. We

propose the gene symbol *pl* (*purple leaf*) for the recessive allele originating from *H.*

androsaemum 'Albury Purple'. Purple (or red) foliage variants, resulting from foliar

accumulation of anthocyanins, have variously been described in agronomic crops and are

important in ornamental plant breeding programs. Cadic (1992) reported inheritance of

purple foliage in barberries (*Berberis* L.) due to a single recessive gene, as we observed in

H. androsaemum. In peach [*Prunus persica* (L.) Batsch.] red foliage is controlled by a

single locus, *Gr*, and with the *Gr* allele incompletely dominant (Chaparro et al., 1995), as is

the bronze-leaf (*Rt*) in *Malus* (Alston et al., 2000; Sampson and Cameron, 1965). The lack

of purple pigmentation in our F_1s and no variation in purple foliage intensity in our

segregating progeny suggests totally recessive gene action. In other woody plant genera,

inheritance of purple foliage is dominant as in *Betula pendula* Roth. 'Purpurea' (Hattemer et

al., 1990), *Fagus sylvatica* L. (Blinkenberg et al., 1958; Heinze and Geburek, 1995), and

Corylus L. (Smith and Mehlenbacher, 1996; Thompson, 1985), though variation in

coloration exists in *Corylus* due to different alleles and complimentary gene action.

Backcrossing to AP (BC_{1P2}) resulted in the recovery of more purple seedlings than expected;

however, these may be explained by accidental self-pollination. The recovery of a few

spontaneous variegated progeny was not expected in BC_{1P2}, and not explained by selfing or

pollen contamination. Phenotypes were scored at the three-leaf stage, and these seedlings

may represent the accumulation of deleterious alleles unrelated to the variegated locus or

instability of our variegated locus due to a transposable element.

The recovery in the F_2s of individuals showing both variegated and purple foliage at

the expected ratio indicates that the two traits (loci) are not closely linked. Based on a

simple recessive model of inheritance for both variegated and purple foliage, phenotypes

displaying the novel combination of variegation on purple foliage have the genotype

plplvarvar.

Both traits behaved as expected at the triploid level, but at the tetraploid level we

recovered more variegated progeny than expected. At the tetraploid level, segregation ratios

for the variegated trait fell between that expected with random chromosome assortment and

random chromatid assortment for autotetraploids. Assuming random pairing between

homologous chromosomes and independent assortment, we would expect perfect tetrasomic

inheritance and 35 green :1 variegated progeny in the $F_2[4x]$. However, the prevalence of

multivalent formation and frequency of recombination will influence double reduction and

affect segregation ratios in autotetraploids (Allard, 1960; Ramsey and Schemske, 2002).

Random chromatid assortment predicts that crossing-over occurs between the locus and the

centromere in tetraploids, which allows for double reduction (sister chromatids in the same

gamete). Thus, if we assume random chromatid assortment (quadrivalents with at least one

chiasma) we would expect 20.8 green : 1 variegated progeny in the $F_2[4x]$. Our observed

ratio was $\approx$ 10 green : 1 variegated, falling between the two models. Assuming preferential

pairing is limited in recently induced autotetraploids (Wu et al., 2001), the frequency of double reduction would then have a larger impact on allele frequencies and gametic genotypes (Ramsey and Schemske, 2002). In our induced tetraploid $F_2[4x]$s, double reduction would increase the production of homozygous gametes and thus increase the number of nulliplexes (homozygous recessive with four alleles) and the number of variegated progeny. Double reduction depends on the location of a locus on a chromosome relative to the centromere and the rate of chiasma formation, thus double reduction rates will vary among loci. This may explain the increased recovery of variegated progeny and the unaffected frequency of purple progeny if the purple locus is located closer to the centromere. In autotetraploid Easter cactus (*Hatiora ×graeseri* Barthlott ex D. Hunt) isozyme segregation ratios fit random chromosomal or chromatidal assortment, depending on the specific loci (Karle et al., 2002), and in allotetraploid quinoa (*Chenopodium quinoa* Willd) discrepancies in tetrasomic segregation ratios for the *R* locus controlling inflorescence color were attributed to variable multivalent formation (Ward, 2000).

Inducing tetraploidy apparently had little effect on male fertility, with pollen staining similar to diploids, however there was a slight decrease in pollen germination compared to that of the diploid. Female fertility, measured as percent fruit set, was lower in the tetraploids when selfed or crossed with diploids than for diploid self's. In reciprocal crosses between diploid and tetraploid inbred lines of *Salpiglossis sinuata* R. & P., fruit set did not differ between crosses, however, the number of seed produced was significantly lower in selfed tetraploids (Needham and Erickson, 1992). For autotetraploids reductions in pollen and seed fertility are usually reduced compared to their diploid progenitor (Ramsey and Schemske, 2002) which is often attributed to the production of multivalents, the frequency

of which is negatively correlated with fertility (Sybenga, 1996). In our study, tetraploid selfs had lower percent fruit set, but average seed set per fruit and percent seed germination were not significantly different.

Diploid *H. androsaemum* pollinated with tetraploid pollen ($2x$ x $4x$) failed to set fruit. Crosses in this direction commonly fail and violate the endosperm balance number theory, which predicts embryo abortion for crosses that violate a 2 maternal : 1 paternal genomic ratio in developing endosperm (Carputo et al., 1999; Johnston et al., 1980; Sanford, 1983). Crosses with tetraploid *H. androsaemum* as the female ($4x$ x $2x$) were successful in producing triploids.

Triploids are expected to be highly infertile owing to uneven segregation from increased multivalent formation and partner exchange during meiosis (Sybenga, 1996) leading to abortive gamete development. Triploids had low pollen germination, even though pollen staining was 40.6%. Pollen staining often results in an overestimation of pollen viability (Olsen et al, in prep.) with germination and successful pollination more indicative of viability. Triploid pollen failed to initiate fruit set in self's and in crosses with tetraploid females in the current study. An additional 139 triploid flowers were selfed on various triploid plants after the study, resulting in a total of five fruit averaging 41.8 seed per fruit (data not shown). Diploids pollinated with triploid pollen resulted in just two fruit with reduced seed set and low germination (1%), which may have been the result of diploid pollen contamination or selfing. One fruit was initiated on $3x$ x $4x$ crosses, which contained four seed that failed to germinate. We were unable to recover any viable seeds from triploid female parents regardless of the ploidy level of the male parent. The fertility of triploids are dependent on the production of euploid gametes ($n = x$, $2x$, or $3x$) or tolerance of gametes

and zygotes to aneuploidy. Triploid blueberries (*Vaccinium corymbosum* L.) are highly

sterile, though crosses with tetraploids and hexaploids are variably successful due to the

production of 2*n* gametes and tolerance of aneuploid gametes above 2*x* level (Vorsa and

Ballington, 1991). Triploid bananas are regarded as sterile, although Ortiz and Vuylsteke

(1995) were able to identify clones with residual fertility due to production of euploid

gametes from selective elimination of one chromosome set during meiosis. The

identification of fertile triploids are valuable for breeding programs; however, selecting

infertile triploids are just as valuable for limiting the unwanted spread of ornamental plants

in the landscape. The above studies in blueberries and bananas, as well as triploid *Lathyrus*

pratensis L. (Khawaja et al., 1997), *Salpiglossis sinuata* (Needham and Erickson, 1992) and

our triploid *H. androsaemum* suggest that hybrid triploids can be screened for infertility as a

viable approach for developing new non-invasive cultivars.

Purple (*pl*) and variegated (*var*) foliage in *H. androsaemum* are both simple recessive

traits and are not linked, allowing the recovery of the two parental phenotypes as well as the

novel combined phenotype at the triploid and tetraploid levels. The greatly reduced male

fertility and apparent lack of female fertility among triploids should allow for selection of

novel, non-invasive *Hypericum androsaemum* cultivars.

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Table 1. Crosses between *Hypericum androsaemum* 'Glacier' (G) and 'Albury Purple' (AP) phenotypes and families produced.

Cross	Ploidy of progeny	Families
G x AP	$2x$	$F_{1P1[2x]}$, $F_{2P1[2x]}$[z]
AP x G	$2x$	$F_{1P2[2x]}$, $F_{2P2[2x]}$
G x $F_{1P1[2x]}$	$2x$	BC_{1P1}
AP x $F_{1P1[2x]}$	$2x$	BC_{1P2}
G $F_{2P1[2x]}$ selfed	$2x$	S_0
AP $F_{2P1[2x]}$ selfed	$2x$	S_0
$F_{1P1[4x]}$ x $F_{1P1[2x]}$	$3x$	$F_{2[3x]}$
$F_{1P1[4x]}$ selfed	$4x$	$F_{2[4x]}$

[z]Ploidy level in brackets for each filial generation for clarity in describing subsequent crosses.

Table 2. Segregation for foliage traits in *Hypericum androsaemum* across ploidy levels in families derived from 'Glacier' (G) and 'Albury Purple' (AP).

Cross	Families	Progeny (no. seedlings)[z]				Expected Ratio	χ^2	P
		Gr	Var	Pur	Var:Pur			
G x AP	$F_{1P1[2x]}$[y]	130	3[x]	0	0	1:0:0:0	0.00	1.00
AP x G	$F_{1P2[2x]}$	162	0	1[x]	0	1:0:0:0	0.00	1.00
G x AP	$F_{2P1[2x]}$	218	71	88	20	9:3:3:1	3.69	0.30
AP x G	$F_{2P2[2x]}$	214	77	81	28	9:3:3:1	1.43	0.70
G x $F_{1P1[2x]}$	BC_{1P1}	200	196	0	0	1:1:0:0	0.04	0.84
AP x $F_{1P1[2x]}$	BC_{1P2}	178	4[w]	221	3[w]	1:0:1:0	4.63	0.03
G selfed	S_0	0	200	0	0	0:1:0:0	0.00	1.00
AP selfed	S_0	0	0	200	0	0:0:1:0	0.00	1.00
$F_{1P1[4x]}$ x $F_{1P1[2x]}$	$F_{2[3x]}$	573	46	49	3	121:11:11:1	1.38	0.71
$F_{1P1[4x]}$ selfed	$F_{2[4x]}$	701	76	22	1	1225:35:35:1	141.20	< 0.001

[z]Number of progeny for each phenotypic class (Gr = green; Var = variegated; Pur = purple; Var:Pur = variegated and purple combined).

[y]Ploidy level in brackets for each filial generation for clarity in describing subsequent crosses.

[x]Unexpected and inconsistent with proposed simple recessive model, due to, possible accidental self-pollination. Data not used in chi-square calculations.

[w]Unexpected and unexplained variegation, which can not be explained by self- or cross-pollen contamination. These seedlings were scored as either green or purple for chi-square calculations.

Table 3. Fertility as a function of ploidy in *Hypericum androsaemum* reciprocal interploid crosses.

Female parent (♀)	Male parent (♂)	Fruit developed			Seed germination (%)[w]
		No.	(%)[y]	Avg seed[w]	
2x	2x	32	64.0 a[v]	632 a	26.3 a
2x	3x	2	2.0 b	138 ab	1.0 a
2x	4x	0	0.0	---	---
3x	2x	0	0.0	---	---
3x	3x	0	0.0	---	---
3x	4x	1	4.2 b	4.0 b	0.0 a
4x	2x	7	10.8 b	260 ab	5.7 a
4x	3x	0	0.0	---	---
4x	4x	15	20.7 b	264 ab	8.4 a
LSD$_{0.05}$			23.7	567	28.4

[z]Means for single plant replicates (n = 6), with ≥ 100 pollen grains scored per replicate per viability test.
[y]Means for single plant replicates (n = 5) with 10 subsamples (flowers pollinated) per replicate, except 3x x 3x (n = 3) and 4x x 4x (n = 4).
[w]n = total number of fruit harvested per cross.
[v]Means followed by the same letter, within a column, not significantly different based on Fisher's protected LSD.