INTERSPECIFIC HYBRIDIZATION, PLOIDY MANIPULATION, AND CYTOLOGICAL AND GENETIC ANALYSES AS TOOLS FOR BREEDING AND IMPROVEMENT OF *CALLICARPA* L., *CRYPTOMERIA* D. DON, *HIBISCUS* L., AND *TECOMA* JUSS.

by

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(Under the Direction of John M. Ruter)

ABSTRACT

Research was conducted as part of breeding programs to improve *Callicarpa*, *Cryptomeria*, *Hibiscus*, and *Tecoma* including field evaluation, polyploidization, genetic and cytogenetic studies, and interspecific hybridization. These techniques were successfully integrated into existing improvement programs. Polyploidy was induced in *Hibiscus acetosella* 'Panama Red' PP20121 to develop a more compact plant. Plant height and fertility was reduced, leaves were smaller, internodes were shorter, and canopy volume were reduced in the induced octoploid. Japanese cedar chromosomes were doubled by spraying seedlings with oryzalin. Eighty-three percent of selected seedlings were tetraploids, 9.3% were cytochimeras, and 7.6% were diploids. Performance of Japanese cedar cultivars was evaluated by measuring chlorophyll and carotenoids and assigning color ratings. There were differences in chlorophyll, ratio of chlorophyll *a*:*b*, carotenoids, and color rating (greeness). There were no consistent trends for differences between winter and summer or between traits. Crosses were performed to

investigate the genetics of fruit color, leaf variegation, self-compatibility, and apomixis in American beautyberry. Crosses between purple and white fruit showed white fruit is recessive and is controlled by a single recessive gene called *white fruit* (*wf*). Progeny developed sexually and all genotypes were self-compatible. We propose that purple, pink, and white fruit are allelic with proposed symbols $Wf > wf^{p} > wf$. Maternal effects appear to be involved in leaf variegation; however, germination was too low to draw conclusions. *Callicarpa americana* seeds were treated with sulfuric acid to increase germination. The control, 15 min, and 30 min treatments germinated at 8.9%, 57.8%, and 48.9%, respectively, indicating that scarification benefits germination. Genome sizes of *Callicarpa* were calculated using flow cytometry and ranged from 1.34 to 3.11 pg. Chromosome counts revealed most were diploid ($2n = 2x = 34$) but two were tetraploid. Interspecific hybridization in *Tecoma* was conducted. Fertile hybrids between *T. garrocha* and *T. stans* (F_1) were backcrossed to parents and self-pollinated. *Tecoma garrocha*, *T. stans*, and *T. guarume* 'Tangelo' were self-fertile. Complex hybrids were developed: F_1 x *T. capensis*; F_1 x *T. guarume* 'Tangelo'. Leaf morphology of F_1 was intermediate. GISH successfully identified hybridity. Four copies of rDNA were observed in F_1s using FISH.

INDEX WORDS: Induced polyploidy, cytogenetics, genome size, inheritance, ornamental plant breeding, in situ hybridization

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DEDICATION

To

Carol English Contreras

and

Ronald Ramon Contreras

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Introduction and Literature Review

Many ornamental cultivars in the nursery trade have arisen by selection and propagation of chance mutations (sports) or selection of unique/superior forms from open pollinated seedlings. The primary skill involved has been the ability to identify traits lacking in commonly available material. Indeed, the difference between a successful ornamental breeder and an unsuccessful one is often determined by plant knowledge. This point is universal for breeders; however, and is exemplified by the late Nobel Laureate Norman Borlaug's response when asked about becoming a successful wheat breeder, "How do you become a successful wheat breeder? Well, you go to the field. You go to the field, you go to the field, you go to the field again, and then you go to the field. When the wheat plants start to talk to you, you know you have made it." Dr. Borlaug's quote indicates the importance of familiarity with the plant material. Technical expertise is of little use without being able to identify the areas that need improvement in the crop and sources of genetic diversity for the trait of interest. However, ornamental cultivar development has long utilized many techniques for improvement and this trend continues today. This review is not meant to be comprehensive of all previous research, rather it is meant to serve as an illustration of the breeding work that has been used to improve extremely diverse taxa. The principle techniques that will be discussed are the use of induced polyploidy, genetic studies to investigate the inheritance of ornamentally important traits, and cytogenetic and molecular cytogenetic studies, concentrating on research involving hybrids or synthesized polyploids. Other cytogenetic and molecular cytogenetic studies have been used to investigate evolutionary relationships; however, this review will focus on studies that have used these techniques as breeding tools.

Induced polyploidy. Induced polyploidy has been used extensively in plant development since the late 1930's following the work of Blakeslee and Avery (1937). In their seminal work, Blakeslee and Avery (1937) described methods of application of colchicine and successful chromosome doubling in the following diverse herbaceous genera: *Datura* L., *Portulaca* L., *Cosmos* Cav., *Phlox* L., *Stellaria* L., *Petunia* Juss., *Nicotiana* L., *Digitalis* L., *Mirabilis* L., *Tropaeolum* L., *Cheiranthus* L., *Raphanus* L., *Cucurbita* L., *Trifolium* L., *Medicago* L., *Zea* L., and *Allium* L. Following the discovery that colchicine induced polyploidy there was a great deal of effort to utilize induced polyploidy for improvement in fruits, vegetables, ornamentals, and agronomic crops with varying degrees of success (Hancock, 1997). One group of plants that benefited from induced polyploidy during this era was floriculture crops (Emsweller and Ruttle, 1941). Chromosome doubling has been used for a number of reasons in ornamentals including restoring fertility in wide hybrids (Contreras et al., 2007; Hadley and Openshaw, 1980; Olsen et al., 2006b; Ranney, 2006; Sanford, 1983; van Tuyl an De Jeu, 1997; van Tuyl and Lim, 1997), developing sterile cultivars (Ranney, 2006), overcoming hybridization barriers (Kehr, 1996; Ranney, 2006), and altering morphology of leaves, flowers, fruits, or overall vigor (Contreras et al., 2009; Kehr, 1996a; 1996b).

Chromosome doubling was traditionally accomplished using colchicine which acts by disrupting microtubular structure of dividing cells in both plants and animals (Bartels and Hilton, 1973). The mode of action of dinitroaniline herbicides such as oryzalin have also been determined (Bartels and Hilton, 1973; Upadhyaya and Noodén, 1977; Upadhyaya and Noodén, 1980) where the herbicide has been found to bind more specifically to plant tubulin in vitro than colchicine (Yemets and Blume, 2008). In

addition to more specific binding, dinitroaniline herbicides have been found to be less toxic and to act at 100- (Yemet and Blume, 2008) to 1000-times (Upadhyaya and Noodén, 1977) lower concentrations than colchicine. The discussion below will include both studies that used colchicine and oryzalin to double chromosomes and will be grouped generally by the purpose of inducing polyploidy.

A majority of studies using induced polyploidy have been conducted to restore fertility in sterile F_1 hybrids. Ackerman and Dermen (1972) used colchicine to double chromosomes of PI 315906 'Fragrant Pink', a sterile F_1 hybrid of *Camellia rusticana* Honda x *C. lutchuensis* Itô. Drop treatment with 0.5% colchicine applied to buds every two days (three to six applications) resulted in cytochimeras that had L-I, L-II, and L-III histogenic layers with the ploidy composition of 2-4-4, which exhibited 77% normal pollen compared to 5% normal pollen in diploids (Ackerman and Dermen, 1972). Zadoo et al. (1975) treated vegetative cuttings of the three sterile diploid *Bougainvillea* cultivars 'Mrs. McClean', 'Roosvelt', and 'Shubhra' with a 0.5% aqueous colchicine solution for two days. Pollen staining was increased from 0% to over 90% in all cultivars and normal seed set was also restored (Zadoo et al., 1975). In vitro polyploidization was performed by Griesbach (1990) on sterile *Anigozanthos* Labill. 'Bush Ranger' using 0.1% colchicine. The resulting tetraploids were fertile and exhibited larger florets and taller inflorescences (Griesbach, 1990). Fertility is not guaranteed to be restored in all sterile hybrids as illustrated by Lu and Bridgen's (1997) work on Peruvian lily (*Alstroemeria* L.). Forty-one percent of plants were tetraploid after in vitro application of 0.2 to 0.6% colchicine for 6 to 24 h; however, despite restoration of proper meiotic pairing and an increase from 0% to 12% pollen staining the tetraploids remained sterile (Lu and

Bridgen, 1997). While colchicine historically has been used to induce polyploidy, dinitroaniline herbicides including oryzalin have been shown to be effective mitotic inhibitors. Van Tuyl et al. (1992) restored fertility in an F_1 hybrid of *Lilium henryi* Bak. x *L. candidum* L. using oryzalin and found that it was more effective and could be used at lower concentrations than colchicine. Also of importance was the observation that mutation induction may be lower when using oryzalin which would allow effective polyploidization while maintaining the desired phenotype. Oryzalin was used to double the sterile diploid cultivars *Rosa* L. 'Pink Surprise' and 'Mermaid' and the resulting tetraploid forms have been successfully used in controlled crosses (Kermani et al., 2003). Contreras et al. (2007) reported restoration in the intersubgeneric hybrid *Rhododendron* L. 'Fragrant Affinity' following chromosome doubling using a 150 μM oryzalin solution to treat shoot tips for 24 h. Restoration of fertility was confirmed by successful seed set and an increase of pollen staining and germination from 4% to 68% and 0% to 45% in diploids and allotetraploids, respectively (Contreras et al., 2007). Olsen et al. (2006b) restored fertility in the sterile intergeneric hybrid ×*Chitalpa tashkentensis* Elias & Wisura. 'Pink Dawn'. Cytochimeras that had tetraploid L-II histogenic layers were recovered and exhibited restored pollen viability documented by staining and germination (Olsen et al., 2006b).

The use of induced polyploidy followed by backcrossing to develop sterile triploids has not been common in ornamental horticulture. The most well known and successful example is the Greek goddess cultivar series of rose-of-sharon (*Hibiscus syriacus* L.) developed by Don Egolf at the U.S. National Arboretum. In 1960, development of the teteraploid that would ultimately serve as a parent for all cultivars

was performed by treating germinating seedlings of 'William R. Smith' with an aqueous solution of 0.05% colchicine. The resulting tetraploid was then backcrossed with the diploids to form 'Diana' (Egolf, 1970), 'Helen' (Egolf, 1981), 'Minerva' (Egolf, 1986), and 'Aphrodite' (Egolf, 1988). Olsen et al. (2006a) developed triploids of the potentially invasive tutsan (*Hypericum androsaemum* L.) by treating seedlings for 24 h with a 150 µM oryzalin solution to develop tetraploids and backcrossing to diploids. Pollen staining and germination was greatly reduced in the triploid compared to diploids and tetraploids, as was reproductive success as a pollen or seed parent in vivo (Olsen et al., 2006a).

Leaf morphology is commonly altered in induced polyploids, with the resulting polyploids typically exhibiting thicker and darker leaves, and may have an altered length to width ratio (Contreras et al., 2009; Thao et al., 2003). Alteration of floral morphology, growth rate, and internode length have also been facilitated using induced polyploidy. Pryor and Frazier (1968) doubled the chromosomes of a number of *Rhododedron* cultivars and observed a great increase in flower size in the cytochimeral tetraploid (4-4-2) form of 'Sun Valley' compared to the diploid. An increase in the thickness of cell walls of flower petals in the tetraploid form of *Rhododendron* 'Gloria' was reported (Pryor and Frazier, 1968), an alteration that has the potential for producing forms with improved flower texture. Kermani et al. (2003) reported that induced hexaploid *Rosa* forms had shorter internodes than triploids, although the relationship was reversed between tetraploids and dipoids. The number of petals was also nearly double in the tetraploid form of *Rosa* 'Thérèse Bugnet' compared to the diploid (Kermani et al., 2003). Rose et al. (2000a) reported that in vitro application of colchicine resulted in a tetraploid form of a lilac (*Syringa* L. sp.) hybrid that was shorter than the diploid. Tetraploid

Buddleia globosa Hope were reported to have shorter internodes and a more compact habit (Rose et al., 2000b). Stanys et al. (2006) reported there was not an increase in fruit size in colchicine-induced flowering quince [*Chaenomeles japonica* (Thunb.) Lindl. ex Spach]; however, they observed there was only one-fourth as many seeds produced in tetraploids as in diploids. Similarly, Contreras et al. (2009) observed greatly reduced seed set and little seed germination in an induced octoploid form of *Hibiscus acetosella* 'Panama Red' PP20121 that was also reported to have shorter internodes, smaller canopy volume, and shorter plant height. Flower spikes of tetraploid *Buddleia madagascarensis* Lam. x *B. crispa* Benth developed by in vitro application of oryzalin were twice the size of diploids (Dunn and Lindstrom, 2007).

Researchers have developed induced polyploids with the ultimate goal of making crosses not possible at the original ploidy level of the species (e.g. Dhooghe et al., 2009); however, reports of successful implementation of this technique in ornamentals are rare. Kehr (1996a) reported success when crossing tetraploid *Rhododendron agustinii* Hemsl. by tetraploid *R. minus* var. *minus* Michx. Carolinianum Group 'Epoch' (Kehr, 1971), a cross that is extremely difficult when using a diploid form of *R. minus* var. *minus* Carolinianum Group. Kehr (1996b) also reported producing many viable seeds when crossing the deciduous azalea *R. calendulaceum* (Michx.)Torr. (natural tetraploid) and tetraploid forms of the evergreen azaleas 'Tahei', Gettsu-toku', 'Banka', and 'Wako'; crosses reported as difficult or impossible when evergreen parents are diploid (Kehr, 1996b). Dunn and Lindstrom (2007) successfully crossed the intersectional hybrid *Buddleia madagascarensis* x *B. crispa* with *B. davidii* var. *nanhoensis* Rehd. 'Alba' after inducing polyploidy in the former species.

Traditional genetic studies. Most ornamental crops have not experienced the intensive research that a crossover crop such as *Amaranthus* has enjoyed, and as such, studies have focused on qualitative traits of economic importance such as flower, fruit, and foliage color. Bright pink flowers in crimson clover (*Trifolium incarnatum* L.) were identified as a simply inherited trait controlled by a recessive allele at a single locus that was designated *bp* (Mosjidis, 2000). Wannakrairoj and Kamamoto (1990) reported that the three genes control spathe color in *Anthurium* Schott. are *M*, *O*, and *P* and described the epistatic interaction among the loci and the combinations that result in various spathe colors. Genetic control of several ornamental traits in hazelnut (*Corylus avellana* L.) have been investigated. Chlorophyll deficiency is controlled by a single recessive gene designated as *chlorophyll deficient #1* and is independent of the dominant gene (*A*) that controls red foliage (Mehlenbacher and Thompson, 1991). Mehlenbacher and Smith (1995) also determined that the *cutleaf* trait in hazelnut, represented as *cf*, is a single recessive gene that is independent of the loci that control red leaf color and chlorophyll deficiency. Contorted growth in hazelnut was found to be independent of the gene for red foliage and is controlled by a single recessive gene designated as *twisted* with the symbol *tw* (Smith and Mehlenbacher, 1996).

Kloos et al. (2004) reported on the inheritance of major flower type in gerbera daisies [*Gerbera hybrida* (*G. jamesonii* Bolus ex Adlam x *G. viridifolia* Shultz-Bip)]. It was proposed that three alleles (Cr^d, *Cr*, *Sp*) with incomplete dominance control the major flower types in gerbera daisy (Kloos et al., 2004). Control of dark disk color in gerbera flowers was also investigated and determined to be a single gene dominant (*Dc*)

to light disk color (*dc*) (Kloos et al., 2005). Kulkarni et al. (2005) reported that five interacting but independently inherited genes *R*, *W*, *E*, *J*, and *O* were involved in production of previously reported corolla colors in periwinkle [*Catharanthus roseus* (L) G. Don] and that the *O* gene was allelic (O^m and *o*). Inheritance of flower color in stokes aster [*Stokesia laevis* (J. Hill) Greene] was determined to be controlled by three loci; *A*, *Y*, and *P* (Barb et al., 2008a). Barb et al. (2008a) determined that *aa* plants produce a reduced amount of anthocyanin and *yy* plants do not produce anthocyanins and the recessive *y* locus is epistatic to the *A* locus when homozygous (*yy*). Ranney and Olsen (2009) determined that pink flower color and production of sterile florets are both recessive in *Hydrangea arborescens* L. A three-gene model (*A*, *B*, *C*) with two alleles at each locus was proposed to control flower color in *Anagallis monelli* L. (Freyre and Griesbach, 2004) but upon the discovery of a white phenotype, a four-gene model was proposed (Quitanan et al., 2008). White flower color in *Buddleia fallowiana* var. *alba* was determined to be homozygous for the recessive gene *alb-1* and *B. davidii* 'Nanhoensis Alba' was heterozygous for a dominant gene controlling white flower color, *Alb-2* (Tobutt, 1993). A gene controlling compact habit, *Con-1*, was tightly linked to *Alb-2* and *B. davidii* 'Nanhoensis Alba' was also heterozygous at this locus, as was *B. fallowiana* var. *alba* for a complementary gene for compact habit, *Con-2* (Tobutt, 1993). Davidson and Lenz (1990) proposed a four-gene model (W_1, W_2, Y_1, Y_2) for flower color and three-gene model (D_1, D_2, Dm) for inheritance of extra petals in *Potentilla fruticosa* L. Purple leaf (*pl*) and variegated (*var*) foliage were found to be controlled by single recessive genes that are unlinked in *Hypericum androsaemum* (Olsen et al., 2006). Jaynes (1981) reported that nine flower and foliage forms in *Kalmia latifolia* L. appear to

be under single gene control. Deng and Harbaugh (2006) reported that vein color in *Caladium* ×*hortulanum* Birdsey was controlled by a single locus with three alleles ($V^r > V^w > V^g$) for red, white, and green veins, which is independent of the co-dominant alleles *F* and *f* that control leaf shape. Leaf spots in *Caladium* ×*hortulanum* are controlled by a single locus with two alleles, *S* for presence of spots and *s* for absence of spots, that are independent of leaf shape by linked to vein color (Deng et al., 2008).

Cytogenetics and molecular cytogenetics in hybrids. Cytogenetics and molecular cytogenetics have been used in ornamental breeding programs principally to determine the mode of sterility in hybrids by observing meiotic pairing and confirm hybridity. Palmer et al. (2009) confirmed hybrids of *Rudbeckia subtomentosa* Pursh. x *R. hirta* L. 'Toto Gold' based on the size of chromosomes donated from each parent and that the hybrids also had intermediate genome sizes as determined by flow cytometry. Of interest from their study was the fact that these two cross compatible species are both in subgenus *Rudbeckia*, yet have gross differences in chromosome size that makes identification possible using standard staining methods (Palmer et al., 2009). Zadoo et al. (1975) reported that sterile diploid cultivars of *Bougainvillea* in cultivation exhibit aberrant meiotic pairing including the production of univalents. There was a low percentage of multivalent formation in induced tetraploids and fertility was restored (Zadoo et al., 1975). Zadoo et al. (1975) noted that tetraploids developed from diploids with greater heterozygosity had higher fertility. The cultivar *Narcissus* L. 'Whitewell' is a triploid hybrid of *N. pseudonarcissus* x *N. poeticus* that was found to have a high percentage of trivalent formation during meiosis (Karihaloo and Koul, 1985). The high degree of

similarity observed between the parental species in addition to pairing during meiosis may provide evidence for alteration of the current taxonomic grouping in *Narcissus* (Karihaloo and Koul, 1985). Krebs (1997) reported that sterility in three interspecific rhododendron hybrids was not due to abnormal meiosis. This finding indicates that chromosomal hybrid sterility is not involved; however, Contreras et al. (2007) reported apparent meiotic abnormalities in intersubgeneric rhododendron hybrids and restoration of fertility in induced polyploids. These contrasting findings indicate that the degree of wide hybridization (intra- vs. intersubgeneric) in rhododendron may play a role. Bolaños-Villegas et al. (2008) found a positive relation between high seed set, high frequency of viable tetrads, high degree of chromosome pairing, and low chromosomal aberrations in hybrids of *Doritaenopsis* Guillaum. & Lami orchids. In contrast, Lu and Bridgen (1997) reported that fertility was not restored in *Alstroemeria* hybrids after chromosome doubling even though meiotic pairing was mostly normal in tetraploids. Reed (2005) reported than an interspecific hybrid between *Clethra alnifolia* L. 'Hokie Pink' ($2n = 32$) x *C. pringlei* S. Wats. ($2n = 16$) had $2n = 32$ chromosomes which exhibited primarily bivalent pairing in pollen mother cells (PMCs). The author proposed that the hybrid resulted from the union of a reduced egg from 'Hokie Pink' and an unreduced male gamete from *C. pringlei* (Reed, 2005). Barb et al. (2008b) reported normal pairing during meiosis in diploid stokes aster, and while there was quadrivalent formation in the natural tetraploid 'Omega Skyrocket' it ultimately exhibited equal disjuntion. On the other hand, newly synthesized tetraploids and interploid crosses of stokes aster had meiotic configurations from uni- to pentavalents and numerous

abnormalities such as laggards, unequal disjunction, and chromosome bridges were present (Barb et al., 2008b).

Molecular cytogenetics has been used to investigate ornamental hybrids to confirm hybridity and investigate parental contribution. Primary techniques that have been used in ornamentals are genomic in situ hybridization (GISH) and fluorescent in situ hybridization (FISH). GISH uses total genomic DNA, while FISH uses sequence information; however, both methods use biotinylated or digoxigenin-labeled DNA to probe chromosome preparations. Marasek et al. (2004) successfully used GISH to probe lily hybrids with genomic DNA of the pollen parent from crosses to confirm hybridity. FISH using 5S and 25S rDNA probes identified marker chromosomes unique for each parent, and was also used in confirmation of lily hybrids (Marasek et al., 2004). Karlov et al. (1999) also used GISH and FISH in lily to identify complex hybrids between *Lilium longiflorum* Thunb., Asiatic, and Oriental lilies. GISH was used to investigate nine cultivars of *Allium* L. subgenus *Melanocrommyum* of uncertain parentage and successfully identified the parentage of seven, with two cultivars for which the second parent was not identified (Friesen et al., 1997). Takahashi et al. (1997) demonstrated recombination in an intergeneric hybrid between *Gasteria* Duval. x *Aloe* L. Differentiation was achieved when either parent was used as the probe, and the genomic differences were great enough that blocking DNA was not used which is not surprising in a bigeneric hybrid; however, the hybrid is partially fertile and there is extensive recombination between parental chromosomes, which is uncommon (Takahashi et al., 1997). In contrast, to differentiate F_1 hybrids in *Clivia* Lindl. by GISH required high stringency and a ratio of 1 probe : 90 blocking DNA (Ran et al., 2001).

This dissertation presents the results of using polyploidy, genetic studies, and cytogenetics, field evaluation, and interspecific hybridization as tools in breeding in breeding programs to improve *Hibiscus* L., *Callicarpa* L., *Cryptomeria* D. Don., and *Tecoma* Juss.

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Chapter 1

AN ORYZALIN-INDUCED AUTOALLOOCTOPLOID OF *HIBISCUS ACETOSELLA* WELW. EX HIERN. 'PANAMA RED' PP20121 (MALVACEAE)[1]

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An Oryzalin-Induced Autoallooctoploid of *Hibiscus acetosella* Welw. ex Hiern. 'Panama Red' (Malvaceae)

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Abstract. *Hibiscus acetosella* Welw. ex Hiern. 'Panama Red' PP20,121 has generated public and grower interest due to its attractive red foliage and vigorous growth, however a horticultural goal is to develop more compact forms. Even though organs of induced polyploids are often larger than the wild-type, whole plants are often shorter in stature. Three studies were conducted to induce polyploidy and evaluate the growth and reproductive potential of the resulting polyploids. In study 1, seeds were soaked for 24-hours in aqueous solutions of 0%, 0.2%, 0.4%, or 0.5% colchicine (w/v) plus 0.5% dimethyl sulfoxide. In studies 2 and 3, apical meristems of seedlings at the cotyledon stage were treated for 1 or 3 days with 0, 50, 100, or 150 μM oryzalin solidified with 0.8% agar. Visual observations and measurement of guard cells were used to identify plants that potentially had their chromosome number doubled. Flow cytometry of nuclei stained with DAPI was used for confirmation of polyploidy. No induced polyploidy was observed following seed treatment with colchicine at the rates and duration used in this study. One-time application of 50 μM oryzalin resulted in a single mixoploid ($4x + 8x$) in which the ploidy of the L-I, L-II, and L-III histogenic layers were identified as a 4-4-4+8, respectively. Three-day applications with 100 and 150 μM oryzalin resulted in an octoploid ($8x$) and a mixoploid ($4x + 8x$), respectively. The mixoploid from the three day

treatment stabilized at the 8*x* level prior to flowering, but was identified as a 4+8-*x*-4 cytochimera. Plant height was reduced, leaves were smaller, internodes were shorter, and canopy volume were reduced in the octoploid (8*x*) form compared to the tetraploid (4*x*) form. Furthermore, in contrast to the tetraploid, the octoploid has produced no self-pollinated seed and has performed poorly both as a staminate and pistillate parent in controlled crosses. This represents the first time oryzalin has been reported to induce polyploidy in *Hibiscus* L. section *Furcaria* DC. *Hibiscus acetosella* is an allotetraploid species with the genome composition AABB. The resulting autoallooctoploid (AAAABBBB) form of 'Panama Red' exhibits a more compact habit and reduced production of seed.

chemical names: oryzalin: 3,5-dinitro-N^4N^4-dipropylsulfanilamide; DAPI: 4',6-diamidino-2-phenylindole; DMSO: dimethyl sulfoxide

Introduction

The genus *Hibiscus* L. (tribe Hibisceae) is comprised of approximately 200 (Fryxell, 1988; Hochreutiner, 1900) to 250 (Bates, 1965) annual and perennial species in ten sections (Fryxell, 1988). Section *Furcaria* DC is a circumglobal tropical and subtropical (rarely temperate) group (Wilson and Menzel, 1964) comprised of more than 100 species (Wilson, 1994). This group includes important fiber, food, and medicinal plants such as kenaf (*H. cannabinus* L.) and roselle (*H. sabdariffa* L.). Many species in this section have been used as ornamentals for their large, showy flowers (Wilson, 1994). The base chromosome number in section *Furcaria* is commonly regarded as x=18 (Menzel and Wilson, 1961; Menzel and Wilson, 1963a; Menzel and Wilson, 1963b; Skovsted, 1941) but x=9 has also been proposed (Sanyal and Kundu, 1959). Most species in section *Furcaria* are allopolyploids ranging from $2n=4x=72$ to $2n=10x=180$ with representative species on all continents with tropical or subtropical regions (Menzel et al., 1986).

There are a number of species in section *Furcaria* with ornamental potential. One that is of interest and was utilized in the current research is *H. acetosella* Welw. ex Hiern. *Hibiscus acetosella* is a tetraploid ($2n=4x=72$; Akpan, 2000; Menzel and Wilson, 1961) of African origin known only in cultivation (Siemonsma and Piluek, 1993; Wilson, 1994). Based on the prevalence of bivalents at metaphase I (MI) (Akpan, 2000; Kuwada, 1977) and genome studies (Menzel and Wilson, 1961), *H. acetosella* has been determined to be an allotetraploid with the genome composition AABB (Menzel and Wilson, 1961). It is widely grown as an ornamental in tropical and subtropical regions (Wilson, 1994). It is an annual or perennial herb or undershrub, typically with red foliage. Solitary flowers of wine-red, rarely yellow, are formed in the axils (van Borssum Waalkes, 1966). A

number of selections have been released over the years including a recent release from The University of Georgia, *H. acetosella* 'Panama Red' PP20,121. This release has generated interest in the green industry due to its attractive foliage and vigorous growth; however, evaluator and consumer comments have indicated that 'Panama Red' could be improved by developing more compact forms.

Induced polyploidy often results in the gigas effect of individual organs; particularly those with determinate growth such as sepals, petals, fruits, and seeds (Stebbins, 1950). However, in the case of induced autopolyploids the growth rate of whole plants is often slower (Stebbins, 1950). Kumar Sen and Chheda (1958) found that induced tetraploids of black gram (*Phaseolus mungo* L.) were stunted and exhibited reduced fertility. Induced polyploids of oil palm (*Elaeis guineensis* Jacq.) also were reported to be shorter than diploids (Madon et al., 2005). The current research was conducted to take advantage of the reduction in plant size and vigor often associated with induced polyploidy. The principle objectives were to induce polyploidy in seedlings from *H. acetosella* 'Panama Red' and evaluate the effects on growth, morphology, and fecundity. Upon discovering that treatments induced two cytochimeras, ploidy of the histogenic layers of these plants was investigated.

Materials and Methods

Plant Material. Seeds from autonomously self-pollinated flowers were collected from *H. acetosella* 'Panama Red' plants grown in a glasshouse. Plant material was previously described as a hybrid between *H. acetosella* x *H. 25adiates* Cav. (Contreras and Ruter, 2009), however the seed source was later correctly identified as 'Panama Red' based on

morphology (personal observation). Plants resulting from self-pollination of 'Panama Red' are uniform and are nearly identical to the cultivar.

Chromosome doubling studies.

Seed treatment with colchicine. Seed were pre-treated by soaking in an aqueous solution of 0.1% Triton® X-100 (v/v; Integra Chemical Company, Renton, WA) for 24-h on a rotary shaker (Model G-33, New Brunswick Scientific, Edison, NJ) at 150 rpm. Following pre-treatment, seeds were transferred to solutions containing 0%, 0.2%, 0.4%, or 0.6% colchicine [(w/v) (Sigma-Aldrich Chemical Co., St. Louis, MO)], plus 0.5% dimethyl sulfoxide [(v/v) (DMSO; Sigma-Aldrich)] as an adjuvant. Treatments (75 seed/treatment) were applied for 24-h on the rotary shaker at 150 rpm. Following treatment, seeds were rinsed under running tap water for 15-min and sown in 200-cell polystyrene tobacco float trays containing Pro-Mix BX with Biofungicide (Premier Tech Ltd., Quakertown, PA). Trays were placed in a glasshouse with natural daylength and set day/night temperatures of 27/20 °C.

One-day shoot tip treatment with oryzalin. Seeds were pre-treated by soaking in water and surfactant, and then germinated as described above. Seedlings were then transferred to 0.2-L containers filled with Pro-Mix BX with Biofungicide. Treatments consisted of 0, 50, 100, or 150μM oryzalin solutions (supplied as Surflan® AS, United Phosphorus Inc., Trenton, NJ) solidified with 0.8% (w/v) agar (Becton, Dickinson and Company, Sparks, MD). Approximately 25 μL of each solution was added while still liquid and allowed to solidify. Each treatment was applied to 25 seedlings. A single application

was made in the laboratory to the shoot tip prior to emergence of the first true leaves. Twenty-four hours after treatment, the seedlings were moved to a glasshouse.

Three-day shoot tip treatment with oryzalin. Seedlings were prepared and treated as in Study 2; however, three applications were applied on consecutive days. Fifty-two total seedlings were treated with 13 seedlings per treatment.

Identification of polyploids. Phenotypic observations such as thicker leaves and stems and increased guard cell length were used to identify plants that potentially had a doubled chromosome number. Guard cell lengths were measured by applying ink to the abaxial side of leaves using a felt tip marker, pressing with clear tape, and then placing the tape on a microscope slide. Slides were then observed under a light microscope equipped with an ocular micrometer and the ink outline of guard cells was measured at ×400 magnification. Three replicates (leaves) and 10 subsamples (guard cells) per taxon were measured.

Putatively doubled plants were then screened using flow cytometry. Approximately 1 cm^2 of newly expanded leaf tissue was finely chopped with a razor blade in a petri dish with 400 µL of nuclei extraction buffer (CyStain UV Precise P Nuclei Extraction Buffer, Partec GmbH, Münster, Germany). The solution was filtered using Partec CellTrics™ disposable filters with a pore size of 50 µm to remove leaf tissue. Nuclei were stained with 1.6 mL 4',6-diamidino-2-phenylindole (DAPI) staining buffer (CyStain UV Precise P Staining Buffer, Partec GmbH) and incubated for 1 to 2 min at approximately 25 °C. The suspension was analyzed using a flow cytometer

(Partec PAS-III, Partec) to determine mean relative DNA fluorescence (mean relative fluorescence, MRF). Ploidy and genome size were determined by comparing the MRF of each sample with the 2C peak of diploids and an internal standard of known genome size. *Pisum sativum* L. 'Ctirad', with a genome size of 8.76 pg (Greilhuber et al., 2007), was used as an internal standard to calculate nuclear DNA content [(2C DNA content of sample in pg = 8.76 pg × (MRF sample/ MRF standard)]. For analysis of leaf tissue the coefficient of variation percentage (CV%) was ≤2.00 and at least 2,500 nuclei were analyzed with the exception of the untreated control (1,393 nuclei). For analysis of root tissue CV% was ≤3.50 and 5,000 nuclei were analyzed.

Cytological Analysis. Cuttings were taken from tetraploid *H. acetosella* 'Panama Red' and placed in rooting substrate composed of 1 pine bark: 2 perlite (by volume) under intermittent mist at a rate of 4 s every 15 min. Rooted cuttings were transplanted into 3.8-L containers and grown in trays containing vermiculite. Roots were allowed to grow out of containers into vermiculite for collection. Actively growing, healthy root tips were collected on sunny mornings before 1000 HR and pre-treated for 1 to 2-h in an aqueous solution of 2 mM 8-hydroxyquinoline (Fisher Scientific Company, Suwanee, GA) + 0.24 mM cycloheximide (Acros Organics, Morris Plains, NJ) at 4 °C. Following pre-treatment, roots were transferred to Carnoy's solution [6 100% EtOH : 3 chloroform : 1 glacial acetic acid (by volume)] and fixed overnight at 25 °C. Roots were rinsed with deionized water and transferred to 70% EtOH (v/v) and stored at 4 °C until observation. Roots were hydrolyzed for 30 to 45 s in an aqueous solution containing 1 concentrated (12 N) HCl : 3 100% EtOH (by volume). Root tips were removed with a razor blade and

cells were spread by pressing under a cover slip and chromosomes stained using modified carbol fuchsin (Kao, 1975). Chromsomes from five cells were counted.

Ploidy of histogenic layers of cytochimeras. Plants that were identified as cytochimeras, also referred to as mixoploids, using flow cytometry on leaf material were further investigated to determine which histogenic layer(s) were doubled. L-I (Dermen, 1960) was evaluated by measuring guard cell length as above. Utility of guard cell length as a tool to discern 4*x* from 8*x* in the L-I histogenic layer was confirmed by comparing the untreated control to 8*x*. Ploidy of the L-III histogenic layer (Dermen, 1960) was evaluated by flow cytometry of root tissue, as described above for leaf tissue. Pollen was measured in an attempt to determine the ploidy of L-II (Dermen, 1960), but the diameter of pollen from 4*x* and 8*x* plants was not different. Therefore, L-II was evaluated by germinating seeds from mixoploids and measuring the ploidy of their progeny. It has been previously established that the epidermis is derived from L-I, the germ line is derived from L-II, and adventitious roots from stem cuttings are derived from L-III (Dermen, 1960). Data were subjected to analysis of variance (ANOVA) and mean separation was conducted using Duncan's multiple range test (MRT).

Growth and foliar measurement of tetraploid and octoploid. Cuttings from tetraploid and octoploid *H. acetosella* 'Panama Red' were collected and rooted as described above at the same time. Rooted cuttings were then transferred into #1 containers (2.8-L) containing 8 pine bark : 1 sand amended with 0.91 $kg \cdot m^{-3}$ dolomitic lime and 0.45 $kg \cdot m^{-3}$ Micromax (The Scotts Co., Marysville, Ohio) and fertilized with 15 g of Osmocote Plus

15-3.96-9.13 (The Scotts Co.) and grown for 2 months. Plants were pruned to approximately 25 cm at the time of field planting to provide uniform material. Two and three plants of tetraploid and octoploid 'Panama Red', respectively, were transplanted to the field on 14 May 2009 at The University of Georgia, Tifton. On 15 July 2009, plant height, plant widths taken perpendicular to each other (width 1 and width 2), internode lengths, and leaf lengths and widths were measured. Plant height was measured a second time on 3 September 2009. Crown volume was calculated by multiplying plant height × width 1 × width 2 and expressed in m^3. Internodes on three stems were measured between the sixth and eleventh (five internodes per stem) nodes basal from the shoot tip. Leaves six through ten (five leaves per stem) basal from the shoot tip were measured on three stems. Data were analyzed using a t-test for pair wise comparison.

Results

Chromosome Doubling Studies.

Seed treatment with colchicine. No induced polyploidy was observed with the rate of colchicine tested. Germination was uniform and percentage was unaffected by treatment; all treatments germinated at 100%. Seedlings exhibited no phenotypic evidence of induced polyploidy.

One-day shoot tip treatment with oryzalin. No mortality was observed in any of the single applications of oryzalin. A single plant treated with 50 μM showed altered

morphology typical of induced polyploidy. Flow cytometric analysis suggested that it was a 4*x*+8*x* cytochimera (Fig. 1A). This plant hereafter will be referred to as HIB-33.

Three-day shoot tip treatment with oryzalin. No mortality was observed in any of the treatments following application of oryzalin on three consecutive days. Two polyploids were identified. One plant each from the 100 and 150 μM treatments were determined to be 8*x* (Fig. 2B-C; 3) and 4*x*+8*x* (Fig 1B), respectively. Genome sizes were calculated to be 2C = 7.27 pg and 2C = 14.85 pg for tetraploid (4*x*) and octoploid (8*x*) ***H. acetosella*** 'Panama Red' (Fig. 2C); a ratio of 2.05. The cytochimera induced in this study will hereafter be referred to as HIB-41.

Cytological analysis. Chromosome spreads from root tips showed that the untreated control was a tetraploid [(2*n*=4*x*=72) (Fig. 3)]. This was used as a basis to set the fluorescence channel for the tetraploid at 100 and allowed us to determine that tissue that had twice the fluorescence was octoploid.

Ploidy of histogenic layers of cytochimeras. HIB-33 was a cytochimera in which the L-I, L-II, and L-III histogenic layers were, 4-4-4+8, respectively . The L-I histogenic layer was not different from the control (Table 1) and the root tissue was found to be mixoploid (Fig. 4A). Thirty-two seedlings resulting from self-pollination of HIB-33 were found to be tetraploid using flow cytometry (data not shown). HIB-41 was a 4+8-*x*-4 cytochimera. Mean guard cell length of HIB-41 was intermediate between tetraploid and octoploid means (Table 1) and root tissue contained only tetraploid cells (Fig. 4B). Prior to flowering, HIB-41 stabilized at the 8*x* level with no evidence of chimerism (data not shown), therefore the L-II could not be identified.

Growth and foliar measurement of tetraploid and octoploid. In morphological comparison of field grown material, the octoploid was shorter on both measurement dates, had a smaller canopy volume, shorter internodes, and smaller leaves than tetraploid *H. acetosella* 'Panama Red' (Table 2; Fig. 5).

Discussion

Cytological analysis confirmed that *H. acetosella* 'Panama Red' is a tetraploid ($2n=4x=72$) which allowed for accurate identification of induced polyploids with flow cytometry when 'Panama Red' was used as a standard. Even though only five cells provided unambiguous counts due to the high number of chromosomes, flow cytometric data showed no evidence of chimerism in the tetraploid (Fig. 2A). The genome size calculated for the tetraploid in the current study is slightly larger (7.27 pg), but similar to the 4C = 6.1 pg calculated for *Hibiscus cannabinus* by Bennett et al. (2000); a difference of less than 20%. The discrepancy could be attributed to interspecific variation. For example, in the related genus *Gossypium* L., twenty-five records compiled for 1C DNA ranged from 1.20 pg to 3.23 pg (Bennett and Leitch, 2005). Differences may also be attributed to technique. Our study was conducted using DAPI which binds preferentially to AT, while Bennett et al. (2000) used propidium idodide (PI), a DNA intercalator that binds uniformly. DAPI has the potential to overestimate genome sizes due to the fact that many families and genera have greater than 50% AT base composition (Meister and Barow, 2007).

Colchicine treatment of seeds of *H. acetosella* 'Panama Red' was unsuccessful in inducing chromosome doubling in the current study. Treating seeds with similar rates of colchicine has been successful in related diploid species *Gossypium herbaceum* L. and *G. arboreum* L. (Omran and Mohammad, 2008). With a 16-h treatment of 0.2%, 0.4%, and 0.6% colchicine, Omran and Mohammad (2008) reported 1.0%, 6.6%, and 92.5% of seedlings had tetraploid cells. Furthermore, in their study, viable seeds were reduced to 60.8% after treatment with 0.6% colchicine for 16-h, while in our study, seed treated at the same concentration for 24-h germinated at 100%.

A single application of oryzalin yielded one cytochimera, which resulted from the 50 μM treatment. Application of oryzalin on three consecutive days yielded one octoploid and one cytochimera from the 100 and 150 μM treatments, respectively. Van Laere et al. (2006) treated seedlings of *Hibiscus syriacus* L. 'Oiseau Blue' and 'Woodbride' for 10 consecutive days with 0.2% colchicine + 2% DMSO to obtain 24.5% and 41.8% doubling in the two cultivars, respectively. The greater success obtained in the study by Van Laere et al. (2006) may be attributed to the longer treatment duration. Furthermore, a lower concentration of agar and preventing the agar droplet from desiccating may have allowed greater uptake of oryzalin. Jones et al. (2008) achieved as high as 41% induction of stabile tetraploids of *Rhododendron* L.'Summer Lyric' by treating seedlings with 50 μM oryzalin solidified with 0.55% agar and maintaining seedlings in a humid chamber (100% humidity) to maintain the integrity of the agar droplet.

The length, width, and chloroplast number in guard cells have been positively correlated to ploidy in *Hibiscus schizopetalus* Hook.f., *H. mutabilis* L., and *H. rosa-*

sinensis L. (Zhuang and Song, 2005). Colchiploids of *Hibiscus syriacus* also exhibit longer guard cells (Lee and Kim, 1976). Similar correlation was found in the current study between guard cell length and ploidy (Table 1), indicating measurement of guard cell length is a useful tool in preliminary screening for polyploids. However, the time taken to prepare slides is similar to that for preparation of flow cytometry samples. Therefore, if a flow cytometer is readily available, the utility of measuring guard cells is in identification of the ploidy of L-I histogenic layer. Guard cell length for the tetraploid was statistically different from the octoploid. Mean guard cell length of HIB-33 was not statistically different from the tetraploid which indicates that the L-I histogenic layer of HIB-33 is tetraploid. However, mean guard cell length of HIB-41 was intermediate and statistically different from the tetraploid and octoploid. The L-I histogenic layer of HIB-41 appears to be a composite of tetraploid (smaller) and octoploid (larger) cells, resulting in an intermediate mean size.

In contrast to the findings of Zhuang and Song (2005) and Lee and Kim (1976), our research found no correlation between pollen size and ploidy. The lack of correlation between pollen size and ploidy precluded using pollen size to determine ploidy of L-II, leading to the evaluation of progeny resulting from self-pollination. Inference about the ploidy of the L-II histogenic layer of cytochimeras has been drawn by evaluating the ploidy of self-pollinated progeny. Olsen et al. (2006) reported that the L-II was tetraploid in cytochimeras of ×*Chitalpa tashkentensis* Elias & Wisura 'Pink Dawn' after flow cytometric analysis of progeny revealed that they were tetraploid. Identification of the ploidy of L-III in cytochimeras was previously accomplished by mitotic chromosome counts of root meristematic cells in the genus *Arachis* L. (Singsit and Ozias-Akins, 1992).

Our study used flow cytometry to determine ploidy of the L-III (Fig. 4), which provides a more rapid method.

Plant height was reduced, leaves were smaller, internodes were shorter, and canopy volume was reduced in the octoploid form of *H. acetosella* 'Panama Red' when compared to the tetraploid. Morphology of the induced polyploid compared to the standard cytotype is similar to that described by Menzel and Wilson (1963b). A spontaneous allododecaploid (2*n*=12*x*=216) arose from a hybrid between *H. 35adiates* x *H. diversifolius* Jacq. that Menzel and Wilson (1963b) reported as having reduced vigor and smaller leaves than the standard F_1. The agreement between the morphology of the study by Menzel and Wilson (1963b) and the current study suggests that a similar response in form may be expected throughout section *Furcaria*. Reduced overall plant size has been reported frequently among other induced polyploids. In his review of the use of induced polyploidy in breeding of agronomic crops, Randolph (1941) reported that dodecaploid (12*x*) wheat (*Triticum aestivum* L.), *Solanum tuberosum* L., and *S. andigenum* Juz. & Bukasov were all less vigorous than standard cytotypes. Conversely, tetraploids of *Solanum jamesii* Torr., *S. chacoense* Bitter, and *S. bulbocastanum* Dunal were as vigorous, or more so than diploids. Tetraploid strains of maize (*Zea mays* L.) are also often taller than their diploid progenitors; however, octoploid forms are much less vigorous than tetraploids and are sterile.

Rigorous and formal investigation of fertility of the induced polyploids was not conducted, however much anecdotal evidence has been observed. *Hibiscus acetosella* 'Panama Red' produces large amounts of autonomously self-pollinated seed when grown in a glasshouse. The induced octoploid has produced no autonomously self-pollinated

seed to date, and has performed poorly as a staminate and pistillate parent in a limited number of controlled crosses. Eleven flowers on the octoploid were pollinated using pollen from *H. acetosella* 'Panama Red' in 2008 and only a single, abnormal seed was obtained, which did not germinate. Further evaluation of the octoploid using a greater number of controlled crosses with fertile and compatible mates is warranted. *Hibiscus acetosella* is an allotetraploid with the genome composition AABB and on average produces 34 bivalents (II) per pollen mother cell [(PMC) (Menzel and Wilson, 1961)]. We propose that the resulting induced polyploid is an autoallooctoploid with the genome composition AAAABBBB.

It is likely that the induced autoallooctoploid produces an increased number of 36adiates36nt, which contributes to its sterility. Increased multivalent production in induced polyploids has been observed by Menzel and Wilson (1961), who reported bivalent pairing (17.9 II per cell) in diploid *Hibiscus cannabinus* (2*n*=2*x*=36) but in the induced tetraploid (2*n*=4*x*=72) there were nearly 10 quadrivalents (IV) per cell. Unfortunately, they did not report on the fertility of the autotetraploid. Tetraploid *H. acetosella* has been reported to produce an average of 34II, 0.1III, and 0.6IV per cell (Menzel and Wilson, 1961). Based on these findings, it is probable that an increase in the production of 36adiates36nt has occurred in the induced autoallooctoploid *H. acetosella* that has led to the reduction in fertility. Support for this idea has been observed in autotetraploid forms of maize (Gilles and Randolph, 1951), pearl millet [(*Pennisetum glaucum* (L.) R. Br.) (Gill et al., 1969; Jauhar, 1970)] and safflower [(*Carthamus tinctorius* L.) (Schank and Knowles, 1961)] where it was observed that after selection for increased fertility, there was a corresponding decrease in multivalent formation; thus

showing an inverse relationship between multivalent formation and fertility. However, analysis of meiotic pairing in the autoallooctoploid is necessary to draw final conclusions as to the mechanism of sterility at work. Regardless of the mode of sterility, it is of utility to have reduced fertility in *H. acetosella*. In many areas where it will be grown, the flowers would experience freezing prior to seed set; however, in extreme southern climates it has the potential to become a prolific seed producer. With reduced fertility there is reduced potential for invasion of landscapes or native ecosystems. Due to the fact that 'Panama Red' and the resulting autoallooctoploid are propagated by stem cuttings, reduced fertility does not affect commercial production.

This study is the first time oryzalin has been successfully used to induce polyploidy in *Hibiscus* sect. *Furcaria*. The resulting autoallooctoploid was more compact, had shorter internodes, had smaller leaves, and exhibited a reduction in fertility. Of these characters, the more compact habit, shorter internodes, and reduction in fertility represent marked improvements over commercially available cultivars of *H. acetosella.* Oryzalin may be regarded as a preferred chromosome doubling agent because it is safer for researchers to use than colchicine. Additionally, colchicine requires a 1000-fold higher concentration to produce quantitatively similar effects (Upadhyaya and Noodén, 1977). Optimization of the protocol, including reducing the concentration of agar and increasing the number or duration of applications could lead to higher incidence of chromosome doubling.

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Table 1.1. Results of measurements of guard cells and pollen grains of tetraploid (4*x*), octoploid (8*x*), and cytochimeric (4*x* + 8*x*) *Hibiscus acetosella* 'Panama Red' PP20,121 plants using an ocular micrometer.

Ploidy	Pollen diameter (μm)[z,y]	Guard cell length (μm)[y]
4*x*	173.9a	25.1c
8*x*	169.5a	36.1a
4*x* + 8*x* (HIB-33)	173.2a	24.4c
4*x* + 8*x* (HIB-41)	175.5a	27.3b

[z]Mean pollen diameter including the projections of the exine.

[y]Different letters within a column indicated statistical difference based on Duncan's multiple range test (MRT). Data presented as means of three replicates of 25 and 10 subsamples for pollen diameter and guard cell length, respectively.

Table 1.2. Morphological comparison of tetraploid (4*x*) *Hibiscus acetosella* 'Panama Red' PP20,121 to induced octoploid (8*x*). All material was propagated at the same time and transplanted to the field on 14 May 2009 and grown under the same conditions at The University of Georgia, Tifton, Ga., USDA Zone 8a. For plant height and canopy volume n=2 for 4*x* and n=3 for 8*x*, n=3 (five subsamples) for internode length, leaf length, and leaf width. All data presented as means; pair wise comparisons conducted using a t-test.

	8*x*	'Panama Red'	*P*
Plant height 1 (cm)[z]	95.7	117.5	0.0370
Plant height 2 (cm)[z]	146.2	196.1	0.0022
Canopy vol. (m^3)[y]	1.16	4.14	0.0002
Internode length (cm)[x]	2.25	4.09	<0.0001
Leaf length (cm)[w]	7.03	9.66	<0.0001
Leaf width (cm)	6.35	10.87	<0.0001

[z]Tallest branch on each plant measured. Plant height 1 recorded 15 July 2009, plant height 2 recorded 3 September 2009.

[y]Canopy volume calculated by multiplying height (h) × width 1 (w1) × width 2 (w2). w1 and w2 were measured perpendicular to each other.

[x]Five internodes (subsamples) were measured between the sixth and eleventh nodes basal from the shoot tip on three stems.

[w]The sixth to eleventh leaves (5 subsamples) basal from the shoot tip were measured on three stems.

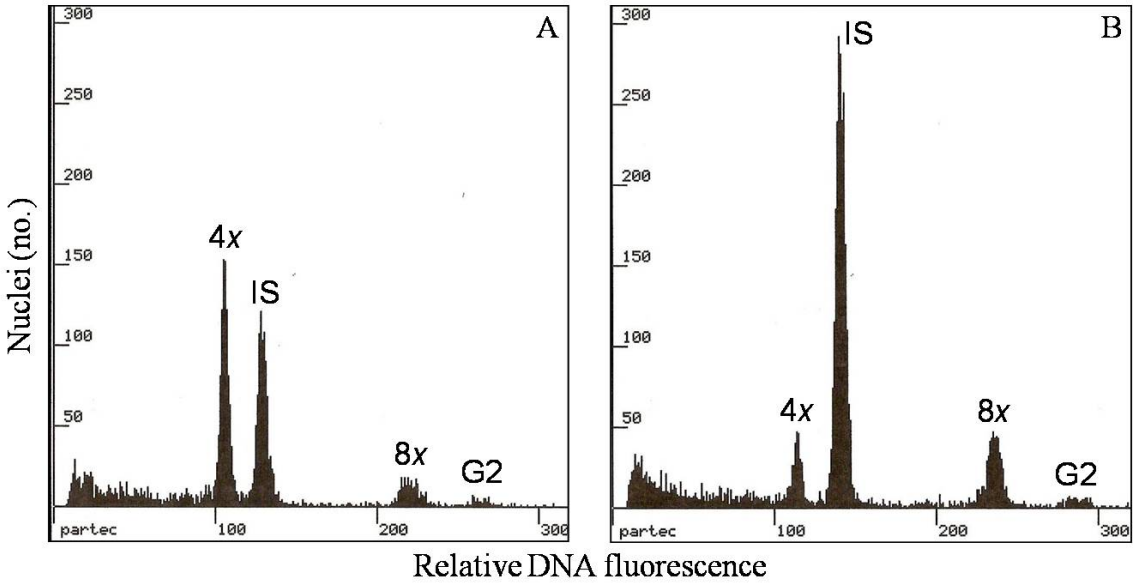

Figure 1.1. Histograms generated using flow cytometry on leaves from the two cytochimeras (HIB-33, HIB-41) of *Hibiscus acetosella* 'Panama Red' PP20,121 composed of both tetraploid (4*x*) and octoploid (8*x*) cells. (A) HIB-33; 4*x* mean relative fluorescence (MRF) = 106, IS MRF = 128, 8*x* MRF = 216; and (B) HIB-41; 4*x* MRF = 114.5; IS MRF = 140.5; 8*x* MRF = 235.5. (IS, *Pisum sativum* 'Ctirad') and its G2 peak is present in (A) and (B).

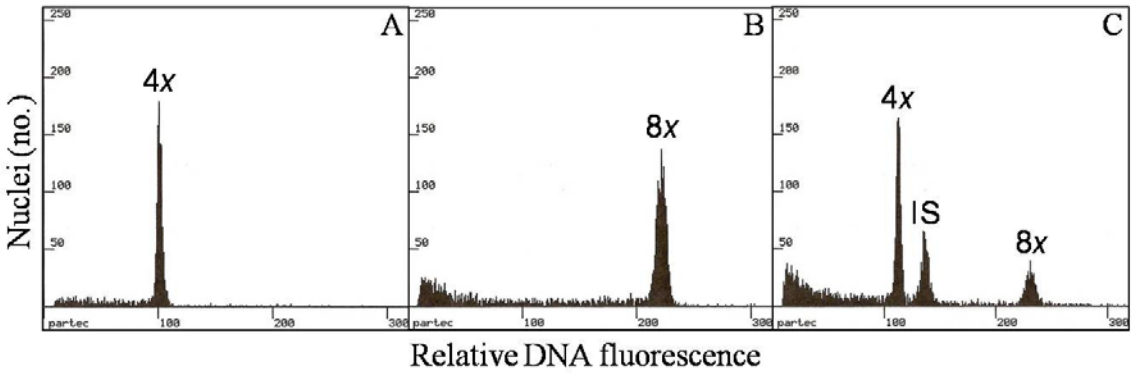

Figure 1.2. Histograms generated using flow cytometry from leaves of (A) untreated tetraploid (4*x*) with a mean relative fluorescence (MRF) = 101.5, (B) successfully doubled octoploid (8*x*) with a MRF = 222, and (C) combination of leaf tissue from an untreated tetraploid (4*x*, MRF = 113), an internal standard (IS, *Pisum sativum* 'Ctirad'; 8.76 pg; MRF = 136.5), and the octoploid (8*x*; MRF = 231.5). In (C) the ratio of the MRF of 8*x* to 4*x* was 2.05; and genome sizes were calculated as 7.27 pg and 14.85 pg for 4*x* and 8*x*, respectively.

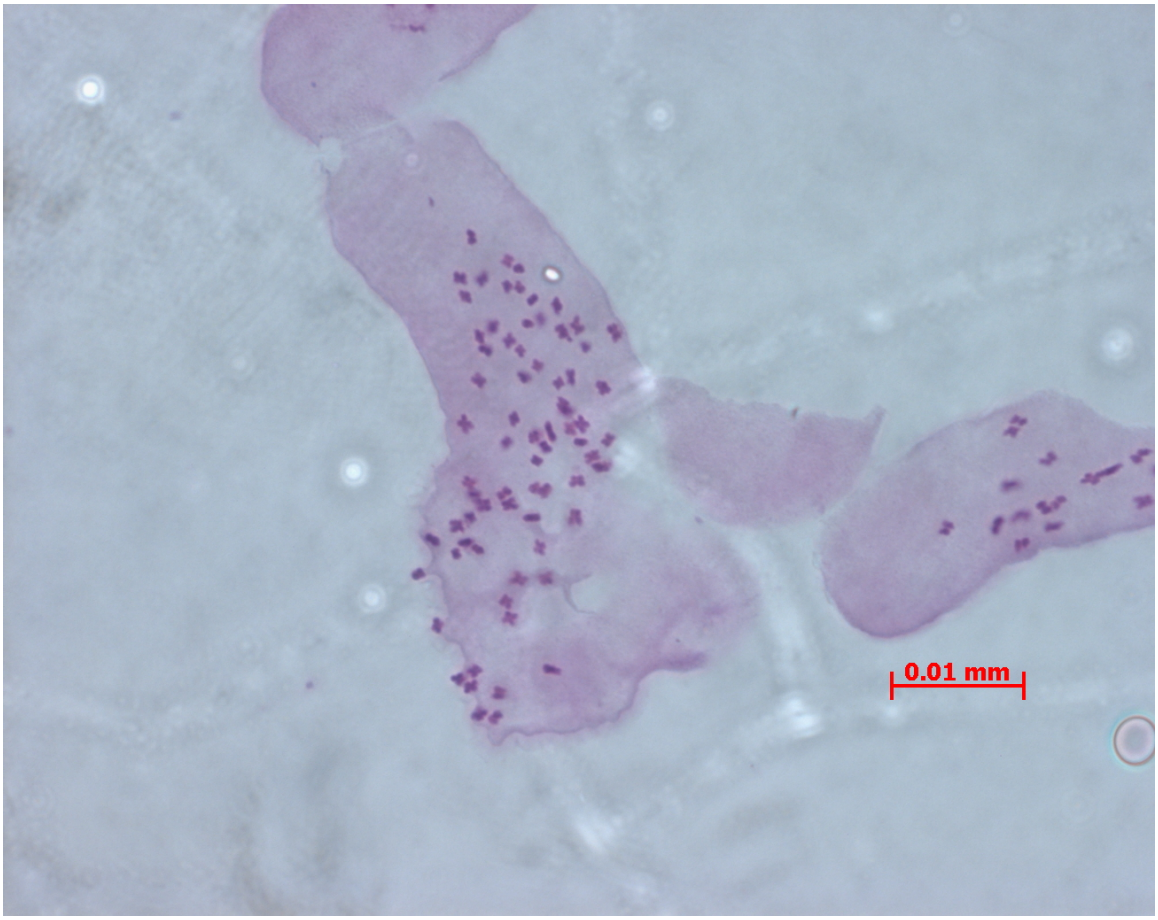

Figure 1.3. Photomicrograph of a root tip squash of 2*n*=4*x*=72 *Hibiscus acetosella* 'Panama Red' PP20,121 stained using modified carbol fuchin technique and taken at ×1000 magnification. Chromosomes of five unambiguous cells counted.

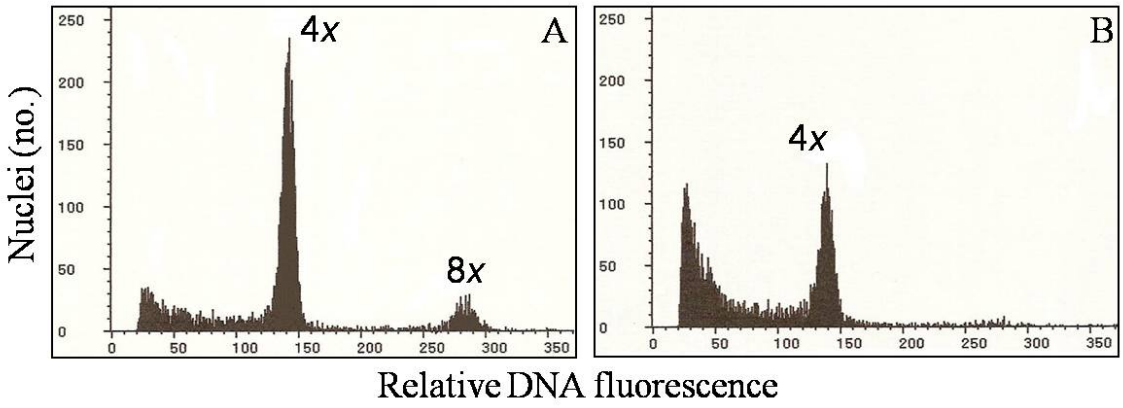

Figure 1.4. Histograms generated using flow cytometry on root tissue of cytochimeras (HIB-33, HIB-41) of *Hibiscus acetosella* 'Panama Red' PP21,120. (A) HIB-33 is L-III 4*x*+8*x*; 4*x* mean relative fluorescence (MRF) = 141 and 8*x* MRF = 282 and (B) HIB-41 is L-III 4*x*; 4*x* MRF = 138.

Figure 1.5. Container grown octoploid (left) and tetraploid (right) *Hibiscus acetosella* ‘Panama Red’ taken 13 August 2009.

Chapter 2

ORYZALIN-INDUCED TETRAPLOIDY IN *CRYPTOMERIA JAPONICA* (THUNB. EX L.F.) D. DON[1]

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Oryzalin-Induced Tetraploidy in *Cryptomeria japonica* (Thunb. ex L.f.) D. Don (Cupressaceae)

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Subject Category: Breeding, Cultivars, Rootstocks, and Germplasm Resources

Oryzalin-Induced Tetraploidy in *Cryptomeria japonica* (L.f.) D. Don (Cupressaceae)

Additional index words. cytology, flow cytometry, gymnosperm, japanese cedar

Abstract. Japanese cedar [*Cryptomeria japonica* (Thunb. ex L.f.) D. Don] represents an alternative to Leyland cypress [×*Cuprocyparis leylandii* (A.B.Jacks. & Dallim.) Farjon] as an evergreen screen or specimen plant for landscapes. It performs well under a range of soil and environmental conditions but has been underutilized, due in part, to unsightly winter browning caused by photoinhibition. In previous studies, chance seedlings that did not exhibit winter browning were identified as tetraploids. The current study was conducted to induce polypoidy in japanese cedar. Approximately 600 seedlings were sprayed with 150 μM oryzalin + 0.1% SilEnergy™ for 30 consecutive days under laboratory conditions. Two-hundred thirty-seven seedlings with thickened and twisted leaves were selected, transplanted, and grown in a glasshouse for 120 days. Seedling ploidy levels were analyzed using flow cytometry 180 days after treatment (DAT), identifying 197 (83.1%) tetraploids, 22 (9.3%) cytochimeras, and 18 (7.6%) diploids. Morphology of induced tetraploids was similar to that previously described and provided a phenotypic marker during selection that was over 92% accurate. A random subset of 20 tetraploid individuals was analyzed 270 DAT and were found to contain only tetraploid cells in the leaves analyzed, confirming stability over this period. This study

demonstrated the utility of oryzalin for inducing tetraploids in japanese cedar, which we predict will also be effective in other gymnosperms.

Cryptomeria D. Don is a monotypic genus comprised of *C. japonica* (Thunb. ex L.f.) D. Don and its two varieties: *C. japonica* var. *japonica* and *C. japonica* var. *sinensis* Miq. *Cryptomeria* is native to China (var. *sinensis*) and Japan (var. *japonica*) and is an important timber tree of the latter, reaching heights of 36 to 46 m in the wild (Dallimore and Jackson, 1967) but is typically 15 to 25 m in gardens (Tripp and Raulston, 1992).

Cryptomerias, also commonly called japanese cedars or sugi, offer an alternative to Leyland cypress due to their limited pest problems (Tripp, 1993; Tripp and Raulston, 1992), ability to perform well under the hot, humid conditions of the southeastern U.S., and tolerance of heavy soils during both wet and dry conditions (Tripp, 1993). Cultivars are numerous and varied and include forms that are dwarf, have irregular branching, and have varying foliage colors including variegated (Rouse et al., 2000). However, in full sun they exhibit a browning in winter that can be undesirable (personal observation). Winter browning in japanese cedar occurs in sun-exposed leaves during periods of low temperature indicating that photoinhibition plays a role (Han and Mukai, 1999; Ida, 1981). Ida (1981) reported that chloroplasts in sun-exposed leaves were transformed into rhodoxanthin-containing chromoplasts during winter, resulting in the brown-red color.

In Japanese forestry nurseries, tetraploid forms of japanese cedar have been identified based on their morphology (Chiba, 1951). Tetraploids have been shown to be more resistant to oxidative damage from UV light than diploids through increased antioxidant levels (Niwa and Sasaki, 2003). However, these seedlings have been rogued from nurseries based on their unfamiliar appearance (Chiba, 1951) and saplings that readily change needle color from green to brown or orange-yellow have been recommended as being superior individuals for silviculture (Han and Mukai, 1999).

Negative selection has been imposed based on the lack of utility of tetraploids in a silviculture but they may have great potential for use in the nursery and landscape industries in the U.S., by providing non-winter-browning forms that consumers find more attractive.

Polyploidy in gymnosperms is rare (Ahuja, 2005; Khoshoo, 1959; Khoshoo, 1961) but following the work of Blakeslee and Avery (1937) there were a number of studies that attempted to induce polyploidy. The primarily goal of those studies was to develop more vigorous trees for timber production. Research included attempts to induce polyploidy in pines (Hyun, 1953; Mergen, 1959, Mirov and Stockwell, 1939), japanese cypress [(*Chamaecyparis obtusa* Endl.) (Kanezawa, 1951)], and giant sequoia [(*Sequoiadendron giganteum* (Lindl.) J. Bucholz (Jensen and Levan, 1941)]. These studies used a variety of delivery methods at various stages of development including treatment of seeds, newly germinated seedlings, and shoot tips; however, all previous studies to induce polyploidy have used colchicine. Colchicine acts by disrupting microtubular structure of dividing cells in both plants and animals (Bartels and Hilton, 1973). The mode of action of dinitroaniline herbicides such as oryzalin have also been determined (Bartels and Hilton, 1973; Upadhyaya and Noodén, 1977; Upadhyaya and Noodén, 1980) and have been found to bind more specifically to plant tubulin in vitro than colchicine (Yemets and Blume, 2008). In addition to more specific binding, dinitroaniline herbicides have been found to be less toxic and to act at 100- (Yemet and Blume, 2008) to 1000-times (Upadhyaya and Noodén, 1977) lower concentrations than colchicine. Due to the reduced toxicity and more effective binding, dinitroaniline

herbicides such as oryzalin may be preferable for inducing polyploidy. The objective of the current study was to induce polyploidy in *Cryptomeria japonica* using oryzalin.

Materials and Methods

Plant material. Wild-collected seed from China of *Cryptomeria japonica* were received from Lawyer Nursery, Inc., Plains, Mont. Based on the collection site, the seed used may have been *C. japonica* var. *sinensis*; however, this was not able to be confirmed. Approximately 1,000 seeds were sown in germination trays containing 8 pine bark : 1 sand (v/v) amended with 0.91 $kg \cdot m^{-3}$ dolomitic lime and 0.45 $kg \cdot m^{-3}$ Micromax (The Scotts Co., Marysville, Ohio) and allowed to germinate in a glasshouse with day/night set temperatures of 27/20 °C. Approximately 600 seedlings germinated and were moved to the laboratory and grown under constant light (28 $\mu mol \cdot m^{-2} \cdot s^{-1}$) supplied by cool-white fluorescent lamps at 25 °C.

Cytology. Roots were harvested and pretreated for 24 h in 3 mM 8-hydroxyquinoline (Fisher Scientific, Suwanee, Ga.) + 0.24 mM cycloheximide (Acros Organics, Morris Plains, N.J.) at 4 °C. Following pre-treatment, roots were transferred to Carnoy's solution [6 100% ethyl alcohol : 3 chloroform : 1 glacial acetic acid (by volume)] and fixed overnight at 25 °C. Roots were rinsed with deionized water and transferred to aqueous 70% ethyl alcohol (v/v) and stored at 4 °C until observation. At the time of observation, roots were rinsed in deionized water for 30 min and root tips were excised on a glass slide using a razor. Root tips were then incubated at 37 °C in an enzyme mixture [0.5% cellulase (Karlan Research, Torrance, Calif.), 0.5% cytohelicase (Sigma-

Aldrich Co., St. Louis, Mo.), and 0.5% pectolyase Y-23 (Karlan Research), in 50 mM citrate buffer at pH 4.5] for 4.5 h. Following digestion, root tips were rinsed, transferred to a glass slide using a Pasteur pipette, and liquid removed by wicking with a one-ply tissue. Meristematic cells were separated from root cap and other non-dividing cells by gently pressing the root tip with a dissecting probe. Before allowing cells to dry, a drop of modified carbol fuchsin stain (Kao, 1975) was added and cells were spread under a cover glass by applying even pressure. Slides were scanned at ×100 magnification; counting and photomicrography were performed at ×1,000 magnification using a compound light microscope (Fisher Micromaster® I, Fisher Scientific). Chromosomes of five cells were counted.

Inducing polyploidy. Seedlings were sprayed to run-off daily for 30 d with an aqueous solution containing 150 μM oryzalin (supplied as Surflan® AS, United Phosphorus, Trenton, NJ) + 0.1% SilEnergy™ (Brewer International, Vero Beach, Fla.), an organosilicate surfactant, using a standard spray bottle (Model P-32, Sprayco, Farmington Hills, Mich.). The rate of oryzalin was selected based on several previous studies that induced polyploidy using 150 μM solutions (Contreras et al., 2007; Contreras et al., 2009; Olsen et al., 2006). After 30 days, approximately 500 surviving seedlings were moved to a glasshouse and transplanted into six trays containing the pine bark substrate described above. Seedlings were allowed to grow for four months and then transplanted into 0.2 L-containers filled with 1 Pro-Mix BX with Biofungicide (Premier Horticulture, Quakertown, Pa.) : 1 pine bark/sand mixture described above (v/v), and fertilized weekly at a rate of 100-ppm N using TotalGro 20N-4.4P-17.6K water soluble

fertilizer (SDT Industries, Winnsboro, La.). Morphology indicative of polyploidy in japanese cedar (Chiba, 1951) was used to select 237 seedlings for transplantation. After one month of growth [180 days after treatment (DAT)], the ploidy level of the seedlings were determined using flow cytometry.

Identification of polyploidy. Flow cytometry, as described in Contreras et al. (2009) with modifications, was conducted on the 237 selected seedlings with altered leaf morphology. Five plants were evaluated during each analysis by bulking leaves. For bulked samples, a comparable amount of mature leaf tissue from five plants was collected and analyzed. In bulked samples where a single peak was observed, all plants were recorded as being composed solely of tissue at the ploidy level corresponding to the fluorescence channel. For bulked analyses in which there were multiple peaks, plants were analyzed individually to determine ploidy of individual plants. The channel corresponding to diploid mean relative DNA fluorescence (MRF) was determined based on an individual that was confirmed as diploid ($2n = 2x = 22$) using cytology as described above. The diploid channel served as a standard for comparison to determine ploidy for most samples. In some cases, the peak was ambiguous due to peak shifting and ploidy level was not clear. These samples were analyzed individually using an internal standard [(*Pisum sativum* L. 'Ctirad'; 2C = 8.76 pg) (Greilhuber et al., 2007)]. The internal standard was used to calculate the 1C DNA content of the diploid [1C DNA (pg) = (MRF Sample/MRF Standard) × 8.76 pg)/2)]. DNA content of the unknown samples was calculated and ploidy determined based on the 1C DNA content of the diploid. A random

subset of 20 individuals identified as tetraploid 180 DAT were re-analyzed using flow cytometry 270 DAT to determine if they remained stable tetraploids.

Results and Discussion

Results from chromosome counts on *Cryptomeria japonica* revealed a diploid complement of $2n = 2x = 22$ (Fig. 1). This finding agrees with earlier results (Khoshoo, 1961; Sax and Sax, 1933). Due to the large size and difficulty in spreading chromosomes, it was necessary to use the enzyme mixture described above for digestion as opposed to a more standard HCl hydrolysis.

Treating *C. japonica* seedlings for 30 consecutive days with 150 μM oryzalin + 0.1% SilEnergy™ successfully induced tetraploidy. Numerous preliminary experiments were unsuccessful in developing tetraploids, including soaking seeds in various rates of colchicine, germinating seeds in various rates of colchicine, and treatment of shoot tips of seedlings and stem cuttings (Contreras and Ruter, 2008) with various rates of oryzalin. We hypothesized that previous applications of oryzalin were unsuccessful because they were too short (one to five days) and did not contain a surfactant to penetrate the cuticle. A total of 237 seedlings were analyzed using flow cytometry and of these, 219 (92.4%) contained tetraploid cells (Table 1). Flow cytometric analysis of seedlings revealed 197 (83.1%) tetraploids, 22 (9.3%) cytochimeras, and 18 (7.6%) diploids (Table 1; Fig. 2). Morphology of induced tetraploids (Fig. 3), primarily thickened and twisted leaves, was similar to that previously described (Chiba, 1951) and provided a phenotypic marker for selection during transplantation that was over 92% accurate. Tetraploids had thicker leaves but leaf length and growth rate displayed variation typical of a seedling

population. Other populations grown from the same seed source had no individuals that exhibited leaf morphology indicative of polyploidy (personal observation), which provides strong indication that the tetraploids recovered were the result of the treatment and not chance seedlings. Also, Chiba (1951) reported that recovery of polyploids as chance seedlings was several orders of magnitude lower (0.0005%) than observed in the current report.

Compared to the number of studies on induced polyploidy in angiosperms, there have been few in gymnosperms and no reports of induced polyploidy in japanese cedar (Ahuja, 2005). This is the first study reporting the use of oryzalin as a mitotic inhibitor to induce polyploidy in a gymnosperm. Previous studies on gymnosperms used colchicine to treat seed (Hyun, 1953; Jensen and Levan, 1941; Kanezawa, 1951; Mirov and Stockwell, 1939), shoot-tips of seedlings (Hyun, 1953), or injection into vascular tissue (Jensen and Levan, 1941). Jensen and Levan (1941) induced polyploidy in *Sequoia gigantea* (Lindl.) Decne. (= *Sequoiadendron giganteum*) by germinating seeds on filter paper moistened with 0.1% and 0.2% colchicine. Polyploidy was confirmed through chromosome counts in a plant from the 0.2% treatment and 25% of *S. gigantea* plants treated with 0.1% exhibited a phenotype indicative of polyploidy (Jensen and Levan, 1941). Hyun (1953) reported on numerous studies to induce polyploidy in pines but had greatest success by treating seeds of *Pinus ponderosa* Douglas ex Loudon with 0.2% colchicine for four or six days and seeds of *P.* ×*attenuradiata* Stockw. & Righter with 0.2% colchicine for four days. Treatment of shoot-tips of *P. ponderosa*, *P. jeffreyi* Balf., and *P.* ×*attenuradiata* seedlings produced cytochimeras, but no stable tetraploids (Hyun, 1953). Kanezawa (1951) obtained an induced tetraploid of *Chamaecyparis obtusa* by

soaking seeds in 0.3% colchicine for 24 h and then treating seedlings with the same solution every two or three days over a period of 20 days.

Plants were initially evaluated using flow cytometry 180 DAT and then a random subset of 20 tetraploid plants were reassessed 270 DAT which were all found to contain only tetraploid cells. Hyun (1953) reported some reversion of cytochimeras to diploids; however, most polyploids remained stable over one year after treatment. Johnsson (1975) reported that colchicine-induced tetraploids of *Pinus sylvestris* L., *Larix sibirica* Ledeb., and *Picea abies* (L.) H.Karst. contained only tetraploid cells 30 years after polyploid induction. It appears that if induction of polyploidy is successful and the resulting individual contains only polyploid cells (is not a cytochimera), that it is likely to remain stable over time. However, continued evaluation of the induced tetaploids in the current study will be required to determine long term stability. Furthermore, since shoot tips were treated, the roots of individuals with tetraploid leaves are presumed to be diploid. Therefore, to obtain plants with only tetraploid cells, tetraploids will be propagated by stem cuttings when sufficient material is available.

This research demonstrates that oryzalin is effective in developing tetraploids in japanese cedar and we predict that similar treatments may be effective in other gymnosperms. Applying 150 μM oryzalin + 0.1% SilEnergy™ for 30 d using a spray bottle, along with selection based on phenotype resulted in a high percentage of induced tetraploids. Long-term evaluation will be conducted at multiple sites to determine if tetraploids remain stable over time and to evaluate their growth and ornamental potential.

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Table 2.1. Results of treating *Cryptomeria japonica* seedling shoot tips with 150 μM oryzalin + 0.1% SilEnergy™ for 30 consecutive days to induce tetraploids.

Ploidy	No. seedlings	Percent of total
2*x*	18	7.6
2*x* + 4*x*	22	9.3
4*x*	197	83.1
Total[z]	237	100

[z]237 seedlings selected based on foliar morphology from ≈500 surviving seedlings after beginning 30 d treatment on ≈600 seedlings.

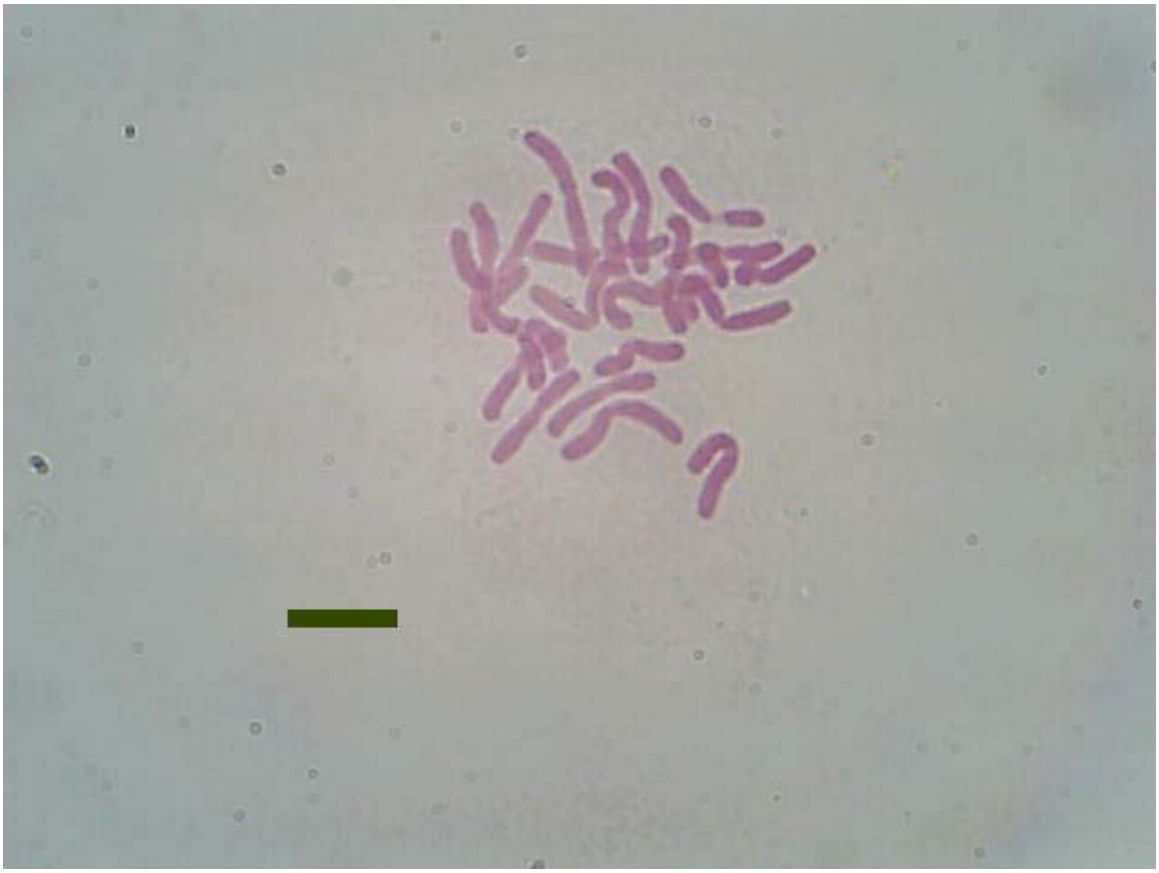

Figure 2.1. Photomicrograph of a chromosome spread from a root tip squash of diploid (2*n*=2*x*=22) *Cryptomeria japonica* taken at ×1,000 magnification. Scale bar 10 µm.

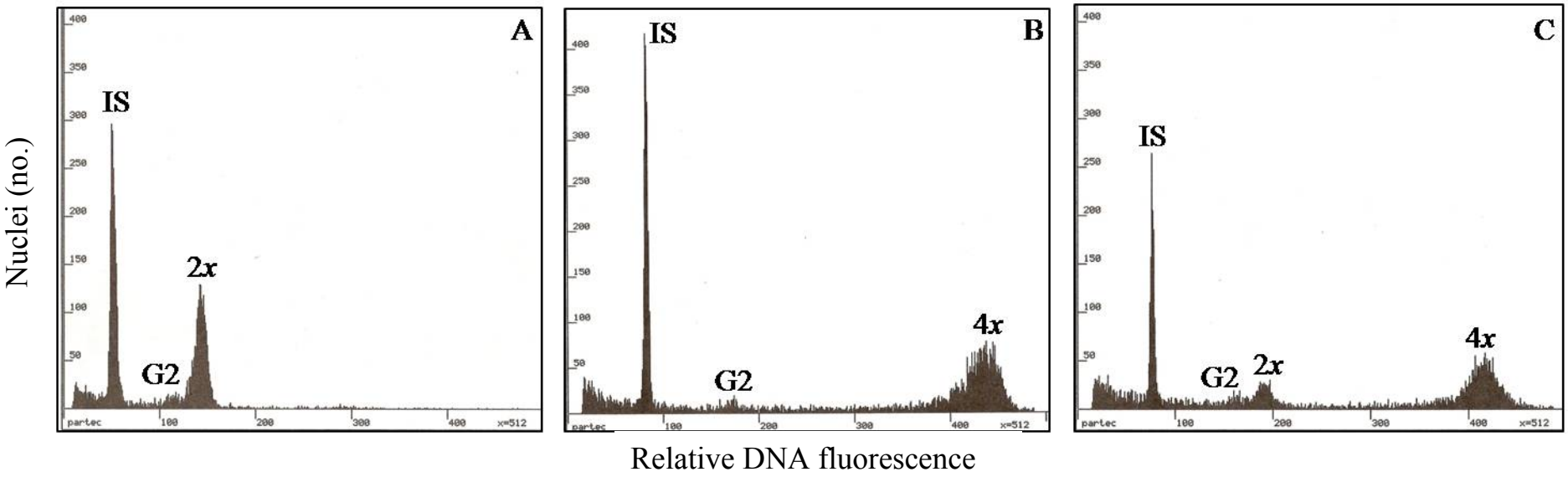

Figure 2.2. Representative histograms generated by analyzing leaves of *Cryptomeria japonica* from seedlings treated for 30 consecutive days with 150 μM oryzalin + 0.1% SilEnergy™ using flow cytometry for diploid (2*x*) (A), tetraploid (4*x*) (B), and mixoploid (2*x* + 4*x*) (C). (A) Internal standard (IS; *Pisum sativum* 'Ctirad') mean relative fluorescence (MRF) = 52, G2 peak from cell cycling of IS, and 2*x* MRF = 143; (B) IS MRF = 83 and 4*x* MRF = 437; (C) IS MRF = 77, 2*x* MRF = 191, 4*x* MRF = 416.

Figure 2.3. Two leaves from an induced tetraploid of *Cryptomeria japonica* (left) exhibiting thickened and twisted morphology and two leaves of diploid (right) with wild-type phenotype.

Chapter 3

PIGMENT ANALYSIS AND EVALUATION OF JAPANESE CEDAR CULTIVARS [*CRYPTOMERIA JAPONICA* (L.F.) D. DON (CUPRESSACEAE) IN SOUTH GEORGIA[1]

[1]Contreras, R.N. and J.M. Ruter. To be submitted to Scientia Horticulturae.

Pigment analysis and evaluation of Japanese cedar cultivars [*Cryptomeria japonica* (L.f.) D. Don (Cupressaceae)] in south Georgia

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Abstract. Japanese cedar, or cryptomeria, [*Cryptomeria japonica* (L.f.) D. Don] has been underutilized in landscapes of the U.S. until recent years. There are now over 100 cultivars, many of which are grown in the southeastern U.S. Performance of cultivars has been described from USDA Zone 6b to USDA Zone 7b; however, there are no reports on how cultivars perform in USDA Zone 8. The current study was conducted to evaluate the performance of 16 taxa of Japanese cedar with regard to winter browning by measuring chlorophyll and carotenoid content and assigning visual color ratings. Differences in amount of total chlorophyll, ratio of chlorophyll *a*:*b*, amount of total carotenoids, and visual color rating were observed among taxa within each season and among seasons within taxa. There were no consistent trends for differences between winter and summer for the two years of the study. The highest and lowest performing taxa for all traits were not consistent over all seasons. Correlations were observed between chlorophyll, ratio of chlorophyll *a*:*b*, carotenoid, ratio of chlorophyll : carotenoid, and rating; however, relationships were not consistent over all seasons. There were large differences in rainfall between the two years and this may have accounted for a portion of the variation.

Introduction

Japanese cedar, or cryptomeria, [*Cryptomeria japonica* (L.f.) D. Don] has been underutilized in landscapes of the U.S. until recent years. It has traditionally been used as screening or specimen planting; however, there are now a large number of cultivars displaying varying forms and growth rates (Rouse et al., 2000; Tripp, 1993) that may be used in a number of landscape situations. Reports differ, but it is estimated that there are over 100 different ornamental cultivars (R. Determann, personal communication; Erhardt, 2005), and approximately 45 of these are grown in the eastern U.S. (Rouse et al., 2000). Cryptomerias are native to the warm-temperate zones of south China and Japan. In the latter, they are generally limited to north-facing slopes that receive more than 180 cm but less than 300 cm of rainfall per year (Tsukada, 1967b); although the majority of the native distribution extends over a range that receives between 120 cm to 180 cm per year (Tsukada, 1982). Japanese cedars perform well under a number of environmental and soil conditions including the hot, humid summers and heavy clay soils of the southeastern U.S. (Tripp and Raulston, 1992). Due to this fact, Japanese cedar has been promoted as an alternative to Leyland cypress [×*Cuprocyparis leylandii* (A.B.Jacks. & Dallim.) Farjon; (Farjon et al., 2002)], on which numerous problems now occur including bagworms [*Thyridopteryx ephemeraeformis* (Haworth); (Lemke et al., 2005)], fungal cankers caused by *Seiridium* Nees ex Link spp. and *Botryosphaeria dothidea* (Moug.) Ces. et De Not., and cercospora needle blight (*Cercosporidium sequoiae* Ellis and Everh.) (Martinez et al., 2009). Japanese cedar exhibits less susceptibility to bagworm infestations, and cultivars are available that have reduced interior dieback (Tripp and Raulston, 1992); however, it is not immune from problems. Redfire (*Phyllosticta aurea*

C.Z. Wang) is a fungal pathogen that can attack stressed plants and cause stem death; particularly on older foliage (Tripp, 2005). Also, as Dirr (1998) notes, there is not a fast growing, tree-like cultivar that remains green during winter. Winter browning in cryptomerias is often unsightly and undesirable to consumers, presumably one of the reasons it has remained underutilized in landscapes.

Winter browning in Japanese cedar occurs through the conversion of chloroplasts to chromoplasts during winter (Ida, 1981). This transition takes place only in sun-exposed leaves during periods of low temperature indicating that photoinhibition likely plays a role (Han and Mukai, 1999; Ida, 1981). Mechanisms that plants use to deal with excess light during periods of low temperature when Calvin cycle activity is limiting include reduction of chlorophyll, activation of the pH dependent xanthophyll cycle, increased levels of carotenoids, and production of antioxidants or reactive oxygen species (ROS) scavenging enzymes. Japanese cedar has been shown to demonstrate several mechanisms during winter to minimize the effects of photoinhibitory conditions. The amount of chlorophyll decreases during winter (Han and Mukai, 1999; Ida, 1981) in both sun- and shade-exposed leaves (Han et al., 2004); as well as in both wild-type and non-browning mutants (Han et al., 2003) thus reducing the amount of energy absorbed. Chloroplasts in sun-exposed leaves are transformed into rhodoxanthin containing chromoplasts during the winter (Ida, 1981).

In a study on 15-year-old trees in Shizuoka Prefecture, Japan, Han et al. (2004) reported accumulation of rhodoxanthin in sun-exposed leaves beginning in January, reaching maximum levels in February, decreasing significantly in March, and decreasing

to zero by April; although the timing of discoloring in winter and restoration in spring is highly variable and location specific (personal observation).

Han et al. (2003) showed that wild-type leaves that accumulated rhodoxanthin maintained higher levels of photosynthetic activity with lower levels of zeaxanthin- and antheraxanthin- dependent thermal dissipation than mutants that remained green all winter. It is proposed that rhodoxanthin intercepts a portion of incident light to help maintain an appropriate balance between light absorption, thermal dissipation, and photosynthesis (Han et al., 2003). Japanese cedars also accumulated substantial levels of xanthophyll cycle pigments and lutein during winter (Han and Mukai, 1999; Han et al., 2003). Among cultivars there appears to be a large degree of variation in accumulation of pigments based on winter color (personal observation).

Rouse et al. (2000) provided a description of a number of cultivars grown in the eastern U.S. based on collections at the Atlanta Botanical Garden (Atlanta, Ga., USDA Zone 7b), Morris Arboretum (Philadelphia, Pa., USDA Zone 6b), the JC Raulston Arboretum at North Carolina State University (Raleigh, N.C., USDA Zone 7b), and the U.S. National Arboretum (Washington, D.C., USDA Zone 7a) (USDA, 1990); however, cultivars have not been critically evaluated for their performance in USDA Zone 8. The objective of the current study was to evaluate 16 taxa of Japanese cedar for amount of total chlorophyll (C_{a+b}), ratio of chlorophyll *a* (C_a): chlorophyll *b* (C_b), total carotenoids (C_{x+c}), ratio of (C_{a+b}): (C_{x+c}), and visual color rating at the University of Georgia Tifton Campus (USDA Zone 8a).

Materials and Methods

Plant material and growing conditions. Plants were maintained in field plots at the University of Georgia Tifton Campus. Field soil was a Tifton loamy sand (fine-loamy, siliceous, thermic Plinthic Paleudult), pH 5.2. Plots were fertilized at a rate of 22.7 kg N·acre^{-1} in March using Super Rainbow 16-1.8-6.6 plus minor elements (Agrium U.S. Inc., Denver, CO). An additional 11.4 kg N·acre^{-1} was applied in late August using the product above. The sixteen taxa, field location, planting date, and height are found in Table 1. Southeast facing branches were flagged during winter 2007-08 and material used for the duration of the study was collected from the same branches. Leaves were collected 8 February 2008, 17 August 2008, 9 February 2009, 5 May 2009 and frozen at -80 °C until analysis. Weather data at the Tifton Campus for the duration of the study is included in Table 2. Supplemental irrigation was used only at the time of new plant establishment within plots.

Chlorophyll and carotenoid extraction, analysis, and calculations. Total chlorophyll and carotenoids were extracted by grinding 85 mg leaf tissue three times in 3.33 mL 80% acetone and the extract was transferred to a test tube for a final volume of 10 mL. After the third grind in acetone the leaf material remaining was transferred to the test tube containing the extract and maintained in the dark at 4 °C for 1-h. Two mL of the extract was centrifuged for 30 s at 6,800 g_n. The supernatant was then transferred to a cuvette and absorbance was measured at 470 nm, 646 nm, and 663 nm using a GENESYS™ 10 Spectrophotometer (Thermo Electron Corp., Madison, WI). Absorbance for all samples was between 0.2 and 0.8.

Determination of chlorophyll *a* (C_a), chlorophyll *b* (C_b), and carotenoids (C_{x+c}) was performed using calculations from Lichtenthaler and Wellburn (1983). Chlorophyll *a* content was calculated using the formula: C_a (mg/L) = (12.21 × A_{663}) – (2.81 × A_{646}). Chlorophyll b content was calculated using the formula: C_b (mg/L) = (22.13 × A_{646}) – (5.03 × A_{663}). Total chlorophyll content was determined by summing chlorophyll *a* /*b* values. Total carotenoid content was determined using the formula: C_{x+c} (mg/L) = [1000 × A470 – (3.27 × C_a) – (81.4 × C_b)]/227. Water content was determined at the time of collection for all taxa at each collection time and used to calculated dry weight (DW). Chlorophyll and carotenoid contents are expressed in mg·g DW^{-1}.

Color rating. Plants were observed within one week of the four leaf collection dates. Ratings for color were assigned by five individuals from 1 (very brown/yellow; off color) to 5 (very green). All plants were evaluated from the southeast side, directly in front of flagged branches.

Chlorophyll fluorescence. Dark-adapted chlorophyll fluorescence measurement was attempted using a portable chlorophyll fluorometer (MINI-PAM Photosynthesis Yield Analyzer (Heinz Walz GmbH, Effeltrich, Germany). Measurements were collected before dawn on 18 February 2008.

Design and statistical analysis. The experimental design was randomized complete block design with four blocks (season). Data were analyzed using analysis of variance (PROC GLM, SAS version 9.1; SAS Institute Inc., Cary, NC). Mean effect of taxa on

the four dependent variables within a season (between taxa, within season) was separated using Tukey's honestly significant difference (HSD). Mean effect of season on the dependent variables (within taxa, between seasons) was separated using orthogonal contrasts (SAS version 9.1). There were signifcant differences observed between all seasons (blocks) therefore, it was not appropriate to pool data to increase. Correlation between dependent variables within each season and over all seasons was also evaluated (PROC CORR, SAS version 9.1). All SAS code for the above analysis is included in Appendix A. Linear regression was also used to investigate the relationship between total carotenoids and total chlorophyll.

Results

Chlorophyll and carotenoid quantitation. Differences in amount of total chlorophyll, ratio of chlorophyll *a*:*b*, and amount of total carotenoids, were observed among taxa within each season and among seasons within taxa (Table 3). The three taxa with the highest and lowest mean total chlorophyll content are reported in Table 4 and varied with each season. 'Gyokruga' was among the highest of three cultivars in three of four seasons; 'Cristata' and 'Tansu' were among the highest two of four seasons; however, no other cultivar was among the top three more than once. 'Barabit's Gold' and 'Sekkan', two yellow-leaf forms, were among the cultivars with the lowest total chlorophyll content for three and two seasons, respectively. Orthogonal contrasts were used to compare taxa between seasons and revealed significant ($P < 0.01$) differences between all seasons for total chlorophyll content (Table 3). The three taxa with the highest and lowest mean total carotenoids for each season are reported in Table 5. Similar to total chlorophyll content,

there was little consistency regarding cultivars with highest and lowest carotenoid content among seasons. Within each season a moderate to strong relationship was observed between total chlorophyll and carotenoid content (Figure 1).

Color rating. Differences in mean visual color rating were observed among taxa within each season and among seasons within taxa (Table 3). Taxa with the highest and lowest visual color rating for each season are reported in Table 6. 'Radicans' was among the highest rated taxa in all four seasons of evaluation and 'Gyokruga' was among the highest in three seasons. 'Araucariodes' was among the lowest rated taxa for three seasons, while 'Barabit's Gold', 'Black Dragon', 'Sekkan', and 'Yaku' were all among the lowest rated taxa two seasons.

Chlorophyll fluorescence. Measurements collected using the MINI-PAM were erratic for all taxa. We believe the shape of the leaves affected measurements and resulted in variation between subsamples of a taxon that were so great that the data were considered uninformative and measurements were only collected for Winter 2007-08 (data not shown).

Correlation. There were many statistically significant correlations observed in the study (Table 7). In the winter of 2007-08 there was a strong negative correlation between $C_{(x+c)}$ and $C_{(a+b)}$: $C_{(x+c)}$ (-0.83, $P < 0.0001$). In summer 2008 there was a strong positive correlation between $C_{(a+b)}$ and $C_{(x+c)}$ (0.89, $P < 0.0001$). In winter 2008-09 strong positive correlations were found between $C_{(a+b)}$ and $C_{(x+c)}$ (0.92, $P < 0.0001$) and between $C_{(a+b)}$ and $C_{(a+b)}$: $C_{(x+c)}$ (0.70, $P < 0.0001$). In summer 2009, a strong positive correlation was found between $C_{(a+b)}$ and $C_{(x+c)}$ (0.98, $P < 0.0001$). Strong negative correlations were found between $C_{(a+b)}$ and C_a: C_b (-0.76, $P < 0.0001$) and between C_a: C_b and $C_{(x+c)}$ (-0.74,

$P < 0.0001$). When data were pooled over all seasons, a positive correlation was found between $C_{(a+b)}$ and $C_{(x+c)}$ (0.64, $P < 0.0001$.

Discussion

Overall, cultivars considered industry standards performed well in the current study. However, beginning in early summer 2009, extensive damage from redfire has been observed on 'Radicans'. This may be because it was planted close to the initiation of the study and has been more susceptible to disease due to the stress of establishment. However, 'Barabit's Gold', 'Tarheel Plum', and UGA5-15 were all planted at approximately the same time but have shown no incidence of redfire. Table 1 shows the planting dates and plant heights recorded fall 2009. The fastest growing cultivars were 'Ben Franklin', 'Tarheel Blue', and 'Yoshino'. In the current study, 'Yoshino' was superior to the others with regard to winter habit and to date is the best of the standard cultivars evaluated. Tripp (2005) reported that 'Yoshino' is cold-hardy in USDA Zone 6, indicating that it has wide adaptability.

The slow-growing (referred to as dwarf or semi-dwarf) cultivars in the study included 'Black Dragon', 'Cristata', 'Gyokruga', and 'Tansu'. In this study, these cultivars exhibited growth rates and/or morphology different than previously reported. For example, 'Black Dragon' and 'Tansu' were reported to reach heights of 2 m and 0.5 m (Rouse et al., 2000); however, individuals in the current study are 3.4 m and 6.0 m tall after 12 years. Height differences may be attributed to age differences since Rouse et al. (2000) reported that materials used in their study were at least 4-years-old. Also, 'Cristata' exhibits a reduced degree of fasciation on shoots compared to other specimens

(personal observation). Performance of 'Cristata' was poor overall, which may be responsible for the reduction in fasciation. Initially, 'Gyokruga' was thought to be a synonym of 'Giokumo' due to the confusion surrounding this cultivar reported by Rouse et al. (2000) and the similarity between names. The height of the specimen in the current evaluation is 3.3 m, compared to 2 m mature height reported by Rouse et al. (2000). In addition to height, Rouse et al. (2000) reported 'Giokumo' as a conical shrub with a height to width ratio of 2 : 1; while the specimen in the current evaluation is roughly 1 : 1. It is not uncommon to observe differences in growth rate under different environments; however, it is unlikely that the overall habit of the cultivar would be altered. The taxonomy surrounding cultivars of Japanese cedar have been reported to be often confused (Rouse et al., 2000; Tripp, 1993), and it is possible that the material we received was mislabeled prior to obtaining for this study, is a cultivar not described in the literature, or mature heights and growth forms are different in USDA Zone 8 after 12 years compared to USDA Zone 6 or 7 as reported in Rouse et al. (2000).

There were significant differences in mean total chlorophyll between all seasons; however, there was higher content in summer than winter when taxa were pooled (Table 3). The mean total chlorophyll content was similar to previous values for Japanese cedar (Ida, 1981). Previous studies also have observed reduction in total chlorophyll content during winter in Japanese cedar (Han et al., 2003; Han et al., 2004) and sitka spruce [(*Picea sitchensis* (Bong.) Carr.)] (Lewandowska and Jarvis, 1977). However, no increase in chlorophyll or alteration of C_a: C_b ratio (discussed below) was observed during spring recovery of Scots pine from winter stress (Ottander and Öquist, 1991).

In winter 2007-08 chlorophyll fluorescence was measured to evaluate the efficiency of PSII to evaluate damage. However, the values collected were extremely inconsistent (data not shown) and measurements were discontinued. Previous studies have successfully measured chlorophyll fluorescence (Han and Mukai, 1999; Han et al., 2003; Han et al., 2004); however, the data we collected were not useful in evaluation of efficiency, or damage to PSII. Therefore, we observed the relationship between chlorophyll *a*/*b* in an attempt to estimate the status of the photosynthetic apparatus. The ratio of C_a: C_b was statistically different among some seasons and not others, and showed no clear pattern for differences between winter and summer (Table 3). Han and Mukai (1999) reported a slight decrease in the C_a: C_b ratio of Japanese cedar during the period in which chlorophyll was being lost (early winter). A rapid loss of C_a compared to C_b during fall (period of chlorophyll degradation) agrees with the findings of Wolf (1956) who observed this phenomenon in 25 tree species; although that study was conducted solely on angiosperms. The reverse was observed in sitka spruce, which showed a slight increase in C_a: C_b ratio during winter (Lewandowska and Jarvis, 1977). An increased ratio of chlorophyll *a*/*b* indicates that light-harvesting complexes are affected more than PSII reaction centers, since chlorophyll *b* is found only in the light-harvesting complexes and chlorophyll *a* is part of the PSII reaction center (Hoober, 1998). Furthermore, characterization of the subunits of PSII reaction center has identified the D1 subunit's involvement in photoinhibition (He and Malkin, 1998) and Sandmann and Scheer (1998) reported that pheophytin *a* (demetalated chlorophyll *a*) may be used as a marker for PSII. The fact that there is not a consistent increase or decrease in the ratio of chlorophyll *a*/*b*

precludes using these data to draw conclusions regarding the status of damage to the light-harvesting complexes compared to the PSII reaction centers.

Differences were observed between seasons for $C_{(x+c)}$ but there was no clear trend to explain differences between winter and summer (Table 3). Robakowski (2005) observed a reduction in total carotenoid content in *Picea abies* (L.) Karst. and *Pinus mugo* Turra during winter but carotenoid content remained stable for *Abies alba* Mill. The author attributed the difference to the latter species' ability to maintain high photosynthetic capacity during winter as its mechanism for preventing oxidative stress (Robakowski, 2005). The current study measured total carotenoid content in cryptomeria leaves, while previous studies separated carotenoids (Han and Mukai, 1999; Han et al., 2003; Han et al., 2004); however, the value for yellow carotenoids presented by Ida (1981) appears comparable to values presented herein.

A positive, linear relationship was found between total chlorophyll and carotenoids in the current study in all seasons (Fig. 1). Similarly, when data were pooled over 36 taxa belonging to Gymnospermae, Ida (1981) found a similar relationship. This relationship seems counterintuitive since it has been established that chlorophyll decreases and carotenoids increase during winter (Han et al., 2003; Han et al., 2004). However, in the current study, this relationship is attributed to differences between taxa. Taxa differ in total chlorophyll and carotenoid content and taxa that had a high amount of total chlorophyll tended to have a high amount of total carotenoids. Oserkowsky (1932) reported data that appear to fit a positive linear relationship between yellow pigments (xanthophyll) and chlorophyll of hardy pear (*Pyrus communis* L.). That study cites other research (Euler and Hellström, 1929; Sjöberg, 1931) that reported a simultaneous

increase in green and yellow pigments in etiolated seedlings. The physiological basis for the concomitant increase in chlorophyll and carotenoids has been established as a means to prevent photooxidative damage due to reactive oxygen species (ROS; Lambers et al., 1998). Excess energy is present, particularly during winter, because light capture and energy transfer occurs more rapidly than the downstream biochemical reactions; however, carotenoids accumulate during winter and prevent damage by dissipating this excess energy as heat (Lambers et al., 1998).

There was a statistically significant correlation between total chlorophyll content and visual rating; however, it was not a strong relationship and did not hold over all seasons. The hypothesis at the beginning of the experiment was that total chlorophyll content may be used as a predictor of eventual field performance; the higher total chlorophyll content, the more green the plants would remain in winter. However, the data did not support this and there was variability in the relationship between total chlorophyll content and greenness. Previous studies have observed linear (Marquard and Tipton, 1987; Yadava, 1986) and polynomial quadratic (Netto et al., 2005) relationships between SPAD readings [SPAD-501 (Marquard and Tipton, 1987; Yadava, 1986); SPAD-502 (Netto et al., 2005)] and total chlorophyll content determined spectrophotometrically. The current study used visual rating to determine greenness, principally because the leaf shape of cryptomeria made SPAD readings inconsistent. In addition to leaf shape, the thickness of cryptomeria leaves precluded use of an instrument such as a SPAD meter. In spite of using visual ratings in lieu of instrumentation, there does not appear to be the same relationship between greenness and total chlorophyll. This may be explained by the increase in carotenoids discussed above.

There was a substantial difference in rainfall between the two years of the study. From November 2007, to September 2008, the evaluation plots received 112 cm of precipitation; while from October 2008, to August 2009 it received 143 cm of rain. Supplemental irrigation was only used for short periods during new plant establishment; therefore, it is likely that the 30 cm difference in rainfall between the two years had a confounding effect on the study. For instance, during winter 2007-08, UGA5-15 was among the plants with highest total chlorophyll content; however, the next season (summer 2008) it was among the lowest. This taxon was planted in early 2008, and received supplemental irrigation during that period, but it is likely that it experienced drought conditions during summer 2008. The sensitivity of Japanese cedar to lack of precipitation is exemplified by the use of its population size during geologic time to identify wet periods; larger population size corresponding to wetter periods (Tsukada, 1967a). Most of the native range of cryptomeria receives between 120 cm and 180 cm of rainfall; however, the optimum growing conditions appear to be the north facing slopes of Japan that receive between 180 and 300 cm of precipitation. The Tifton, Ga. site has a south facing slope and receives approximately 120 cm; the very low end of rainfall reported to sustain cryptomeria. Furthermore, the field soil [Tifton loamy sand (fine-loamy, siliceous, thermic Plinthic Paleudult)] has low water holding capacity, further limiting the available water. Other studies also have reported that drought stress was a major limiting factor in the survival of plants from northern Japan (Widrlechner et al., 1998). Growers in the southeastern U.S. have reported that increased irrigation on Japanese cedar prior to, and during, winter maintains greener foliage than when the

substrate/soil is allowed to dry in both containerized and field grown plants (personal communication).

The current research was conducted to evaluate biochemical properties of 16 taxa of Japanese cedar grown in USDA Zone 8 and determine if there was a relationship with winter color. If a correlation could be established, measurement of a single biochemical property would allow early screening of progeny to predict landscape performance. A weaker correlation was observed between total chlorophyll content and visual color rating than between total chlorophyll content and total carotenoid content. Due to the importance of drought stress in landscape performance of Japanese cedar, different results may be obtained if consistent supplemental irrigation is used such that water is not limiting. Also, performance of many cultivars evaluated in the current study is altered when grown in cooler climates that are more similar to the native range of the species (personal observation). Japanese cedar has a native range that includes Akita Prefecture, Japan which has a mean yearly temperature, mean yearly high temperature, and mean yearly low temperature of 11.1 °C, 15.1 °C, and 7.4 °C compared to 18.6 °C, 24.4, °C and 12.6 °C for Tifton, Ga. The increased temperature coupled with the relatively low rainfall of Tifton, Ga. and other USDA Zone 8 sites often results in poor performance of cultivars. Some cultivars that languished in the current study have performed much better in cooler climates (USDA Zone 6-7; personal observation). Many cultivars perform better overall in cooler zones, but all selections exhibit increased winter browning in cooler climates. Even in areas as near as Athens, Ga. (USDA Zone 7b), 10-year-old specimens of ‘Yoshino’ planted on the University of Georgia campus exhibit a marked increase in winter browning compared to comparable size plants in Tifton, Ga.

This may be because the lower temperatures are more limiting to the biochemical pathways, causing the plant to rely more heavily on the xanthophyll cycle to prevent oxidative damage. The current research provides a formal evaluation on the performance and pigment analysis of field grown plants of selected cultivars in USDA Zone 8, several of which were observed to perform well in spite of less than favorable conditions.

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Table 3.1. The sixteen taxa evaluated during winter 2007-08, summer 2008, winter 2008-09, and summer 2009 at the University of Georgia Tifton Campus, Tifton, Ga. (USDA Zone 8a) including field location, planting date, and height.

Taxon	Field Location	Planting Date	Height (m)[z]
Araucariodes	4-6	1997	4.0
Barabit's Gold	6-10	2006	2.0
Ben Franklin	6-11	1997	8.3
Black Dragon	6-6	1997	3.3
Cristata	4-4	1997	4.0
Gyokruga	4-8	1997	2.7
Radicans	6-14	2007	1.0
Rasen	6-12	1997	6.1
Sekkan	6-9	1997	5.6
Tansu	6-4	1997	5.5
Tarheel Blue	6-13	1997	8.3
Tarheel Plum	5-19	2007	1.0
UGA5-15	5-15	2008	0.8
var. *sinensis*	6-7	1997	6.4
Yaku	6-16	1997	6.2
Yoshino	6-5	1997	8.2

[z]Height measured fall 2008.

Table 3.2. Weather data at the University of Georgia Tifton Campus for the duration of the study to evaluate 16 taxa of *Cryptomeria japonica*.

Month	Avg. daily max. temp (°C)	Avg. daily min. temp. (°C)	Avg. daily temp. (°C)	Rainfall (cm)
November 2007	20.8	7.4	14.1	2.3
December 2007	19.4	8.0	13.7	14.4
January 2008	14.0	3.9	8.9	8.7
February 2008	18.9	5.6	12.2	19.9
March 2008	20.9	8.5	14.7	8.1
April 2008	24.1	13.0	18.6	7.7
May 2008	28.8	17.1	22.9	4.7
June 2008	32.5	21.0	26.7	5.8
July 2008	32.3	21.8	27.0	8.4
August 2008	30.7	21.9	26.3	31.3
September 2008	29.8	19.3	24.5	0.4
October 2008	23.7	12.5	18.1	16.3
November 2008	18.5	6.7	12.6	9.9
December 2008	18.8	7.5	13.1	7.9
January 2009	15.7	4.7	10.2	4.2
February 2009	17.1	4.3	10.7	5.5
March 2009	20.9	9.6	15.3	20.9
April 2009	24.0	12.6	18.3	21.0
May 2009	27.6	18.6	23.1	16.6

June 2009	32.8	22.3	27.6	5.0
July 2009	31.7	21.6	26.6	17.6
August 2009	31.5	22.0	26.7	17.8

Table 3.3. Mean total chlorophyll ($C_{(a+b)}$), ratio of chlorophyll *a* (C_a) : chlorophyll *b* (C_b), total carotenoid ($C_{(x+c)}$), and visual rating of 16 taxa of *Cryptomeria japonica* evaluated in Tifton, Ga. during winters of 2007-08 and 2008-09 and summers of 2008 and 2009.

Season	Taxon	$C_{(a+b)}$[z]	C_a:C_b[y]	$C_{(x+c)}$[x]	$C_{(a+b)}$: $C_{(x+c)}$	Rating[w]
Winter						
			2007-08			
	Araucariodes	2.81	2.53	0.75	3.8	2.60
	Barabit's Gold	2.45	2.53	0.70	3.5	2.00
	Ben Franklin	1.10	2.31	0.55	2.0	2.60
	Black Dragon	3.03	2.23	0.52	11.7	2.60
	Cristata	3.15	1.71	0.53	11.1	3.00
	Gyokruga	3.65	2.20	0.57	13.9	3.20
	Radicans	3.30	2.66	0.69	4.8	4.20
	Rasen	3.07	2.22	0.75	4.1	3.40
	Sekkan	2.56	2.77	0.68	3.8	2.40
	Tansu	4.13	2.35	0.91	4.5	3.60

	Tarheel Blue	2.84	2.52	0.69	4.1	2.60
	Tarheel Plum	3.55	2.44	0.85	4.2	2.80
	UGA5-15	3.58	2.84	0.49	17.2	4.40
	var. *sinensis*	3.36	2.59	0.73	4.6	4.00
	Yaku	2.13	2.57	0.57	3.8	2.20
	Yoshino	2.74	2.43	0.72	3.8	4.00
HSD[v]		0.61	0.16	0.51	21.6	1.28

	2008-09				
Araucariodes	1.52	2.28	0.48	3.2	1.2
Barabit's Gold	1.98	2.66	0.59	3.4	1.8
Ben Franklin	1.12	2.65	0.42	2.7	1.4
Black Dragon	2.71	2.32	0.57	4.8	2.0
Cristata	3.39	1.63	0.97	3.5	2.0
Gyokruga	3.58	2.70	0.83	4.3	3.6
Radicans	2.65	2.59	0.71	3.8	4.6

	Rasen	2.16	2.70	0.59	3.7	3.2
	Sekkan	1.95	2.75	0.53	3.7	2.0
	Tansu	2.06	2.40	0.55	3.8	3.4
	Tarheel Blue	2.36	3.02	0.69	3.4	2.2
	Tarheel Plum	2.20	3.17	0.60	3.6	2.8
	UGA5-15	1.64	3.04	0.49	3.4	3.4
	var. *sinensis*	3.39	2.88	0.76	4.4	3.8
	Yaku	1.44	2.89	0.41	3.5	3.2
	Yoshino	1.86	2.55	0.52	3.6	3.0
HSD		0.48	0.28	0.11	0.30	1.4
Summer						
			2008			
	Araucariodes	3.67	2.55	0.75	5.1	2.4
	Barabit's Gold	3.45	2.93	0.54	6.4	2.8
	Ben Franklin	3.74	2.98	0.61	6.1	3.2
	Black Dragon	4.85	2.54	0.86	5.7	2.4

	Cristata	4.31	1.73	0.78	5.4	3.2
	Gyokruga	5.20	2.57	0.91	5.7	4.0
	Radicans	3.90	2.83	0.61	6.4	4.0
	Rasen	4.13	2.54	0.71	5.9	3.6
	Sekkan	3.56	2.76	0.61	5.8	2.6
	Tansu	3.78	2.34	0.61	6.2	3.6
	Tarheel Blue	4.77	2.62	0.77	6.2	4.0
	Tarheel Plum	5.42	2.75	0.84	6.4	3.6
	UGA5-15	2.84	3.04	0.50	5.7	3.6
	var. *sinensis*	4.29	2.70	0.69	6.2	3.8
	Yaku	2.52	2.60	0.43	5.9	2.6
	Yoshino	3.78	2.68	0.62	6.1	3.4
HSD		1.21	0.14	0.25	1.14	1.6
			2009			
	Araucariodes	6.14	2.58	0.92	6.7	2.2
	Barabit’s Gold	3.32	2.99	0.51	6.5	3.4

	Ben Franklin	3.70	2.92	0.57	6.5	3.4
	Black Dragon	4.92	2.46	0.77	6.4	2.6
	Cristata	6.32	2.05	1.0	6.3	3.8
	Gyokruga	5.35	2.67	0.84	6.4	5.0
	Radicans	4.77	2.58	0.74	6.4	4.8
	Rasen	4.14	2.53	0.65	6.4	3.4
	Sekkan	4.53	2.84	0.73	6.2	2.8
	Tansu	5.94	2.25	0.84	7.1	4.4
	Tarheel Blue	4.51	2.70	0.68	6.6	4.4
	Tarheel Plum	3.35	2.96	0.55	6.1	4.0
	UGA5-15	4.28	2.85	0.64	6.7	4.2
	var. *sinensis*	3.52	2.69	0.54	6.5	3.8
	Yaku	4.37	2.61	0.66	6.7	3.2
	Yoshino	5.28	2.62	0.81	6.5	4.2
HSD		0.59	0.14	0.11	0.43	1.3

Orthogonal contrasts used for comparison within taxa between seasons

Winter 07-08 vs. Winter 08-09[u]	**	**	**	**	**
Winter 07-08 vs. Summer 2008	**	**	NS	NS	NS
Winter 07-08 vs. Summer 2009	**	**	*	NS	*
Winter 08-09 vs. Summer 2008	**	NS	**	**	**
Winter 08-09 vs. Summer 2009	**	NS	**	**	**
Summer 2008 vs. Summer 2009	**	NS	NS	NS	**

[z]Mean total chlorophyll in mg · g DW^{-1}; n=3.

[y]Mean ratio of chlorophyll *a* : chlorophyll *b*; n=3.

[x]Mean total carotenoids in mg · g DW^{-1}; n=3.

[w]Visual rating based on color with 1=yellow or brown and 5=green; n=5.

[v]Tukey's honestly significant difference (HSD); $P<0.05$. Test compares between taxa, within season.

[u]*, ** indicate significance at $P<0.05$ and $P<0.01$ levels, respectively. NS indicates no significant difference.

Table 3.4. Three taxa of *Cryptomeria japonica* containing the highest and lowest mean total chlorophyll (mg · g DW^{-1}) for each season of evaluation at the University of Georgia Tifton Campus (USDA Zone 8a).

	Winter 2007-08	Summer 2008	Winter 2008-09	Summer 2009
High				
	Tansu	Tarheel Plum	Gyokruga	Cristata
	Gyokruga	Gyokruga	Cristata	Araucariodes
	UGA5-15	Black Dragon	var. *sinensis*	Tansu
Low				
	Yaku	UGA5-15	Ben Franklin	Barabit's Gold
	Barabit's Gold	Barabit's Gold	Yaku	Tarheel Plum
	Sekkan	Sekkan	Araucariodes	var. *sinensis*

Table 3.5. Three taxa of *Cryptomeria japonica* containing the highest and lowest mean total carotenoids (mg · g DW^{-1}) for each season of evaluation at the University of Georgia Tifton Campus (USDA Zone 8a).

	Winter 2007-08	Summer 2008	Winter 2008-09	Summer 2009
High				
	Tansu	Gyokruga	Cristata	Cristata
	Tarheel Plum	Black Dragon	Gyokruga	Araucariodes
	Araucariodes	Tarheel Plum	var. *sinensis*	Gyokruga
Low				
	UGA5-15	Yaku	Yaku	Barabit's Gold
	Black Dragon	UGA5-15	Ben Franklin	var. *sinensis*
	Cristata	Barabit's Gold	Araucariodes	Tarheel Plum

Table 3.6. Three taxa of *Cryptomeria japonica* receiving the highest and lowest mean visual color rating for each season of evaluation at the University of Georgia Tifton Campus (USDA Zone 8a).

	Winter 2007-08	Summer 2008	Winter 2008-09	Summer 2009
High				
	UGA5-15	Gyokruga	Radicans	Gyokruga
	Radicans	Tarheel Blue	var. *sinensis*	Radicans
	Yoshino	Radicans	Gyokruga	Tansu
Low				
	Sekkan	Yaku	Araucariodes	Araucariodes
	Yaku	Araucariodes	Ben Franklin	Black Dragon
	Barabit's Gold	Black Dragon	Barabit's Gold	Sekkan

Table 3.7. Correlation between total chlorophyll ($C_{(a+b)}$), ratio of chlorophyll *a* : chlorophyll *b* (C_a:C_b), total carotenoids ($C_{(x+c)}$), ratio of $C_{(a+b)}$: $C_{(x+c)}$, and visual color rating (Rating) of 16 taxa of *Cryptomeria japonica* evaluated at the University of Georgia Tifton Campus during winters of 2007-08 and 2008-09 and summers of 2008 and 2009.

Winter 2007-08

	$C_{(a+b)}$	C_a:C_b	$C_{(x+c)}$	$C_{(a+b)}$: $C_{(x+c)}$	Rating
$C_{(a+b)}$	1.00	-0.042	0.294*[z]	0.193	0.413**
C_a:C_b		1.00	0.139	-0.155	0.5429
$C_{(x+c)}$			1.00	-0.834***	-0.074
$C_{(a+b)}$: $C_{(x+c)}$				1.00	0.288*
Rating					1.00

Summer 2008

	$C_{(a+b)}$	C_a:C_b	$C_{(x+c)}$	$C_{(a+b)}$: $C_{(x+c)}$	Rating
$C_{(a+b)}$	1.00	-0.251	0.887***	0.159	0.358*
C_a:C_b		1.00	-0.389**	0.343*	-0.088
$C_{(x+c)}$			1.00	-0.307*	0.298*
$C_{(a+b)}$: $C_{(x+c)}$				1.00	0.084
Rating					1.00

Winter 2008-09

	$C_{(a+b)}$	C_a:C_b	$C_{(x+c)}$	$C_{(a+b)}$: $C_{(x+c)}$	Rating
$C_{(a+b)}$	1.00	-0.248	0.922***	0.700***	0.246

C_a:C_b		1.00	-0.340*	-0.036	0.269
$C_{(x+c)}$			1.00	0.383**	0.114
$C_{(a+b)}$: $C_{(x+c)}$				1.00	0.379**
Rating					1.00

Summer 2009

	$C_{(a+b)}$	C_a:C_b	$C_{(x+c)}$	$C_{(a+b)}$: $C_{(x+c)}$	Rating
$C_{(a+b)}$	1.00	-0.758***	0.979***	0.260	0.061
C_a:C_b		1.00	-0.735***	-0.247	-0.138
$C_{(x+c)}$			1.00	0.059	0.042
$C_{(a+b)}$: $C_{(x+c)}$				1.00	0.083
Rating					1.00

Seasons combined

	$C_{(a+b)}$	C_a:C_b	$C_{(x+c)}$	$C_{(a+b)}$: $C_{(x+c)}$	Rating
$C_{(a+b)}$	1.00	-0.134	0.643***	0.234**	0.369***
C_a:C_b		1.00	-0.266**	-0.087	0.056
$C_{(x+c)}$			1.00	-0.401***	0.146*
$C_{(a+b)}$: $C_{(x+c)}$				1.00	0.228**
Rating					1.00

[z]*, **, *** indicate significance at $P \leq 0.05$, $P \leq 0.01$, and $P < 0.0001$, respectively

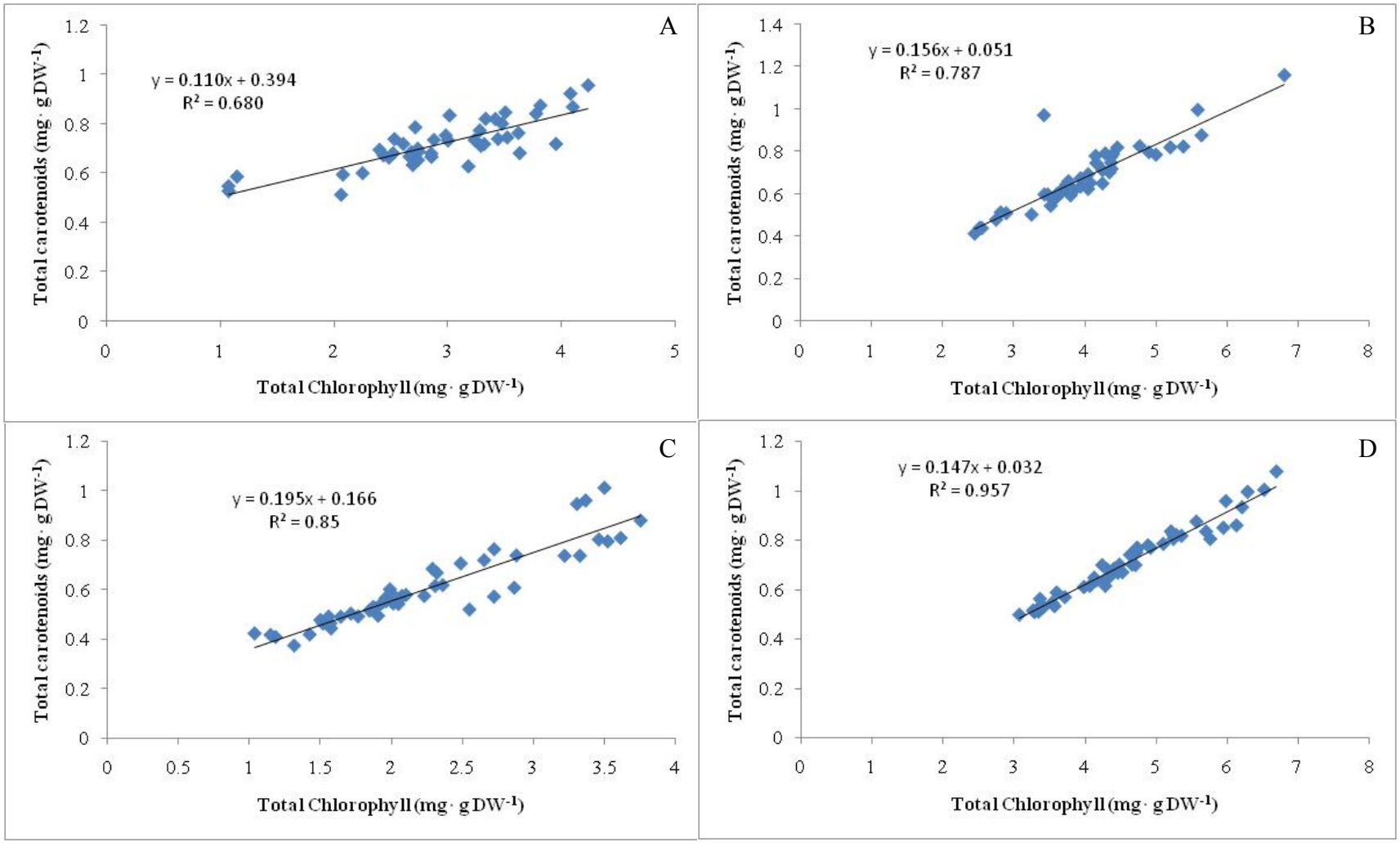
A
y = 0.110x + 0.394
R² = 0.680
Total carotenoids (mg · g DW-1)
Total Chlorophyll (mg · g DW-1)
B
y = 0.156x + 0.051
R² = 0.787
Total carotenoids (mg · g DW-1)
Total Chlorophyll (mg · g DW-1)
C
y = 0.195x + 0.166
R² = 0.85
Total carotenoids (mg · g DW-1)
Total Chlorophyll (mg · g DW-1)
D
y = 0.147x + 0.032
R² = 0.957
Total carotenoids (mg · g DW-1)
Total Chlorophyll (mg · g DW-1)

Figure 3.1. Linear regression of total chlorophyll content over total carotenoid content of 16 taxa of Cryptomeria japonica evaluated at the University of Georgia Tifton Campus (USDA Zone 8a). (A) Winter 2007-08, (B) summer 2008, (C) winter 2008-09, and (D) summer 2009.

Chapter 4

GENETIC STUDIES INVESTIGATING THE INHERITANCE OF FRUIT COLOR AND LEAF VARIEGATION IN *CALLICARPA AMERICANA* L. (LAMIACEAE)[1]

[1]Contreras, R.N., J.M. Ruter, and D.A. Knauft. To be submitted to J. Amer. Soc. Hort. Sci.

Genetic studies investigating the inheritance of fruit color and leaf variegation in *Callicarpa americana* L. (Lamiaceae)

Ryan N. Contreras, John M. Ruter, and David A. Knauft

Abstract. American beautyberry (*Callicarpa americana* L.) is a deciduous shrub native to the southeast U.S. and is grown primarily for its metallic-purple fruit that develop in fall. There are also pink- and white-fruiting and variegated forms but these traits are rare in nature and there is no information available regarding their inheritance. Also, there is confusion regarding self-compatibility and the presence of apomixis in *Callicarpa* L. As part of the cultivar development program at the University of Georgia Tifton Campus, crosses were performed to investigate the genetics of fruit color, leaf variegation, self-compatibility, and apomixis in American beautyberry. Test crosses between *C. americana* (CA) and *C. americana* 'Lactea' (CAL) showed that white fruit is recessive to purple. White fruit appears to be controlled by a single recessive gene for which we propose the name *white fruit* and the gene symbol *wf*. While there were only a limited number of progeny grown, crosses between CA and 'Welch's Pink'suggest that purple is dominant to pink. Test crosses between CAL and 'Welch's Pink' are needed to draw conclusions; however, we propose that purple, pink, and white fruit are controlled by an allelic series for which we suggest the gene symbols $Wf > wf^{p} > wf$. Seed from test crosses to investigate leaf variegation germinated poorly, so no conclusions were possible, but maternal effects appear to be involved. Segregation ratios showed that all

progeny in the study developed through sexual hybridization. All genotypes used in the current study were self-compatible.

Introduction

Callicarpa L. is a genus of ~150 species of shrubs and trees distributed throughout the world including warm-temperate and tropical America, SE Asia, Pacific Islands, and Australia (Harden, 1992) with the greatest concentration of species found in southeast Asia, specifically the Philippine Islands (Atkins, 1999). There are approximately 28 New World species, of which 16 are endemic to Cuba (Moldenke, 1936). The native distribution of American beautyberry (*C. americana* L.) in the U.S. ranges from Maryland in the north, west to Missouri and south along the Gulf Coast to south Texas and Florida (USDA, 2009). American beautyberry produces a berry-like drupe in axillary cymes that encircle the stem and ripen in fall. The wild-type color is metallic-purple to magenta but there are cultivars with white ('Lactea' and 'Bok Tower') and pink ('Welch's Pink') fruit; both of which are rare in nature. There are also leaf-variegated forms of American beautyberry such as 'Berries and Cream', which exhibit a mottled and unstable variegation pattern. To our knowledge, there is no information in the literature on the inheritance of either trait for any species of *Callicarpa*, including *C. americana*.

There is confusion about the self-compatibility and presence of apomixis in *Callicarpa*. Dirr (1998) reported that *C. dichotoma* (Lour.) K. Koch produces fruit consistently every year even when isolated from other seedlings or species (self-compatible) but *C. japonica* Thunb. produces fruit only when planted in a group, possibly indicating self-incompatibility. Three species of beautyberry endemic to the Bonin Islands of Japan have been found to be functionally dioecious (Kawakubo, 1990). However, *C. longissima* (Hemsl.) Merr. and *C. pedunculata* R. Br. produced viable seed after self-pollination in a glasshouse (unpublished data). Populations resulting from

open-pollination of *C. dichotoma* 'Issai' or *C. americana* 'Welch's Pink' were very uniform; appearing almost clonal (M. Dirr, personal communication). This lack of diversity in seedling populations provides evidence that apomixis may be present since either self- or cross-pollination should result in variation from the parental type (Ozias-Akins, 2006), although it may also be because both species are highly homozygous. Tsukaya et al. (2003) confirmed that *C. ×shirasawana* Makino is a natural hybrid of *C. japonica* and *C. mollis* Sieb. et Zucc. Fertility was confirmed by pollen staining and seed germination of the F_1 as well as successful backcrossing to *C. japonica* (Tsukaya et al., 2003). These results indicate that sexual reproduction exists in the genus and at least some level of outcrossing is found.

An improvement program was initiated at the University of Georgia, Tifton Campus, Tifton, Ga., with the goal of developing novel forms of beautyberries that are well adapted to a variety of USDA Hardiness Zones. In addition to development of cultivars for the nursery industry, various aspects of the genetics of *Callicarpa* were investigated. The goal of the current research was to use controlled crosses to investigate the genetics of fruit color and leaf variegation in *C. americana*. Results of crosses and segregation ratios of progeny were also used for inference about apomixis and self-incompatibility in American beautyberry.

Materials and Methods

Plants of *Callicarpa americana* (CA; Accession no. GEN08-0036), *C. americana* 'Berries and Cream' (CBC), *C. americana* 'Lactea' (CAL), *C. americana* 'Welch's Pink' were maintained at the University of Georgia Tifton Campus in 11.4-L containers filled

with substrate containing 8 pine bark : 1 sand amended with 0.91 kg·m^{-3} dolomitic lime and 0.45 kg·m^{-3} Micromax (The Scotts Co., Marysville, Ohio) and topdressed with 45 g of Osmocote Plus 15-4.0-9.1 (The Scotts Co.). Controlled crosses were conducted in a glasshouse with day/night set temperatures of 27/20 °C. For cross-pollination and emasculation only (EO) treatments, emasculation was performed at least one day prior to anthesis. For self-pollination, emasculation was not performed and pollen was applied to the stigma by direct contact with an anther of the same flower. For cross-pollination, pollen was collected by tapping inflorescences over a Petri dish and was then applied to receptive stigmas using brushes. After ripening, fruit were scored as purple, pink, or white (Figure 1), collected and counted, and then seed were cleaned by hand and counted. Seed were then subjected to cold, moist stratified at 4 °C for 60 d and sown in the same pine bark substrate as above. Controlled crosses performed to investigate fruit color and leaf variegation may be found in tables 1 and 2, respectively. In addition to these crosses, 113 flowers of CA were subjected to EO to determine if pollination was necessary for fruit development. Crosses were also made between CA and *C. americana* 'Welch's Pink' to investigate the genetics of pink fruit. Chi-square analysis was conducted to test for goodness-of-fit to theoretical ratios (PROC FREQ; SAS version 9.1, SAS Institute Inc., Cary, N.C.).

Results

Fruit color. All progeny resulting from self-pollination of CA and CAL had fruit that were purple and white, respectively (Table 1) indicating that both are homozygous. Reciprocal crosses between CA and CAL yielded all purple fruit with the exception of

four white individuals that were obtained when CAL was used as the pistillate parent and are likely the result of self-pollination. These four individuals were not included in chi-square analysis. Both F_2 families fit the expected 3 : 1 ratio (F_{2P1} $P = 0.50$; F_{2P2} $P = 0.48$) and all three backcross (BC) families fit the expected 1 : 1 ratio [(BC1P1 $P = 0.32$; BC1P2 $P = 0.67$; BC2P2 $P = 1.00$) (Table 1)]. These results support the hypothesis that white fruit is a simple recessive trait. Furthermore, recovery of white fruit in both F_2 and all BC families indicates that maternal inheritance is not involved. All twelve plants resulting from crosses using 'Welch's Pink' as the pistillate parent and CA as the pollen parent were purple. Three plants resulting from self-pollination of 'Welch's Pink' were pink (data not shown). Recovery of only purple (no intermediates) progeny from crosses between 'Welch's Pink' and CA indicate that purple is dominant to pink.

Fruit color and petiole color were inherited together in 100% of progeny (Fig. 1). Individuals with purple fruit had dark pigmented petioles, individuals with white fruit had green petioles, and individuals with pink fruit had petioles of intermediate pigmentation. Flower color also corresponded with fruit color. Individuals with pink flowers had purple fruit, while individuals with white flowers had white fruit. Flower color was not recorded for plants with pink fruit. The lack of segregation between flower, fruit, and petiole color suggests that they are likely controlled by a single pleiotropic gene or by two very tightly linked genes.

Leaf variegation. Results of crosses to investigate the genetics of leaf variegation were confounded by low seed germination in most families. Only two families, F_{2P2} and BC1P2, had greater than 35% germination and were the only families that were subjected

to chi-square analysis (Table 2). In both of these families the putative homozygous recessive white phenotype had larger numbers than the putative heterozygous variegated condition. Therefore, epistatic interactions resulting in three phenotypic classes (recessive and dominant epistasis and duplicate interaction) were tested for goodness of fit. The F_{2P2} family fit a 9 : 6 : 1 (green : white : variegated) distribution ($P = 0.63$) and the BC1P2 family fit a 12 : 3 : 1 distribution ($P = 0.57$).

Self-compatibility and apomixis. Self-pollination of *C. americana* in a glasshouse produced viable seed indicating self-compatibility. The 113 flowers subjected to EO treatment produced only 0.3 seed per pollinated flower (data not shown), which was lower than the mean seed set per pollinated flower for all other pollination treatments (2.9). The seed produced from EO treatment is likely to be produced from accidental self-pollination. Segregation for purple pigmentation in the petioles and fruit of F_2 and BC families is indicative of amphimixis.

Discussion

There was neither variation in intensity of purple color, nor were intermediates between purple and white or purple and pink recovered. This confirms that incomplete (partial) dominance is not involved in the gene controlling fruit color. Alternatively, Honda et al. (1990) present evidence that fruit color in beefsteak plant (*Perilla frutescens* Britton) is controlled by a single incomplete dominant gene (*W*) that results in three phenotypic classes with white being recessive. Mature fruit color in *Capsicum annuum* L. is reported to be controlled by three genes and is also recessive; identified genetically

as $yyc_1c_1c_2c_2$ (Shifriss and Pilovsky, 1992). Fruit color is controlled by two genes with dominant epistasis in summer squash [(*Cucurbita pepo* L.)(Globerson, 1969)] and hybrid grapes [(*Vitis* spp. L.)(Barritt and Einset, 1969)]. In both examples, white fruit is recessive and is identified as *ccrr* in summer squash (Globerson, 1969) and *bbrr* in grapes (Barritt and Einset, 1969). In the current study, control of white fruit color appears to be controlled by a single recessive gene for which we propose the name *white fruit* and the symbol *wf*. The lack of intermediates between the three classes (purple, pink, white) indicates that there are three alleles for fruit color. All progeny (F_1, F_2, and BC families) have shown that purple is dominant to white and a limited number of progeny tested indicate that purple is dominant to pink. We propose the gene symbols for the allelic series controlling fruit color as $Wf > wf^p > wf$ for purple (wild-type), pink, and white fruit, respectively. In order to confirm this hypothesis, F_2 seed were collected from F_1 progeny resulting from crosses between 'Welch's Pink' x CA and are currently being germinated. We expect that F_2 progeny will segregate in a 3 purple : 1 pink. Reciprocal crosses will also be conducted between CAL and 'Welch's Pink' with the expectation that F_1 progeny will all be pink and progeny resulting from backcrosses to CAL will segregate 1 pink : 1 white.

Flower, petiole, and fruit color cosegregated in all F_1, F_2, and BC families suggesting either a single pleiotropic gene or tight linkage between genes controlling these traits. Dirr (1998) also noted a correlation between flower and fruit color in American beautyberry. Pleiotropic genes controlling pigment production in multiple organs was described previously by Evans et al. (1984), who reported monogenic control of flower, fruit, and leaf color in tomato (*Solanum lycopersicum* L.). Linkage cannot be

ruled out completely; however, the lack of recombinant progeny makes pleiotropy a more likely scenario. The cosegregation of petiole and fruit color may be a useful tool in early screening of American beautyberry progeny.

The current research used an EO treatment and reciprocal crosses between white and purple fruited plants to determine if apomixis is present in American beautyberry. The EO treatment resulted in reduced fruit and seed set. The fruit and seed that were produced after emasculation can be attributed to accidental self-pollination. These results indicate that pollination is required for seed set, but does not rule out pseudogamy. However, F_1, F_2, and BC families fit the expected Mendelian segregation ratios for fruit color and showed that all progeny resulted from sexual reproduction. Ozias-Akins (2006) indicated that controlled crosses using a dominant marker for red vegetative or reproductive organs, as used in the current study, are an effective means for determining the relative rates of apomixis vs. amphimixis. This technique has been used to assess apomixis among hybrids involving pearl millet [*Pennisetum glaucum* (L.) R. Br.] and *P. squamulatum* Fresen. (Roche et al., 2001) using the single dominant gene Rp^1 for red leaf (Hanna and Burton, 1992).

Variegation was only recovered when CBC was used as the pistillate parent, including both families subjected to chi-square analysis. These results suggest reciprocal differences are involved; meaning that inheritance of variegation may be controlled by a non-nuclear gene that is inherited maternally (Tillney-Bassett, 1978). Low germination prevented recovery of variegated progeny in all families resulting from crosses using CBC as pistillate parent; however, it seems likely that maternal effects are involved. Even though the two families analyzed were found to fit theoretical ratios (Table 2), there

was not a single mechanism that could explain all data. Reciprocal crosses between CA and CBC resulted in all green progeny in the F_1 and a segregating F_2 family [(CBC x CA) selfed], which is indicative of Mendelian inheritance of a nuclear gene (Tillney-Bassett, 1978). However, the low germination percentage of most families does not capture the genetic diversity and likely omits potential phenotypes. The results obtained from this study lead to the conflicting inferences above (Mendelian vs. non-Mendelian inheritance). As a result, it is not possible to draw conclusions regarding the inheritance of leaf variegation in American beautyberry from the current data.

The current research presents evidence supporting an allelic series for fruit color in American beautyberry. We propose the symbols for purple, pink, and white fruit as $Wf > wf^{p} > wf$. Furthermore, the data suggested that the gene controlling fruit color is pleiotropic and also controls flower and petiole color. Test crosses and emasculation also showed that all of the progeny produced in the current study developed through sexual hybridization and that all genotypes used in the study were self-compatible. Due to low seed germination in the test crosses to investigate inheritance of leaf variegation, no conclusions were possible; however, the data suggest that maternal effects are involved.

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Table 4.1. Controlled crosses between *Callicarpa americana* (CA) and *C. americana* 'Lactea' (CAL) to determine inheritance of fruit color and the number of wild-type, purple individuals (+/-) and white individuals (*wf*/*wf*).

		Progeny (no. plants)				
Cross (♀ x ♂)	Families	+/-	*wf*/*wf*	Exp. Ratio	χ^{2z}	*P*
CA selfed	S_0	65	0	1 : 0	0.00	1.00
CAL selfed	S_0	0	46	0 : 1	0.00	1.00
CA x CAL	F_{1P1}	114	0	1 : 0	0.00	1.00
CAL x CA	F_{1P2}	88	4[y]	1 : 0	0.00	1.00
F_{1P1} selfed	F_{2P1}	34	14	3 : 1	0.44	0.50
F_{1P2} selfed	F_{2P2}	23	10	3 : 1	0.49	0.48
F_{1P1} x CAL	BC1P1	15	21	1 : 1	1.00	0.32
F_{1P2} x CAL	BC1P2	10	12	1 : 1	0.18	0.67
CAL x F_{1P2}	BC2P2	10	10	1 : 1	0.00	1.00

[z]$\chi^2_{0.05, 1} = 3.841$.

[y]Unexpected phenotype that deviates from disomic-monogenic model; likely due to accidental self-pollination. These data not included in chi-square analysis.

Table 4.2. Crosses between *Callicarpa americana* (CA) and *C. americana* 'Berries and Cream' (CBC) to determine inheritance of leaf variegation.

Cross (♀ x ♂)	Families	Seed	green	white	variegated	Exp. Ratio	χ^{2z}	*P*
		no.						
CA x CBC	F_{1P1}	168	33	0	0	---	---	---
CBC x CA	F_{1P2}	205	4	0	0	---	---	---
CBC selfed	S_0	102	0	0	6	---	---	---
F_{1P1} selfed	F_{2P1}	162	17	0	0	---	---	---
F_{1P2} selfed	F_{2P2}	208	84	43	10	9 : 6 : 1[y]	0.92	0.63
F_{1P1} x CBC	BC1P1	225	19	0	0	---	---	---
F_{1P2} x CBC	BC1P2	172	50	13	2	12 : 3 : 1[x]	1.13	0.57
CBC x F_{1P1}	BC2P1	133	0	11	0	---	---	---
CBC x F_{2P2}	BC2P2	236	3	0	0	---	---	---

$\chi^2_{0.05,\,2} = 5.991$

[y]Theoretical phenotypic ratio for duplicate interaction between two unlinked genes.

[x]Theoretical phenotypic ratio for dominant epistasis between two unlinked genes.

Figure 4.1. The three phenotypic classes of *C. americana* (A) for fruit and petiole color and (B) close up of petiole color.

Chapter 5

SULFURIC ACID SCARIFICATION OF *CALLICARPA AMERICANA* (LAMIACEAE) SEEDS IMPROVES GERMINATION[1]

[1]Contreras, R.N. and J.M. Ruter. Accepted by Native Plants Journal. Reprinted here with permission of publisher.

Sulfuric acid scarification of *Callicarpa americana* (Lamiaceae) seeds improves germination

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Sulfuric acid scarification of *Callicarpa americana* (Lamiaceae) seeds improves germination

Additional index words. beautyberry, propagation, sexual propagation

Abstract. An experiment was conducted to determine if sulfuric acid scarification improved seed germination of *Callicarpa americana* L. Treatments included a control (0 min), 15 min, and 30 min soaks in concentrated (18N) sulfuric acid followed by a 15 min rinse in tap water. The 30 min treatment had the earliest germination with seedlings appearing 18 days after treatment (DAT). The 15 min treatment had seedlings emerge at 26 DAT while seedlings in the control did not begin to emerge until 60 DAT. After 60 days, seed from the acid treatments had approximately 50% germination while the control had less than 10%. At the conclusion of the study the control, 15 min, and 30 min acid treatments germinated at 8.9%, 57.8%, and 48.9%, respectively. This study shows the benefit of sulfuric acid scarification in the germination of *Callicarpa americana.* Recommendations should be amended to include a 15 to 30 min soak in concentrated sulfuric acid to promote rapid and more uniform germination for this species.

Introduction

Callicarpa L. (commonly called beautyberry) is a genus of ~150 species of shrubs and trees distributed throughout the world including warm-temperate and tropical America, SE Asia, Pacific Islands, and Australia (Harden 1992). Beautyberries are grown primarily for their handsome fruit, typically purple, produced in late summer to fall (Figure 1). *Callicarpa americana* L. is an attractive native shrub that is underutilized in the nursery and landscape industries. Selection, the sole means of improvement to date, has been primarily for pink or white-fruiting forms and increased fruit production. To increase available diversity, a program has been initiated at the University of Georgia, Tifton Campus with the goal of developing novel forms of beautyberry through interspecific hybridization and investigating inheritance of ornamental characters to assist in the breeding process. During this program, seed were germinated after 60-d cold, moist stratification. Subsequent germination was observed to be slow, sporadic, and at low percentages.

The fruit of *Callicarpa* is a berry-like drupe with a fleshy exocarp and hard endocarp separated into four pyrenes, each containing a single seed (Harley and others 2004; Moldenke 1936). *Callicarpa americana* exhibits seedcoat dormancy, but this point has not been taken into account for previous recommendations on germinating beautyberry seed (Connor 2004; Dirr and Heuser 2006). In nature, seed distribution is facilitated by a number of mammals (Connor 2004; Halls 1977) and birds, particularly quail (Halls 1977) and mockingbirds (Moldenke 1936). Fruits are primarily utilized during winter months when other food sources are scarce (Halls 1977). During passage through the gut of birds and mammals the seed becomes scarified and germinates the

following spring when temperatures are not limiting. In addition to natural scarification via animals, laboratory acid scarification has also been used to improve germination of native species such as *Sophora secundiflora* (Ruter and Ingram, 1991). Germination of American beautyberry is reported to be slow but occurs without scarification or stratification treatment (Connor 2004). Dirr and Heuser (2006) report that seed sown in the fall has excellent germination the following spring. This delayed germination has also been observed in our program at the University of Georgia and has impeded the speed of research. Therefore, the objective of this study was to determine a more effective protocol for germinating *Callicarpa americana* seeds. It was hypothesized that scarification with sulfuric acid will aid in breaking seedcoat dormancy thereby resulting in more rapid and uniform germination.

Materials and Methods

Fruit resulting from open-pollination was collected on 28 January 2009 from container grown *Callicarpa americana* plants at the Coastal Plain Experiment Station, Tifton, Ga. Parent plants were from a north Ga. provenance (USDA Hardiness Zone 7; USDA, 1990). Plants were grown in isolation blocks to prevent pollination from other species of *Callicarpa*. Seeds were cleaned by hand and treatments were applied immediately after collection. The three treatments consisted of a control (direct sow) and either 15 min or 30 min soak in concentrated sulfuric acid (18N H_2SO_4). During acid treatment, seeds were gently stirred periodically with a glass rod. Following acid treatment, seeds were rinsed in running tap water for 15 min. After rinsing, seeds were sown in 1.28 L (12.7 cm top diameter) containers filled with 8 pine bark : 1 sand (by volume) amended with

1.2 kg dolomitic limestone and 0.59 kg Micromax® (The Scotts Company, Marysville, Ohio) per m^3. Seeds were covered lightly with substrate when sown. Containers were maintained in a glasshouse at 27/20 °C day/night set temperatures and hand watered as needed. The experiment was completely randomized with 3 replications (15 seeds/replication). Seeds were considered germinated if cotyledons had emerged by the end of the study. The study was terminated 60 days after treatment (DAT). Data were analyzed using Analysis of Variance and means were separated by comparing acid treatments to the control using Dunnett's procedure in SAS 9.1 (SAS Institute, Cary, N.C.).

Results and Discussion

Seeds of *Callicarpa americana* germinated more quickly after treatment with sulfuric acid. The 30 min treatment had seedlings emerging 18 DAT and seeds from the 15 min treatment began emerging 26 DAT, while the control had no germination until 60 DAT. The 30 min treatment reached maximum germination of 49% 50 DAT and showed no further germination at 60 DAT at which time the experiment was concluded (Table 1). The 15 min treatment exceeded 50% germination 50 DAT and reached 58% at 60 DAT (Table 1). The control had the lowest germination rate of only 9% at 60 DAT. Both acid treatments were statistically different from the control from 40 DAT until the end of the study (Table 1). No distorted or abnormal growth was observed among seedlings in any of the treatments.

These results show the benefit of acid scarification in germinating *Callicarpa americana* seed. Overall germination may ultimately be similar between control and

acid-treated seed; however it could take six months for untreated or stratified seeds to reach 50% germination. In a previous study on eight species of *Callicarpa*, seeds were collected 28 November 2007 and cold-moist stratified for 60-d prior to sowing. The study was concluded 28 May 2008. Germination ranged from 6% to 45% with a mean of 27% (data not shown). Bonner (2008) reports that a sample of *C. americana* seed stratified for 30-d resulted in 22% germination after 90-d. In the current study, the seeds were collected in January, which exposed them to at least some degree of stratification while on the plant. This in vivo stratification is unlikely to have had an affect on the results. Haywood (1994) reports that *C. americana* seeds are unaffected by stratification and found that germination of seeds planted in a forest site increased over five years, reaching 100% at year five. The ability of *C. americana* to remain viable for a number of years and increase germination rate is likely due to its hard seedcoat slowly breaking down over time to become permeable to water.

For production scheduling, it may be desirable to delay germination until tender seedlings can survive outside of heated glasshouses. Therefore, growers may not necessarily benefit from rapid germination. However, for a research and breeding program, it is desirable to decrease time from seed to seed; that is the time it takes from germination of a seedling to crossing and collection of the next generation's seed. Currently, it is possible to obtain one generation per year, but with acid scarification it may be possible to collect seed in August, scarify and sow, and have the next generation flowering in a glasshouse by early spring. This could allow our program to obtain two generations per year as compared to the current scheduling which is only slightly more rapid than in a natural setting, even with the use of heated glasshouses.

This study shows the benefit of acid scarification of seeds of *C. americana*. Due to the limited treatments applied, relatively small number of seeds, single seed source, and short duration, it is necessary to conduct further work to optimize treatments. *Callicarpa americana* has a native range from Maryland to Texas in the US. While our study was conducted using a north Ga. seed provenance, further work with other germplasm over the extent of its range may be necessary to optimize germination protocols for this species. Previous attempts to germinate *C. americana* under conditions similar to those described by Baskin and Baskin (unpublished results; see McDonald and Young, 2005) resulted in sporadic emergence and low percentages even though they obtained germination as high as 90%, possibly providing evidence for differences in germination requirements of different seed sources. However, even with the small scale of this study, significant differences were observed between treatments and the control. Therefore, we predict these findings will also extend to other species of *Callicarpa*. The 15 min and 30 min scarification treatments were both superior to the control; therefore, germination of *Callicarpa* seeds can be improved by including a 15 to 30 min scarification treatment with concentrated sulfuric acid followed by rinsing and direct sowing.

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Table 5.1. Percent germination of *Callicarpa americana* seeds for each treatment (n=3; 15 seeds/replication).

Acid scarification	Days after treatment (DAT)				
treatment (minutes)	18	28	40	50	60
0	0.0	0.0	0.0	0.0	8.9
15	0.0	20.0	37.8*	53.3*	57.8*
30	4.7	31.1*	46.7*	48.9*	48.9*

*Means significantly different from control at α=0.05 using Dunnett's procedure for mean separation.

Figure 5.1. *Callicarpa americana* in early fall exhibiting mature fruit

Chapter 6

GENOME SIZE AND CHROMOSOME NUMBER IN *CALLICARPA* L. (LAMIACEAE)[1]

[1]Contreras, R.N. and J.M. Ruter. To be submitted to HortScience.

Genome size and chromosome number in *Callicarpa* L. (Lamiaceae)

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Abstract. Genome size estimates and chromosome number information can be useful for studying the evolution or taxonomy of a group and also has applied use for plant breeders. *Callicarpa* L. is a group of approximately 140 species with nearly worldwide distribution. There are no reports for genome size estimates in the literature and the information on chromosome numbers is limited. Genome size estimates were calculated based on flow cytometry of DAPI stained nuclei extracted from leaf tissue of 15 taxa of *Callicarpa*. Chromosome counts were conducted on six species of *Callicarpa* by staining meristematic cells of roots tips using modified carbol fuchsin. Holoploid genome size estimates ranged from 1.34 pg to 3.11 pg with a mean of 1.62 pg. One tetraploid ($2n = 4x = 68$; *C. salicifolia* P'ei & W. Z. Fang in W. Z. Fang) was identified based on holoploid genome size and was confirmed by chromosome counts. There was little variation between species for monoploid genome size. 1Cx-values ranged from 0.67 pg to 0.88 pg with a mean of 0.76 pg. Chromosome counts for all six species studied revealed a base chromosome number of $x = 17$. *Callicarpa chejuensis*, *C. japonica* 'Leucocarpa', *C. longissima*, and *C. rubella* were confirmed as diploids ($2n = 2x = 34$). In addition to *C. salicifolia*, *C. macrophylla* GEN09-0081 was confirmed as a tetraploid ($2n = 4x = 68$).

Introduction

Callicarpa L. is composed of ≈140 species found in Asia, Africa, Australia, and America; however, most species are distributed in tropical and subtropical Asia (Shou-liang and Gilbert, 1994). Centers of diversity have been identified as the Philippine Islands for Old World species and Cuba for New World species; the former comprising a much larger group (Moldenke, 1936). *Callicarpa* was previously placed in the Verbenaceae; however, it was recently transferred into Lamiaceae along with several other genera (Cantino, 1992; Harley et al., 2004). Most species are shrubs but there are also tree and subscandent members of the genus (Moldenke, 1936). Leaves are typically decussate with axillary inflorescences that are most often cymose (Bramley, 2009; Moldenke, 1936). Beautyberries, as they are commonly referred, are grown primarily for their showy berry-like drupes produced in fall. However, species have been found to contain a number of compounds that have allelochemical activity (Cruz-Ortega et al., 2002), mosquito repellent properties (Cantrell et al., 2005), and act as a cyanobactericide (Tellez et al., 2000). In addition to landscape use an ornamental, *Callicarpa* spp. have been grown for use as cut stems for the florist's trade (Bir and Conner, 1997; Greer and Dole, 2009).

In recent years there has been a great increase in the number of genome size estimates available for both plants and animals (Bennett and Leitch, 2005; Gregory, 2005). For plants, there has been progress in documenting genome sizes for diverse groups (Bennett and Leitch, 2005) and angiosperms, in particular, have received much attention (Bennett and Leitch, 1995; Bennett and Leitch, 1997; Bennett et al., 2000; Bennett and Leitch, 2005). The Plant DNA C-values Database (Bennett and Leitch,

2005) currently contains data for 5,150 species; however, no genome size estimates have been reported for *Callicarpa*.

Chromosome numbers have been a useful tool for researchers investigating evolutionary relationships (Guerra, 2008; Levin and Wilson, 1976), particularly at the generic level (Goldblatt, 2007). Chromosome number data compliments genome size estimates by allowing calculation of chromosome size, which has been correlated with evolutionary age (Mehra and Bawa, 1972). Knowledge of chromosome numbers is also a useful tool for breeders (Fehr, 1991). Chromosome numbers can affect inbreeding depression and potential for introgression of traits via interspecific hybridization, among other factors that can alter breeding strategy (Fehr, 1991). Unfortunately, relative to the number of species and in the genus, the cytological information is sparse for *Callicarpa*.

The first beautyberry chromosome count reported was for *C. japonica* Thunb. in Murr. ($n = 16$) by Sugiura (1936); a count that appears to have been incorrectly cited numerous times, as indicated below. In the seminal compilation of Darlington and Wylie (1956), *C. japonica* is cited from Sugiura's (1936) publication; however, the count was reported as $2n = 16$. Another count attributed to Patermann (1938; see Darlington and Wylie, 1956) for *C. japonica* ($2n = 18$) is not included in the bibliography, therefore, should not be considered reliable. Lewis (1961) cited Darlington and Wylie (1956) and concluded that $2n = 18$ was the correct count for *C. japonica* and also provided the first account for *C. americana* L. as $n = 18$. Furthermore, he reported that *C. americana* was a tetraploid; concluding that $x = 9$ in *Callicarpa*. The next species reported on was *C. macrophylla* Vahl ($2n = 34$) by Sharma and Mukhopadhyay (1963), who reported a variant number of $2n = 32$ in addition to the normal complement. Chuang et al. (1963)

reported different base chromosome numbers for *C. formosana* Rolfe (2*n* = 36) and *C. loureiroi* Hook. and Arn. (2*n* = 34). Chromosome number for *C. americana* (2*n* = 36) was apparently confirmed by Santamour (1965), who also reported *n* = 18 for *C. japonica* and *C. dichotoma* (Lour.) K. Koch, providing the first account of the latter. Chromosome counts for *C. tomentosa* Murr. (= *C. arborea* Roxb.) (*n* = 68, 85), *C. psilocalyx* Clarke (*n* = 17), and *C. acuminata* Wall. (*n* = 17) were reported by Mehra and Bawa (1969). Federov (1969) published a compilation of counts and correctly cited Sugiura (1936) by reporting 2*n* = 32 for *C. japonica*, as well as citing the works of Lewis (1961), Santamour (1965), Chuang et al. (1963), and Sharma and Mukhopadhyay (1963). Ono (1975) reported chromosome numbers of *C. glabra* Koidz. collected at three sites (2*n* = 32, 34), *C. subpubescens* Hook. et Arn. collected at two sites (2*n* = 30, 34), and of *C. nishimurae* Koidz. (2*n* = 34) collected at a single site. Chromosome numbers are reported by Yamazaki (1993); however, there is no indication of the source of the counts. Species include *C. kochiana* Makino (2*n* = 34), *C. formosana* (2*n* = 36), *C. japonica* (2*n* = 32, 36), and *C. dichotoma* (2*n* = 36). Similarly, Harley et al. (2004) reported the generic chromosome complement of *Callicarpa* as 2*n* = 16 or 18 with no reference, although it is likely that their source was Darlington and Wylie (1956).

There has been a lack of reports in recent years for chromosome numbers of *Callicarpa*. The *Index to Plant Chromosome Numbers* (*IPCN*) online database (Goldblatt and Johnson, 1979--) publishes newly reported chromosome counts from 1979 onward and does not contain any counts for *Callicarpa*. Santamour (1965) called for a "critical cyto-taxonomic treatment study of a large number of species." The current research does

not provide the necessary comprehensive treatment; rather, the goal was to contribute information regarding chromosome number and genome size of *Callicarpa*.

Materials and Methods

Plant material. All plant material was maintained in containers (described below) at the University of Georgia, Tifton Campus. The 16 taxa, including 14 species, that were studied along with their accession numbers that we assigned are found in Table 1.

Genome sizing.

Leaf tissue preparation and nuclei staining was performed according to Contreras et al. (2009). *Pisum sativum* L. 'Ctirad', with a genome size of 8.76 pg (Greilhuber et al., 2007) was used as an internal standard to calculate holoploid genome size [(2C DNA content of sample (pg) = 8.76 pg × (mean DNA fluorescence (MRF) sample/ MRF standard)]. Monoploid (1Cx-value; Greilhuber et al., 2005) genome sizes were calculated by dividing by the number of chromosome sets. Analysis was conducted using a Partec PA II (Partec, Münster, Germany). Three replicates were used for each taxon. Data for holoploid DNA content and coefficient of variation % (CV%) are presented as means ± SE.

Cytological Analysis. Plants were grown in 2.4-L or 11.4-L containers filled with substrate containing 8 pine bark : 1 sand amended with 0.91 $kg \cdot m^{-3}$ dolomitic lime and 0.45 $kg \cdot m^{-3}$ Micromax (The Scotts Co., Marysville, Ohio) and topdressed with 15 g (2.4-

L) or 45 g (11.4-L g) of Osmocote Plus 15-4.0-9.1 (The Scotts Co.). Containers were placed in trays filled with vermiculite and roots were allowed to grow out of containers into vermiculite for easy collection. Collection of roots and cytological analysis were performed according to Contreras et al. (2009) and chromosomes of 10 to 20 cells counted for each taxon.

Results and Discussion

Holoploid genome size estimates ranged from 1.34 pg to 3.11 pg with a mean of 1.62 pg (Table 1). Little variation in holoploid genome size was observed, with the exception of *C. salicifolia* P'ei & W. Z. Fang in W. Z. Fang, which had twice the number chromosomes (Table 1; Fig. 1D) as did *C. macrophylla* GEN09-0081 (Fig. 1E), which was omitted from the genome size study because it was received after the study had been conducted. Monoploid genome size was also calculated and values ranged from 0.67 pg to 0.88 pg with a mean of 0.76 pg. The lack of variation in monoploid genome size indicates that, within the taxa evaluated, there is not a large difference in chromosome size. This conclusion is based on the observation that all species have the same, or similar, base chromosome number ($x = 17$), discussed below. Furthermore, all species subjected to genome size estimation were diploid, with the exception of *C. salicifolia*. As indicated above, there are no published estimates for *Callicarpa*; however, Bennett and Leitch (2005) reported 21 estimates for taxa in Lamiaceae. These monoploid genome size estimates ranged from 0.33 pg to 5.65 pg with a mean of 1.56 pg. The current estimates fall within the range of values previously reported for the family and provides the first report of cytological or genome information for a number of species including:

C. cathayana Chang., *C. chejuensis* Y. H. Chung & H. Kim, *C. ferruginea* Sw., *C. kwangtungensis* Chun, *C. longissima* Merr., *C. rubella* Lindl., *C. shikokiana* Makino, and *C. ×tosaensis* Makino. *Callicarpa ×tosaensis* is reported to be a hybrid of *C. kochiana* and *C. japonica* (Yamanaka, 1988). All of these species are native to Southeast Asia except *C. ferruginea*, which is endemic to Cuba. This report is the first account of genome information from the New World center of genetic diversity.

Chromosome counts are reported for six species (Table 1; Fig. 1A-F). Four taxa appear to be diploid with the chromosome complement $2n = 2x = 34$: *C. chejuensis*, *C. japonica* 'Leucocarpa', *C. longissima*, and *C. rubella*. *Callicarpa macrophylla* GEN09-0081 and *C. salicifolia* both had twice the number of chromosomes and were interpreted as tetraploids ($2n = 4x = 68$). These counts agree with previous reports of Mehra and Bawa (1969) and Sharma and Mukhopadhyay (1963) who reported $x = 17$ for *C. tomentosa*, *C. psilocalyx*, *C. acuminata*, and *C. macrophylla*. Base chromosome number in *Callicarpa* has often been reported as $x = 8$ or 9; however, this seems to be largely based on the incorrect citation of Darlington and Wylie (1956). The erroneous citation of Sugiura (1936) is the only report of *Callicarpa* below $2n = 32$. It is likely that *C. japonica*, *C. americana*, and *C. dichotoma* are diploid, not tetraploid as previously reported (Lewis, 1961; Santamour, 1965). The current study reported two tetraploid species (*C. macrophylla* and *C. salicifolia*). Two accessions of *C. macrophylla* were included; one tetraploid determined by chromosome counts and one diploid determined by genome size estimation. Sharma and Mukhopadhyay (1963) previously reported a diploid count of $2n = 34$ for *C. macrophylla* as well as a variant that was an apparent nullisomic aneuploid ($2n = 32$). Previous accounts of chromosome numbers of *C.*

acuminata, *C. psilocalyx*, and *C. tomentosa* reported $x = 17$; furthermore, three collection sites of *C. tomentosa* were assessed and two were octoploid ($n = 68$) and one was decaploid ($n = 85$) (Mehra and Bawa, 1969). The current research supports the report of Mehra and Bawa (1969) regarding the presence of a polyploid series in *Callicarpa*. Although it is not completely clear, dysploidy, not just isolated cases of aneuploidy, appears to be present in *Callicapra*. Dysploidy is the step-wise loss of chromosomes within a genus and its occurrence in *Callicarpa* is supported by the fact that Santamour (1965), Sugiura (1936), Lewis (1961), Chuang et al. (1963), and Ono (1975) all reported chromosome counts in the genus that can be interpreted as varying from $x = 17$ and include $x = 15, 16, 18$. Multiple base chromosome numbers have been reported elsewhere. *Latana* L. is reported to be based on at least two numbers ($x = 11, 12$) and polyploid series are found in both basic lines (Sanders, 1987).

All of the species included in the current study have similar distributions in southeast and eastern China (Shou-liang and Gilbert, 1994) indicating that the development of their current base chromosome number likely took place before their divergence. If the reports of Sugiura (1936) and Santamour (1965) are true then *C. japonica* also has aneuploid populations. Lewis (1961) and Santamour (1965) both report the chromosome number of *C. americana* as $n = 18$. Since the distribution of *C. americana* is separated from other species that have been reported, it is likely that its genome evolution and polyploidization/diploidization occurred independently. Similarly, Ono (1975) reported aneuploidy in two of three species found on the Bonin Islands. These species have evolved in isolation as the islands have never been connected to any landmass (Kawakubo, 1990; see Asami, 1970).

The current research provides genome size estimates using flow cytometry on DAPI stained nuclei for *Callicarpa*. Genome sizes all lie within the range of previous reports for Lamiaceae. Chromosome numbers are also reported for six species and the base chromosome number of $x = 17$ is common for all. Two tetraploid species are reported. The current research agrees with previous reports on the base chromosome number and presence of a polyploid series in *Callicarpa*. Accounts for species reported herein that differ from previous reports warrant further investigation of a number of populations. In addition to multiple populations of each species, we hope to document chromosome numbers for all species in Table 1. Additional genome sizing is also planned. Material will be analyzed on a 2008 CyFlow PA (Partec) using both DAPI and propidium idodide (PI). This will allow 1) estimation of variation between laboratories and 2) determination of base pair composition since DAPI is AT specific and PI is a DNA intercalator.

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Table 6.1. Genome sizes calculated using flow cytometry on DAPI stained nuclei and chromosome numbers counted using light microscopy of 15 taxa of *Callicarpa* maintained at the University of Georgia, Tifton Campus.

Taxon	Accession No.[x]	2C-value (pg)[z]	1Cx-value (pg)[y]	CV%	Chromosome
C. acuminata	GEN08-0041	1.54 ± 0.02	0.77	6.75 ± 0.76	
C. americana 'Lactea'	---	1.56 ± 0.02	0.78	6.08 ± 0.50	
C. cathayana Chang	GEN08-0030	1.52 ± 0.03	0.76	5.94 ± 0.15	
C. chejuensis	GEN08-0040	1.44 ± 0.02	0.72	6.13 ± 0.46	$2n = 2x = 34$
C. ferruginea Sw.	GEN08-0038	1.62 ± 0.01	0.81	6.46 ± 0.39	
C. formosana	GEN08-0029	1.34 ± 0.02	0.67	4.86 ± 0.36	
C. japonica	GEN08-0034	1.45 ± 0.01	0.72	4.75 ± 0.36	
C. japonica 'Leucocarpa'	---	1.52 ± 0.01	0.76	7.02 ± 0.80	$2n = 2x = 34$
C. kwangtungensis Chun	GEN08-0035	1.64 ± 0.01	0.82	5.93 ± 0.31	
C. longissima	GEN08-0042	1.63 ± 0.03	0.82	5.82 ± 0.68	$2n = 2x = 34$
C. macrophylla	GEN08-0039	1.76 ± 0.03	0.88	5.01 ± 0.44	
C. macrophylla	GEN09-0081	---[w]	---	---	$2n = 4x = 68$

C. rubella	GEN08-0033	1.39 ± 0.04	0.69	7.21 ± 0.52	$2n = 2x = 34$
C. salicifolia	GEN08-0031	3.11 ± 0.05	0.78	4.45 ± 0.34	$2n = 4x = 68$
C. shikokiana Makino	GEN08-0032	1.44 ± 0.01	0.72	5.35 ± 0.24	
C. tosaensis Makino	GEN08-0037	1.42 ± 0.02	0.71	5.26 ± 0.09	

[z]Holoploid genome size; determined using *Pisum sativum* 'Ctirad' as an internal standard with known genome size of 8.76 pg; n=3. Calculated using the formula: 2C DNA content of sample = 8.76 × (mean relative fluorescence of sample (MRF)/MRF standard).

[y]Monoploid genome size; calculated as above and divided by number of sets of chromosomes.

[x]Mitotic spreads of meristematic root tip cells prepared and stained using modified carbol fuchsin technique (Kao, 1975); counted using light microscopy at ×1,000 magnification.

[w]Not included for genome size estimation.

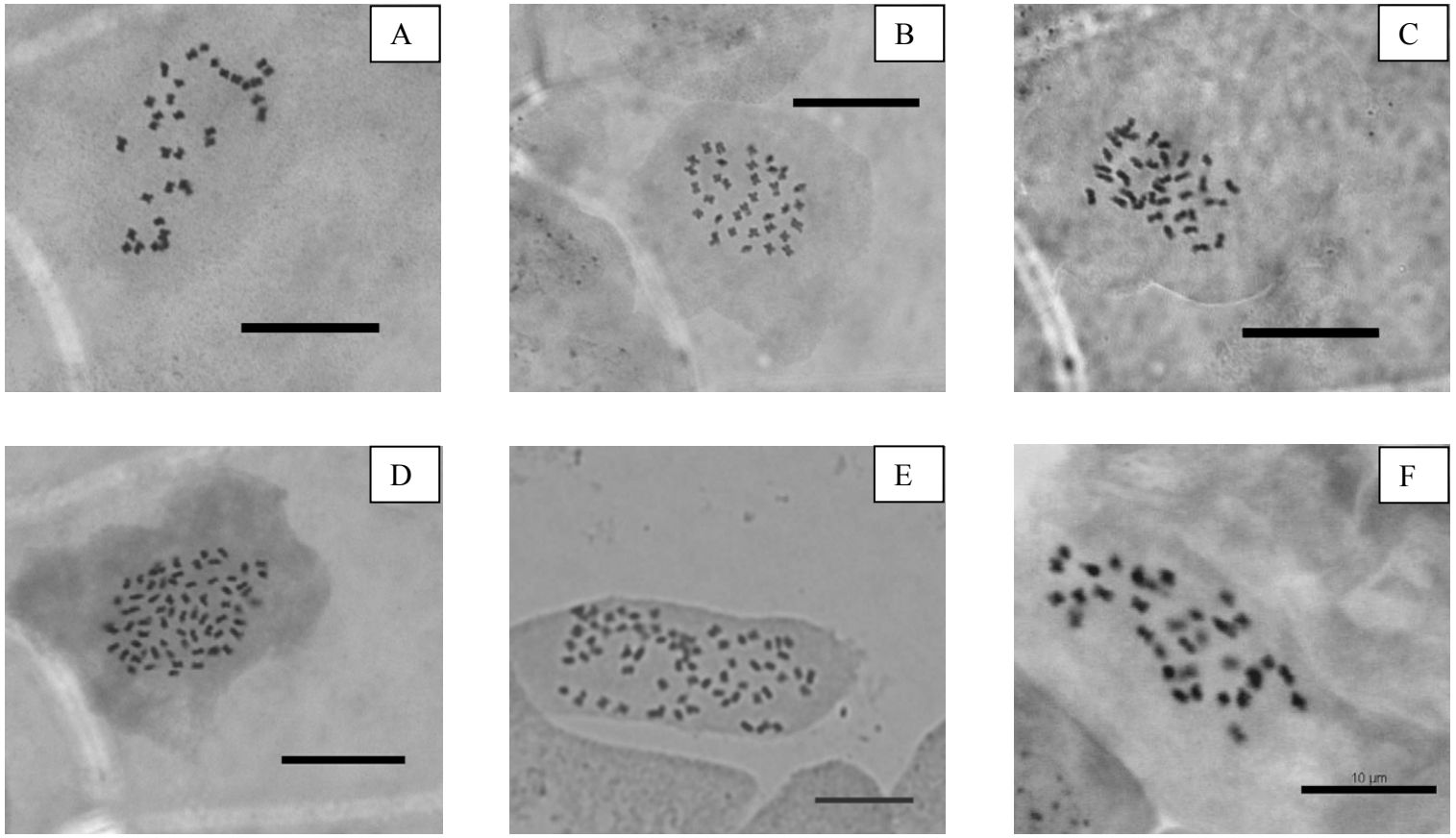

Figure 6.1. Photomicrographs of root tip squashed of (A) *Callicarpa chejuensis*, (B) *C. japonica* 'Leucocarpa', (C), *C. rubella*, (D) *C. salicifolia*, (E) *C. macrophylla* GEN09-0081, and (F) *C. longissima*. Scale bar 10 µm.

Chapter 7

INTERSPECIFIC HYBRIDIZATION IN *TECOMA* JUSS. (BIGNONIACEAE): CONFIRMATION OF HYBRIDITY USING GISH AND DETERMINATIN OF 18S rDNA COPY NUMBER USING FISH IN F_1 HYBRIDS[1]

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Interspecific hybridization in *Tecoma* Juss. (Bignoniaceae): Confirmation of hybridity using GISH and determination of 18s rDNA copy number using FISH in F_1 hybrids

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Interspecific hybridization in *Tecoma* Juss. (Bignoniaceae): Confirmation of hybridity using GISH and determination of 18S rDNA copy number using FISH in F_1 hybrids

Abstract. Interspecific hybridization of several species of *Tecoma* Juss. was conducted to develop novel forms for the nursery industry. Morphology and genomic in situ hybridization (GISH) were used to confirm hybridity and fluorescence in situ hybridization (FISH) was used to identify copy number of the 18S region. We report the development of fertile hybrids from the cross between *T. garrocha* Hieron. and *T. stans* (L.) Juss. ex H.B.K. that were successfully backcrossed to both parents and self-pollinated to produce F_2 progeny. *Tecoma garrocha*, *T.stans*, and *T. guarume* A. DC 'Tangelo' were identified as self-fertile. The current research reports the first three species hybrids in *Tecoma* by crossing F_1 progeny of *T. garrocha* x *T. stans* with *T. capensis* (Thun.) Spach. and *T. guarume* 'Tangelo', respectively. Leaf morphology of the F_1 hybrids of *T. garrocha* x *T. stans* was intermediate between the parents. GISH was also successful in identifying hybridity. FISH conducted on F_1 hybrids identified four copies of the 18S region. The current research reports on the first three species hybrids in *Tecoma* and on the utility of GISH in identifying hybrids. Further analysis using FISH has the potential to provide information on the evolution of Bignoniaceae and the potential role of polyploidy.

Introduction

Tecoma Juss. is a member of tribe Tecomeae within the Bignoniaceae. Gentry (1992) described *Tecoma* as a genus comprised of 14 species of shrubs or small trees; two being native to Africa and 12 naturally occurring in the Neotropics ; however, Wood (2008) regards a number of taxa as subspecies. The African species includes *T. capensis* (Thun.) Spach.; a native of South Africa which was previously described as *Tecomaria capensis*. This species was separated because the apical portion of the anthers is fused; however, Gentry (1992) justified the transfer into *Tecoma* by noting that there are other genera in Bignoniaceae that contain species with both fused and free anthers. The neotropical species range from the extreme southern U.S. to northern Argentina, with a high concentration in Andean South America (Gentry, 1992).

Gentry (1992) divided the genus into two groups based on floral morphology and pollinator type; one with narrowly tubular orange or orange-red hummingbird-pollinated flowers and the other with campanulate yellow bee-pollinated flowers. Three hummingbird-pollinated and one bee-pollinated species of interest for breeding are *T. capensis*, *T. garrocha* Hieron., *T. guarume* A. DC., and *T. stans* (L.) Juss. ex H.B.K. *Tecoma capensis* is a shrub or subshrub that has glossy foliage with 7 to 11 leaflets and red or red-orange narrowly tubular flowers with corollas typically 4 to 5 cm long. *Tecoma garrocha* is a shrub or small tree (2 to 5 m) with 5 to 9 leaflets and very narrowly tubular flowers with yellow to orange corollas and red or orange-red lobes. *Tecoma guarume* is a shrub (2 to 3 m) with 5 to 11 leaflets and broadly salverform-tubular flowers of variable color. *Tecoma stans* is a shrub or small tree to 10 m tall with 3 to 9 leaflets with a coarse texture and campanulate yellow flowers of 4 to 6 cm.

Neotropical Bignoniaceae species have been reported as self-incompatible and obligatory outcrossers (Bawa, 1974; Stephenson and Thomas, 1977), including *Tecoma* (Singh and Chauhan, 1994). Singh and Chauhan (1994) reported *Tecoma stans* exhibited gametophytic self-incompatibility (GSI). However, their account is unclear because it reports that autogamous pollination aborted five to seven days after pollination but geitonogamous pollination resulted in 65% fruit set (Singh and Chauhan, 1994); results that are inconsistent with true self-incompatibility. Regardless of the self-incompatibility mechanism (sporophytic vs. gametophytic), geitonogamous pollination should not result in production of viable seed in a self-incompatible individual (de Nettancourt, 1972). The data of Singh and Chauhan (1994) indicates that *T. stans* is self-fertile, but their interpretation of geitonogamous pollinations as cross-pollination led to the erroneous conclusion that it was self-incompatible. Dutra and Machado (2001) reported that *Stenolobium stans* (Juss.) Seem (= *Tecoma stans*) was self-compatible and produced fertile seed via autogamous, geitonogamous, and xenogamous pollinations, but that this species required pollinators. Self-fertility was also confirmed in *T. stans* by Raju et al. (2001). Pelton (1964) reported that while autogamy is not usually shown in *T. stans*, it was observed that in *T. stans* var. *velutina* cultivated in California, the stigma was on the same level, and within 1 to 2 mm, as dehiscing anthers, which may facilitate autogamous pollination.

Interspecific crosses in *Tecoma* have been reported for over a century. *Tecoma* ×*smithii* Hort. is an interspecific cross between *T. velutina* [= *T. stans* var. *velutina* (A.P. DC.) Fabris] x *T. capensis* (Smith, 1894; Watson, 1893). Smith (1894) reported that it flowered as early as six months from seed and produced flowers year-round in South

Australia. More recently, controlled crosses have resulted in an interspecific hybrid of *T. stans* x *T. garrocha* (Kobayashi et al., 2004). Fruit set was observed when *T. garrocha* was used as the pistillate parent; however, fertile seed were only obtained when *T. stans* was used as the pistillate parent (Kobayashi et al., 2004). Also, Gentry (1990) has reported successful hybridization between bee-pollinated (yellow) flowered species and hummingbird-pollinated (orange to red-orange) flowered species. Natural hybridization between sympatric species has been reported in Bolivia, particularly where *T. tenuiflora* (A. DC.) Fabris grows with *T. stans* or *T. beckii* J. R. I. Wood (Wood, 2008).

Identification of hybrids traditionally has been performed through morphological comparison, including in *Tecoma* spp. hybrids (Kobayashi et al., 2004). However, this may sometimes be difficult when the morphology of the parental species overlaps. *Tecoma* has been described as a, "...taxonomically difficult group with poorly demarcated species mostly differentiated by variable and often complexly overlapping vegetative characters" (Gentry, 1992), therefore other methods would be useful in identifying hybrids. Karyotype markers such as distinctive chromosomes or specific banding pattern produced from Giemsa staining can be useful; however, the chromosomes of *Tecoma* are extremely small, which could make comparison of banding patterns difficult. Genomic in situ hybridization (GISH), which uses labeled total genomic DNA as a probe (Anamthawat-Jónsson et al., 1990), has been used to successfully identify interspecific hybrids in numerous diverse crops including hybrids of teosinte [*Zea perennis* (Hitch.) Reeves & Mangelsd.] and maize (*Z. mays* L.) (Tang et al., 2005), tomato (*Lycopersicon esculentum* Mill. = *Solanum lycopersicum* L.) (Ji et al., 2004), and ornamentals such as

Clivia spp. Lindley (Ran et al., 2001) and *Lilium* spp. L. (Karlov et al., 1999; Marasek et al., 2004).

There have been conflicting reports on chromosome numbers; however, Goldblatt and Gentry (1979) reported $2n = 36$ and concluded that the base chromosome number of *Tecoma* is likely $x = 18$. It was suggested that $n = 20$, a prevalent complement in Bignoniaceae, developed by the formation of a hexaploid that lost a chromosome and the base chromosome number of the family is likely $x = 7$(Goldblatt and Gentry, 1979). Therefore, it appears that *Tecoma* is a polyploid genus. GISH has been used to confirm polyploidy and to identify diploid progenitor species (Bennett et al., 1992); however, this is only useful in cases such as *Milium* L., where there are putative progenitor species based on chromosome numbers or morphology. In *Tecoma*, there are no species reported with chromosome numbers that suggest a hybridization event followed by polyploidization (e.g., $2n = 18$). As a result, a different technique to investigate the presence of polyploidy is required. Copy number of 18S rDNA, most often found with other rDNA in a cluster referred to as the nucleolar organizing region (NOR) (Long and Dawid, 1980), has been associated with ploidy. Copy number of the NOR has been correlated to ploidy level in taxa as diverse as wheat [(*Triticum aestivum* L. 'Chinese Spring); Mukai et al., 1991), salmonids [(*Oncorhynchus* spp. Suckley); (Lozano et al., 1992)], and *Musa* L. (Osuji et al., 1998). Lozano et al. (1992) and Osuji et al. (1998) both used fluorescence in situ hybridization (FISH) (Bauman et al., 1980) which is similar to GISH, but uses specific sequence information as opposed to genomic DNA.

Hybridization between morphologically diverse species such as *T. garrocha*, *T. guarume*, *T. stans*, and *T. capensis* as described by Gentry (1979, 1990) offers potential

to develop novel cultivars with unique combinations of flower and foliage characters. The objectives of this study were to 1) perform crosses including interspecific and self-pollinations to evaluate crossability, 2) confirm hybridization using morphology and GISH, and 3) determine copy number of the 18S region in hybrids using FISH.

Materials and Methods

Plant materials. A selection of *T. capensis* that was found to be cold-hardy at the University of Georgia, Tifton Campus was used in the current research. Two genotypes of *T. stans* were used, one form was selected because it was more compact and will be referred to as *T. stans* DS (dwarf selection). The form of *T. garrocha* used in the current study had fine textured foliage and flowers with corollas and lobes of red-orange. *Tecoma guarume* 'Tangelo' (Meerow and Ayala-Silva, 2008) was also used in the current research. All material was maintained at the University of Georgia, Tifton Campus in 2.4-L or 11.4-L containers filled with substrate containing 8 pine bark : 1 sand amended with 0.91 $kg \cdot m^{-3}$ dolomitic lime and 0.45 $kg \cdot m^{-3}$ Micromax (The Scotts Co., Marysville, Ohio) and topdressed with 15 g (2.4-L) or 45 g (11.4-L g) of Osmocote Plus 15-4.0-9.1 (The Scotts Co.). Plants used for controlled crosses and cytogenetic analysis were grown in a glasshouse with day/night set temperatures of 27/20 °C. Plants that were grown for cytogenetic analysis were placed in trays filled vermiculite and roots were allowed to grow out of containers into vermiculite for easy collection. Root tips were collected prior to 1000 HR and pre-treated for 1 to 2-h in an aqueous solution of 2 mM 8-hydroxyquinoline (Fisher Scientific Company, Suwanee, GA) + 0.24 mM cycloheximide (Acros Organics, Morris Plains, NJ) at 4 °C. Following pre-treatment, roots were

transferred to Carnoy's solution [6 100% EtOH : 3 chloroform : 1 glacial acetic acid (by volume)] and fixed overnight at 25 °C. Roots were rinsed with deionized water and transferred to 70% EtOH (v/v) and stored at 4 °C.

Controlled crosses. Controlled crosses conducted in 2008-09 are found in Table 1. Cross-pollination was performed by emasculating the pistillate parent at least two days prior to anthesis to prevent self-pollination. Pollen of the selected staminate parent was applied by touching an anther directly to a receptive stigma. Stigmas of *Tecoma* are thigmotropic and remain closed after successful pollination, which provided a simple means of ensuring that application of pollen was not a limiting factor in the success of crosses. If pollen was not adequately applied the stigma would reopen after ≈10 min. Self-pollination was conducted on the morning of anthesis in the same manner as cross-pollination. Glasshouse-grown plants of *T. garrocha* also set fruit without supplemental pollination; presumably through autogamous self-pollination.

Comparison of leaf morphology. Ten leaves of *T. garrocha*, an F_1 hybrid of *T. garrocha* x *T. stans* DS, and *T. stans* DS were measured to determine if the hybrid exhibited intermediate morphology. Total leaf length, terminal leaflet length, terminal leaflet width, and number of leaflets were recorded. Data were subjected to analysis of variance (ANOVA) and means were separated using Duncan's multiple range test (MRT), $\alpha = 0.05$.

Chromosome preparations. Roots were rinsed for 15 min in deionized water and then root caps were removed with a scalpel. Root tips were placed into a previously described

enzyme mixture (Akiyama et al., 2004) with modifications. The mixture consisted of 2.3% Cellulase Onozuka RS (Karlan Research, Torrance, CA), 0.9% Macerozyme R-200 (Karlan Research), 0.7% Pectolyase Y-23 (Karlan Reserach), and 0.6 mM EDTA adjusted to pH 4.2 at 37 °C for 1.5 h. The enzyme mixture was then removed by rinsing root tips in deionized water for 15 min. Individual root tips were then transferred to a glass slide, water was removed, and the root tip was macerated in 17 µL of 3 100% EtOH : 1 glacial acetic acid using forceps. Before the slide was allowed to dry, it was exposed to steam using a water bath set to 65 °C. Finally, the slide was dried on a heat block at 85 °C.

Genomic DNA extraction. Genomic DNA for probe preparation was extracted from *T. stans* DS and *T. garrocha* using a DNeasy® Plant Mini Kit (Qiagen Inc., Valencia, CA) according to the bench protocol. Blocking DNA was extracted from the same taxa as above using a modified version of the protocol described by Afanador et al. (1993). Approximately 150 mg of newly expanding leaves were collected on ice and ground in a mortar using liquid nitrogen. The powder was then transferred to a 1.5 mL Eppendorf tube and 600 µL of CTAB buffer (65 °C) was added. The amount of chloroform : isoamyl alcohol (24 : 1) and isopropanol was increased from 400 µL to 600 µL. Ammonium acetate (1/10 volume) was added to the final precipitation step and samples were suspended in water.

Blocking DNA preparation. Genomic DNA from *T. garrocha* and *T. stans* DS were diluted to 50 ng·µL^{-1} and autoclaved at 105 °C for 15 min in order to generate fragments of ≈200 bp.

18S rDNA preparation. The 18S rDNA region from the obligate apomictic buffelgrass (*Cenchrus ciliaris* L.) accession B12-9 (Goel et al., 2003) was amplified using PCR. Each reaction mixture was composed of approximately 10 ng of template DNA, 0.5 μM primer (Forward 5'-AACGGCTACCACATCCAAGGAAGGC-3'; Reverse 5'-GCGCGTGCGGCCCAGAACATCTAAG-3'), 0.25 mM dNTPs, 0.5 units JumpStart™ *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, MO), 1× PCR buffer (Sigma-Aldrich) in HPLC grade water for a final volume of 20 μL. The primers were designed from the 18S sequence from maize (*Zea mays* L.). Reactions were conducted under the following conditions: 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 90 s, for 35 cycles. After confirmation of successful amplification using 1% agarose gel, the PCR product was run on a 0.8% agarose gel and the band was excised to ensure only 18S rDNA was recovered. Purification was performed using a QIAquick® Gel Extraction Kit (Qiagen Inc.) and then rDNA was diluted 1 : 10 in HPLC grade water and amplified under the same conditions as above. The amplification product was purified using QIAquick® PCR Purification Kit (Qiagen Inc.).

Probe labeling and hybridization. Genomic DNA from *T. stans* DS, *T. garrocha*, and the 18S region were labeled with either biotin (Biotin-16-dUTP; Roche, Indianapolis, IN) or digoxigenin (DIG-11-dUTP; Roche), using a nick translation kit (Roche). Reactions were incubated at 15 °C for 4 h and unincorporated dNTPs were removed by ethanol precipitation in the presence of ammonium acetate. DNA was suspended in HB50 (2× SSC, 50% (v/v) formamide) and stored at -20 °C.

To prepare slides for hybridization, 500 µL of 100% ethanol was applied to each slide and dried on a heat block at 85 °C and then the protocol for RNA and protein digestion described by Zhong et al. (1996) was followed. A number of combinations of probe mixtures were used for double target hybridization including biotinylated 18S + digoxigenin-labeled *T. garrocha* DNA, digoxigenin-labeled 18S + biotinylated *T. stans* DS DNA, and biotinylated *T. stans* + digoxigenin-labeled *T. garrocha*. Hybridization experiments were also conducted by probing slides with a combination of unlabeled *T. garrocha* DNA and biotinylated *T. stans* DS DNA and also with unlabeled *T. stans* DS DNA and digoxigenin-labeled *T. garrocha* DNA using approximately 5× to 10× blocking DNA. Hybridization was conducted as previously described by Goel et al. (2003) with modifications. Hybridization mixtures for each slide consisted of 1.3 to 2.5 ng·µL^{-1} probe, 50% formamide, 5% dextran sulfate, 100 to 300 ng·µL^{-1} salmon sperm DNA and 2× SSC in a final volume of 15·µL. In some GISH experiments, blocking DNA was included in the hybridization mixture (described above). The hybridization mixtures were denatured at 85 °C for 10 min and chilled on ice. Slides were incubated at 37 to 39 °C for 16 to 20 h in a humid chamber. Post hybridization washes were conducted as described by Goel et al. (2003).

Probe detection. Digoxigenin labeled probes were detected using a signal-amplification kit (Molecular Probes, Eugene, OR) and biotinylated probes were detected using Texas Red® streptavidin (Vector Laboratories, Burlingame, CA). All slides were blocked for nonspecific binding and washes were performed as in Goel et al. (2003) with modifications. An additional blocking step was done using blocking buffer (Roche).

Incubation was conducted in the dark in three steps: fluorescein-conjugated anti-dig (Roche) and Texas Red®-conjugated streptavidin, then rabbit anti-fluorescein and biotinylated anti-streptavidin, followed by goat anti-rabbit IgG and Texas Red®-conjugated streptavidin. Slides were rinsed 2× for 5 min in T-PBS [0.2% Triton-X 100 (by volume) in 1× PBS; Sigma-Aldrich] and then rinsed in an alcohol dehydration series [70%, 95%, and 100% ethanol (by volume)] 1× for 5 min in each solution. Finally, slides were mounted in Vectashield (Vector Laboratories) containing DAPI (4', 6-diamidino-2-phenylindole (DAPI; 1.5 ng/µL). Slides were examined using the equipment and conditions described by Goel et al. (2003). At least 20 cells from each hybridization mixture were observed.

Results

Crossing studies. Results of crossing studies conducted in 2008 to 2009 are presented in Table 1. In 2008, seed was recovered from crosses between *T. garrocha* x *T. capensis* UGA4-3 and *T. garrocha* x *T. stans* DS; however, only seed from the latter germinated. There was no fruit set from any crosses using *T. capensis* UGA4-3 as a pistillate parent, therefore in 2009 it was only used as a staminate parent. The reproductive efficiency, calculated as the number of progeny per pollinated flower, of the cross between *T. garrocha* x *T. stans* DS was 46; meaning that 46 hybrids were recovered for each flower pollinated. An F_1 individual was selected from the above seedlings and used in crosses in 2009. It was self-pollinated and used as a pistillate parent in crosses with *T. capensis*, *T. garrocha*, *T. stans* DS, and *T. guarume* 'Tangelo' and had fertility similar to its parents (Table 1). When the F_1 hybrid was used as the pistillate parent in crosses with *T.*

guarume ‘Tangelo’ it had a reproductive efficiency of 36.8, compared to 6.8 and 23.3 when pollinated with *T. garrocha* or *T. stans* DS, respectively. The F_1 produced the only seedling in crosses with *T. capensis* UGA4-3; producing only one seedling from 25 pollinated flowers. The F_1 hybrid was also successfully used as the staminate parent in crosses with *T. garrocha*, *T. guarume* ‘Tangelo’ and *T. stans* DS and had reproductive efficiencies of 13.9, 19.0 and 28.7 in the respective crosses. Self-pollination of the F_1 resulted in a reproductive efficiency of 16.0. *Tecoma garrocha* was also self-fertile and produced 17.0 plants per pollinated flower. In addition, four flowers of *T. garrocha* produced 139 seed with no pollination, 56 of which germinated. Crosses between *T. garrocha* and *T. capensis* UGA 4-3 produced a large number of seed, none of which germinated. However, when *T. garrocha* was pollinated with *T. guarume* ‘Tangelo’ it produced nearly 48 plants per pollinated flower. *Tecoma guarume* ‘Tangelo’ and *T. stans* DS were found to be self-fertile, producing 6.2 and 11.6 plants per self-pollinated flower, respectively.

Comparison of leaf morphology. Leaves of the F_1 hybrids of *T. garrocha* x *T. stans* DS were morphologically intermediate between their parents (Table 2, Fig. 1). Length and width of the terminal leaflet was statistically different from both parents and serration of leaflets appeared intermediate.

Confirmation of hybridity using GISH. Mitotic chromosome preparations were made for an F_1 hybrid of *T. garrocha* x *T. stans* DS and investigated using GISH (Figure 2). When blocked with *T. stans* DS genomic DNA the *T. garrocha* probe generally hybridized with

18 or 20 chromosomes, the latter of which is seen in Figure 2B-C. When *T. garrocha* was used to block, the *T. stans* DS probe hybridized to approximately 18 to 20 chromosomes (Fig.2E-F) but in some cells hybridized to as many as 22. There was more noise in most experiments using the *T. stans* DS probe (Fig. 2E, I; 3C); potentially due to nonspecific binding or the need for long exposure time during imaging because of poor labeling. When cells were dual probed with labeled DNA of both parents, the Dig-labeled *T. garrocha* most often hybridized to 18 chromosomes (Fig. 2H) but hybridized to 20 at times. The *T. stans* DS probe hybridized to 18 to 22 chromosomes (Fig. 2I). There were also chromosomes that hybridized with both probes, indicating a high degree of similarity in some regions of the parental genomes.

FISH using 18S rDNA and GISH. Mitotic chromosome preparations were probed using FISH and GISH simultaneously to investigate hybridity as well as copy number of the 18S region in F_1 hybrids. As in previous experiments, there was less nonspecific binding with Dig-labeled probes than biotinylated probes; however, Figure 3B, D shows that there was some nonspecific binding using Dig-labeled probes as well. Dig-labeled *T. garrocha* genomic DNA hybridized to 18 chromosomes (Fig. 3F, H), while the *T. stans* DS probe was less specific (Fig. 3C-D). Biotinylated and Dig-labeled 18S rDNA probes each hybridized to four chromosomes in the F_1 hybrid (Fig. 3B,D,G,H) indicating that the 18S region is found on two chromosomes in the hybrid studied. Chromosomes that hybridized to the 18S probe also hybridized with the *T. stans* DS probe (Figure 3B-D) and with the *T. garrocha* probe (Figure 3F-H).

Discussion

In 2008, interspecific hybrids were developed between *T. garrocha* and *T. stans* DS. There was a mean of 46 seedlings per pollinated flower resulting from this cross indicating that these species are closely related. Reciprocal crosses were not conducted due to limited number of flowers produced by *T. garrocha* when *T. stans* DS was flowering. The current research differs from previous attempts to cross these two species. Kobayashi et al. (2004) reported viable seed set only when *T. stans* was used as the pistillate parent; however, reciprocal crosses set fruit with no viable seed. Also in 2008, *T. capensis* was self-pollinated and used in crosses with *T. stans* and *T. garrocha*; setting fruit only when used to pollinate *T. garrocha*. Due to the lack of fruit set using *T. capensis* as a seed parent it was only used as a pollen parent in 2009. Crosses set seed when it was used to pollenize *T. garrocha*, *T. guarume* 'Tangelo' and the F_1 hybrid of *T. garrocha* x *T. stans* DS; however, only a single seedling from the latter germinated. Interspecific hybrids have been developed using *T. capensis* (Smith, 1894; Watson, 1893); however, the current research reports the first instance of its use in development of a three species hybrid. Reciprocal crosses were also used to develop three species hybrids between the F_1 developed in 2008, and *T. guarume* 'Tangelo'. *Tecoma guarume* 'Tangelo' was reported to produce abundant fruit in the landscape; however, no seedlings were observed (Meerow and Ayala-Silva, 2008). Even though seedlings have not been observed, there is still an opportunity for non-native species such as *T. guarume* to hybridize with wild populations of *T. stans* since our research has clearly demonstrated the crossability between species. We hope that the complex hybrids developed will exhibit sterility or highly reduced fertility due to chromosomal hybrid sterility. Hybrids

including *T. guarume* 'Tangelo' are unlikely to have the desired level of sterility in the current generation due to the ease of the cross; however, the hybrid developed using *T. capensis* is more likely to exhibit reduced fertility based on the relative difficulty of the cross. The three species hybrids developed in 2009 have yet to flower. Relative fertility will be evaluated in 2010.

Self-fertility was observed in *T. garrocha*, *T. guarume* 'Tangelo', *T. stans* DS, and *T. garrocha* x *T. stans* DS. Our findings agree with previous reports indicating self-compatibility in *T. stans* (Dutra and Machado, 2001; Raju et al., 2001). *Tecoma garrocha* also set autogamous seed without supplemental pollination in a glasshouse. Pelton (1964) reported a close association of anthers and stigma in cultivated *T. stans* var. *velutina*, indicating the potential for autogamy; however, no reports of autogamous seed production without pollination are available for *T. garrocha*.

Leaf morphology of F_1 hybrids of *T. garrocha* x *T. stans* DS was compared to the parents and was determined to be intermediate. Previous reports on hybrids between these species also exhibited intermediate floral morphology (Kobayashi et al, 2004). In contrast to the report of Kobayashi et al. (2004), our research found that the cross was successful using *T. garrocha* as the pistillate parent. Furthermore, it was reported that *T. stans* required uniconazole treatment to induce flowering (Kobayashi et al., 2004), while in our study *T. stans* DS flowered freely and *T. garrocha* was more reticent to flower, which prevented reciprocal crossing.

To determine the utility of using GISH to identify hybrids in *Tecoma*, species were selected that produce interspecific hybrids with intermediate morphology such that confirmed hybrids could be selected for GISH. Results of GISH agreed with morphology

and demonstrate the utility of this technique for identification of even closely related species. Parental probes often hybridized to more than half of the chromosomes in the hybrid. In particular, parental probes hybridized to all four ribosomal chromosomes (Fig. 3), an indication of conservation between the species. However, even in experiments which used blocking DNA, there was often hybridization with more than 18 chromosomes. It seems apparent that these species have extremely similar genomes; however, it is probable that an increased ratio of block : probe would produce a better result. The amount of blocking DNA used in the previous reports varies widely from 10 or 20 : 1 (Karlov et al., 1999) to 100 : 1 (Ran et al., 2001). There are numerous reports on the utility of GISH to identify hybrids between closely related species used as ornamentals (e.g. Karlov et al., 1999; Ran et al., 2001) and our research confirms the utility of GISH in *Tecoma* hybrids. Molecular genetics have previously been used to investigate the phylogeny of Bignoniaceae (Spangler and Olmstead, 2006) and genetic diversity at the generic level (Jain et al., 1999) but this is the first time that molecular cytogenetics have been used in the family. The current study showed that ribosomal DNA appears to be located on two chromosomes in hybrids of *T. garrocha* and *T. stans*. Copy number of the NOR, which contains the 18S region used in the current study, has been shown to be correlated to ploidy in taxa such as *Musa* (Osuji et al., 1998) and wheat (Mukai et al., 1991). Further investigations on diverse species of *Tecoma* as well as other genera in Bignoniaceae are warranted. Goldblatt and Gentry (1979) hypothesized that Bignoniaceae is originally based on $x = 7$ due in part to the prevalence of $n = 20$, and perhaps more importantly on the genus *Oroxylum* Vent. which is $n = 14$. A more

complete analysis of the family using FISH to determine copy number of the 18S region may help to identify polyploidy and provide information on the family's evolution.

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Table 7.1. *Tecoma* spp. crosses conducted in 2008-09.

Cross (♀ x ♂)	Flowers Reproductive pollinated	Fruit set	Seed set/ poll. flw.	Germination	efficiency[z]
		no.		%	no.
	2008				
T. stans x *T. capensis* UGA 4-3	13	0	---	---	---
T. capensis UGA 4-3 x *T. stans* Dwarf Selection (DS)[y]	10	0	---	---	---
T. capensis UGA 4-3 x *T. stans*	6	0	---	---	---
T. capensis UGA 4-3 selfed	12	0	---	---	---
T. garrocha x *T. capensis* UGA 4-3	5	4	36.6	0	0
T. garrocha x *T. stans* DS	5	5	49.6	92.7	46.0
T. stans DS x *T. capensis* UGA 4-3	23	0	---	---	---
T. stans x *T. capensis* UGA 4-3	2	0	---	---	---
	2009				
T. garrocha x *T. capensis* UGA 4-3	11	11	43.7	0	0

T. garrocha selfed	8	7	37.4	45.5	17.0
(*T. garrocha* x *T. stans* DS) x *T. capensis* UGA 4-3	25	8	4.8	0.8	0.04
(*T. garrocha* x *T. stans* DS) selfed	7	6	23.7	67.5	16.0
T. guarume 'Tangelo' selfed	5	3	23.8	26.1	6.2
T. garrocha x (*T. garrocha* x *T. stans* DS)	10	4	15.0	92.7	13.9
T. garrocha x *T. guarume* 'Tangelo'	5	5	56.2	84.7	47.6
T. guarume 'Tangelo' x *T. capensis* UGA 4-3	10	2	6.1	0	0
T. guarume 'Tangelo' x (*T. garrocha* x T. *stans* DS)	4	4	36.0	52.8	19.0
(*T. garrocha* x *T. stans* DS) x *T. garrocha*	4	2	15.0	56.7	6.8
(*T. garrocha* x *T. stans* DS) x *T. stans* DS	3	2	25.7	90.9	23.3
(*T. garrocha* x *T. stans* DS) x *T. guarume* 'Tangelo'	6	5	46.8	78.6	36.8
T. stans DS x (*T. garrocha* x *T. stans* DS)	3	2	37.0	77.5	28.7
T. stans DS selfed	9	4	26.4	43.7	11.6
T. stans DS x *T. capensis* UGA 4-3	5	0	---	---	---

[z]Plants obtained per pollinated flower.

[y]Dwarf selection made at the University of Georgia, Tifton Campus

Table 7.2. Leaf morphology of *Tecoma garrocha*, F_1 hybrid of *T. stans* (UGA dwarf selection, DS) x *T. garrocha*, and *T. stans* DS. All data presented as means (n=10) separated using Duncan's multiple range test (MRT).

Taxon	Leaf length (cm)	Term. leaflet length (cm)	Term. leaflet width (cm)	No. leaflets
T. garrocha	13.4b[z]	6.4c	1.4c	7.4a
F_1	16.0a	8.1b	2.5b	6.5a
T. stans DS	16.9a	11.4a	4.5a	4.4b

[z]Means within columns followed by different letters are significantly different based on MRT, $P \leq 0.05$

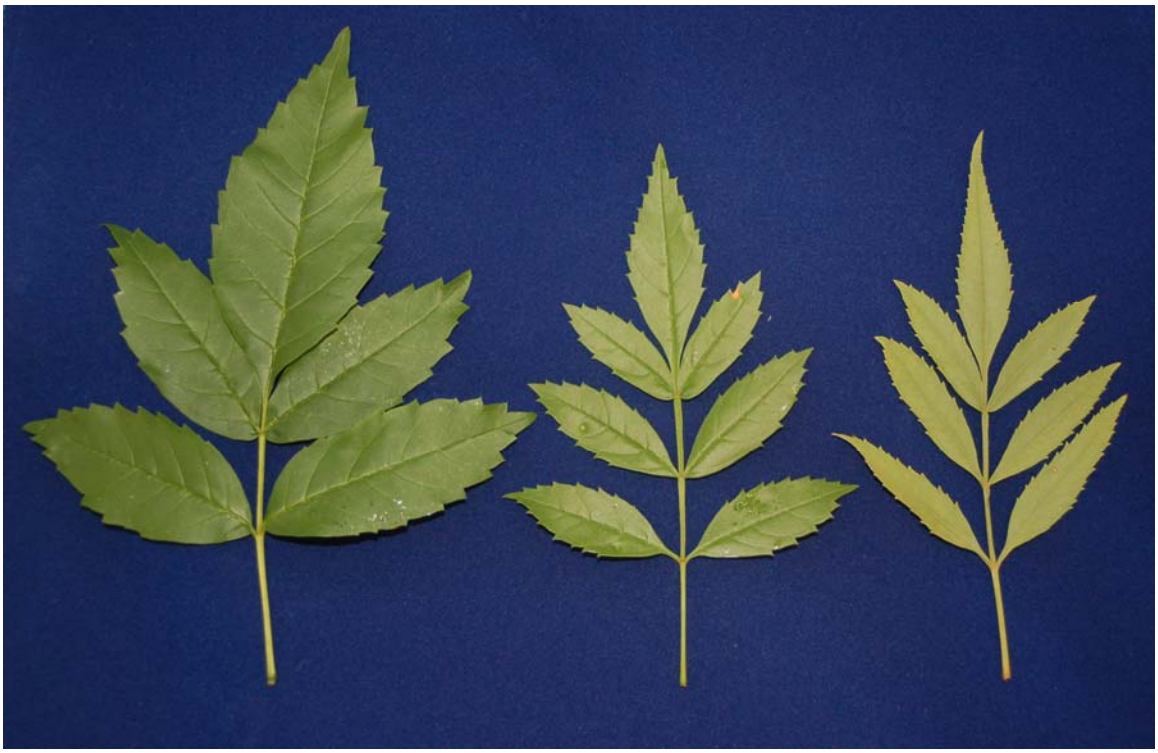

Figure 7.1. Leaves of *Tecoma stans* (UGA dwarf selection) (left), F1 hybrid of *T. stans* x *T. garrocha* (center), and *T. garrocha* (right).

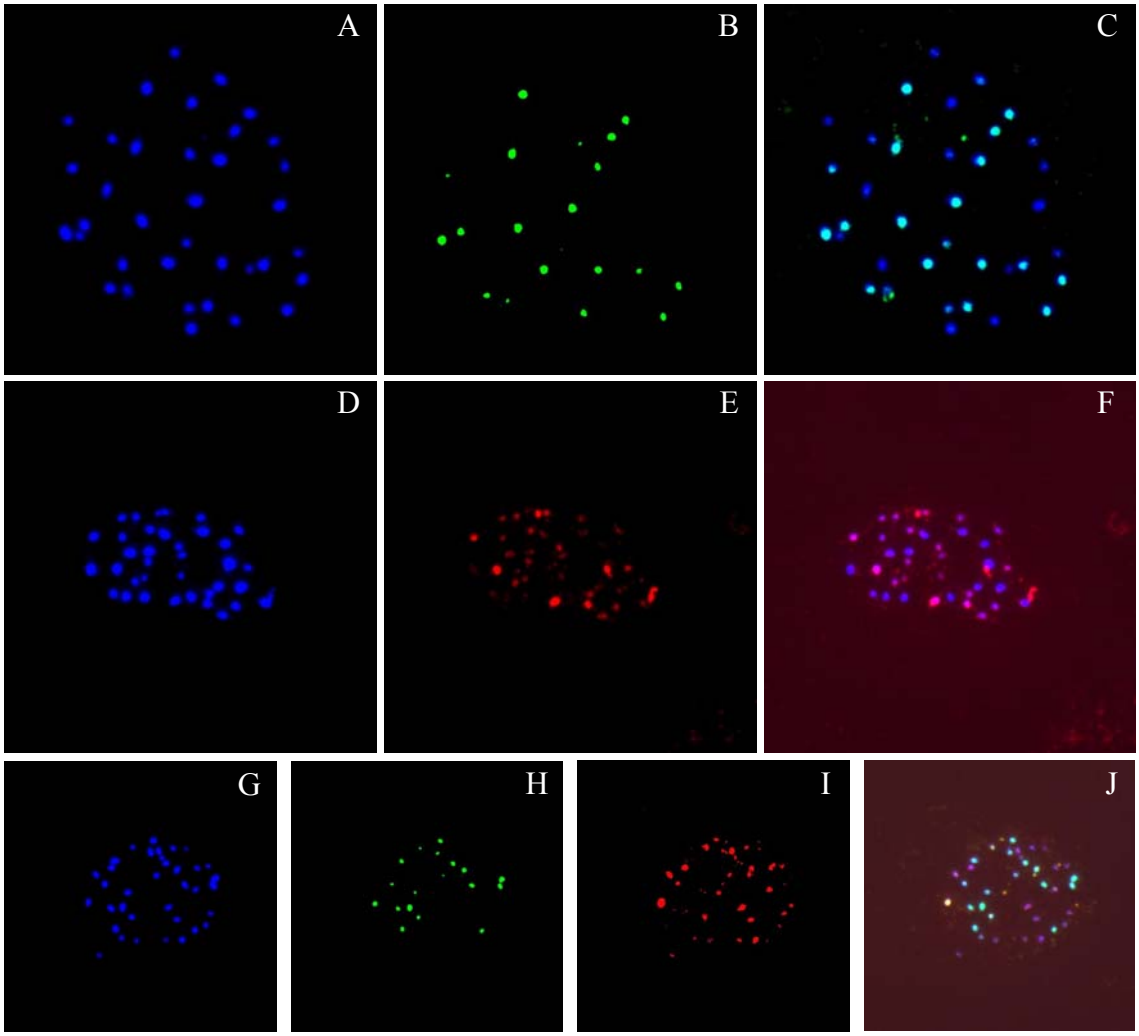

Figure 7.2. Results of GISH on mitotic chromosomes of an F_1 hybrid of *T. garrocha* x *T. stans* DS probed with Dig-labeled *T. garrocha* and blocked with sheared genomic DNA of *T. stans* DS (B-C), biotinylated *T. stans* DS and blocked with sheared genomic DNA of *T. garrocha* (E-F), and probed with both parental probes without blocking DNA (H-J). Chromosomes counterstained with DAPI (A,C,D,F,G,J).

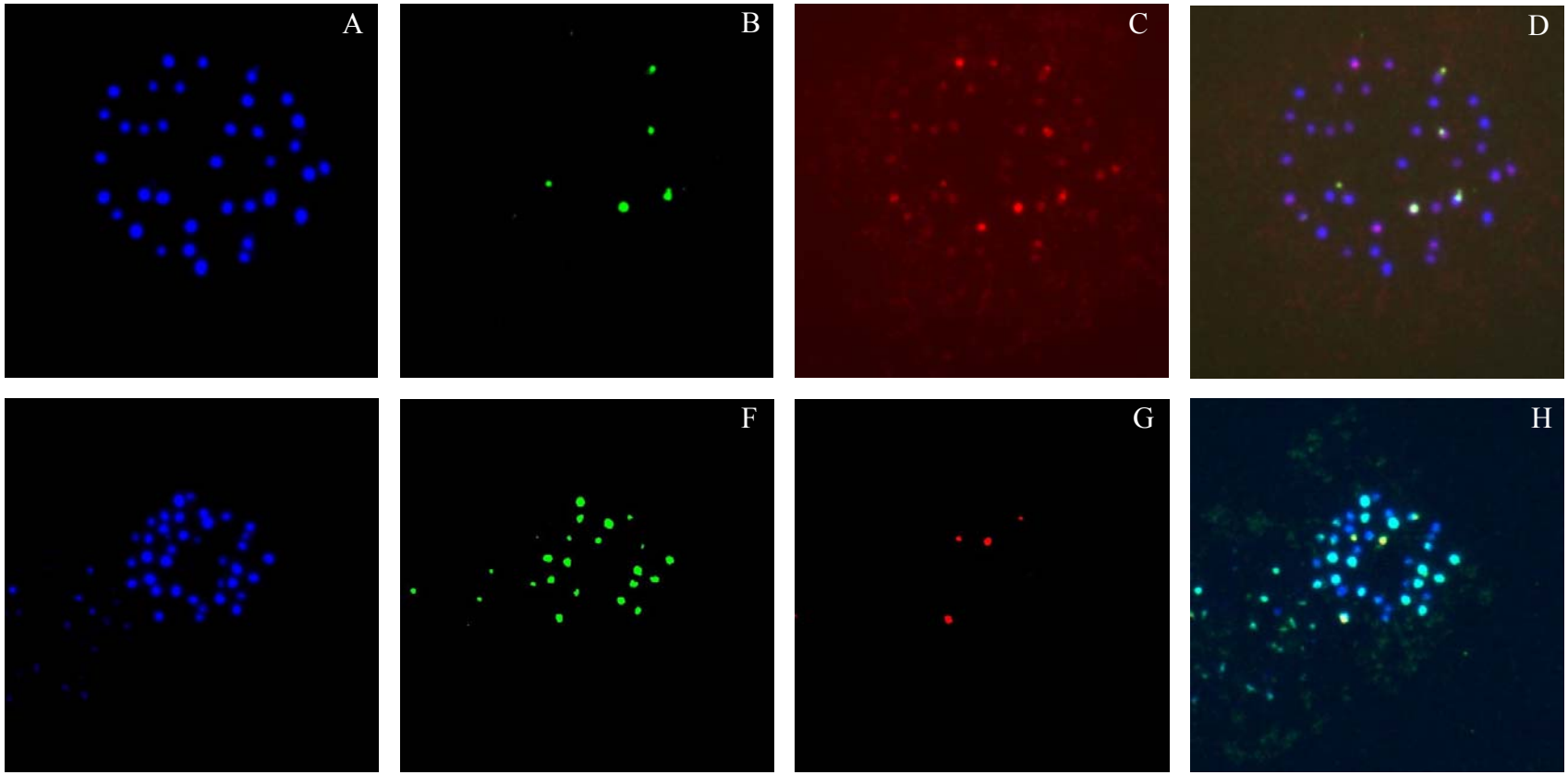

Figure 7.3. Results of GISH (C-D, G-H) and FISH (B,D,G,H) on mitotic chromosomes of an F_1 hybrid of *T. garrocha* x *T. stans* DS. The cell in A-D was dual probed with Dig-labeled 18s rDNA from buffelgrass (*Cenchrus ciliaris*) and biotinylated genomic DNA of *T. stans* DS. The cell in E-F was dual probed with Dig-labeled genomic DNA of *T. garrocha* and biotinylated 18s rDNA from buffelgrass. Chromosomes counterstained with DAPI (A,D,E,H).

Conlusion

This dissertation has presented reserach ornamental cultivar development in *Callicarpa*, *Cryptomeria*, *Hibiscus*, and *Tecoma*. Brief results and implications of each chapter will be discussed below. Chapter 1 was a study attempting to develop a more compact form of *Hibiscus acetosella* 'Panama Red' PP20121 by inducing polyploidy. Treatment of seedlings with oryzalin was found to be an effective means of inducing polyploidy and the resulting polyploids were smaller than tetraploids. The polyploids had shorter internodes as well as several other morphological characters that differentiated the octoploid from the tetraploid. Chapter 2 also attempted to use polyploidy, this time in Japanese cedar. Thirty day application of oryzalin combined with a surfactant was found to be successful in developing tetraploids. This study was the first time that polyploids have been induced in Japanese cedar and the first time that oryzalin has been used to induce polylpoidy in a gymnosperm. Leaf morphology was an excellent phenotypic marker for selection of tetraploids and was found to be greater than 92% accurate. Chapter 3 evaluated 16 taxa of Japanese cedar for performance in south Georgia, the first extensive evaluation in USDA Zone 8. Differences in amount of total chlorophyll, ratio of chlorophyll *a*:*b*, amount of total carotenoids, and visual color rating were observed among taxa within each season and among seasons within taxa. There were no consistent trends for differences between winter and summer for the two years of the study. The highest and lowest performing taxa for all traits were not consistent over all seasons. Correlations were observed between chlorophyll, ratio of chlorophyll *a*:*b*, carotenoid, ratio of chlorophyll : carotenoid, and rating; however, relationships were not consistent over all seasons. There were large differences in rainfall between the two years

and this may have accounted for a portion of the variation. Chapter 4 evaluated the potential benefit of scarification in germinating American beautyberry and acid scarification for 15 to 30 minutes was found to be beneficial in germination. Chapter 5 presents research on American beautyberry using crossing studies to investigate the inheritance of fruit color and leaf variegation, the presence of apomixis, and self-compatibility. Test crosses between *C. americana* (CA) and *C. americana* 'Lactea' (CAL) showed that white fruit is recessive to purple. White fruit appears to be controlled by a single recessive gene for which we propose the name *white fruit* and the gene symbol *wf*. While there were only a limited number of progeny grown, crosses between CA and 'Welch's Pink'suggest that purple is dominant to pink. Test crosses between CAL and 'Welch's Pink' are needed to draw conclusions; however, we propose that purple, pink, and white fruit are controlled by an allelic series for which we propose the gene symbols $Wf > wf^{p} > wf$. Seed from test crosses to investigate leaf variegation germinated poorly, so no conclusions were possible, but maternal effects appear to be involved. Segregation ratios showed that all progeny in the study developed through sexual hybridization. All genotypes used in the current study were self-compatible. Chapter 6 was conducted to generate information regarding genome sizes and chromosome numbers in *Callicarpa*, data that was severely lacking. Chromosome counts were conducted on six species of *Callicarpa* by staining meristematic cells of roots tips using modified carbol fuchsin. Holoploid genome size estimates ranged from 1.34 pg to 3.11 pg with a mean of 1.62 pg. One tetraploid ($2n = 4x = 68$; *C. salicifolia* P'ei & W. Z. Fang in W. Z. Fang) was identified based on holoploid genome size and was confirmed by chromosome counts. There was little variation between species for monoploid genome size. 1Cx-values ranged from of

0.67 pg to 0.88 pg with a mean of 0.76 pg. Chromosome counts for all six species studied revealed a base chromosome number of $x = 17$. *Callicarpa chejuensis*, *C. japonica* 'Leucocarpa', *C. longissima*, and *C. rubella* were confirmed as diploids ($2n = 2x = 34$). In addition to *C. salicifolia*, *C. macrophylla* GEN09-0081 was confirmed as a tetraploid ($2n = 4x = 68$). Chapter 7 was conducted to develop new interspecific hybrids in *Tecoma* and confirm their hybridity using GISH. Also, FISH was conducted to determine the copy number of rDNA in hybrids and assess self-compatibility in the genus. We report the development of fertile hybrids from the cross between *T. garrocha* and *T. stans* that were successfully backcrossed to both parents and self-pollinated to produce F_2 progeny. *Tecoma garrocha*, *T.stans*, and *T. guarume* 'Tangelo' were identified as self-fertile. The current research reports the first three species hybrids in *Tecoma* by crossing F_1 progeny of *T. garrocha* x *T. stans* with *T. capensis* and *T. guarume* 'Tangelo', respectively. Leaf morphology of the F_1 hybrids of *T. garrocha* x *T. stans* was intermediate between the parents. GISH was also successful in identifying hybridity. FISH conducted on F_1 hybrids identified four copies of the 18S region. The current research reports on the first three species hybrids in *Tecoma* and on the utility of GISH in identifying hybrids. Further analysis using FISH has the potential to provide information on the evolution of Bignoniaceae and the potential role of polyploidy.

Appendix A

Study 1. Cuttings were collected 29 May 2007, and treated with an oryzalin solution solidified by agar. The experiment was a full factorial and consisted of 0 µM, 20 µM, 40 µM, and 60 µM oryzalin solutions that were applied to shoot tips of cuttings for 0, 6, 12, or 24 h. There were four replications and 18 cuttings per replication (4 concentrations × 4 durations × 4 replications = 64 experimental units). No differences were observed for survival between treatments and there were no differences in morphology of surviving cuttings between treated and untreated.

Study 2. Seed was collected from 'Tarheel Blue' and surface sterilized using 20% bleach and 0.1% Triton X-100 as a surfactant. Seeds were rinsed for 15 min in deionized water and placed on germination blotters in Petri dishes wetted with 7.0mL of 0%, 0.05%, 0.1%, or 0.25% aqueous solutions of colchicine. The experiment was completely randomized with 15 dishes per concentration and 10 seeds per dish (4 concentrations × 15 replications = 60 experimental units). Dishes were placed on growth racks at 25 °C under constant light supplied by cool-white fluorescent lamps. Only eight seeds germinated in the study and none displayed a phenotype indicative of induced polyploidy.

Study 3. Seed from 'Tarheel Blue' were collected and sterilized as in Study 2. Seeds were then soaked in 0%, 0.05%, 0.1%, 0.25% aqueous colchicine solutions with 0.1% Triton-X 100 as a surfactant. The experiment was a full factorial with four concentrations × four durations × 5 replicates = 80 experimental units. There were 50 seed per replicate. After treatments were applied, seeds were sown in Petri dishes on germination blotters moistened with 7.0 mL deionized water. Dishes were placed on growth racks at 25 °C under constant light supplied by

cool-white fluorescent lamps. Only six seeds germinated in the study and none displayed a phenotype indicative of induced polyploidy.

Study 4. Twenty newly germinated seedlings from seed collected from 'Tarheel Blue' were treated with 150 μM oryzalin solidified with 0.8% agar. A drop of solution was applied to shoot tips of seedlings on three consecutive days. No seedlings exhibited morphology indicative of induced polyploidy.

Study 5. Seed received from Lawyer Nursery as described in Chapter 2 were treated with 0%, 0.2% colchicine, or 0.2% colchicine with 0.5% DMSO; all solutions solidified with 0.8% agar. Twenty-five plants were treated with each solution by applying one drop on three consecutive days. No plants exhibited morphology typical of induced polyploidy; however, two seedlings were kept due to unique leaf morphology.

Study 6. Seedlings were treated as in Study 5 but the solutions were applied for five consecutive days. There were 25 seedlings per treatment. No plants exhibited morphology typical of induced polyploidy.

Study 7. Seedlings were inverted in 25 mL of deionized water, 0.2% colchicine, 0.5% DMSO, 0.2% colchicine with 0.5% DMSO. The beaker was placed in a humid chamber to prevent dessication of roots. Treatments were applied to 25 seedlings each. The control, colchicine, DMSO, and colchicine + DMSO treatments had 12, 17, 13, and 18 surviving seedlings. No plants exhibited morphology typical of induced polyploidy.

Study 8. One hundred seedlings were etiolated by germinating and growing in darkness. Seedlings were inverted in 25 mL of 0.4% colchicine with 0.5% DMSO. The beaker was placed in a humid chamber to prevent the roots from dessicating. No plants exhibited morphology typical of induced polyploidy.

Study 9. Fifty seedlings were treated germinated and treated with a solution of 150 μM oryzalin + 0.1% SilEnergy solidified with 0.8% agar. A single drop of the solution was applied to shoot tips while still liquid on three consecutive days. Following treatment, the seedlings were not able to be screened until the plants had become very large and I believe a number of seedlings that were potentially tetraploid had reverted; therefore, it is difficult to determine the efficacy of the treatment. However, three seedlings were retained due to unusual leaf morphology and determined to be tetraploids after submission of the manuscript presented in this dissertation as Chapter 2.

Appendix B

```
Title 'Evaluation of 16 Cryptomeria';
options ls=72 formdlim='*';
data 'cryptomeria';
input season$ rep$ taxa$ chlAB carot chl chl_carot rating;
cards;

1 1 Araucariodes 2.515111199 0.784557728 2.712893426 3.457863366 4
1 2 Araucariodes 2.537113279 0.832950221 3.013837094 3.618267959 2
1 3 Araucariodes 2.542353892 0.631625828 2.691129424 4.260638662 3
1 4 Araucariodes . . . . 2
1 5 Araucariodes . . . . 2
1 1 BarabitsGold 2.508284604 0.693462857 2.399589121 3.460299421 2
1 2 BarabitsGold 2.552046891 0.73698167 2.530543715 3.43365896 2
1 3 BarabitsGold 2.536594964 0.670990443 2.434307609 3.627931865 3
1 4 BarabitsGold . . . . 2
1 5 BarabitsGold . . . . 1
1 1 BenFranklin 2.321700694 0.525978633 1.075747451 2.045230325 2
1 2 BenFranklin 2.353787761 0.584762585 1.148534577 1.964104076 2
1 3 BenFranklin 2.268999134 0.546134938 1.074205153 1.966922604 4
1 4 BenFranklin . . . . 3
1 5 BenFranklin . . . . 2
1 1 BlackDragon 2.205893873 0.1113112 2.959029418 26.58339339 4
1 2 BlackDragon 2.20549117 0.771652162 3.27965572 4.250173695 2
1 3 BlackDragon 2.266558692 0.664628908 2.854767413 4.295280238 2
1 4 BlackDragon . . . . 3
1 5 BlackDragon . . . . 2
1 1 Cox 2.750588854 0.0770686 3.147999074 40.84671415 5
1 2 Cox 2.919646364 0.679781244 3.629388668 5.339053849 5
1 3 Cox 2.842558735 0.717259212 3.948342518 5.504763764 4
1 4 Cox . . . . 4
1 5 Cox . . . . 4
1 1 Cristata 1.702795766 0.1309264 3.286272079 25.10014847 3
1 2 Cristata 1.706355336 0.651503091 2.737271161 4.201470724 3
1 3 Cristata 1.708917863 0.818051208 3.41595863 4.175727138 4
1 4 Cristata . . . . 3
1 5 Cristata . . . . 2
1 1 Gyokruga 2.147486078 0.1103282 3.567893928 32.3389118 4
1 2 Gyokruga 2.286352739 0.839569708 3.773442105 4.4944953 4
1 3 Gyokruga 2.164677193 0.761233154 3.618879037 4.753969288 3
1 4 Gyokruga . . . . 3
1 5 Gyokruga . . . . 2
1 1 Radicans 2.654083688 0.70929403 3.288482507 4.636275462 5
1 2 Radicans 2.733743597 0.737516433 3.438591319 4.662392815 4
1 3 Radicans 2.604802257 0.626275372 3.179134269 5.076256243 4
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1	4	Radicans	.	.	.	.	4	
1	5	Radicans	.	.	.	.	4	
1	1	Rasen	2.19683172	0.751451534	2.981053151	3.96705977	4	
1	2	Rasen	2.213457539	0.798998814	3.474410727	4.348455423	3	
1	3	Rasen	2.263451632	0.686178137	2.74279839	3.997210404	4	
1	4	Rasen	.	.	.	.	3	
1	5	Rasen	.	.	.	.	3	
1	1	Sekkan	2.75022802	0.679205634	2.518791219	3.708436874	3	
1	2	Sekkan	2.759038608	0.684149042	2.678715094	3.915396982	2	
1	3	Sekkan	2.804278071	0.661819378	2.481632241	3.749712267	3	
1	4	Sekkan	.	.	.	.	3	
1	5	Sekkan	.	.	.	.	1	
1	1	Tansu	2.348139119	0.92065937	4.074675886	4.425823514	4	
1	2	Tansu	2.352657444	0.954197963	4.230898461	4.433983958	4	
1	3	Tansu	2.338998466	0.866810976	4.096055316	4.725430837	4	
1	4	Tansu	.	.	.	.	3	
1	5	Tansu	.	.	.	.	3	
1	1	TarBlue	2.445698313	0.666049113	2.663039716	3.99826329	3	
1	2	TarBlue	2.473174544	0.678663945	2.853425136	4.204474331	2	
1	3	TarBlue	2.629289865	0.731232961	3.001444081	4.104634556	3	
1	4	TarBlue	.	.	.	.	3	
1	5	TarBlue	.	.	.	.	2	
1	1	TarPlum	2.427998393	0.844668182	3.503944835	4.148309254	3	
1	2	TarPlum	2.494635681	0.818886949	3.331084535	4.067819786	2	
1	3	TarPlum	2.403942643	0.872467652	3.810619409	4.367634034	3	
1	4	TarPlum	.	.	.	.	3	
1	5	TarPlum	.	.	.	.	3	
1	1	varsinensis	2.667355597	0.733090129	3.233956835	4.411404145	5	
1	2	varsinensis	2.571621456	0.743279215	3.5202929	4.73616486	4	
1	3	varsinensis	2.529739758	0.716586442	3.317649326	4.629796393	3	
1	4	varsinensis	.	.	.	.	4	
1	5	varsinensis	.	.	.	.	4	
1	1	Yaku	2.50906789	0.511284758	2.06227191	4.033509464	2	
1	2	Yaku	2.627350943	0.592991061	2.078482709	3.505082699	2	
1	3	Yaku	2.567650651	0.599005629	2.251027049	3.757939728	3	
1	4	Yaku	.	.	.	.	2	
1	5	Yaku	.	.	.	.	2	
1	1	Yoshino	2.398579028	0.716771132	2.607303407	3.637567545	4	
1	2	Yoshino	2.368450954	0.733414841	2.877079268	3.922853897	4	
1	3	Yoshino	2.520539433	0.697681291	2.735197507	3.920411145	5	
1	4	Yoshino	.	.	.	.	4	
1	5	Yoshino	.	.	.	.	3	
2	1	Araucariodes	2.519820647	0.972852359	3.43733767	3.533257268	3	
2	2	Araucariodes	2.554092011	0.627533939	3.797235772	6.051044469	3	
2	3	Araucariodes	2.590422295	0.660697069	3.7823439	5.724777778	2	

2	4	Araucariodes	.	.	.	.	2
2	5	Araucariodes	.	.	.	.	2
2	1	BarabitsGold	2.917733148	0.502771627	3.264338753	6.492686898	3
2	2	BarabitsGold	2.932099619	0.544315404	3.533363986	6.49139076	2
2	3	BarabitsGold	2.938543228	0.585615011	3.610459166	6.165243541	3
2	4	BarabitsGold	.	.	.	.	4
2	5	BarabitsGold	.	.	.	.	2
2	1	BenFranklin	2.96622563	0.63488105	3.950873576	6.223013865	3
2	2	BenFranklin	2.878820546	0.57618561	3.589167946	6.229187059	2
2	3	BenFranklin	3.095588381	0.614087262	3.678081846	5.989510079	4
2	4	BenFranklin	.	.	.	.	3
2	5	BenFranklin	.	.	.	.	4
2	1	BlackDragon	2.555429631	0.745994435	4.17336996	5.594371434	2
2	2	BlackDragon	2.495467332	0.825998934	4.781432208	5.788666321	3
2	3	BlackDragon	2.559032095	0.997584115	5.596973133	5.61052752	2
2	4	BlackDragon	.	.	.	.	2
2	5	BlackDragon	.	.	.	.	3
2	1	Cox	2.979643015	0.478607669	2.766966251	5.781282731	4
2	2	Cox	3.000747555	0.509190371	2.907976618	5.710981167	3
2	3	Cox	3.140176812	0.513028395	2.831114915	5.51843708	4
2	4	Cox	.	.	.	.	3
2	5	Cox	.	.	.	.	4
2	1	Cristata	1.675925775	0.792075909	4.298677354	5.427102766	3
2	2	Cristata	1.736718724	0.779563556	4.163656051	5.341009104	5
2	3	Cristata	1.773872085	0.819794134	4.468011999	5.450163416	3
2	4	Cristata	.	.	.	.	2
2	5	Cristata	.	.	.	.	3
2	1	Gyokruga	2.61196647	0.789013048	4.408039507	5.58677644	4
2	2	Gyokruga	2.572291056	0.77497381	4.380517241	5.652471327	4
2	3	Gyokruga	2.522112839	1.161847851	6.815186548	5.865816718	5
2	4	Gyokruga	.	.	.	.	4
2	5	Gyokruga	.	.	.	.	3
2	1	Radicans	2.78151959	0.623705381	4.058724006	6.507437851	3
2	2	Radicans	2.824007939	0.593755955	3.811639298	6.419538645	4
2	3	Radicans	2.896628115	0.602865135	3.814616787	6.327479501	4
2	4	Radicans	.	.	.	.	5
2	5	Radicans	.	.	.	.	4
2	1	Rasen	2.533336378	0.674417927	3.952064196	5.859963145	4
2	2	Rasen	2.536331485	0.69362165	4.057708542	5.850031558	3
2	3	Rasen	2.554341821	0.748093694	4.378732569	5.853187379	3
2	4	Rasen	.	.	.	.	5
2	5	Rasen	.	.	.	.	3
2	1	Sekkan	2.789894244	0.597344118	3.491710776	5.84539241	3
2	2	Sekkan	2.765919998	0.597684984	3.445436259	5.764635806	2
2	3	Sekkan	2.729538159	0.643739203	3.748186359	5.822523069	3
2	4	Sekkan	.	.	.	.	3

2	5	Sekkan	.	.	.	2		
2	1	Tansu	2.309174624	0.617576997	3.85341834	6.239575562	3	
2	2	Tansu	2.316086517	0.604447728	3.675398279	6.080589122	4	
2	3	Tansu	2.409258768	0.621915903	3.824303075	6.149228627	4	
2	4	Tansu	.	.	.	4		
2	5	Tansu	.	.	.	3		
2	1	TarBlue	2.663713343	0.719058583	4.387733264	6.102052555	4	
2	2	TarBlue	2.524292075	0.785661971	5.011107414	6.378197749	4	
2	3	TarBlue	2.668099076	0.797486895	4.911833209	6.159139717	5	
2	4	TarBlue	.	.	.	4		
2	5	TarBlue	.	.	.	3		
2	1	TarPlum	2.695165211	0.824203815	5.395665038	6.546517913	3	
2	2	TarPlum	2.739654254	0.877442775	5.651042581	6.440354562	4	
2	3	TarPlum	2.819983118	0.820557973	5.213719391	6.35387086	4	
2	4	TarPlum	.	.	.	4		
2	5	TarPlum	.	.	.	3		
2	1	varsinensis	2.665280538	0.650009788	4.25901091	6.552225814	4	
2	2	varsinensis	2.715339954	0.712276232	4.260418667	5.981413492	3	
2	3	varsinensis	2.732107578	0.702096808	4.358195808	6.207400118	3	
2	4	varsinensis	.	.	.	5		
2	5	varsinensis	.	.	.	4		
2	1	Yaku	2.576232337	0.412239513	2.465786193	5.981440683	2	
2	2	Yaku	2.602562326	0.440316065	2.53636737	5.760333481	2	
2	3	Yaku	2.630456224	0.438535215	2.567906893	5.8556458	3	
2	4	Yaku	.	.	.	3		
2	5	Yaku	.	.	.	3		
2	1	Yoshino	2.700609972	0.593334654	3.636107038	6.128256644	4	
2	2	Yoshino	2.675505516	0.602001897	3.626578421	6.024197664	3	
2	3	Yoshino	2.674043802	0.652359154	4.08903553	6.26807412	3	
2	4	Yoshino	.	.	.	4		
2	5	Yoshino	.	.	.	3		
3	1	Araucariodes	2.264049934	0.491144606	1.556921585	3.169986125	2	
3	2	Araucariodes	2.313012075	0.463415886	1.516608329	3.272672292	1	
3	3	Araucariodes	2.266171634	0.477347918	1.498897651	3.140052767	1	
3	4	Araucariodes	.	.	.	1		
3	5	Araucariodes	.	.	.	1		
3	1	BarabitsGold	2.765807963	0.588469013	1.996341977	3.392433473	2	
3	2	BarabitsGold	2.590893441	0.565217751	1.955847251	3.460342937	2	
3	3	BarabitsGold	2.620226681	0.602140381	1.988310309	3.302071029	1	
3	4	BarabitsGold	.	.	.	2		
3	5	BarabitsGold	.	.	.	2		
3	1	BenFranklin	2.63734871	0.416796563	1.14833843	2.75515331	2	
3	2	BenFranklin	2.500815839	0.408572267	1.182802688	2.894965673	1	
3	3	BenFranklin	2.802252632	0.423048135	1.035482701	2.447671115	1	
3	4	BenFranklin	.	.	.	2		

3	5	BenFranklin	.	.	.	.	1	
3	1	BlackDragon	2.301770693	0.608217231	2.864263116	4.709276507	2	
3	2	BlackDragon	2.328031212	0.519821376	2.54852732	4.902698193	3	
3	3	BlackDragon	2.327564186	0.572246871	2.721280845	4.755431584	2	
3	4	BlackDragon	.	.	.	.	1	
3	5	BlackDragon	.	.	.	.	2	
3	1	Cox	3.063050012	0.49064193	1.642412213	3.347476258	3	
3	2	Cox	2.969029572	0.464122537	1.569152828	3.380902032	3	
3	3	Cox	3.080730867	0.5033328	1.713103211	3.403519918	4	
3	4	Cox	.	.	.	.	4	
3	5	Cox	.	.	.	.	3	
3	1	Cristata	1.618269786	1.013808666	3.499517002	3.451851539	2	
3	2	Cristata	1.613763047	0.962640776	3.366402129	3.497049171	2	
3	3	Cristata	1.648482662	0.948100713	3.305323757	3.486258066	1	
3	4	Cristata	.	.	.	.	3	
3	5	Cristata	.	.	.	.	2	
3	1	Gyokruga	2.720892941	0.804006938	3.459062176	4.302279013	4	
3	2	Gyokruga	2.663661955	0.796632358	3.522608704	4.421874994	4	
3	3	Gyokruga	2.729999231	0.881114501	3.753860868	4.260355337	3	
3	4	Gyokruga	.	.	.	.	3	
3	5	Gyokruga	.	.	.	.	4	
3	1	Radicans	2.83248645	0.739301996	2.879511146	3.894905144	4	
3	2	Radicans	2.31165865	0.619220617	2.360176043	3.811526904	5	
3	3	Radicans	2.634122092	0.764393912	2.72237794	3.561485641	5	
3	4	Radicans	.	.	.	.	5	
3	5	Radicans	.	.	.	.	4	
3	1	Rasen	2.650417909	0.579093934	2.101295608	3.628591988	3	
3	2	Rasen	2.698518861	0.615462838	2.30677573	3.748034141	3	
3	3	Rasen	2.736888228	0.574797155	2.07288458	3.606288866	4	
3	4	Rasen	.	.	.	.	3	
3	5	Rasen	.	.	.	.	3	
3	1	Sekkan	2.813934288	0.529625263	1.870616059	3.531961541	2	
3	2	Sekkan	2.520258706	0.494723118	1.905131327	3.850904188	2	
3	3	Sekkan	2.930077012	0.565489486	2.062983058	3.648136895	1	
3	4	Sekkan	.	.	.	.	2	
3	5	Sekkan	.	.	.	.	3	
3	1	Tansu	2.387797834	0.542162776	2.048121275	3.777687011	3	
3	2	Tansu	2.394996367	0.52424019	1.889571446	3.604400201	5	
3	3	Tansu	2.419816886	0.574972543	2.230376411	3.879100727	3	
3	4	Tansu	.	.	.	.	3	
3	5	Tansu	.	.	.	.	3	
3	1	TarBlue	3.045753813	0.68503262	2.288383413	3.340546635	2	
3	2	TarBlue	2.962255466	0.706975694	2.487077896	3.517911461	2	
3	3	TarBlue	3.049712484	0.668766138	2.318297495	3.466529423	3	
3	4	TarBlue	.	.	.	.	2	
3	5	TarBlue	.	.	.	.	2	

3	1	TarPlum	3.204033638	0.720053138	2.651695857	3.682639124	3
3	2	TarPlum	3.052795854	0.54441658	2.012521615	3.696657465	3
3	3	TarPlum	3.267337042	0.548998309	1.937783918	3.529671927	3
3	4	TarPlum	.	.	.	.	2
3	5	TarPlum	.	.	.	.	3
3	1	varsinensis	2.897009551	0.810360755	3.613128602	4.458666808	3
3	2	varsinensis	2.881328568	0.738327901	3.325953662	4.504710787	4
3	3	varsinensis	2.853456991	0.737857759	3.218484196	4.361930411	4
3	4	varsinensis	.	.	.	.	4
3	5	varsinensis	.	.	.	.	4
3	1	Yaku	2.908510588	0.418509327	1.423562828	3.401508008	3
3	2	Yaku	2.891658071	0.373719283	1.314240766	3.516652271	3
3	3	Yaku	2.865217873	0.443128469	1.574243238	3.552566237	4
3	4	Yaku	.	.	.	.	3
3	5	Yaku	.	.	.	.	3
3	1	Yoshino	2.577826827	0.553518437	1.961307359	3.543346034	4
3	2	Yoshino	2.550425888	0.491827787	1.76429828	3.587227737	4
3	3	Yoshino	2.521997266	0.515682954	1.843682921	3.575225646	2
3	4	Yoshino	.	.	.	.	3
3	5	Yoshino	.	.	.	.	2
4	1	Araucariodes	2.63195025	1.001714432	6.519875795	6.508717044	2
4	2	Araucariodes	2.566116019	0.931539918	6.206953556	6.663110654	3
4	3	Araucariodes	2.546401727	0.833529118	5.70064018	6.839161416	2
4	4	Araucariodes	.	.	.	.	2
4	5	Araucariodes	.	.	.	.	2
4	1	BarabitsGold	2.916620766	0.509523375	3.288189662	6.453461853	3
4	2	BarabitsGold	2.916744091	0.519621821	3.320797954	6.390797727	3
4	3	BarabitsGold	3.134111506	0.511053152	3.344019805	6.543389451	3
4	4	BarabitsGold	.	.	.	.	4
4	5	BarabitsGold	.	.	.	.	4
4	1	BenFranklin	2.965093045	0.636392413	4.152277024	6.5247117	3
4	2	BenFranklin	2.944418769	0.529341687	3.414181535	6.449863326	3
4	3	BenFranklin	2.847978741	0.546234747	3.534736645	6.47109446	4
4	4	BenFranklin	.	.	.	.	4
4	5	BenFranklin	.	.	.	.	3
4	1	BlackDragon	2.487806055	0.783927121	5.098917865	6.504326395	2
4	2	BlackDragon	2.464880527	0.768601624	4.730769231	6.155034134	3
4	3	BlackDragon	2.427200584	0.768189831	4.920063112	6.404749079	3
4	4	BlackDragon	.	.	.	.	3
4	5	BlackDragon	.	.	.	.	2
4	1	Cox	2.888193822	0.669470303	4.52399656	6.757576157	3
4	2	Cox	2.859777674	0.614920507	4.065557298	6.611516856	4
4	3	Cox	2.799293923	0.634667282	4.255029628	6.704346901	5
4	4	Cox	.	.	.	.	4
4	5	Cox	.	.	.	.	5

4	1	Cristata	2.044687747	1.075407457	6.690965381	6.221795597	3
4	2	Cristata	2.058551189	0.956296428	5.979588451	6.252860801	5
4	3	Cristata	2.044339883	0.993443092	6.284451809	6.32593035	3
4	4	Cristata	.	.	.	.	4
4	5	Cristata	.	.	.	.	4
4	1	Gyokruga	2.68500847	0.874060406	5.564970608	6.366803218	5
4	2	Gyokruga	2.666783837	0.834062504	5.205841426	6.241548326	5
4	3	Gyokruga	2.653420317	0.818820684	5.285494442	6.455008461	5
4	4	Gyokruga	.	.	.	.	5
4	5	Gyokruga	.	.	.	.	5
4	1	Radicans	2.510294338	0.698475991	4.707561623	6.739761541	5
4	2	Radicans	2.605760421	0.777603393	4.882066365	6.278350133	5
4	3	Radicans	2.614782309	0.749542804	4.718710908	6.295452219	5
4	4	Radicans	.	.	.	.	4
4	5	Radicans	.	.	.	.	5
4	1	Rasen	2.585970542	0.680951224	4.30961497	6.32881595	3
4	2	Rasen	2.5279149	0.610230977	3.981638418	6.524805474	3
4	3	Rasen	2.47905965	0.647918119	4.126662168	6.369110615	4
4	4	Rasen	.	.	.	.	4
4	5	Rasen	.	.	.	.	3
4	1	Sekkan	2.843114433	0.761255786	4.7248216	6.206615028	3
4	2	Sekkan	2.82735119	0.739987037	4.629733134	6.256505725	3
4	3	Sekkan	2.863438901	0.698218645	4.238815047	6.07089925	2
4	4	Sekkan	.	.	.	.	3
4	5	Sekkan	.	.	.	.	3
4	1	Tansu	2.243137701	0.803743834	5.759899403	7.16633728	5
4	2	Tansu	2.257439783	0.848910236	5.945434274	7.003607713	5
4	3	Tansu	2.238146484	0.858374801	6.129494798	7.140814	4
4	4	Tansu	.	.	.	.	4
4	5	Tansu	.	.	.	.	4
4	1	TarBlue	2.74033017	0.700562635	4.658789542	6.650068544	5
4	2	TarBlue	2.68916625	0.66783664	4.45907139	6.676889416	4
4	3	TarBlue	2.654870322	0.683686683	4.404264871	6.441934558	4
4	4	TarBlue	.	.	.	.	5
4	5	TarBlue	.	.	.	.	4
4	1	TarPlum	2.986172223	0.5878431	3.598521031	6.121567189	4
4	2	TarPlum	2.938165235	0.562352288	3.364076407	5.982151186	4
4	3	TarPlum	2.954831541	0.498268439	3.072725366	6.16680714	4
4	4	TarPlum	.	.	.	.	4
4	5	TarPlum	.	.	.	.	4
4	1	varsinensis	2.71362134	0.569388378	3.712153915	6.519546343	3
4	2	varsinensis	2.675932737	0.533197419	3.567676338	6.691098292	3
4	3	varsinensis	2.686433423	0.515192957	3.265474646	6.338352659	5
4	4	varsinensis	.	.	.	.	4
4	5	varsinensis	.	.	.	.	4
4	1	Yaku	2.61012892	0.615025408	4.281923455	6.962189529	3

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4	2	Yaku	2.620435384	0.651248019	4.341750118	6.666815085	3
4	3	Yaku	2.587427895	0.700347161	4.481335314	6.398734176	3
4	4	Yaku	.	.	.	.	3
4	5	Yaku	.	.	.	.	4
4	1	Yoshino	2.645786991	0.816498629	5.35419232	6.557503137	4
4	2	Yoshino	2.634122092	0.803919933	5.240059002	6.518135437	4
4	3	Yoshino	2.60549311	0.820250948	5.23727983	6.384972603	4
4	4	Yoshino	.	.	.	.	5
4	5	Yoshino	.	.	.	.	4

;

proc glm;
class rep taxa season chlAB carot chl chl_carot rating;
model chlAB=rep season taxa season*taxa;
contrast 'winter 07 vs. winter 08' season 1 0 -1 0;
contrast 'summer 08 vs. summer 09' season 0 1 0 -1;
contrast 'winter 07 vs. summer 08' season 1 -1 0 0;
contrast 'winter 07 vs. summer 09' season 1 0 0 -1;
contrast 'winter 08 vs. summer 08' season 0 1 -1 0;
contrast 'winter 08 vs. summer 09' season 0 0 1 -1;
run;

proc glm;
class rep taxa season chlAB carot chl chl_carot rating;
model carot=rep season taxa season*taxa;
contrast 'winter 07 vs. winter 08' season 1 0 -1 0;
contrast 'summer 08 vs. summer 09' season 0 1 0 -1;
contrast 'winter 07 vs. summer 08' season 1 -1 0 0;
contrast 'winter 07 vs. summer 09' season 1 0 0 -1;
contrast 'winter 08 vs. summer 08' season 0 1 -1 0;
contrast 'winter 08 vs. summer 09' season 0 0 1 -1;

proc glm;
class rep taxa season chlAB carot chl chl_carot rating;
model chl=rep season taxa season*taxa;
contrast 'winter 07 vs. winter 08' season 1 0 -1 0;
contrast 'summer 08 vs. summer 09' season 0 1 0 -1;
contrast 'winter 07 vs. summer 08' season 1 -1 0 0;
contrast 'winter 07 vs. summer 09' season 1 0 0 -1;
contrast 'winter 08 vs. summer 08' season 0 1 -1 0;
contrast 'winter 08 vs. summer 09' season 0 0 1 -1;
run;

proc glm;
class rep taxa season chlAB carot chl chl_carot rating;
```

```
model chl_carot=rep season taxa season*taxa;
contrast 'winter 07 vs. winter 08' season 1 0 -1 0;
contrast 'summer 08 vs. summer 09' season 0 1 0 -1;
contrast 'winter 07 vs. summer 08' season 1 -1 0 0;
contrast 'winter 07 vs. summer 09' season 1 0 0 -1;
contrast 'winter 08 vs. summer 08' season 0 1 -1 0;
contrast 'winter 08 vs. summer 09' season 0 0 1 -1;

proc glm;
class rep taxa season chlAB carot chl chl_carot rating;
model rating=rep season taxa season*taxa;
contrast 'winter 07 vs. winter 08' season 1 0 -1 0;
contrast 'summer 08 vs. summer 09' season 0 1 0 -1;
contrast 'winter 07 vs. summer 08' season 1 -1 0 0;
contrast 'winter 07 vs. summer 09' season 1 0 0 -1;
contrast 'winter 08 vs. summer 08' season 0 1 -1 0;
contrast 'winter 08 vs. summer 09' season 0 0 1 -1;

proc sort;
by season;
proc glm;
by season;
class rep taxa;
model chlAB=rep taxa;
means taxa/tukey lines;

proc sort;
by season;
proc glm;
by season;
class rep taxa;
model chl=rep taxa;
means taxa/tukey lines;

proc sort;
by season;
proc glm;
by season;
class rep taxa;
model carot=rep taxa;
means taxa/tukey lines;

proc glm;
by season;
class rep taxa;
model chl_carot=rep taxa;
```

```
means taxa/tukey lines;

proc sort;
by season;
proc glm;
by season;
class rep taxa;
model rating=rep taxa;
means taxa/tukey lines;

proc sort by season;
proc corr;
by season;
var chlAB chl carot chl_carot rating;
run;

proc corr;
var chlAB chl carot chl_carot rating;
run;
```