TOOLS FOR ORNAMENTAL BREEDING: INTRASPECIFIC CROSSING, TRIPLOID DEVELOPMENT, INSECT RESISTANCE EVALUATION, INHERITANCE TESTING, SOMATIC EMBRYOGENESIS, PLANT GROWTH REGULATORS, AND PHYSICAL MUTAGENESIS WITH *HIBISCUS* SPP., *LIQUIDAMBAR FORMOSANA* 'FORMOSAN GOLD', *HELIANTHUS SIMULANS,* AND *ILLICIUM PARVIFLORUM*

by

KAITLIN BARRIOS

(Under the Direction of John M. Ruter)

ABSTRACT

Hardy hibiscus (*Hibiscus moscheutos*) was the main species worked with to develop elite selections based on foliage color, flower color and pest resistance through traditional breeding, ploidy manipulation, and inheritance testing. Induction of polyploidy was tested by soaking 1,397 seedlings in an oryzalin solution over several dates during Spring and Summer 2017. Approximately 6.6% of treated diploid seedlings were converted to tetraploids and were crossed with select genotypes of their natural diploid form. Approximately 5,300 putative triploid seed were recovered from crosses, and from the 2,004 seed sown 2018 and 2019 there was a total of 433 seedlings identified as triploid (~22%). The remaining approx. 2,300 seed were not sown due to constraints of resources and time, and the priority of seed that was sown was based on likelihood of triploid status. Two trial plots were planted with replicates of 25 *Hibiscus* spp. and *H. moscheutos* hybrid genotypes at the University of Georgia's research sites in Blairsville and Watkinsville, GA in 2017. These plants were evaluated during the growing seasons of 2017 and

2018 for feeding damage from the hibiscus sawfly (*Atomacera decepta*), which can devour susceptible genotypes if populations are left unchecked. Genotypes having a greater amount of pubescence on foliage had less feeding damage than those with glabrous leaves. The inheritance of several phenotypic traits (e.g., foliage color, foliage pubescence, flower color) of intraspecific *H. moscheutos* subsp. *moscheutos* hybrids and interspecific hybrids of *H. moscheutos* subsp. *moscheutos* with H. *moscheutos* subsp. *lasiocarpos* and *H. grandiflorus* were evaluated. Results indicate a red foliage phenotype is controlled by a single locus with a dominant allele for red foliage to green foliage among and within the two subspecies of *H. moscheutos*. Outside of the work focusing on *Hibiscus* spp., the tissue culture propagation method of somatic embryogenesis was evaluated using dormant buds and immature fruit of a select genotype of *Liquidambar formosana* but was not successful. This selection was released as the cultivar 'Formosan Gold' from the ornamental breeding program in 2018. The efficacy of two plant growth regulators (PGRs) at different rates via treatment of the native swamp sunflower (*Helianthus simulans*) was investigated for reducing overall plant size for greenhouse production. The first experiment was initiated 25 June and the second 7 Sept. 2018 and results from these experiments suggest a substrate drench application of paclobutrazol at 6.0 or flurprimidol at 4.0 mg a.i./pot can be used to produce smaller plants compared to non-treated plants, which are ideal for the ornamental market. Lastly, the visually-uniform shrub *Illicium parviflorum* was subjected to gamma irradiation to induce mutations. Cuttings at three different stages of growth (i.e., soft-, semi-hard-, and hard-wood) over two years were irradiated and evaluated as rooted cuttings. Two irradiated clones have demonstrated a slight degree of distinct morphology.

INDEX WORDS: traditional breeding, polyploidy induction, gamma irradiation, oryzalin, leaf color, leaf pubescence, stem and petiole color, flower color, flow cytometry, paclobutrazol, flurprimidol, dormant buds, immature fruit, Cobalt-60

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Introduction and Literature Review

***Hibiscus* spp.** The *Hibiscus* species utilized for the breeding and hibiscus sawfly (*Atomacera decepta*) resistance studies in this dissertation were *H. moscheutos* subspecies *moscheutos*, *H. moscheutos* subsp. *lasiocarpos*, and *H. grandiflorus*. The aforementioned species were chosen for these projects based on their ranges of natural distribution, vigor, native status, and morphological traits. Common rosemallow (*Hibiscus moscheutos* L.) is a perennial shrub native to wetland areas of North America where it blooms throughout the summer bearing showy flowers in shades of pink to white. Because of its prolific blooming, it has been used in ornamental cultivation in the U.S. since the early 19th century when the American horticulturalist John Bartram listed it in his 1807 catalog. *Hibiscus moscheutos* has been used in many interspecific crosses with several breeders, professional nurserymen, and home gardeners having developed cultivars starting in the early 1900's (Winters, 1970). Various breeders in the first half of the 1900's conducted crosses and made selections around the country in states such as Pennsylvania, Maryland (MD), New Jersey (NJ), California, and Georgia (GA), and are identified by Winters (1970) in his discussion on hybridization of ornamental hardy hibiscus species. Parents utilized were reported as *H. moscheutos*, *H. coccineus* and *H. militaris* (Winters, 1970). In 1952, breeders in Florida (FL) began to cross *H. moscheutos* with other natives from their region such as *H. grandiflorus* and (what they identified as) *H. incanus* to breed a version of rose mallow better adapted to FL (McFadden, 1955, 1959). Their 20 advanced selections, named "gator hybrids," were chosen for their large, midsummer flowers and variability in height and form (McFadden, 1959). Much of the appeal of breeding with *Hibiscus moscheutos* and

closely related *Hibiscus* spp. is likely the vast amount of variability that appears in the traits of the plants, e.g. foliage shape, color, and texture; flower color, size and openness; and the overall form of the plant. This can be gleaned from sources describing in detail the hybrids they observed, such as from A.B. Stout (1917) at the New York Botanical Garden, C.S. Kennedy (1960) in Ohio and many more mentioned by H.F. Winters (1970). *Hibiscus moscheutos* continues to be used as a parent today for the same reasons it was used over past decades in the U.S. and abroad.

The genus *Hibiscus* is a member of the Malvaceae family and consists of over two hundred species of mostly small annuals or medium to large perennial shrubs from different parts of the world, mainly tropical or sub-tropical regions (Godfrey and Wooten, 1981; Flora of China, 2007; Wise and Menzel, 1971). Several species of *Hibiscus* are native to North America and comprise the section Muenchhusia (Heister ex Fabricius) O. J. Blanchard. The five species in this section are *H. moscheutos*, *H. coccineus*, *H. grandiflorus*, *H. laevis*, and *H. dasycalyx* (Blanchard, 2008; Small, 2004). Molecular comparisons using nuclear and chloroplast DNA relatively recently confirmed this group, commonly known as the Rose mallows, as monophyletic within *Hibiscus* (Small, 2004). In addition to the species in sect. Muenchhusia sharing a basic number of chromosomes (n = 19), they are often found in similar environments (wetlands or rivers' edge), are herbaceous perennials, and have similar native ranges in North America (Small, 2004; Wise and Menzel, 1971). Section Muenchhusia was initially defined by O. J. Blanchard (1976) and his taxonomic descriptions separated the five species from sect. Trionum. Contemporaries of Blanchard, Wise and Menzel (1971), similarly grouped four of the five same *Hibiscus* species together based on results from making numerous inter- and intra-specific crosses. Wise and Menzel (1971) observed two sub groups within the four species they

studied that did not successfully cross. This observation was later supported by Small (2004) with nuclear genomic information. Small (2004) incorporated the fifth Rose mallow species and determined the two clades, partly on similar nuclear genes, as: 1) *Hibiscus grandiflorus* and *H. moscheutos* and 2) *H. coccineus*, *H. dasycalyx*, and *H. laevis*. Kuligowska et al. (2016) conducted interspecific crosses between cultivars of *H. moscheutos*, *H. coccineus*, and *H. laevis* and their findings partly supported those of Small (2004) and Wise and Menzel (1971). The genus *Hibiscus* has and will continue to incur much fluctuation and reassignment of species, and the Rose mallow group has been no exception (Flora of China, 2007; Pfeil and Crisp, 2005; Pfeil et al., 2002; Skovsted, 1935). Although they are native to North America, taxonomists have had difficulty defining the species partly because of the overlap in their ranges, because some species easily hybridize, and possibly due to early gardeners and plant enthusiasts cultivating plants from the wild and hybridizing them for decades (Winters, 1970; Wise and Menzel, 1971).

Despite said complications, the current taxonomy of *Hibiscus moscheutos* divides the species into two subspecies: *moscheutos* and *lasiocarpos* (Cavanilles) O. J. Blanchard (Blanchard, 1976, 2008; FNA, 2019a). Both subspecies are diploid (2n = 38) and have been introduced into other U.S. states as well as parts of Europe and Asia (FNA, 2019a; Skovsted, 1935; Wise and Menzel, 1971). In past years, some taxonomists similarly described the subspecies *moscheutos*, however there were varying names (subsp. *lasiocarpos*, *palustris*, and *incanus*) and descriptions of a second subspecies of *H. moscheutos* (Bates, 1965; Clausen, 1949; Godfrey and Wooten, 1981). The distinctions of the current classifications are based on the presence (subsp. *lasiocarpos*) or absence (subsp. *moscheutos*) of hairs on the adaxial leaf surface, capsules (fruits), and bracts of the involucel (epicalyx), as well as their geographic ranges (FNA, 2019a). Subspecies *moscheutos* is found in the wild from Ontario to New Hampshire, south to

FL and west to Texas (TX). *Hibiscus moscheutos* subsp. *lasiocarpos* exists naturally from Indiana, south to Alabama (AL), and west to TX, including the mid-western states of Kansas and Oklahoma with disjunct populations in FL, New Mexico, and northern Mexico (Chihuahua) (Blanchard, 2008; FNA, 2019a). Although the subspecies' ranges overlap, the Mississippi River serves as a general border with subsp. *moscheutos* found mostly to its east and subsp. *lasiocarpos* to its west (FNA, 2019a). Previous taxonomists attempted to separate the subspecies into northern and southern groups (Bates, 1965; Winters, 1970), rather than the current west-east ranges. Given these native ranges, *H. moscheutos* is hardy from the United States Department of Agriculture (USDA) zones 4a to 9b, hence its other common name of hardy hibiscus (Winters, 1970). Plants typically sprout stems from underground storage structures during March in GA with flowers first appearing in May, peak bloom occurring late June/early July, and blooming sporadically into August and September. *Hibiscus moscheutos* is classified as a long-day plant, i.e. flowers emerge when the plant is exposed to ≥ 12 hr of light, as supported by studies using two *H. moscheutos* cultivars (Runkle et al., 1998; Warner and Erwin, 2001). Flowers are described as either white, pink or of shades in between, and with or without a red spot at the flower base (eye) (FNA, 2019a). The inflorescence is a perfect, solitary flower on a long pedicel (2-15 cm) held in the leaf axil (Flora of China, 2007; FNA, 2019a; Giles et al., 1980). The flowers can be quite large, some reaching 30 cm (12 in) in diameter, and typically with five petals each 7-10 cm long. The corolla shape can range from funnel form to opening flat, which some liken to 'dinner plates' (Clausen, 2014; Clausen and Ekstrom, 1989; FNA, 2019a; Godfrey and Wooten, 1981; Hawke, 1993). As a member of the genus *Hibiscus*, the flowers typically last one day and have a staminal column, petals which are fused basally to the staminal column, an involucel of bractlets (or epicalyx), and ovaries with five carpels. The staminal column is white

or cream in color and approx. half the length of the corolla with typically creamy white to yellow stigmas. The bractlets of the involucel typically number between 10 and 14, are separate, and are linear or lanceolate in shape. The calyx measures 1.5-4 cm in length and is usually campanulate shaped with five triangular to triangular-ovate lobes having acute apices with pubescent surfaces. The fruits have five locules, each of which can develop many seed (FNA, 2019a; Giles et al., 1980; Godfrey and Wooten, 1981; Pfeil et al., 2002). *Hibiscus moscheutos* populations studied in wetlands of MD over a three-year period had full seed set when a flower was pollinated with approx. 360 pollen grains (Spira et al., 1992). The researchers explained this estimation by reporting that a typical ovary of *H. moscheutos* has 139 ovules and 2.6 pollen grains were required to obtain a seed (Spira et al., 1992). Plants set fruit as dehiscent capsules into late summer and fall, senesce in the fall, and remain dormant during the winter until sprouting new, herbaceous growth from the ground in the spring. Leaf morphology varies much within the genus and this can also be seen with *H. moscheutos* which can vary in shape (broadly lanceolate to triangular-ovate), base (cuneate to cordate), lobing (three-or five-lobed or unlobed), and margins (crenate to serrate). Leaves typically measure 8-20 cm long and 3-13 cm wide (FNA, 2019a; Giles et al., 1980; Godfrey and Wooten, 1981). As stated earlier, subsp. *lasiocarpos* typically has leaves with a pubescent upper surface that give them a silver-gray appearance, which explains one of its common names of woolly rose mallow. Subsp. *moscheutos* is known to have glabrous upper leaf surfaces. Leaves of subsp. *lasiocarpos* are also described as typically unlobed (Blanchard, 2008; FNA, 2019a). Plants can measure 0.9 - 2.4 m (3-8 ft) tall and their form is generally upright to a rounded shrub with a few to several stalks. As a wetland native, though, its shape can appear asymmetric, leggy or floppy when planted alone. Another name used sometimes for *H. moscheutos* is swamp rose-mallow since it can be found growing

naturally in wetland settings and is sometimes used in wetland restoration projects in the eastern U.S. (Liu and Spira, 2001). The stems are quite fibrous and a study in a brackish marsh near the Chesapeake Bay found stems take approx. seven to eight years to decompose, which is five times slower than typical brackish marsh species (Cahoon and Stevenson, 1986). The roots of common rose mallow can be thick and abundant, contributing to its vigor and adaptability as seen in a natural brackish marsh population that reportedly had a root:shoot ratio of 2.3, which is comparable to fresh water marsh plants but low for high-salinity marshes (Cahoon and Stevenson, 1986). Swamp rose-mallow can tolerate many growing conditions, performing well in non-wetland landscapes and best in full sun (FNA, 2019a; Godfrey and Wooten, 1981; Hawke, 1993). Some diseases have been occasionally noted on *Hibiscus* such as, stem and root rot, fungal leaf spot, rust and viruses (Clausen and Christopher, 2014; Hawke, 1993; NC State Extension, 2019). Plants can be propagated vegetatively from cuttings during the growing season with a high rooting percentage from softwood cuttings taken ~ May through July, or by division of the crown (Clausen and Christopher, 2014; Epping, 1993; Giles, 1980; Kennedy, 1966; Winters, 1970). Sexual propagation can occur from seed which typically require scarification due to their hard coat to overcome physical dormancy before imbibition can occur (Clausen and Christopher, 2014; Liu and Spira, 2001). A very small amount of seed will germinate readily in the field (personal observation) and Giles et al. (1980) claimed plants freely self-sow, however Hawke (1993) reported no seed were observed to germinate during a two-year study. Plants from seed which germinate in spring typically produce flowers within the first year (Giles et al., 1980; Winters, 1970).

Common rose mallow is beneficial to various wildlife species; attracting hummingbirds and over 30 species of moths and butterflies, which use this taxa as a host plant (Clausen and

Christopher, 2014; NC State Extension, 2019; NWF, 2015). The main pollinator of common rose mallow is a species of bee, *Ptilithrix bombiformis*, whose range coincides with the native range of *Hibiscus* and its active pollen-foraging period is timed with the flowering period of *Hibiscus* (Rust, 1980; Spira et al., 1992). Rust (1980) observed this bee population over a four-year period in salt marshes of Delaware and noticed rose mallow was the only taxa it visited despite there being other flora in bloom. Interestingly, this bee is described as being able to walk on water (Rust, 1980). In addition to *Ptilithrix bombiformis*, other bee species (*Bombus* spp.) were noticed by Spira et al. (1992) to visit the flowers of *H. moscheutos* over a three-year study of populations in wetlands of MD. Spira et al. (1992) reported the frequency of visits to individual flowers by these bee species was two to four every 15 mins, and a later study (Spira et al., 1996) of plants in the same area saw a visit every 15 mins in more than 50% of their observations. When a pollinator contacted the stigma of the flower, a median of 70 pollen grains were deposited (with a wide range in number of pollen grains) (Spira et al., 1992). Other insects are attracted to common rose mallow as a food source. Reports of pests include prevalence of Japanese beetles and occasionally whiteflies, hibiscus sawfly (*Atomacera decepta*), aphids and scale (Clausen, 2014; Clausen and Christopher, 2014; NC State Extension, 2019; Russ, 2004; Tippins, 1965). Researchers in the Chesapeake Bay area observing natural stands of *H. moscheutos* reported herbivore feeding mainly from hibiscus sawfly (*Atomacera decepta*), a leafroller (*Chionodes hibiscella*) which fed on the foliage and seed, and a species of beetle (*Althaeus hibisci*) which attacked the seed, leading to a 15% loss in biomass (Cahoon and Stevenson, 1986). Spira (1989) reported to observe similar pests in a related location of the Chesapeake Bay a few years later with an additional species of weevil, *Conotrachelus fissunguls*, which also infested developing fruit and seed. Spira (1989) quantified the collective reduction in viable seed due to the beetle

and weevil as ~53% for 1985 and ~89% for 1986. Some 30 insects were identified (at the Family or Genus level) on and in flowers, stems and leaves of nursery and wild populations of *H. moscheutos* in NJ by Weiss and Dickerson (1919). A few birds in different parts of rose mallow's native range use the seed for food, such as Northern bobwhites (*Colinus virginianus*) and some water fowl (Clausen, 2014).

The fertilization of flowers of *H. moscheutos* in natural wetland populations of MD has been studied in detail. Pollen grains of *H. moscheutos* are quite large (150 μm in diameter), as is the flower structure, likely facilitating the amount of pollination and fertilization research that has occurred. *H. moscheutos* in the wild has been described as self-compatible by means of flowers on the same plant being fertilized by each other (via insect pollinator), known as geitonogamy, rather than a single flower pollinating itself (Spira, 1989). Spira (1989) observed a natural stand of common rose mallow in wetlands of MD and attributed the spatial separation between the stigma and anthers, also known as herkogamy, to preventing selfing of an individual flower. Snow and Spira (1991) found that once pollen reached the stigmatic surface, it germinated within approx. 1 h and by 3 hrs the pollen tube had reached the ovary. Given that pollinator bees often deposited six times more pollen than is needed to fertilize all the ovules (Snow and Spira, 1991), there is substantial competition among pollen tube growth to reach the ovary. Snow and Spira (1991) found that genotypes having pollen with a faster pollen tube growth rate had a higher number of successfully created seed, which led the researchers to conclude that fertilization was not random, meaning certain genotypes contributing pollen led to more seed than other genotypes. Additionally, pollen tube growth rate from self-fertilization varied among individuals (some slower, others faster) compared to out-crossing (Snow and Spira, 1991, 1993). The timing of pollen contact (deposited by pollinators) with the stigma

relative to later pollen 'loads' was found to affect the success of seed set in *H. moscheutos* (Spira et al., 1996). Spira et al. (1996) reported that when pollen was deposited 15 mins after a previous pollen deposit, the amount of seed produced from the later pollen was reduced by 13-30%. For pollen arriving 30 mins after a prior load, seed set by the later pollen was reduced by 21-57%. These results demonstrate it is possible for pollen deposited on stigmas after previous pollen (up to approx. 2 hrs) to compete with earlier arriving pollen. A later study (Snow et al., 2000) similarly found that pollen deposited 15 or 30 mins after a previous load typically led to fewer seed than the earlier arriving pollen. However, after examining pollen tube growth rates of the later arriving pollen, Snow et al. (2000) discovered that the advantage of a faster growth rate of some pollen donors was ineffective by arriving 15 or 30 mins after a prior load. The authors concluded that differences in pollen competition in natural populations on the fitness of individuals was more complex than initially expected and potentially difficult to detect or insignificant for *H. moscheutos* (Snow et al., 2000).

Seed longevity was evaluated in a study comparing one, two, three, and five-year-old seed collected from a wild population of *H. moscheutos* in MD. From the evaluations, it was found that the older the seed, the lower the germination percent, the longer the germination period, and the higher the amount of non-viable seed once seed was sown (Liu and Spira, 2001). Inbreeding depression was investigated in natural populations in MD by Snow and Spira (1993) who found variability in the amount of seed set and in seed size among individual maternal plants. Seedling progeny from selfing, however, had reduced biomass at six weeks after germination. Inbreeding depression was also evaluated and found to have a negative influence on percent germination and dry weight of seedlings, somewhat similar to results from Snow and Spira (1993) (Liu and Spira, 2001). The authors speculated that individuals from younger seed

resulting from outcrossing likely dominate a natural population than plants from older, inbred seed (Liu and Spira, 2001).

Much research has been done with natural stands of *Hibiscus moscheutos* regarding its population dynamics and the role of seed dispersal by water (hydrochory) on genetic variability (Kudoh and Whigham, 1997; Kudoh et al., 2006; Shimamura et al., 2007). From studying allozyme polymorphisms within and between 10 isolated populations of *H. moscheutos* in an approx. 1.5 km^2 area of freshwater to brackish estuary in MD, Kudoh and Whigham (1997) discovered the gene flow between populations was higher than expected. It was anticipated that since flowers are pollinated by bees travelling short distances, the gene flow would have been lower, however the effect of hydrochory (dispersal of seed by water) during high tide and/or flooding led to a greater mixing and spatial distribution of the genotypes (Kudoh and Whigham, 1997). A study revisiting these same populations reaffirmed the importance of hydrochory both on the scale of a single year and a multi-year scale (Kudoh and Whigham, 2001). Kudoh and Whigham (2001) found the populations adjacent to the tidal stream had more seed exchange than populations farther from the stream, although the more isolated non-tidal wetland populations did contribute to the floating seed bank but on a less frequent occurrence. Kudoh et al. (2006) reviewed research on the importance of hydrochory in *H. moscheutos* metapopulations studied in wetlands of MD as well as other *Hibiscus* spp. The importance of secondary seed dispersal, which is seed relocation after the primary dispersal by gravity (falling from the dehiscent fruit), was stated by Shimamura et al. (2007) after studying the same natural populations of *H. moscheutos* in MD as Kudoh and Whigham (1997, 2001). Shimamura et al. (2007) found less change genetically over seasons in seed found in non-tidal populations as compared to the seed

bank in tidal areas, which experienced frequent inundation; again, reaffirming the importance of secondary hydrochory on the spatial and genetic distribution of the population.

Hibiscus grandiflorus Michaux

Hibiscus grandiflorus is the species most closely related to *H. moscheutos*, hence it is a member of the Malvaceae family and section Muenchhusia within the *Hibiscus* genus (Small, 2004; Wise and Menzel, 1971). Early descriptions (Bates, 1965; Winters, 1970) list it as native to marshes along the Gulf of Mexico coast of Mississippi (MS), FL and GA, and more recently it is reported to grow naturally in fresh and brackish marshes of southeast North America in peninsular FL, AL, MS, Louisiana (LA) and TX and to some extent north along the Atlantic coast in GA and South Carolina (SC) (Blanchard, 1976; Christman, 2008; FNA, 2019b; USDA NRCS, 2019). There is also some evidence that it may grow in Cuba, or a transient population of it, under the synonym *Hibiscus urbanii* (Blanchard, 1976, 2008). Also called giant rose mallow, velvet mallow, and/or swamp rose mallow, this taxon is considered an obligate wetland species, meaning it almost always occurs in wetlands, however, it can grow in non-wetland cultivated landscapes. *H. grandiflorus* prefers well-drained but consistently moist soil, full sun, and is hardy in USDA zones 7-10 (Christman, 2008; Clausen, 2014; USDA NCRS, 2019). Giant rose mallow is known for its large size (to 3 m, or 10 ft, tall), leaves and flowers (Christman, 2008; Winters, 1970). Leaves are typically wider (10-30 cm) than long (10-18 cm) with an ovate shape, have a cordate to truncate base, are three-lobed (occasionally five-lobed), and have triangular lobes with acute or acuminate apices. Leaf margins have been described as irregularly toothed, crenate or crenate-dentate. Petioles are typically half to ¾ the length of the blade (5-16 cm long) (Bates, 1965; Blanchard, 1976; Clausen and Ekstrom, 1989; Godfrey and Wooten, 1981). As the name velvet mallow denotes, the foliage has an abundance of pubescence on both the adaxial and

abaxial surfaces giving the plant a velvety, silver-green appearance. In addition to the foliage, the involucellar bractlets (epicalyx), calyx, capsule (fruit) and to some extent the young stems have hairy or tomentose surfaces (Blanchard, 1976; FNA, 2019b). The involucellar bractlets number about 10 (9-13), are linear to subulate in shape and measure approx. 13-30 mm long. The calyx is typically campanulate shaped with lobes starting around the middle of its length, has deltoid-shaped apices, and measures 3-6 cm long (Bates, 1965; Blanchard, 1976; Godfrey and Wooten, 1981). The showy inflorescence is a solitary flower held horizontally or angled slightly up. Flowers of giant rose mallow are large, funnel-form, and have light to pale pink petals with a crimson base (‘eye spot’) that are rounded at the tips (Bates, 1965; Blanchard, 1976; FNA, 2019b; Godfrey and Wooten, 1981). Individual petals can measure 12-15 cm long leading to a front width of 25 cm and are considered the largest blooms of any North American *Hibiscus* spp. (Bates, 1965; Clausen and Christopher, 2014; Gettys et al., 2013; Godfrey and Wooten, 1981). The specific epithet is due to the floriferous nature and large flowers of *H. grandiflorus* (Christman, 2008). The staminal column is about 2/3 the length of the petals and is pink to white in color with yellow stigmas. The 200+ stamens span the length of one side (secund) of the staminal column and bear yellow anthers (Blanchard, 1976; FNA, 2019b). Flowers open in late afternoon to evening emitting a slight fragrance, and individual blooms senesce the following morning with more blooms opening daily from summer to fall (Christman, 2008; Clausen and Christopher, 2014; FNA, 2019b). Each of the five carpels can contain 30-40 ovules, potentially resulting up to 40 seed per locule in the dehiscent fruit (Blanchard, 1976). The fruit is an ovoid-shaped capsule with the apex contracted to a short beak and measures approx. 2.5-3.5 cm long. The fruit surface is covered in many short, stiff hairs of yellow- to red-brown color. Seed are small (3 mm diameter) obovate-shaped and dark brown, brown-red or black in color (Blanchard,

1976; FNA, 2019b; Godfrey and Wooten, 1981). Giant rose mallow produces a large amount of biomass every year and dies back to the crown in late fall, as perennials do. More mature stems can be woody at the base and their surfaces become glabrous with age (Christman, 2008; Godfrey and Wooten, 1981). Plants produce more stems yearly to increase their size, rather than spreading by suckering. Propagation can occur readily by rooting stem cuttings, dividing the crown, or sowing seed (Christman, 2008; Clausen, 2014).

In addition to terrestrial garden settings, *H. grandiflorus* can be used as a large shrub in ponds or water gardens or for wetland restoration and mitigation projects. Although *H. grandiflorus* is categorized as an obligate wetland plant, a study in 2013 found two commercial grower substrates and (drained) sand led to larger, more floriferous plants versus growing in flooded sand (Gettys et al., 2013). Gettys et al. (2013) evaluated practices to produce this native hibiscus for potential nursery and greenhouse growers. Besides substrate, the effect of added fertility was evaluated on growth parameters of *H. grandiflorus* and was found to lead to few differences. The high and medium rates (80 and 66.7 g per container, respectively) of fertilizer resulted in greater stem diameters than the low (40 g) fertilizer treatment after eight weeks of growth but there were no differences in height, shoot dry mass, or number of buds and flowers (Gettys et al., 2013).

As with *H. moscheutos*, giant rose mallow can hybridize with a few other members of the genus. Hybrids of *H. grandiflorus* in the trade are mentioned, having caused some confusion over the years in defining the true species (Bates, 1965; Godfrey and Wooten, 1981). Morphologically, *H. grandiflorus* resembles *H. moscheutos* more than other members of section Muenchhusia (Winters, 1970; Wise and Menzel, 1971). Hybridization with *H. coccineus* has been reported by Wise and Menzel (1971) with limited success and the authors observed the two

species to cohabit near the St. John's River, FL and in southern FL without any evidence of wild hybrids. A natural population of what appeared to be hybrids between *H. moscheutos* and *H. grandiflorus* on Hilton Head Island, SC was observed by Blanchard (1976). This report supports a suggestion by Wise and Menzel (1971) that *H. moscheutos* and *H. grandiflorus* are ecotypes, meaning the two taxa are separated ecologically or geographically and are genetically different, but if they were in close proximity, would hybridize freely (Clausen et al., 1939).

Polyploidy Induction in Plants

Polyploidy is the condition whereby an organism has three or more copies of a complete set of chromosomes. The condition is rare in mammals, but common in the plant kingdom and has enabled the evolution of a number of plant species. It can occur spontaneously in nature in meristematic cells developing into a doubled-chromosome mutant (sport), or from the union of unreduced gametes (2n egg and sperm cells) (Otto, 2007; Ranney, 2006; Yildiz, 2013). Some estimate between 47-70% of angiosperm plants are polyploids (Masterson, 1994; Ramsey and Schemske, 1998). From an evolutionary perspective, the advantages and disadvantages of polyploidization are still being researched and debated (Mayrose et al., 2011, 2015; Otto, 2007; Soltis and Soltis, 1993; Soltis et al., 2014). The artificial induction of plants to become polyploids began in the 1930's with the historic publication by Blakeslee and Avery (1937). The report presented many examples of varying genera (e.g. *Datura*, *Cosmos*, *Portulaca*) which had multiple sets of chromosomes following the application of colchicine (Blakeslee and Avery, 1937). Colchicine can be extracted from the corms of autumn crocus (*Colchicum autumnale*) as a yellow powder and is highly toxic to humans. This alkaloid inhibits the polymerization of subunits into microtubules, which make up the majority of spindle fibers during mitosis, thereby disrupting normal cell division in plant and animal cells (Bartels and Hilton, 1973; Blakeslee and

Avery, 1937; Kehr, 1996a; van Tuyl et al., 1992). Since the replicated chromosomes then fail to separate, one daughter cell often retains double the number of chromosomes as a typical cell (Ranney, 2006). Following the work of Blakeslee and Avery (1937), there was much interest in chromosome doubling via colchicine for a few decades and many agronomic crops were experimented with to develop polyploids for crop improvement. Despite the excitement over the novel tool and its transforming abilities, very few cultivars resulting from polyploid induction made an impact on a large scale. Some aims by early researchers were to increase plant and fruit size due to the enlarged cells resulting from more nuclear material, to make crosses which were previously unattainable ("bridge species"), and to create seedless fruits and vegetables via triploid plant development (Darrow, 1950; Hancock, 1997). Ornamental and floriculture plants also received attention from researchers wanting to modify plant traits by doubling chromosomes. Blakeslee and Avery (1937) worked with several genera grown for their blooms as did Nebel and Ruttle (1938) and Emsweller and Ruttle (1941) with *Antirrhinum, Petunia, Phlox, Chrysanthemum, Begonia,* multiple species of *Lillium,* and others. In later years, the high cost, hazardous nature, and incidence of mutations with colchicine lead researchers to look for alternatives (van Tuyl et al., 1992; Yemets and Blume, 2008). Several herbicides were found to have anti-microtubular effects similar to colchicine but through different specific mechanisms and include: amiprophosmethyl (APM), pronamide, and the dinitroanaline compounds oryzalin, trifluralin, and pendimethalin. Most of these anti-microtubular chemicals have been found to effectively induce polyploidization at higher conversion rates and lower concentrations than colchicine (Yemets and Blume, 2008). The higher polyploidization efficiency of oryzalin over colchicine (and other mitotic inhibitors) has been demonstrated with potato (Sree Ramulu et al., 1991), *Lilium* and *Nerine* spp. (van Tuyl et al., 1992), apple (*Malus* x *domestica*) (Bouvier et al.,

1994), *Rhododendron* cultivars (Väinölä, 2000), *Nepeta* spp. (Mitrofanova et al., 2003), *Alocasia* (Thao et al., 2003), and cork oak (*Quercus suber*) (Pintos et al., 2007). Oryzalin was found to have comparable chromosome doubling effects as colchicine in oil seed rape (*Brassica napus*) (Hansen and Andersen, 1996) and gerbera (*Gerbera jamesonii*) (Tosca et al., 1995), but with lower phytotoxic effects and at 100 times lower concentrations, respectively. Oryzalin induced a higher number of tetraploid plants but lower survival percentages than colchicine on young seedlings of *Hibiscus moscheutos* 'Luna Red' (Li and Ruter, 2017).

Polyploidy induction has been utilized by plant breeders for several reasons; the main purposes being to cause changes in plant morphology (potentially improved), restore fertility in progeny from wide crosses, and develop sterility (Kehr, 1996a; Ranney, 2006). Polyploidy induction can serve various purposes depending on the desired goal, therefore it can be a valuable, albeit time-consuming, tool for breeders. From an ornamental breeding perspective, many modifications to phenotypic and physiological traits have been reported with varying mitotic inhibitors (Hancock, 1997). Reported changes to flower morphology with increasing ploidy level include: larger flowers in marigold 'Gold Guinea' (Nebel and Ruttle, 1938), azalea (Pryor and Frazier, 1968), kangaroo paw (*Anigozanthos* 'Bush Ranger') (Griesbach, 1990), *Rhododendron* and *Magnolia* (Kehr, 1996a,b), *Buddleja* hybrids (Dunn and Lindstrom, 2007), *Rhododendron* 'Fragrant Affinity' (Contreras et al., 2007), and olive (*Olea europaea*) (Caporali et al., 2014); smaller flowers in *Hibiscus moscheutos* 'Luna Red' (Li and Ruter, 2017); an increased number of petals in *Rosa* Thèrése Bugnet (Kermani et al., 2003); and an increased flower thickness and/or texture in azalea (Pryor and Frazier, 1968), *Rhododendron* (Kehr, 1996a) and *Buddleja* hybrids (Dunn and Lindstrom, 2007). A few alterations to flower timing with induced polyploids have been reported such as longer-lasting individual flowers by Kehr

(1996a), delayed bloom initiation in tetraploid *Buddleja* hybrids (Dunn and Lindstrom, 2007), as a general description by Stebbins (1947), and longer bloom period of triploid *Hibiscus moscheutos* 'Luna Red' (Li and Ruter, 2017). Leaf morphology often changes with increased ploidy level with thicker, larger and darker green leaves commonly observed, as well as a crinkly, ruffled or leathery texture on occasion (Kehr, 1996a; Ranney, 2006); this has been reported in *Rhododendron* (Kehr, 1996b, 1971), *Alstroemeria* (Lu and Bridgen, 1997), lilac interspecific hybrids (*Syringa*) (Rose et al., 2000a), *Rosa* cultivars (Kermani et al., 2003), *Rhododendron* 'Fragrant Affinity' (Contreras et al., 2007), *Cryptomeria japonica* (Contreras et al., 2010), Japanese privet (*Ligustrum japonicum*) (Fetouh et al., 2016), and (*Hibiscus moscheutos* 'Luna Red' (Li and Ruter, 2017). However, a decrease in leaf size was observed in an octoploid version of *Hibiscus acetosella* 'Panama Red' compared to the tetraploid (Contreras et al., 2009). An increase in the width to length ratio of leaves, which effectively changes their shape, with artificially increasing ploidy level has been reported in *Rosa* cultivars (Kermani et al., 2003), *Alocasia* (Thao et al., 2003), and *Buddleja* hybrids (Dunn and Lindstrom, 2007). Reports of fewer stomata per leaf area (lower density) and/or larger or longer guard cells with increased ploidy have been made for *Rhododendron* (Kehr, 1971), *Alstroemeria* (Lu and Bridgen, 1997), *Alocasia* (Thao et al., 2003), several species of *Hibiscus* (Zhuang and Song, 2005), japanese quince (*Chaenomeles japonica*) (Stanys et al., 2006), and *Hibiscus acetosella* 'Panama Red' (Contreras et al., 2009). Solo'eva (1990) found the number of chloroplasts in guard cells of crab apple cotyledons increased as ploidy level increased as did Zhuang and Song (2005) in *Hibiscus schizopetalus*, *H. mutabilis*, *H. rosa-sinensis*, and three cultivars of *H. rosa-sinensis*. Internode length can be altered as ploidy increases with a decrease of internodes reported in lilac interspecific hybrids (*Syringa*) (Rose et al., 2000a) and *Hibiscus acetosella*

'Panama Red' (Contreras et al., 2009) but a slight increase of internode length reported in cultivars of *Rosa* (Kermani et al., 2003). An increase in stem thickness with increase in ploidy has been reported in marigold 'Gold Guinea' (Nebel and Ruttle, 1938), kangaroo paw (*Anigozanthos*) (Griesbach, 1990), *Buddleja* hybrids (Dunn and Lindstrom, 2007), Japanese privet (*Ligustrum japonicum*) (Fetouh et al., 2016), and by Kehr (1996a). Overall plant form can be modified with plant height often being reduced and compactness increased as ploidy level increases, as in interspecific hybrids of lilac (*Syringa*) (Rose et al., 2000a), *Hibiscus acetosella* 'Panama Red' (Contreras et al., 2009), japanese privet (*Ligustrum japonicum*) (Fetouh et al., 2016), and *Hibiscus moscheutos* 'Luna Red' (Li and Ruter, 2017). Other plant organs, such as fruit, can display altered size and/or shape as well (Ranney, 2006; Sanford, 1983). Overall, the enlargement of the leaves, flowers, fruit, and stems of a plant treated with an anti-mitotic agent is often referred to as the "gigas effect" (Ranney, 2006).

Cell size has been found to increase in the ovaries of olive flowers (Caporali et al., 2014) and in the leaf epidermis of sugar beet (Beyaz et al., 2013) as ploidy increases. Additionally, the increase in cell size was accompanied by a decrease in cell number in the foliar epidermis of sugar beet and the fruit tissue of olive with increasing ploidy level, also termed 'compensation' because the plant organ remains a similar size as the volume of the cells increases (Beyaz et al., 2013; Caporali et al., 2014; Tsukaya, 2008). Likewise, cell volume typically increases as genomic content increases with the phenomenon of endopolyploidy (Melaragno et al., 1993). The increased amount of nuclear DNA typically requires more time to replicate and divide, as related to the cell cycle (Van't Hof and Sparrow, 1963; Yildiz, 2013), which can manifest in a chemically-induced polyploid plant as slower overall growth (Ranney, 2006). The changes in cell volume and plant organ sizes can be disproportionate within the plant leading to imbalances,

which could be considered beneficial or detrimental (Hancock, 1997; Ranney, 2006). Doubling the chromosomes of an individual leads to an 'autopolyploid,' simply meaning the organism has additional copies of the same chromosomes (Ranney, 2006). This could result in unpredictable outcomes, and evidence exists that with the additional chromosomes in a tetraploid versus a diploid comes "enzyme multiplicity, increased heterozygosity, and increased allelic diversity" (Soltis and Soltis, 1993), all of which are potentially advantageous for breeding. Although autopolyploids have been compared to 'inbred lines,' mutations can take place which could increase heterozygosity, and some argue the additional copies (chromosomes) act as a buffer for the individual during environmental stresses enabling adaptation (Kehr, 1996a). Enhanced resistance to biotic and/or abiotic stresses with increased polyploidization can occur due to a number of unpredictable secondary results, such as an increase in secondary metabolites involved in stress and defense responses or altered leaf morphology; and may be more likely in polyploids which gained additional genomes from other species (allopolyploids) rather than autopolyploids (Levin, 1983; Ranney, 2006). Greater resistance to the pest hibiscus sawfly was observed in triploid forms of *Hibiscus moscheutos* 'Luna Red' as compared to diploid versions (Li and Ruter, 2017). Research is still ongoing to study changes in gene expression, regulatory interactions, and epigenetic changes from increased polyploidy (Osborn et al., 2003).

Changes to the reproductive status of plants have been observed and can be useful or detrimental depending on the research objectives. Induced or enhanced fertility and induced sterility (or reduced fertility) can result from polyploidy induction. 'Restored' fertility has been reported for plants which result from wide (intergeneric or interspecific) or difficult crosses and hence are sterile or have greatly reduced fertility. Increased or restored fertility from polyploidization has been reported in (formerly sterile) interspecific hybrids of *Camellia*

rusticana and *C. lutcheunsis* (Ackerman and Dermen, 1972), interspecific hybrids of kangaroo paw (*Anigozanthos*) (Griesbach, 1990), hybrids of *Lilium henryi* x *L. candidum* (van Tuyl, 1992), interspecific hybrids of lilac (*Syringa*) (Rose et al., 2000a), sterile diploid and triploid David Austin cultivars of *Rosa* (Kermani et al., 2003), intergeneric hybrids of Chitalpa (x*Chitalpa* 'Pink Dawn') (Olsen et al., 2006a), and an intergeneric hybrid of *Rhododendron* L. 'Fragrant Affinity' (Contreras et al., 2007). By restoring or enhancing their fertility, further breeding and/or experimentation can be carried out. Another use for polyploidzation is to "bridge" species by 'equalizing' the ploidy levels of two species so that they can be crossed. Some examples of this approach have been reported in *Rhododendron*. An induced tetraploid Carolina rhododendron (*Rhododendron carolinianum*) will reportedly cross with the tetraploid *R. augustinii* successfully, but not with the natural diploid form of Carolina rhododendron (Kehr, 1996a). The deciduous azalea *Rhododendron calendulaceum* is a natural tetraploid and only successfully crossed with evergreen azaleas (e.g. cultivars 'Tahei' or 'Banka') if they are first doubled from their diploid form (Kehr, 1996a). Successful progeny were obtained by inducing polyploidy of interspecific hybrids of *Buddleja madagascarensis* x *B. crispa* and then crossing with cultivars of the tetraploid *B. davidii* (Dunn and Lindstrom, 2007). Similar to Dunn and Lindstrom (2007), Rose et al. (2000b, 2001) induced polyploidization of *Buddleia globosa* to cross with the tetraploid *B. davidii*.

In contrast to restoring fertility, reducing fertility (or inducing sterility) can be ideal for cultivar development, particularly for preventing invasiveness or unintended hybridization (Ranney, 2004, 2006). Reduced fertility has been reported with an increase in ploidy in Japanese quince as tetraploid plants were observed to have reduced seed set vs. diploids (Stanys, 2006). Contreras et al. (2009) reported the induced octoploid (doubled tetraploid) form of *Hibiscus*

acetosella ‘Panama Red’ had no seed set from self-pollination and had reduced fertility as a male or female parent. Related research by Contreras and Ruter (2009) induced polyploidization of a hybrid from natural tetraploids *Hibiscus acetosella* x *H. radiatus*, thereby gaining sterility and an altered phenotype. Another method to develop sterility, or greatly reduce fertility, from induced polyploids is by crossing plants of unequal ploidy level, often a tetraploid (4x) by a diploid (2x), to obtain triploid (3x) plants, which have difficulty or are impossible to cross. This has been demonstrated in a limited number of ornamental taxa. One of the earliest examples was breeding work conducted by Don Egolf from the U.S. National Arboretum who induced tetraploid forms of a rose-of-sharon (*Hibiscus syriacus*) cultivar via colchicine treatment and then crossed the tetraploids with diploids of the same genotype and obtained several triploid rose-of-sharon cultivars released under names of Greek goddesses (Egolf, 1970, 1981, 1986, 1988). Crossing tetraploid and diploid forms of tutsan (*Hypericum androsaemum*), led to infertile triploid plants that had no observed seed set and very low pollen grain germination (6%), which is ideal since fertile tutsan can be potentially invasive (Olsen et al., 2006b). Olsen et al. (2006a) found that crosses between plants having tissues of differing ploidy levels (as a result of oryzalin treatment) of x*Chitalpa* and diploid plants of either parental species (*Catalpa bignonioides* or *Chilopsis linearis*) led to triploid plants after performing embryo rescue. Tetraploid versions of *Hibiscus moscheutos* ‘Luna Red’ were created by the use of oryazlin or colchicine and crossed with the original diploid form of the plant (Li and Ruter, 2017). Triploid *H. moscheutos* were obtained and displayed a longer bloom period than diploids, were sterile (female infertile and nonviable pollen), and exhibited a resistance to aerial phytophthora not seen in the diploids (Li and Ruter, 2017).

Germinating seed (Kehr, 1971; Lehrer et al., 2008), seedlings (Kehr, 1996b; Li and Ruter, 2017), meristematic shoots (Olsen et al., 2006a; Contreras et al., 2007), buds (Ackerman and Dermen, 1972), or roots can be treated with an anti-mitotic agent to induce polyploidization. Cells must be actively dividing for the chemical to be effective; therefore meristematic tissue is the most common target of treatment. Dry seed or dormant tissue would not be an ideal choice to treat (Kehr, 1996a). Treating young, newly forming tissue is ideal due to the absence of cells which are less likely to undergo polyploidization, and pre-existing lateral shoots can confound the task of identifying converted regions of the plant. Plant material may be treated in a variety of methods including being sprayed (Contreras et al., 2010), soaked (Pereira et al., 2014; Li and Ruter, 2017), or applied with drops (Ackerman and Dermen, 1972; Pryor and Frazier, 1968) of a solution containing the anti-mitotic chemical (Kehr, 1996a; Yemets and Blume, 2008). Additionally, an agar solution containing the anti-mitotic chemical can be applied to the meristematic area and allowed to solidify for several hours or days before washing off (Jones et al., 2008; Conteras et al., 2009; Fetouh et al., 2016). Haploid microspores or anther culture in vitro are other target materials for polyploidization (Yemets and Blume, 2008). Material may be treated in vivo, however many experiments have treated plant material in vitro (Griesbach, 1990; Kermani et al, 2003; Thao et al., 2003; Stanys et al., 2006; Dunn and Lindstrom, 2007; and Dhooge et al., 2009). Following treatment, there is usually a delay in growth while the plant recovers and the duration can vary from a few weeks to months (Ackerman and Dermen, 1972; Li and Ruter, 2017). In addition to recognizing the abovementioned phenotypic traits which are typically altered, another method to identify plants and tissue which have been altered is by flow cytometry. With the use of a known standard (a diploid of the same species or an unrelated species with known genome size) for comparison, samples can be quickly screened for induced

polyploidization (Ranney, 2006). A complication, however, with chemically treating plant tissue is the occurrence of cytochimeras (or mixaploids). Cytochimeras are plants having histogenic layers that are not genetically the same throughout or layers that are not the same ploidy as each other. The plant consists of the L-I, L-II, and L-III layers and differences in the cells, such as different ploidy levels or mutations, within a layer can result in different types of chimeras. The L-1 consists of the epidermal tissue, the L-II consists of the cortical tissue from which reproductive organs arise, and the L-III is the roots, typically (Marcotrigiano, 1997; Ranney, 2006). Another advantage of treating the meristem is that all three layers are present at the growing point, therefore increasing the chances of inducing uniform polyploidization across histogenic layers. Given that cells of differing ploidy levels regenerate at differing rates, it follows that tissue would increase in size at different rates. Cells of lower ploidy require less time to duplicate than cells of higher ploidy, therefore over time chimeral plants may revert back to the lower ploidy level (Jones et al., 2008; Pratt, 1983). If the desired tissue, e.g. a region of tetraploid epidermal cells, is identified, it may be isolated and excised via tissue culture and propagated (Pryor and Frazier, 1968).

Hibiscus Sawfly Study

The hibiscus sawfly (*Atomacera decepta*) is a member of the family Hymenoptera (wasps and bees) and feeds on several species of *Hibiscus* and other perennial plants, like hollyhock (*Alcea rosea*) in regions of the Mid-Atlantic and Midwestern states of the U.S. (Cranshaw, 2004; Hiskes, 2014). The larvae cause the most damage by feeding on the undersides of the foliage creating a windowpane-like effect after eating the epidermal tissue layer. Severe infestations lead to skeletonized leaves with only portions of the midrib and larger veins remaining; even the calyx can be eaten (personal observation) (Boyd, 2005; Tippins, 1965). The lifecycle starts with

the female laying eggs typically on the upper side of older (lower) leaves in rows containing three to six eggs (however numerous eggs can occur on a leaf). The female does this by using her saw-like (hence, sawfly) ovipositor to puncture the foliage and insert the eggs leading to a "blistered" look on the leaves (Boyd, 2005; Gill et al., 1999; Tippins, 1965). About 3-7 d later the larvae emerge from the eggs and the first few instar stages feed on the abaxial (bottom) leaf surface. Once the larvae grow and transition through six instar stages, they feed on both surfaces of the leaf and can potentially defoliate a plant. The bodies of the larvae are yellow-green and somewhat translucent with a dark brown to black head and measure approx. 1.2 cm at the final instar (Tippins, 1965). The hibiscus sawfly larvae resemble moth and butterfly larvae but can be distinguished by their six pair of prolegs whereas the latter have five or fewer pair of prolegs (Hiskes, 2014). Mature larvae pupate by forming a light brown-colored cocoon around themselves which is affixed to lower stems or in leaf litter or soil. After approx. 1.5 weeks, the adult emerges with black wings and body with an orange-red spot on the upper thorax (behind the head) and measure about 4-5 mm long (Hiskes, 2014; Tippins, 1965). To some they resemble love bugs (*Plecia nearctica*) (Boyd, 2005 and personal observation). The adults then mate and the females lay another generation of eggs completing the lifecycle in approximately 28 d. Because the lifecycle is relatively quick, multiple generations often occur, reaching up to as many as six during a growing season. The adults have been reported to emerge in early May in GA and late May in CT, and remain active until the first frost (Hiskes, 2014; Tippins, 1965). Hibiscus sawfly is generally considered a minor pest for *Hibiscus* spp., as it can be managed by scouting and elimination of larvae (if caught early) or with the application(s) of pesticides (Boyd, 2005; Boyd and Cheatham, 2004). However, if left unattended and populations increase over

time, severe defoliation can occur and significant damage has been reported in parts of GA (Tippins, 1965), MD (Cahoon and Stevenson, 1986), MS (Boyd, 2005), and CT (Hiskes, 2014).

The first documentation of the hibiscus sawfly was in 1911 (Rohwer) in "New sawflies in the U.S. National Museum," and feeding damage on different species of *Hibiscus* has been witnessed and reported since at least 1965 (Tippins). An experiment by Boyd and Cheatham (2004) evaluated the number of eggs and larvae found on twelve species and cultivars of *Hibiscus* and rated final feeding damage of plants in an infested greenhouse. The authors concluded that *H. acetosella*, *H. aculeatus*, and *H. grandiflora* (sic) were the least affected by hibiscus sawfly (Boyd and Cheatham, 2004). This observation that certain species of herbaceous *Hibiscus* were less damaged than others by the hibiscus sawfly was reiterated by Boyd (2005). Plants trialed in Blairsville, GA that were part of the UGA ornamental hibiscus breeding program suffered from significant hibiscus sawfly damage in 2013, however differences in damage across phenotypes were observed. Plants in the trial were hybrids containing *Hibiscus moscheutos* subsp. *moscheutos*, *H. moscheutos* subsp. *lasiocarpus* and *H. grandiflorus* with a noticeable trait of increased leaf pubescence, which is a characteristic of *H. moscheutos* subsp. *lasiocarpos* and the velvet-leaved *H. grandiflorus* (NPIN, 2017). Leaf pubescence is comprised of trichomes on the epidermis of a plant which can serve as a barrier to insects in several ways from feeding, attachment, oviposition or movement (Norris and Kogan, 1980). Increased or abundant foliage pubescence has been attributed to reduced insect damage and/or egg oviposition in other species such as soybean from potato leafhopper (*Empoasca fabae*) (Broersma, 1972), wheat from cereal leaf beetle (*Oulema melanopus*) (Gallun et al., 1966; Schillinger and Gallun, 1968) and Hessian fly (*Mayetiola destructor*) (Roberts et al., 1979), domesticated and a wild relative of tomato from whitefly (*Bemisia* spp.) (Sánchez-Peña et al., 2006), cotton from

tarnished plant bug (*Lygus lineolaris*) (Wood et al., 2017), black gram (a pulse crop in India) from whitefly (*Bemisia tabaci*) (Taggar and Gill, 2012) and to some degree in eggplant from whitefly (*Bemisia tabaci*) (Leite et al., 2003). Trichomes can also contain glands which excrete secondary metabolites that can negatively affect herbivores (Norris and Kogan, 1980). Norris and Kogan (1980) reviewed morphologically based resistance of plants to insects including the details of the role of plant pubescence in relation to predatory arthropods with specific crop-insect examples.

Leaf pubescence is an example of antixenosis, which is one term to describe plant traits affecting herbivore behavior that reduces the colonization or acceptance/desirability of a plant as a host. In other words, the plant acts as a poor host and the pest then selects an alternate host. This is also called, 'nonpreference', and could be a morphological or chemical trait of the plant. Other such physiological or morphological examples may be a plant containing a compound that tastes 'bad' to the insect, waxy leaf surface (insect cannot hold on while eating or ovipositing), or thick outer surface layers (insect cannot physically get to the inner tissues of the plant for consumption) (Smith, 2005). The terms antixenosis, antibiosis and tolerance were originally defined by Reginald H. Painter (1951) as mechanisms, but have since been modified by researchers, particularly in viewing the terms more as categories than mechanisms. Antibiosis describes adverse effects of resistant plants on the physiology and life history of an herbivore such as reduced growth, survival and fecundity. In other words, the biology of the arthropod is affected by the plant. Ex: an insect feeding on a plant ingests a phytochemical (or secondary compound) produced by the plant that is toxic to the insect and its survival is compromised. There is a range of how the insect's survival may be affected, from mild to lethal. The stage that an insect is affected in its life cycle can also be a factor. A low concentration of a toxin could

affect young larvae more so than insects at mature stages. The higher concentration of a toxin often leads to mortality in older larvae causing them to not pupate, or for pupae and adults a failure to enclose. In this way, the effects could be prolonged and reduce the overall fecundity of the insect and subsequent generations (Smith, 2005). Tolerance is defined as the ability of the plant to withstand or recover from insect damage to which susceptible plants are also subjected. This describes a highly vigorous plant possessing traits allowing it to outgrow an arthropod infestation or to recover and add new growth after being damaged; yield loss is reduced. This term considers plant characteristics and does not focus on plant/arthropod interaction (Smith, 2005; Stout, 2012). These are the traditional 'categories' and many researchers have over the many years following Painter's definitions argued that there is much overlap, particularly between antibiosis and antixenosis. Additionally, these categories do not specify mechanisms that can vary, hence research continues to develop a consensus on new terminology. The more recent category of resistance focuses on aspects of the plant that limit injury from herbivore attack and has been subdivided into two areas of emphasis: whether the plant has previously been attacked by herbivores or not and how directly the herbivore is affected by the plant under attack. Constitutive resistance is considered defense that a plant expresses regardless of the prior history of herbivore attacks, whereas inducible resistance is only expressed, or expressed to a greater extent, after prior injury from an herbivore (Stout, 2012). Direct plant resistance occurs when the traits of a plant directly affect an herbivore and its behavior or biology, and indirect resistance involves another trophic level or natural enemies of the pest. An example would be a plant releasing volatile organic compounds which attract or alert other predators and parasitoids to the presence of the pest feeding on the plant and therefore a 'third party' predator eliminates the insect rather than the plant doing it directly (Stout, 2012). These terms assist researchers in

describing the interactions between host-plants and arthropod pests. The hypothesis that foliar pubescence on intra- and inter-specific hybrids of *Hibiscus moscheutos* (both subspecies) and *H. grandiflorus*, as well as the species, leads to less feeding and oviposition damage from the hibiscus sawfly (*Atomacera decepta*) than genotypes lacking pubescence will be evaluated.

Traditional Determination of Trait Inheritance

Determination of the mode of inheritance of traits can stimulate a breeding program by making more informed crosses and saving time and resources. For this doctoral research, four initial crosses involving inter-subspecific hybrids of *Hibiscus moscheutos* subspecies *moscheutos* and *lasiocarpus* and *H. grandiflorus* were made and the four subsequent second (F2) generations were observed for traits of interest. The inheritance of these phenotypic traits was evaluated at the F_2 generation and from progeny via selfing of select individuals. Traits focused on were: foliage color, foliage pubescence, stem and petiole color, flower openness, petal overlap and plant compactness. Additionally, crosses were made to evaluate the inheritance of flower color among intraspecific crosses of *H. moscheutos* and a cultivar of *H. moscheutos* ('Robert Fleming').

Studies of the inheritance of some of the above-mentioned traits have been documented. Foliage color has been observed and reported in various genera. Studies evaluating the inheritance of red foliage are focused on in this review of the literature since the inheritance of red foliage in *Hibiscus* was a focus of this doctoral project. Red to purple foliage has been reported to follow a single gene (monogenic) Mendelian 3:1 inheritance as either a dominant or recessive trait in some publications. Red foliage is controlled by a dominant monogenic allele in ornamental coleus (Nguyen et al., 2008) and some woody plants, such as beech (Blinkenberg et al., 1958; Heinze and Geburek, 1995) and birch (Hattemer et al., 1990). In other taxa, red foliage

is inherited in a single-locus recessive fashion; for example, in barberry (Cadic, 1992), redbud (Roberts et al., 2015), and tutsan (Olsen et al., 2006). In other cases, inheritance of red foliage is reportedly controlled by complementary gene action, as in hazelnut (Smith and Mehlenbacher, 1996; Thompson, 1985) and flowering dogwood (Wadl et al., 2010); or by a single gene with incomplete allelic dominance as with the bronze foliage allele (Rt) in crabapple (Alston et al., 2000; Sampson and Cameron, 1965).

The mode of inheritance of pubescence on foliage has been reported in a few agronomic crops and even fewer species having ornamental or ecological importance. Pubescence on lentil (*Lens culinaris* Medik.) plants was found to be inherited monogenically in a dominant (3:1) fashion over non-pubescence, or glabrousness, and given the allelic symbol *Pub* (Hoque et al., 2002). Kumar et al. (2005) supported those findings and described the pubescence on lentil plants as most obvious on tissues at the growing apex and inflorescence. Nawab et al., 2011 reported that the presence of trichomes on leaves of cotton (*Gossypium hirsutum*) was common, but did not present a method of inheritance or number of genes involved. *Brassica incana*, which has pubescent leaves, was crossed with several other *Brassica* species having n = 9 and pubescent foliage was found to be dominant to smooth but controlled by more than one gene (Kianian and Quiros, 1992). On the other hand glabrous, or smooth, leaves are dominant to pubescent leaves in pearl millet (*Pennisetum glaucum*, syn. *P. americanum, P. typhoides*) and controlled by a single gene (Burton and Powell, 1968; Gill et al., 1971; Singh et al., 1968). The allelic notation for pubescent lamina was proposed by Gill et al., 1971 as *hl* (hairy leaf) and found it to be independently inherited from the gene for hairy node (*Hn*). Rao and Koduru (1979) found that the trait of hairy lamina (*hl*) in pearl millet to be part of a linkage group and inherited

along with a hairy stem, hairy sheath, and glabrous leaf margin. Additionally, the gene for hairy lamina was found epistatic to expression of the gene for hairy stem (Rao and Koduru, 1979).

A study reported in 1926 found that the stem pubescence or smoothness of Japanese morning glory (*Pharbitis nil*, syn. *Ipomoea nil*) was inherited in a manner close to a monogenic 3:1 where hairy is dominant to smooth, however the author also stated the trait could be controlled by more than one gene (Imai, 1926). In 1960 inheritance of pubescent leaflets of mature seedlings was reportedly dominant to glabrous plants of *Lotus corniculatus* L., but the specific mode of inheritance was undetermined and suspected to be under multi-genic control (Hinkley and Keim, 1960). Crosses between two species of ornamental pepper (*Capsicum chinense* and *C. annuum*) led to progeny from multiple interspecific crosses and backcrossing that were evaluated for glabrous or pubescent leaves and multiple or single flowers. Results supported a two gene 13:3 model where pubescent leaves are dominant to glabrous and independently inherited from the flower number trait (Shuh and Fontenot, 1990). Pubescent leaves of periwinkle (*Catharanthus roseus*) were found to be controlled by a single gene in a homozygous recessive fashion (Kulkarni et al., 1999). A species endemic to Sweden, *Helianthemum oelandicum* var. *canescens*, exists in two forms, pubescent and non-pubescent on the abaxial leaf surface (Widén, 2018). The segregation in the F2 generation was found to follow a monogenic 3:1 inheritance ratio with a lack of pubescence being the dominant trait over pubescence (Widén, 2018).

There are several examples of taxa with flower color controlled by a single locus. The flower of the agronomic crop, chickpea (*Cicer arietinum*), was found to be controlled by a single gene with pink dominant to white (Hasan and Deb, 2013). Dolichos bean (or hyacinth bean) (*Lablab purpureus*) is mostly grown for human consumption, and the flower color is controlled

by a single gene with purple flower being dominant to white in a Mendelian (3:1) manner (Keerthi et al., 2016). Flower color of the attractive and aquatic pickerelweed (*Pontederia cordata*) is controlled by a single gene (*white flower*) with the dominant color being blue (*WW or Ww*) and the recessive being white (*ww*) (Gettys and Wofford, 2007). Crimson clover (*Trifolium incarnatum*), often used as a cover crop or roadside plant, was found to have flower color controlled by a single gene with crimson being dominant (*Bp_*) to bright pink (*bpbp*) (Mosjidis, 2000). Cultivated genotypes of gerbera daisy [*Gerbera hybrida* (*G. jamesonii* x *G. viridifolia*)] have flowers with either a light or dark central disk which is controlled by a single gene (Kloos et al., 2005). The dominant form of the gene, *Dc*, leads to a dark disk color and the recessive, *dc*, leads to a light-colored disc, which is the wild-type (Kloos et al., 2005).

There are many examples in the literature of flower color under the control of multiple genes with the contributing effect of epistasis. The white-flowered species *Buddleia fallowiana* var. *alba* was crossed with a white-flowered cultivar of *B. davidii*, as well as a few cultivars of *B. davidii* having colored flowers to observe flower color inheritance (Tobutt, 1993). Flower color was reportedly controlled by two loci with different genotypes leading to the white-flowered phenotype for the two species (Tobutt, 1993). Several genotypes of the ornamental and culinary safflower (*Carthamus tinctorius*) were crossed to evaluate spininess and flower color inheritance (Pahlavani et al., 2004). Results demonstrated that flower color is under the control of at least two loci which are epistatic to each other (leading to yellow or orange flowers) and segregate independently from the single dominant gene controlling spininess (Pahlavani et al., 2004). The southeastern U.S. native stokes aster (*Stokesia laevis*) was found to have at least three loci regulating the color which can range from blue, lavender, pale pink, pale yellow or albescent (Barb et al., 2008). Blue was found to be dominant to pale yellow and albescent (white) at one

locus with two alleles with an epistatic interaction occurring when both homozygous recessives are crossed, and blue was found dominant to pink (or producing cyanidin) (Barb et al., 2008). Periwinkle (*Catharanthus roseus*) can have many different flower colors from rose, magenta, or pink with a red eye or white without a red eye (Kulkarni et al., 2005). Kulkarni et al. (2005) found four independent but epistatic genes (*R, W, O^m, J*) to control flower color of periwinkle. Contreras et al. (2014) observed flower color in American beautyberry (*Callicarpa americana*) to be inherited over several generations (F1, F2, BC's) with the traits of fruit and petiole color, indicating the effect of pleiotropy or multiple linked genes.

Flower color of scarlet rosemallow (*Hibiscus coccineus*), a close relative to *H. moscheutos* and *H. grandiflorus*, was found to be controlled by a single diallelic gene known as *white flower* with the allelic symbols *W* and *w* (Gettys, 2012). It follows that a white-flowered phenotype of *H. coccineus* is homozygous recessive (*ww*) and a red-flowered phenotype is either homozygous dominant (*WW*) or heterozygous (*Ww*) (Gettys, 2012). The African-native *Hibiscus cannabinus* is grown for its fibrous stalks, and in 1990 a single plant was observed out of approx. 300,000 to have a white and smaller flower as compared to the standard yellow flower of kenaf (*H. cannabinus*) (Cook and Bañuelos, 1997). Following test crosses, selfing and backcrossing of the species and mutant specimen, flower color was found to be monogenically inherited with yellow dominant to white (Cook and Bañuelos, 1997). Cook and Bañuelos (1997) designated the recessive white-flowered allele *wf*, and did not observe any change in the red eye center, suggesting red eye might be controlled by a separate locus. Stout (1917) noted characteristics of polymorphisms that exist in natural populations of *Hibiscus* which were taken from natural populations (locations unknown) of the eastern U.S. and evaluated at the New York Botanical Garden. Performing self-crosses and crosses between plants of different phenotypes, Stout

(1917) grouped plants into several “races” based on the resulting progeny. Although not a formal genetic study nor concluding with any modes of inheritance, of interest is the variability in flower and foliage color and other traits which are described, as well as the observation that a cross between a white-flowered and a red-flowered (“amaranth pink” to “Tyrian rose,” Stout, 1917) hybrid of *H. moscheutos* led to mostly red-flowered progeny in the second generation (Stout, 1917).

***Liquidambar formosana* Hance**

Formosan Sweetgum (*Liquidambar formosana* Hance) is a handsome tree sometimes used as a landscape plant for its unique 3-lobed leaves, brilliant yellow fall color and tall stately form. It is native to China and distributed in temperate forests across the southern and eastern range of the country, as well as South Korea and Taiwan (Dirr, 1998; Hoey and Parks, 1994). The tree was introduced to North America in 1884 and is considered to grow rapidly and performs well in USDA zones 7-9. Formosan sweetgum belongs to Hamamelidaceae family and is similar to American Sweetgum (*L. styraciflua* L.), which is native to the eastern United States and also found in cloud forests in eastern Mexico and Central America (Hoey and Parks, 1994). Leaves are a lustrous, dark green, with serrated margin, the middle lobe is longer and triangular in shape and petiole is fairly long (8-12 cm). Fall color can vary from yellow-red in the southern U.S. to red in northern regions. Trees can reach up to 30 m, are monoecious, and bear separate male (staminate) and female (pistillate) flowers from March to June. The fruits are globose and somewhat prickly from the persistent stigmas of the 24-43 capsules once they dehisce, giving them the colloquial term ‘gum balls.’ *Liquidambar formosana*, while being an excellent ornamental tree, is also used for the production of timber and balsam (Dirr, 1998; Durkovic et al., 2005; Gilman and Watson, 1993; eFloras, 2003; Zomlefer, 1994).

Traditional/ex vitro methods of propagation of Liquidambar *spp.*

An important aspect to cultivar development and germplasm protection is economic propagation. Few publications reporting ex-vitro propagation with Formosan sweetgum exist, so precedent examples from related species, *L. styraciflua*, are additionally presented. A report from southern Mississippi stated that girdling the stem of the mother plant increased rooting success of cuttings of Formosan sweetgum (Hare, 1976). Girdling was done in May and 90% of cuttings had rooted after one month, compared to cuttings from a non-girdled mother plant having only one rooted cutting. The survival of the propagules, however, was not mentioned. A study in China found the highest attained rooting rate of Formosan sweetgum to be 64% when they applied IBA (indole-3-butyric acid) at 250 $mg \cdot L^{-1}$ to semi-lignified branches (He et al., 2004). There have been mixed reports over a few decades of ex-vitro propagation work with the related *L. styraciflua*. A rooting study in 1973 from Texas concluded it was "a difficult-to-root species" after attempting to root cuttings from shoot and root sections (Bilan, 1974). The article reported varied rooting with age of cuttings, and obtained higher rooting percentages from root pieces than shoots. Another experiment using roots to propagate American sweetgum found 4 in. cuttings to have higher budding success and faster shoot growth from younger plants (three-year-old seedlings vs. 20-year-old trees) after 90 d with no effect from kinetin applied at 10 ppm (Brown and McAlpine, 1964). Another rooting study with *L. styraciflua* used softwood cuttings taken from suckers in mid-August to early September (Farmer, Jr., 1966). Neither age of trees producing the suckers nor application of IBA at 50 ppm was reported to affect rooting success, which reached 100% when cuttings were placed in a 1:1 sand:peat medium for six weeks under mist. In 1999, a study testing the effects of several parameters on rooting American sweetgum found 15 cm long terminal shoots had a higher survival (84%) at 15 weeks after removing from mist than sub-

terminal cuttings of equal length (68% survival) (Rieckermann et al., 1999). The study found that the sub-terminal cuttings had higher dry weights for new shoots and roots and higher shoot number and shoot length, however the survival through Summer and Fall 1994 was lower than for terminal cuttings. A new method was reported in 2010 by researchers in Brazil using "mini-cuttings" to propagate a large number of *L. styraciflua* propagules (Wendling et al., 2010). Cuttings were taken from "mini-stumps" of three different clones and dipped in varying levels of IBA solutions ranging from 0 to 6,000 $mg \cdot L^{-1}$. Survival of cuttings was evaluated of each rooting hormone level at three different stages of the rooting process, specifically, after 60 d in greenhouse, after 30 d in shade house and after 20 d of being subjected to outdoor conditions. Survival was not affected by IBA treatment at any of the three stages and mean survival over the clones at termination (about 17 weeks post initiation) was 62.5%. The mini-stumps also produced 10 rounds of cuttings that were collected during the experiment and each stump yielded an average of 2.84 cuttings per collection. This average can be interpreted as approx. 283.2 cuttings per square meter (over 10 collections). The technique by Wendling et al. (2010) seems to demonstrate a high production rate per area of cuttings with a moderate level of survival of rooted cuttings in a relatively short amount of time.

In vitro methods of propagation of Formosan sweetgum

The alternative to ex vitro propagation is in vitro, of which there is very limited literature pertaining to Formosan sweetgum. A 2005 study reported the use of micropropagation to regenerate plants of Formosan sweetgum from axillary buds and petiole segments (Durkovic et al., 2005). The optimal plant growth regulators for producing shoots were cytokinin-like TDZ (thidiazuron) and the synthetic cytokinin BAP (6-benzylaminopurine) combined. Shoot elongation was observed when BAP was applied alone, regardless of auxin (such as IBA) in the

medium (Durkovic et al., 2005). An interesting observation was also reported of adventitious root development from abaxial leaf tissue not in contact with the rooting media, however, this phenomenon subsided once propagules were grown ex vitro. In 2007, a study investigated plantlet regeneration via adventitious shoots from leaf explants of Formosan sweetgum (Xu et al., 2007). The experiment tested three concentrations of NAA (1-Napthaleneacetic acid) with four concentrations of TDZ on shoot generation of five genotypes of Formosan sweetgum. Xu et al. (2007) determined the optimal additions to WPM (woody plant medium) for generating shoots across all genotypes to be 1.14 µM TDZ and 0.27 µM NAA.

Somatic embryogenesis

Another method of in vitro propagation is somatic embryogenesis. In general, the principle of somatic embryogenesis is to induce plant tissues to produce somatic embryos in vitro via culture medium amendment. Somatic embryos can either arise directly (no callus formed) or indirectly (callus formed) from the explant tissue. The technique of somatic embryogenesis, while having the ability to produce mass quantities of new plants, also restores juvenility or reestablishes the plant to the juvenile phase of its cycle. This can be advantageous for woody plants by priming propagules for micropropagation or other forms of propagation. This method of propagation is particularly well suited for genetically modified plant cells since somatic embryos develop from a few cells or even a single cell (Hartmann et al., 2002; Williams and Maheswaran, 1986). Different explant sources can be utilized, but embryo and seedling tissues tend to have the greatest likelihood to produce somatic embryos. For coercing explant material to undergo induction, forms of auxin such as 2,4-dichlorophenoxyacetic acid (2,4-D) and NAA, and cytokinins are often used. Different types of cell cultures can form, such as callus or proembryogenic masses (PEMs), and suspension culture may aid in streamlining the formation

of distinct somatic embryos (SEs), which are synchronous and of high quality once filtered by size (size-fractionated). Once SEs are obtained, they further develop and mature in medium absent of plant growth regulators before undergoing germination. Some species require modification to their environmental conditions in order to transition to the germination stage (or conversion to seedlings). Common treatments include partial desiccation, cold stratification, and application of gibberellic acid (GA) and/or cytokinin. Finally, once shoots and roots of seedlings have further extended, they are transplanted to soilless medium in a greenhouse for hardening off and ultimately to the field for evaluation (Hartmann et al., 2002).

A study in 2001 used hybrid seed from nine controlled crosses of American and Formosan sweetgum trees to obtain clonal somatic embryos (Vendrame et al., 2001). Tissue from the immature seeds was plated on two induction media containing 2,4-D and seed was collected at two dates in summer 1999. Neither media nor cross had an effect on induction frequency of embryogenic cultures, however, the date of collection did. From the seeds cultured, only 2% resulted in repetitive embryogenic cultures which produced embryos that germinated into somatic seedlings. A later study was carried out to improve on generating somatic embryos and seedlings from the *Liquidambar* hybrids (*L. styraciflua* x *formosana*). Trees displaying ornamental potential were chosen for cloning and it was found that applying cold temperatures (10°C) to cultures for at least eight weeks prior to germination improved average germination and conversion of the embryos as compared to those given only four weeks of cold or none (Merkle et al., 2010). An additional related study using immature seed of hybrid sweetgum evaluated the production of SEs yielding seedlings when PEMs were grown in liquid induction-maintenance medium (IMM). The liquid IMM was also supplemented with amino acids to enhance embryo development (Dai et al., 2004). Many publications present advancements of

somatic embryogenic work with *Liquidambar styraciflua* and have proven useful in designing this experiment (Merkle and Battle, 2000; Merkle et al., 1998; Merkle et al., 2003). These examples demonstrate the capability of somatic embryogenesis to generate new duplicate plants of a genotype from vegetative tissues and/or organs.

***Helianthus simulans* E. Watson**

Swamp sunflower is an underutilized fall-blooming native perennial producing a swath of eye-catching bright yellow inflorescences. Being a member of the Aster family (Asteraceae) it has brilliant golden yellow ray florets surrounding the central disk florets with dark purplish-red corollas. Blooms can be found from August through November making it a uniquely attractive sight in the cooler months. The numerous flower heads reside above the foliage in a corymbose or racemose arrangement each with a medium sized disk about 1.3-2 cm wide surrounded by 12-23 ray florets 2-3 cm long (FNA, 2006a; Heiser et al., 1969; Watson, 1929). The leaves are dark green and rough on the adaxial surface with a prominent mid-vein and a lighter green, slightly tomentose underside. Leaves are firm with an entire margin, long (3.5 to 8.5 in), attached at the base (subsessile) and mostly alternate arrangement. The plant typically has two leaf types: narrow upper leaves (approx. 0.25 to 1.5 inches wide) with linear to lanceolate shape, whereas basal leaves are obovate with short petiole (FNA, 2006; Heiser et al., 1969; NPIN, 2008; Watson, 1929). The plant typically grows about 1-1.8 m tall, but can reach 2.5 m in height making it versatile and fitting for naturalized settings but likely less ideal for formal landscapes. Swamp sunflower's native range is from Georgia and Florida west to Texas and Arkansas. Also called muck sunflower, the common names suggest it is typically found in moist or saturated soils near ponds or along riparian and drainage ways. However, as it is considered a facultative wetland plant it is also found in non-wetland habitats (NPIN, 2008; Wunderlin et al., 2019). The

species grows best in full sun to part shade and tolerates a variety of growing conditions, reportedly staying more compact in denser, saturated soils (NPIN, 2008). Its ability to thrive under such conditions could be attributed to its thick rhizomes, up to 1 cm in diameter, and coarse main roots (Heiser et al., 1969). In addition to swamp sunflower's aesthetic attributes, it has value for wildlife including beneficial insects like native bees that forage the flowers and birds that consume the seed (NPIN, 2008). With the growing interest in natives as ornamentals, swamp sunflower is a strong candidate for wider use in perennial beds and landscapes. Research on nursery management protocol(s) for swamp sunflower is necessary for promoting its use. Shipping and maintenance of ornamentals typically requires costly and labor-intensive pruning, particularly for vigorous growers like swamp sunflower, but a more economical practice is application of plant growth regulators (PGRs) to maintain certain plant dimensions.

Plant growth regulators

PGRs are often used in ornamental plant production to manage growth size and branching, as well as promote or suppress flowering. A common group of PGRs are plant growth retardants, many of which inhibit biosynthesis of the plant hormone gibberellin (GA) leading to a reduction in plant growth (Davis et al., 1988; Rademacher, 2000). Of these types (which inhibit GA biosynthesis), there are four groups, one of which is compounds with an N-containing heterocycle. These compounds inhibit oxidation of *ent*-kaurene to *ent*-kaurenoic acid within the GA biosynthetic pathway, thereby playing a major role in reducing endogenous levels of GA within the plant (Rademacher, 2000). Two such growth retardants are paclobutrazol and flurprimidol.

Effect on plant size of PGRs

Pacbolutrazol is the most widely used plant growth retardant in greenhouse-grown ornamentals to control excessive plant growth and several studies have demonstrated its reduction in height for annual sunflower (*Helianthus annuus* L.) (Ahmad et al., 2015; Barbosa et al., 2008; Dasoju et al., 1998; Davis et al., 1988; Koutroubas et al., 2014; Vernieri et al., 2003; Wample and Culver, 1983; Whipker and Latimer, 2016; Whipker and McCall, 2000). In 1998, Dasoju et al. applied paclobutrazol as a 2 mg a.i. per pot drench on the potted sunflower cultivar 'Pacino' and found height was reduced by 17-25%. A wide range of rates was tested with an observed reduction in plant height and diameter at rates up to 16 mg a.i./ pot but with phytotoxic symptoms observed at rates of 16 and 32 mg a.i./ pot. Similar responses with drench application of paclobutrazol at the rate of 2 mg a.i./ pot were obtained by Whipker and McCall (2000) on five cultivars of pot sunflowers (*H. annuus* L.) and by Ahmad et al. (2015) on 'Pacino Gold.' Plant height was reduced from control by 21-28% and diameter was 12-15% reduced at 1-2 mg a.i./ pot for Ahmad et al. (2015). Whipker et al. (2004) also observed height reductions (20%) with 2 mg a.i./pot drench of paclobutrazol on potted sunflower. Drench application of 4 mg a.i. paclobutrazol led to height reductions from the control for Dasoju et al. (1998) of 26-36%, Whipker and McCall (2000) of 33%, and Ahmad et al. (2015) of 34% for potted annual sunflower. Interestingly, Vernieri et al. (2003) treated four of the five same cultivars as Whipker and McCall (2000) with a drench application at similar rates of paclobutrazol and observed a 50% height reduction at their highest rate (16 mg a.i./ pot) but with some negative effects such as decreased flower size and delayed flowering. Barbosa et al. (2008) observed reduced plant height with increased paclobutrazol concentration and recommended 6 mg a.i. per pot for 'Golden.' Paclobutrazol has also reduced plant height of agronomic sunflower by 11.1% with a single application as foliar spray which helped reduce lodging and facilitate mechanical harvesting

(Koutroubas et al., 2014). A 2-3 ppm (≈ 0.2-0.3 mg a.i.) drench application of paclobutrazol 1-2 weeks after pinching during greenhouse production is suggested for an interspecific hybrid of sunflower (*Helianthus hybrida*), 'Sunfinity' (Syngenta Flowers, 2017). A contributing factor to overall height reduction is shortened length of internodes, which has been reduced by paclobutrazol application in several species (Lever, 1986; Rahman et al., 1989; Richardson and Quinlan, 1986; Tschabold et al., 1970; Wample and Culver, 1983; Wood, 1984). One report of insufficient height reduction by paclobutrazol on 'Pacino' potted sunflower was by Whipker and Dasoju (1998) with a foliar spray application, and they suggested spraying at even higher concentrations (80+ mg a.i./pot) or using a drench application. Recommendations for greenhouse floriculture growers is to apply paclobutrazol as a high-rate drench for enduring effects throughout the growing season, and for sunflower that is a 2-4 mg a.i. drench of 4 fl. oz. per 6-in pot (Whipker, 2015). Application method, particularly of paclobutrazol, in precedent literature of other plant species has largely been by foliar spray or substrate drench with more favorable results from drench (Davis et al., 1988; Hawkins et al., 2015; Keever et al., 1990; Ruter, 1996; Whipker and Dasoju, 1998).

Flurprimidol is another popular plant growth retardant for ornamentals and a pyrimidine compound that has been recently introduced to the US market (Rademacher, 2000; Whipker, 2013). Flurprimidol reduced plant height and diameter of potted sunflower 'Pacino' when applied as a drench or foliar spray by Whipker et al. (2004). Two PGRs were compared to flurprimidol and results showed a drench application of flurprimidol at 2 mg a.i./ pot (which reduced height by 22%) to be similar to paclobutrazol at the same rate (Whipker et al., 2004). Another comparison in 2003 by Vernieri et al. found flurprimidol at 60 mg a.i./ L on sunflower was less effective at height reduction than their lowest studied rate of paclobutrazol (2 mg a.i./

pot). However, the large difference in results was likely due to application method of flurprimidol as a drench (Whipker et al., 2004) versus a spray (Vernieri et al., 2003). Flurprimidol is described as a highly cost-effective drench and recommended rates for sunflower are 1-2 mg a.i. drench for 6-in. pot or 30-50 ppm spray (Whipker, 2015). Application of flurprimidol as a drench rather than spray has been shown to produce more ideal plant effects in other species as well (Barrett and Bartuska, 1982; Krug et al., 2005a; Krug et al., 2005b; Rezazadeh and Harkess, 2015; Whipker et al., 2006).

Movement within plant and soil of PGRs

Research has shown that by applying paclobutrazol to the soil, as long as it is placed in close proximity to the roots, it is then transported acropetally via the xylem and translocated to meristematic regions as it is transported to and accumulates in leaves (Davis et al., 1988; Lever, 1986; Wang et al., 1986). Application of paclobutrazol solution to roots is important since it is relatively immobile in soil (Lever, 1986). By applying as a soil drench, the growth retardant is translocated to more regions of the plant where GA synthesis is occurring, such as developing leaves and elongating internodes (Taiz et al., 2015). Studies researching application of paclobutrazol as a spray to above-ground portions of the plant (e.g., young shoots) found no movement out of the leaf lamina or shoot tip but a small amount was transported acropetally from young stems (Barrett and Bartuska, 1982; Richardson and Quinlan, 1986; Wang et al., 1986). Due to the absence of basipetal movement of paclobutrazol, it is described as xylem-transported (Davis et al., 1988). For continued suppression of GA biosynthesis, drench application of paclobutrazol is typically more effective than foliar spray and reports explain an accumulation of paclobutrazol directly behind shoot apices creates a ‘reservoir’ via a streaming supply transported from roots (Barrett and Bartuska, 1982; Davis et al., 1988). The continued

effects of paclobutrazol when applied as a drench could also be due to it having a half-life of approximately 3-12 months (Lever, 1986). Even though soil drench yields more plant growth reduction than foliar spray, type of media can impact the efficacy of a paclobutrazol drench. Plants growing in pine bark media applied with a paclobutrazol drench led to no or less reduction of height than plants in media without pine bark for Barrett (1982) and Million et al. (1998). Million et al. (1998) found a 3-4 fold increase in the PGR rate required to reach similar height reductions of pine bark media-grown plants as peat-based media plants. Barrett (1982) attributed the effect of media to pine bark having hydrophobic surfaces which growth retardant molecules that are not very water-soluble, like paclobutrazol, adhere to when poured through the media.

Effect of PGRs on foliage

Effects on leaf morphology and physiology have been observed in many studies when applying triazoles and triazole-like compounds that include paclobutrazol and flurprimidol. Reduction in leaf area or size from triazoles has been reported by several studies (Davis et al., 1988; Fletcher et al., 2000; Izumi et al., 1984; Nazarudin et al., 2007; Richardson and Quinlan, 1986; Wample and Culver, 1983; Wood, 1984). Retardants inhibiting GA biosynthesis result in smaller leaves, likely due to restriction of cell elongation and expansion. Thicker leaves have also been reported by several studies from the application of triazoles (Burrows et al., 1992; Gao et al., 1988; Nazarudin et al., 2007; Wood, 1984). This is likely due to increases in the epicuticular wax layer of the epidermis and thicker palisade and mesophyll layers. Burrows et al. (1992) described a 64% thicker palisade layer from application of paclobutrazol resulting in more cell layers (2-3) than the untreated control (1 cell layer), as well as a 72% thicker spongy mesophyll layer of *Chrysanthemum* cv Lillian Hoek. Nazarudin et al. (2007) described a similar observation using scanning electron microscopy (SEM) and observed tighter packed cells of the palisade and

spongy mesophyll layers and a thicker palisade parenchyma layer of *Syzygium campanulatum* when treated with paclobutrazol. Gao et al. (1988) reported an increase in leaf thickness, epicuticular waxes, and mesophyll cell thickness in wheat (*Triticum aestivum*) with the application of a compound similar to paclobutrazol.

Increase of leaf greenness has been noted by many studies from the application of paclobutrazol and other triazoles. Ahmad et al. (2015) observed increased green foliage for potted sunflower 'Pacino Gold' when applied with 2-4 mg paclobutrazol. Barbosa et al. (2008) also reported increased SPAD values with increased rates of paclobutrazol, peaking between 4 to 6 mg a.i./pot on 'Golden.' Increase in chlorophyll content was reported in annual sunflower by El-Kheir et al. (2000) with spray applications of 30 and 60 ppm paclobutrazol. Increased foliar chlorophyll content or darker green appearance with paclobutrazol application, or other triazoles, has been reported in additional plant genera to *Helianthus* (Aly and Latif, 2011; Bañón et al., 2001; Burrows et al., 1992; Dahab et al., 2015; Fletcher and Arnold, 1986; França et al., 2017; Izumi et al., 1984; Kumar et al., 2012; Wood, 1984). Increase in relative chlorophyll and leaf greenness with the application of anti-gibberellin compounds has been explained by several factors, including less leaf expansion leading to increased concentration of chloroplasts per leaf area, a boosting of chlorophyll biosynthesis, and an increase in chloroplast size (Davis et al., 1988; Fletcher et al., 2000; Gao et al., 1988). The increase in chlorophyll biosynthesis has been speculated to result from enhanced cytokinin levels stemming as a secondary effect from paclobutrazol or other triazole application (Aly and Latif, 2012; Fletcher et al., 2000; Grossman, 1992) and this increase in chlorophyll content is thought to be a contributing factor to increased whole-plant photosynthetic capability (Kumar et al., 2012).

Effect of PGRs on flowering

There can be an impact on the initiation of flowering with the application of plant growth retardants, particularly those that inhibit GA. Delays in flowering for potted sunflower by PGRs have been reported by Dasoju et al. (1998) of 4-6 days with paclobutrazol drench at 2-32 mg during winter production and by Whipker and Dasoju (1998) with foliar spray of paclobutrazol at 80 mg/L. Flowering delays of less impact to marketability have been reported by Whikper and McCall (2000) for paclobutrazol 2 and 4 mg a.i./1.2L pot drench on 'Teddy Bear' potted sunflower and by Vernieri et al. (2003) for paclobutrazol (2-16 mg a.i./pot drench) and flurprimidol (7.5-60 mg a.i./L spray) on four cultivars of potted sunflower. However, no delay to anthesis for potted sunflower was observed by Whipker et al. (2004) from the application of paclobutrazol (2 mg a.i./pot drench) or flurprimidol (10-50 mg/L spray or 0.5-4 mg a.i./pot drench) and by Whipker and Dasoju (1998) with paclobutrazol 5-40 mg/L as foliar spray. PGR effect on flowering can vary among species with no effect observed by application of paclobutrazol on *Dissotis rotundifolia* (Hawkins et al., 2015), 'Anna Marie' hyacinth (*Hyacinthus orientalis*) bulbs (Krug et al., 2005b), and begonia (*Begonia semperflorens* 'Red Devil') (Farthing and Ellis, 1990). But delays were observed from paclobutrazol application on cape daisy (*Osteospermum ecklonis*) (Barnes et al., 2009), Bengal rose (*Rosa* hybrid 'Gruss an Teplitz') (Singh and Bist, 2003), geranium (*Pelargonium zonale* F1 'Ringo Scarlet') (Farthing and Ellis, 1990), potted tulips (*Tulipa gesneriana* L. 'Paul Richter' and *Tulipa* hybrid 'Apeldoorn') (McDaniel, 1990; Suh et al., 1992) and *Primula malacoides* (Dogra, 2013) and by flurprimidol on 'Star Gazer' oriental lily (*Lilium* hybrids) (Krug et al., 2005a) and 'Anna Marie' hyacinth bulbs (Krug et al., 2005b), to name a few. Early flowering from the application of paclobutrazol was observed on geranium [*Pelargonium hortorum* (sic) 'Springtime'] and Chinese hibiscus [*Hibiscus rosa-synensis* (sic)] by Andrasek (1989). Early flowering of

geranium (*Pelargonium* × *hortorum* L. H. Bailey 'Ringo White' and 'Ringo Rose') was observed in 1988 as a result of paclobutrazol application, but the following year no effect on flowering date was observed for 'Ringo Rose' (Latimer and Baden, 1994). Initiation of flowering is known to be promoted by endogenous levels of gibberellins, and an exogenous application of gibberellins can promote flowering, particularly for dual-day length plants grown under short-days and rosette long-day plants (Taiz et al., 2015). Therefore, it would follow that the application of anti-gibberellin compounds would alter timing of flowering despite the fact that other factors, such as species, seasonal conditions, and other endogenous hormones, can play roles as well (Hisamatsu et al., 1998; Taiz et al., 2015).

Flower diameter and PGRs

Precedent studies with potted annual sunflower have found no differences of inflorescence diameter from an untreated control when compared to a paclobutrazol drench at 2 or 4 mg a.i./pot (Whipker and McCall, 2000) or as a foliar spray at 5-80mg/L (Whipker and Dasoju, 1998). On the other hand, reductions of inflorescence diameter by higher paclobutrazol drench rates (4-32 mg a.i./pot) and flurprimidol spray (60 mg a.i./L) have been observed, as compared to untreated control (Dasoju et al., 1998; Vernieri et al., 2003).

Secondary effects of PGRs

In addition to the desired ornamental effects that plant growth retardants impart, some triazoles, particularly paclobutrazol, lead to other alterations for plants, such as fungicidal activity, delayed leaf senescence, reduced water use, and tolerance to abiotic stresses (Davis et al., 1988; Fletcher et al., 2000). Several GA-inhibiting plant growth retardants have fungicidal activity for plants due to the fact that GA synthesis in higher plants is similar to GA synthesis in fungi (Coolbaugh et al., 1982; Köller, 1987; Rademacher, 2000). Paclobutrazol can be one of two

diastereoisomeric forms, each having differing activities of either PGR or fungicide for a plant (Sugavanam, 1984). Delay of leaf senescence has been observed in several plant species as a result of applying a GA-inhibiting growth retardant and is thought to be due to an increase in cytokinin levels, which delay senescence, and a decrease of abscisic acid (ABA) and/or ethylene levels, which typically promote senescence (Davis et al., 1988; Grossman, 1990; Grossman, 1992; Kumar et al., 2012). Reports of reduced water use by plants as a result of GA-inhibiting growth retardant application is somewhat mixed in the literature and seems to be species-dependent. Additionally, attributed causes vary with studies, citing reduced leaf area or increased stomatal resistance (Davis et al., 1988; Fletcher et al., 2000; Wample and Culver, 1983). ABA is known to play an important role when plants are under an abiotic stress such as drought, cold or salinity. ABA is reported to initially increase within plants and later decrease with the application of an N-containing heterocycle type of growth retardant. Fluctuations in levels of ABA and/or cytokinins are partially explained by their connection to the same pathway (isoprenoid) as gibberellins, which triazoles affect (Davis, 1988). Research is still ongoing as to the exact cause of increased stress tolerance of plants treated with different growth retardants, largely GA-inhibitors, and various examples exist that demonstrate these tolerances (Asamoah and Atkinson, 1985; El-Kheir et al., 2000; Fletcher and Hofstra, 1985; Fletcher and Nath, 1984; Grossman, 1992; Rademacher, 1991; Shanahan and Nielsen, 1987; Tseng and Li, 1984; Wample and Culver, 1983).

***Illicium parviflorum* Michx. ex Vent.**

The small anise tree (*Illicium parviflorum* Michx. ex Vent.), also called yellow-anise tree, is an evergreen shrub with olive to yellow-green leaves that performs well in many terrains and environmental conditions from USDA hardiness zones 6 to 9 (Dirr, 1998; FNA, 2006b). It can be

found in forested, moist soils of the Coastal Plains region of Georgia (GA) and is native to low woods and swamp areas of peninsular Florida (FL), where it is also listed as endangered (Duncan and Kartesz, 1981; Hardin, 1972; Weaver and Anderson, 2010). It is commonly planted in the southeastern U.S. and prefers shade and moist soil, but can tolerate full-sun and dry soil (FNA, 2006b; Hardin, 1972; NPIN, 2013). Some describe it as faster growing and hardier than other *Illicium* spp. (Dirr, 1986). It has been found to tolerate quite cold temperatures for a southeastern native plant, surviving to -20°C (Lindstrom and Dirr, 1989). The medium to large native shrub is reportedly without any major pest problems (Gilman, 1999) and appropriate for sites with root-knot nematode-infested soils (Sharma and Rich, 2005). Useful for quickly providing a vegetative mass or screen that brings a distinct shade of green, yellow-anise tree has an upright pyramidal form that can be wide-spreading, particularly if allowed to sucker or layer, and is typically 1.8-4.5 m (6-15') in height (up to 6 m, or 20'). Leaves are simple, alternate, 5-10 cm (2-4") long, with entire margins, and oval to elliptic in shape with an obtuse to acute apex. Leaves have a smooth texture and are often held at 45° from the upper stems. When slightly bruised, leaves and stems have a pleasing 'anise' scent, attributed to the dominant essential oil of safrole (Tucker and Maciarello 1999), which also gives sassafras its characteristic 'spicy' scent. Stems are glabrous and somewhat green in color, with inconspicuous lenticels. Flowers are often overlooked as they are small (about 0.2 cm, or ½", wide) and not strongly scented. Flowers are six to 12-tepalled, yellow-green or yellow-cream colored, bell-shaped, and visible from May to July in GA (Dirr, 1986, 1998; FNA, 2006b; Smith, 1947; NPIN, 2013). The solitary, bisexual flower lasts about two to three days and bears 11-14 tightly held carpels each with a single ovule. The six or seven stamens surround the pistils in an outer whorl curving over them so that the anthers reside above the stigmas (FNA, 2006b; Smith, 1947; White and Thien, 1985; Wood, 1985; Zomlefer, 1994).

Small insects, mostly Dipteran, have been observed on the flowers around dusk, attracted by the faint aroma of the nectar (White and Thien, 1985). Although the proximity of the pollen to the stigmas is very close and the insects could potentially act as pollinators, *Illicium parviflorum* is described as self-incompatible (White and Thien, 1985; Zomlefer, 1994). The Illiciaceae family is also referred to as the "Star Anise Family" due to the shape of the fruit which is an aggregate of follicles in a star-like arrangement, each follicle potentially having a single seed (Zomlefer, 1994). Small anise tree has low fruit and seed set and White and Thien (1985) reported a total of five fruit from 100 flowers that matured, and 12 seed were collected from 50-65 follicles (Hopkins, 1972; Roberts and Haynes, 1983). As the fruit dehisces, the smooth and glossy seed is projected out of the follicle in a ballistics-like manner, reportedly shooting an average of 1.2-2.5 m (depending on height) for *I. floridanum* (Florida anise) (Roberts and Haynes, 1983). Bagging fruit once the tepals have dropped is recommended for collecting seed (personal observation) and for best germination, seeds should be moist, cold (5°C) stratified for 90 d prior to sowing (Olsen and Ruter, 2001).

The Illiciaceae family consists of a single genus, *Illicium*, which contains about 37-42 species (depending on the source) native mostly to southeastern Asia and nearby islands with a few scattered in southeastern North America, the Caribbean, and Mexico (Cronquist, 1981; Smith, 1947; Wood, 1958). *Illicium parviflorum* and *I. floridanum* are the only *Illicium* species native to mainland North America and both are from the southeastern U.S. Interestingly, *Illicium parviflorum* has a base chromosome number of 14 and *I. floridanum* has 13 and both are diploid (Stone and Freeman, 1968). The name comes from the Latin *illicio* meaning to allure, entice or seduce, owing to the fragrance of the plant's oils (Hopkins, 1972; Wood, 1958). An early classification by Smith (1947) separated taxa within the genus based on traits of the inner whorls

of the perianth, placing *I. parviflorum* into Section (Sect.) Cymbostemon and *I. floridanum* in Sect. Badiana, later renamed Sect. Illicium (Hopkins, 1972). More recent research has questioned this phylogeny and grouped the North American species separate from the Asian species, based on molecular data (internal transcribed spacers (ITS) of nuclear ribosomal DNA) and a few other traits, such as pollen type and geographical distribution (Hao et al., 2000; Wang et al., 2010). Oh et al. (2003) further supported the phylogeny of Hao et al. (2000) with detailed seed characters and compared those to fossils of *Illicium* spp. There is evidence that the ranges of both the Asian and North American *Illicium* species were more wide-spread in past millennia (Qi, 1995). The shrub of *Illicium parviflorum* is on occasion confused with *I. floridanum* and *I. anisatum* (Dirr, 1986), and the genus has few traits to distinguish between species, according to Oh et al. (2003), citing the uniformity of epidermal features between the species. Within *I. parviflorum* there is little variation and much homogeneity in the foliage. Additionally, a recent study sampling wild populations as well as horticultural stock of *I. parviflorum* found little genetic diversity. Newell and Morris (2010) analyzed plant tissue from four nursery stocks across FL, AL, and GA and found all samples shared the same genotype that was also the same genotype as a wild population. This sameness is likely due to nurseries utilizing a solitary or very few collection events as the source for plant material to vegetatively propagate, which greatly reduces genetic diversity. There are very few cultivars of *Illicium parviflorum*, such as 'Forest Green,' 'Florida Sunshine,' and 'BananAppeal.' 'Forest Green' is described as having a lustrous, darker green foliage and a more rounded leaf shape (Dirr 1998), while 'Florida Sunshine' has bright chartreuse foliage, a reduced size and a slower growth (Monrovia, 2019). A recent introduction 'BananAppeal' (PP28,887) is also reduced in size with yellow-green summer foliage (Dirr and Kardos, 2018). Only a handful of cultivars originating from *I. floridanum* exist

commercially, such as ‘Pink Frost’ (PP21,287) and recent introductions by Star Roses and Plants® ‘Orion’ and ‘Scorpio’ PPTBS, which are described as compact cultivars (Harp, 2010). These scant examples demonstrate the opportunity for introducing phenotypic variation within North American *Illicium* for the ornamental market.

Physical Mutagenesis for Breeding

Ornamental plants have greatly benefited from mutation breeding, particularly in the Netherlands’ market (van Harten, 1998). It was reported in 1990 that there were an estimated 400 cultivars globally that originated from mutation breeding, and the Mutant Variety Database by the Joint FAO/IAEA currently lists over 3,000 cultivars originating from chemical or physical mutagenesis (FAO/IAEA, 2019; Micke et al., 1990). Mutations can be induced via chemical or physical mutagens, and the most common types of physical mutagens are ultraviolet (UV) radiation and ionizing radiation. There are a few sources for ionizing radiation, including X-rays, gamma (γ) rays, protons and neutrons. Gamma rays are commonly used to induce mutagenesis in plants and are typically applied as acute radiation, which is a high dose applied for a relatively short period of time (minutes to hours). Cobalt-60 (or ^{60}Cobalt) is a synthetic radioactive isotope with a half-life of ~5.3 years, and is used commercially for industrial and medical purposes as a source of gamma radiation (EPA, 2017). The International System of Units’ (SI) unit for radiation is currently expressed as a Gray (Gy). The former unit was a rad (R) (abbreviation of ‘radiation absorbed dose’), and 1 Gy is equal to 100 rad or 1 $J \cdot kg^{-1}$ (Predieri, 2001; van Harten, 1998; WHO, 2019). A recommended first step when working with a previously non-irradiated species is to use a broad spectrum of doses for initial studies. The radiosensitivity of the material being irradiated is determined by its physiological response to the radiation. A method to estimate the radiosensitivity is determining the LD 50 (LD = lethal dose), which is the dose rate

at which 50% mortality or reduction in growth of the irradiated sample occurs compared to a control (Predieri, 2001; van Harten, 1998). The prime dose rate for plant material has the highest frequency of mutations with the least detrimental effects on the plant's growth and vigor. As suggested by van Harten (1998), the optimal range of irradiation dose for living, vegetative plant material is 20-80 Gy.

Ionizing radiation does not have a specific target, unlike UV radiation. Research has found that the main target of ionizing radiation is water, since the plant cell consists largely of water. During ionizing radiation, free electrons and radical ions are produced and resultant free radicals are synthesized due to the presence of water molecules. The free radicals from water radiolysis in solution are trapped by the cellular membrane and interact with DNA. This is likely the source of most damage to DNA with ionizing radiation, however the DNA molecules can also be damaged by directly absorbed radiation (Ahnström, 1977; Britt, 1996; Ward, 1975). Tissue with less water, such as seeds, would have less damage to DNA from free radicals but could require higher dose rates to induce mutations (Ahnström, 1977; Britt, 1996). Effects of ionizing radiation to the plant can be categorized by different methods. Van Harten (1998) approached the organization of describing resulting mutations at several 'levels' including: the genome, chromosome and extranuclear mutations. Changes to the number of a chromosome set (aneuploidy) and the number of complete sets of chromosomes (polyploidy and haploidy) are included in genome mutations. Chromosomal changes include single- or double-stranded breaks, inversions, duplications, deletions, translocations or single point mutations (Acquaah, 2007; Britt, 1996). Point mutations could lead to chnages within genes being actively expressed; these would specifically include: a missense mutation, where an amino acid is changed potentially leading to a non-functional protein; a nonsense mutation, where a change in amino acid results in

a stop codon and likely premature termination of protein synthesis; a neutral mutation, where an amino acid change occurs without a protein change; or a silent mutation, where the three-base codon is changed but the amino acid is not (Acquaah, 2007; van Harten, 1998). Insertions or deletions could lead to frameshift mutations leading to 'misreading' of the genetic code and potential protein modification. Because there is DNA not only in the nucleus of a plant cell but also in plastids, these mutations could occur in mitochondrial or chloroplast DNA (extranuclear mutations) (van Harten, 1998). Another aspect to consider is the occurrence of DNA repair that occurs daily in organisms. There are different types of repair including the replacement of damaged nucleotides with new ones; alternatively, other pathways can be created for DNA to tolerate damage (Britt, 1996).

When DNA undergoes a mutation, it may or may not result in a phenotypic alteration, or the alteration might not be observed in that generation of plant but in following generations (Prina et al., 2011). A recessive mutation occurs when a homozygous dominant gene (AA) is mutated to a heterozygous (Aa) individual for a particular locus and the mutated recessive allele would be expressed in the next generation (F_2 population). If the plant were selfed (or crossed with a heterozygous plant for a single gene trait) then there should be approximately 25% of the next generation with a recessive phenotype (aa) for that gene (assuming it is a gene resulting in an observable phenotype). It is more common to get a mutation from a dominant to a recessive allele (A -> a), however there could be a mutation in the other direction (a -> A), known as a dominant mutation. In the latter situation, if the original genotype were homozygous recessive (aa), this type of mutation could lead to an observable phenotype in the irradiated plant. This is more likely when using naturally inbred species, which tend to have a greater number of homozygous recessive genotypes (Acquaah, 2007; van Harten, 1998). Since irradiation is

random and could potentially lead to any number of mutations, observation and selection of plants over several generations is typically necessary to obtain a desired phenotype (Micke and Donini, 1993; van Harten, 1998).

The type of material being irradiated should also be considered, as this can affect the outcome of the treatment. There are several methods and parts of the plant that can be used to generate a uniformly mutated plant from a single-cell mutation event. Since mutations occur at the cellular level, a single cell can give rise to a modified phenotype through multiple rounds of mitosis. One of the main drawbacks to irradiation is not obtaining a homohistont mutant, or a plant with the same mutation in all histogenic tissue layers, i.e. is the same in all cells of the plant. The more common scenario is a chimeric mutant, which is a plant with mutated cells in part of a tissue layer(s) or in an entire layer but not in all tissue layers. There are a few different types of chimeras; including sectorial, mericlinal, and periclinal. The type of chimera depends on which histogenic layer the mutated tissue is in and the extent of the similarly mutated tissue within the plant. In-depth descriptions can be found by Broertjes and van Harten (1978) and Marcotrigiano (1997). Single-celled zygotes or haploid gametes are ideal starting material, however these are not always feasible to obtain or utilize. Tissue cultures of callus and/or single-cell cultures are also appealing for their ease of manipulation. Any meristematic tissue (e.g., apical or axillary bud) could be a viable option because newly emerging tissue post-irradiation could contain mutations and be used for propagation. There is also the possibility of adventitious buds arising from non-meristematic regions, but typically this depends on the species. Tissue culture can be used to generate new, potentially homohistont mutated plants, depending on the location of the mutated cells. Tissue culture and micropropagation of a portion of the mutated tissue could be attempted with continuous rounds of isolating the mutated cells and selecting

against the non-mutated cells. This technique is called “chimera dissociation” and can be done in vitro or with a whole plant in vivo (Suprasanna and Nakagawa, 2011). The desired pheno- and geno-type could then be ‘fixed’ in order to obtain a homohistont mutant with all three tissue layers of the plant mutated throughout (solid mutant). Once in vitro plantlets appear to be mutated and reach a sufficient size, they can gradually be moved out of culture and into soilless media in pots. If plants display different phenotypes in different parts of the plant, shoots can be removed and vegetatively propagated. Rooted cuttings can be transplanted to pots or the field to monitor their performance. Several rounds of vegetative propagation are often required to establish a stable mutant ready for cultivar release. Sexual propagation is another means of obtaining a uniform mutant and is conducted by selfing a genotype. There is the possibility of obtaining a plant that phenotypically appears to be mutated, yet is not mutated throughout all histogenic layers (a chimera). Since reproductive organs arise from the L2 (layer 2), a means of checking the mutated status of the L2 is to self the plant. The progeny could then be evaluated phenotypically and/or via molecular techniques to determine if they are uniformly mutated (Broertjes and van Harten, 1978; Micke and Donini, 1993; Suprasanna and Nakagawa, 2011; van Harten, 1998). An alternative option is genetic screening, or TILLING (Targeting Induced Local Lesion IN Genomes; McCallum et al., 2000). Tissue extracted could be analyzed with PCR-based methods to compare target genes, portions of them, or nearby sequences of putative mutants with un-mutated plants in order if a plant is a homohistont mutant. Literature exists that describes these processes, some of which are applicable to certain types of mutations (SNPs, deletions, etc.) and could prove more reliable and faster than relying on phenotypic data (Bovina et al., 2011; Wilde, 2015). There are many routes to obtain a unique plant derived from chemical or physical mutagenesis and many specific examples can be found in the literature, and the

Mutant Variety Database (online) by the Joint Food and Agriculture Organization of the United Nations/ International Atomic Energy Agency (FAO/IAEA, 2019) can be searched for agricultural and ornamental examples (Broertjes and van Harten, 1978; Micke and Donini, 1993; Suprasanna and Nakagawa, 2011; van Harten, 1998).

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CHAPTER 1

Triploid development of *Hibiscus moscheutos* hybrids

Introduction

Hardy hibiscus cultivars are valued for their large, showy blooms, low maintenance and reliability as perennial landscape plants. Two characteristics which could be improved are fruit production and the duration of bloom period, which is typically June to Aug. (FNA, 2019). Once the abundant blooms are fertilized, dehiscent fruits remain on the stalks and detract from the aesthetics of the plant. A method employed by breeders for reducing or eliminating fruit production is to develop infertile plants. Infertile plants can have different reproductive morphologies, and some sterile plants do not form fruit, which was the desired phenotype for this study. A method to develop infertile plants is by polyploidy induction, whereby the number of copies of the chromosomes is multiplied by some factor of two, followed by crossing with a parent of a lower ploidy level to obtain plants with an intermediate ploidy level (Ranney, 2006). Ornamental polyploid plants have been reported to have a longer bloom period [as with triploid *Hibiscus moscheutos* 'Luna Red' (Li and Ruter, 2017)], longer-lasting individual flowers (Kehr, 1996), and delayed bloom initiation in tetraploid *Buddleja* hybrids (Dunn and Lindstrom, 2007). Li and Ruter (2017) also observed tetraploid forms of *H. moscheutos* 'Luna Red' to lack pollen. Triploid forms had non-viable pollen and pollinated flowers produced no fruit (Li and Ruter, 2017). This study aimed to develop triploid forms of intraspecific hybrids of *Hibiscus moscheutos* subsp. *moscheutos* using advanced selections from the hardy hibiscus breeding program at UGA and a few select cultivars. The predominant species used was *H. moscheutos*

subsp. *moscheutos*, however some hybrids did have *Hibiscus moscheutos* subsp. *lasiocarpos* and *H. grandiflorus* in their genetic backgrounds (Table 1.1). All the afore-mentioned species are diploids (2n=38) (Skovsted, 1935; Wise and Menzel, 1971), therefore chromosome doubling would lead to tetraploids. The study was conducted by first inducing polyploidization and identifying tetraploids. The chemical agent used was oryzalin because it is less hazardous than colchicine (Hassawi and Liang, 1991; Yemets and Blume, 2008) and has been demonstrated to have a higher conversion efficiency over colchicine (and other mitotic inhibitors) for potato (Sree Ramulu et al., 1991), *Lilium* and *Nerine* spp. (van Tuyl et al., 1992), *Rhododendron* cultivars (Väinölä, 2000), *Nepeta* spp. (Mitrofanova et al., 2003), *Alocasia* (Thao et al., 2003), and cork oak (*Quercus suber*) (Pintos et al., 2007). Oryzalin has been used on seedlings at the cotyledon stage to successfully induce polyploidization in *Hibiscus acetosella* 'Panama Red' (Contreras et al., 2009) and *H. moscheutos* 'Luna Red' (Li and Ruter, 2017), therefore a similar protocol was used in this study. Once tetraploids were obtained, the next steps for obtaining triploid plants which are commonly followed would entail crossing with selections of diploid genotypes and screening the resulting seedlings for triploidy.

Materials and Methods

Phase 1. Polyploidy induction and verification

Eleven selected, experimental lines and two commercial cultivars were used to obtain 12 families of seed from controlled crosses made throughout Summer 2016, with the exception of one group of seed from an open pollination. Plants were chosen for aesthetic characteristics including red foliage, red stems and petioles, compact size, and for enhanced tolerance of hibiscus sawfly (*Atomacera decepta*). Details of the seed from which the seedlings for polyploid induction originated can be found in Table 1.1. Seed from multiple fruit of the same cross were bulked to

form the “seed lots” listed in Table 1.1. Several seed lots were sown on each of three dates with the seed lots for each sow date collectively called “rounds”. Seed for Round 1 was sown 9 and 16 Mar. 2017, Round 2 sown 30 May 2017 and Round 3 sown 30 June 2017. Seed was scarified by soaking in 95–98% sulfuric acid (Avantor Performance Materials, LLC, Center Valley, PA) for 10 min and rinsed in a sieve under tap water for approx. 15 s the day prior to sowing. Seed for Round 1 was sown in 100% sand in 1020 trays (21.2 × 10.8 × 2.3 in) (Landmark Plastic, Akron, Ohio) with one tray for 9 Mar. and one for 16 Mar. per seed lot. Seed for Rounds 2 and 3 was sown in potting substrate [Jolly Gardener Pro-line C/L Growing Mix (Oldcastle, Shady Dale, GA)] that was amended with micronutrients (Micromax; Everris NA Inc., Dublin, OH) at 594 $g \cdot m^{-3}$ and used to fill 1020 trays (54 × 27 × 6 cm) (Landmark Plastic, Akron, Ohio). Seed was also sown separately to measure germination percentage for each seed lot: Round 1 had 100 seed for each seed lot sown in two circular 12.7 cm (height) pots (Dillen Products, Middlefield, Ohio) (50 seed per pot) sown 9 Mar. 2017; Rounds 2 and 3 had 25 seed sown per 12.7 cm pot with two pots per seed lot for Round 2 and one pot per seed lot for Round 3 sown on the same start date as seed used for oryzalin treatment. All seed for germination percentages was sown in the same potting substrate as mentioned above [Jolly Gardener Pro-line C/L Growing Mix (Oldcastle, Shady Dale, GA) amended with micronutrients]. Trays and pots were placed under 24 h incandescent light [~55 $\mu mol/m^2/s$ (measured with quantum meter, model: MQ-100, Apogee Instruments, Logan, UT)] in lab conditions (~25°C) at Miller Plant Science Bldg. at the University of Georgia (UGA), Athens, GA. Seedlings were treated once there were several at the same stage of two or more seed lots for Rounds 1, 2, and 3. The stage at which seedlings were removed from the substrate and treated was the “cotyledon stage” (Contreras et al., 2009), where the cotyledons are fully expanded and the apical meristem is visible but before the first true leaf

has emerged. There was variability in the number of treatments per Round, the time between treatment dates and which seed lots were treated; this was due to differences in germination rates and percentages and number of seed sown. Germination percentages were calculated by dividing the number of germinated seedlings by the number of seed sown and expressing as a percent. Treatment was applied on 21 and 28 Mar., and 4 Apr. for Round 1; 14 and 29 June for Round 2; and 11, 14, 19, 25 July, 2, 9, 17 Aug. 2017 for Round 3. Seedlings were treated for 6 h for Round 1 and for Rounds 2 and 3 seedlings were divided equally (± 2 seedlings) between a 2 h and a 4 h treatment. Treatment was applied by submerging seedlings in 100 mL of a 100 μM solution of oryzalin (Surflan A.S.; Southern Agricultural Insecticides, Hendersonville, N.C.) diluted in tap water in glass baby food jars with plastic lids on a rotary shaker (New Brunswick Scientific, Edison, N.J.; Model: G-33) at 150 rpm. Jars were randomly arranged on the shaker. Following treatment, seedlings were placed in 150 mL flasks and rinsed under continuously flowing tap water for ~45 min to remove any oryzalin residue. All seedlings were placed in moist paper towels and brought to UGA's Durham Horticultural Farm in Watkinsville, GA where they were transplanted to 8.8 cm square pots (Kord; The HC Companies, Inc., Twinsburg, OH) filled with the same potting substrate as above [Jolly Gardener Pro-line C/L Growing Mix (Oldcastle, Shady Dale, GA) amended with micronutrients] and maintained under greenhouse conditions. Greenhouse temperature was set to 25°C during the day and 20°C at night with natural lighting. Seedlings from Round 1 were placed on bottom heat mats (~65°F) in an unshaded section of the greenhouse, whereas seedlings from Rounds 2 and 3 were not placed on bottom heat and were in a shaded section of the greenhouse. After approximately 4-5 weeks, seedlings from Rounds 2 and 3 were moved to an unshaded area of the greenhouse. Weekly fertilizer application began soon after transplanting with a 20N-4.4P-16.6K water-soluble liquid fertilizer at 200 $mg \cdot L^{-1}$

nitrogen (Jack's Professional; J.R. Peters, Inc., Allentown, PA). Approximately 8-10 weeks after treatment, surviving seedlings were transplanted to 2.8 L pots filled with potting substrate (pine bark, peat and sand mix; Oldcastle, Shady Dale, GA) and top-dressed with 14 g per pot of 15N-3.9P-10.0K controlled-release fertilizer (Osmocote Plus; ICL Fertilizers, Dublin, Ohio) and maintained under greenhouse conditions. Seedlings were cut back once (when 18-22 cm in height). Several plants from Round 1 were transplanted to 11 L (3 gal) pots in the same potting substrate as 2.8 L pots beginning 12 Aug. 2017 and top-dressed with 28 g per pot of 15N-3.9P-10.0K controlled-release fertilizer. Beginning on 24 Oct. 2017, plants in 11 L pots were brought to a greenhouse at the Trial Gardens at UGA with temperature set to ~24°C and maintained under natural light, as well as extended daylength light (high-pressure sodium lighting set at $10{:}00_{PM}$ to $2{:}00_{AM}$ and $7{:}00$-$8{:}30_{AM}$) to encourage growth and flower initiation. Remaining plants in 2.8 L pots were moved to an overwintering greenhouse at the Durham Farm in early Nov. The overwintering greenhouse was set to 15°C and plants were placed near a few halogen 1,000 watt stand work-lights (Utilitech, China, Model: HW-GZ001) set to 14 h duration ($5{:}00_{AM}$ to $7{:}00_{PM}$). Remaining plants in 8.8 cm pots stayed in the same greenhouse as where they were originally transplanted until 30 Jan. 2018. At that time, they were transplanted to 2.8 L pots (with the same potting substrate and fertilizer as above) and moved to the overwintering greenhouse. On 11 May 2018 the plants in the overwintering greenhouse that had new growth were transplanted to 11 L pots with the same potting substrate and fertilizer as above and brought to the Trial Gardens greenhouse. Plants in the overwintering greenhouse which were not ready for transplanting were moved to the container pad outside at the Durham Farm and brought to the Trial Gardens later if they developed flower buds.

Flow cytometry was used to evaluate the ploidy level of plants treated with oryzalin when they were actively growing yet prior to flower initiation. A CyFlow Ploidy Analyzer (Partec GmbH, Münster, Germany) was used to discern ploidy level and reagants from the manufacturer of the flow cytometer were used to prepare the samples: a nuclei extraction buffer and a nuclei staining buffer (CyStain UV Precise P, Sysmex Partec, Germany). Flow cytometry began in Sept. and continued intermittently until 18 Dec. 2017; with some extending 2 Apr. to 12 June 2018. The method used was the same as described in Li and Ruter (2017), except the filter size was 30 μm, rather than 40 μm mesh. At the point of testing for ploidy, many plants had multiple stems, therefore larger stems were designated with a piece of colored tape and tested separately. If a plant had all stems test as tetraploid, the plant was considered fully tetraploid. If a plant had only one stem test as something other than tetraploid, yet the remaining stems were tetraploid, the plant was considered partially tetraploid and the non-tetraploid stem was cut down to the node and a piece of flagging tape wrapped around to prevent re-growth. If a plant had multiple stems test as mixoploid (having tissue layers with differing ploidy levels), it was discarded. If a plant had all stems test as diploid, it was retained for crossing.

Phase 2. Crossing

Plants that had been moved to UGA's Trial Gardens greenhouses in Fall 2017 were confirmed as tetraploids using flow cytometry. Diploid plants for use as pollinator parents were also brought to the Trial Gardens greenhouses. Approx. 105 tetraploid and 109 diploid plants in 11 L pots were used for crossing. Hand pollinations began 16 Jan. 2018 and were conducted before $10{:}00_{AM}$ by removing a flower from a diploid plant and rubbing the pollen onto a tetraploid flower, fully covering the stigmatic surfaces. Pollinations were conducted in a greenhouse, so risk of pollen contamination was very low. A pollination tag was placed around the flower

pedicel with information of the seed and pollinator parents and pollination date. When fruit were removed (typically one month after pollination), the harvest date (or collection date) was also written on the tag. Fruit that matured were collected and the seed were removed, counted and the seed from a fruit were given an accession number (HibSED18- #) based on the parents. Crosses which aborted were also recorded. Supplemental lighting was stopped 13 Apr. 2018 at the Trial Gardens greenhouse and pollinations continued until 14 Sept. 2018. Once crosses were stopped, 109 surviving tetraploid and 75 selected diploid (used in a cross) plants were moved to the container pad at the Durham Farm until being planted in-ground 8 Dec. 2018 at 1.2 m (4') spacing.

Phase 3. Triploid propagation, verification and planting

The first putative triploid seed were sown 28 Mar. 2018 of seed from three crosses made between 16 Jan. and 27 Mar. 2018. Seed was acid scarified in the same manner as above the day prior to sowing and sown in the same potting substrate as above in 72-cell trays. Trays were placed under mist (10 s every 30 min; adjusted to every 20 min mid-May) in a greenhouse at the Trial Gardens at UGA. After 28 d, seedlings were transplanted to 8.8 cm pots filled with same potting substrate [Jolly Gardener Pro-line C/L Growing Mix (Oldcastle, Shady Dale, GA) amended with micronutrients], brought to the Durham Farm and placed in a shaded section of the greenhouse. Liquid fertilization began when seedlings were transferred to the Durham Farm using the same fertilizer as above. Approx. 10-14 d later, seedlings were moved to an unshaded section of the greenhouse. Approx. four weeks later seedlings were transplanted to 2.8 L pots filled with potting substrate (pine bark, peat and sand mix; Oldcastle, Shady Dale, GA) and top-dressed with ~8 g per pot of 15N-3.9P-10.0K controlled-release fertilizer (Osmocote Plus; ICL Fertilizers, Dublin, Ohio). More putative triploid seed were sown 7 and 13 June and on 12 and 20

July 2018. Testing for triploid status began 7 June 2018 using the same method as for tetraploids and continued intermittently until 9 Sept. 2018. Once seedlings were confirmed as triploid, they were cut back to 2 to 3 nodes to encourage branching. Triploid seedlings were transplanted from 2.8 L pots to 11 L pots later in the growing season as needed. On 8 Dec. 2018, 76 triploid plants were planted in-ground at 1.2 m (4') spacing.

Remaining putative triploid seed was sown in the same manner as above on 1 Mar. and 15 Mar. 2019 in a greenhouse at the Durham Farm in 72-cell trays with a single seed per cell. The number of seed sown was 461 on 1 Mar. and 1,087 on 15 Mar. 2018. A combination (combo) of the seed accession number, cross date and harvest date was used to separate the seed into individual envelopes (see Appendix A for entire list of seed from crossing 4x × 2x). If there were multiple fruit with seed of the same accession no. crossed on the same day and harvested on the same day, the seed was bulked in one envelope. The envelopes were placed in ascending order by the number of seed recovered starting with one, and no more than 25 seed were sown of any specific envelope. Temperature in the greenhouse was set to 22°C, and the same liquid fertilizer as above was diluted to half the concentration and applied to seedlings once they had their first true leaves. The number of germinated seedlings was recorded 28 d after sowing to obtain germination percent. Seedlings were removed from the 72-cell trays once reaching approx. 5 cm in height and transplanted to 8.8 cm pots filled with potting substrate (Pro-mix BX Mycorrhizae; Premier Tech Horticulture, Rivière-du-Loup, QC, Canada) amended with the same micronutrients as above. Once transplanted to 8.8 cm pots, seedlings were placed in a different greenhouse at the Durham Farm set to 24°C and supplied weekly with the same liquid fertilizer as above. Flow cytometry testing of putative triploid seedlings occurred from 11 April to 14 May 2019. Seedlings were tested after being transplanted to 8.8 cm pots and actively growing. Flow

cytometry testing was conducted in the same manner as in 2018, except a different flow cytometer was used (Beckman Coulter CytoFlex, Indianapolis, IN) in the Cytometry Shared Resource Lab. in the Paul D. Coverdell Building on the UGA Athens campus and a 96-well plate (Corning Incorporated, Kennebunk, ME; Model: 3596) was used to test 200 µL of each sample. Seedlings were cut back to 2-3 nodes after testing for ploidy to encourage branching. Planting of 329 triploid seedlings occurred on 16 May 2019 to the same plot and at the same spacing as triploids from 2018 and their parents. Another 29 triploid seedlings and a further 245 seedlings from the putative triploid seed that tested as ranging from diploid to tetraploid were planted in a separate plot at the Durham Farm at the same spacing on the same day as the 329 triploids.

Embryo Rescue/Ovule Culture Work

Due to the observed occurrence of immature fruits occasionally aborting from 4x × 2x crosses, embryo rescue/ovule culture technique was trialed. An immature fruit was taken from 10 crosses (of the above-mentioned crosses) between 15 and 16 Sept. 2018 and brought with a cold-pack to Star Roses and Plants, West Grove, PA by the author. The details of the crosses are in Table 1.8. On 18 Sept. 2018, ovules were removed from the immature fruits and plated onto Differentiation Media (see Appendix B). One fruit from the cross SED2018-96 had further developed embryos (than the other 9 fruits used for this trial) that were able to be excised from the ovules and plated directly. There were 20 embryos plated for SED2018-96. For the other nine immature fruits, the embryos were dissected from the ovules two weeks after the initial plating. This and subsequent tissue culture work as well as transitioning seedlings to hardening off stage was performed by Emily Alff, associate breeder at Star Roses and Plants. About 10 d after initial plating, several embryos from SED2018-96 were transferred to Maturation Media (see Appendix B) and were

starting to develop roots and shoots. Approx. 2 weeks after being transferred to Maturation Media, five seedlings from SED2018-96 were transferred out of tissue culture and into potting substrate to harden off under greenhouse conditions. Approx. 5-6 weeks after plating on Differentiation Media, six more seedlings from SED2018-96 were transferred out of tissue culture and into potting substrate for hardening off. Seedlings were maintained in the greenhouse and each was vegetatively propagated to obtain a second "copy" of each genotype. These second "copies" of plants were shipped to UGA, Athens, GA and received 1 May 2019. They were tested using flow cytometry for ploidy level 10 May 2019 and planted in-ground at the Durham Farm in Watkinsville, GA with other triploid seedlings and parental plants on 26 May 2019.

Results

Seedlings treated with 100 μM oryzalin for 2 h resulted in 28 fully and 30 partially tetraploid plants out of the 571 seedlings that were treated between 14 June and 17 Aug. 2017 (Table 1.2). The combined number of plants with some amount of tetraploid stems (fully or partially) was ~10.1% of the total seedlings treated. Approx. 8-10 weeks after treatment, 61.1% of the seedlings were alive (349 plants). The 4 h treatment resulted in 22 fully and 24 partially tetraploid plants that is ~8.2% of the 563 seedlings that were treated during the same calendar dates as the 2 h treatment (Table 1.3). About 8-10 weeks after the 4 h treatment, ~45.8% of seedlings had survived (258 plants). Seedlings treated with 100 μM oryzalin for 6 h resulted in two fully and two partially tetraploid plants out of the 263 (~1.5%) that were treated from 21 Mar. to 4 Apr. 2017 (Table 1.4). Approx. 10 weeks after treating the seedlings for 6 h, only 8.3% were still alive (about 22 plants).

Approximately 1,070 crosses between tetraploid and diploid plants were made between 16 Jan. and 14 Sept. 2018 and 800 of them aborted (~75%). Of the 2,004 putative triploid seed

sown in 2018 and 2019, a total of 433 seedlings tested as triploid (~22%). Putative triploid seed that was sown 28 Mar. 2018 resulted in approx. 33% of seed germinating after 28 days and 29 triploid seedlings, which is ~40% of the total seed sown that day (Table 1.5). Seed sown 7 and 16 June 2018, had 18% germination after 28 days and 13 triploid seedlings (~11% of the seed sown) (Table 1.5). Seed sown during July 2018 had 9% germination after 28 days and 33 triploid seedlings (~12% of the seed sown) (Table 1.5). The average survival percentages for seedlings which germinated in 2018 were: 100% for seed sowed 28 Mar., 54% for seed sowed in June, and 100% for seed sowed in July. The following year, putative triploid seed that was sown 1 Mar. resulted in a 27% germination at 28 days with a 20% survival and 17 triploid seedlings, which is 3.7% of the seed sown (Table 1.6). For putative triploid seed sown 15 Mar., the germination was 51% at 28 days with a 99% survival and 341 seedlings were triploid (31.4% of the seed sown on that date) (Table 1.7).

Discussion

This project did not have an even number of replicates due to differences in the number of seedlings that germinated, therefore the lack of balanced replicates did not allow for a formal experiment to test the effects of oryzalin treatment or different levels of the treatment. Therefore, no statistics were employed to evaluate the data. The main goal was to obtain many triploid plants, which was accomplished. The first oryzalin treatment applied was for 6 h, which seemed too long of an exposure due to the low survival of seedlings averaged over the five seed lots (8.3%). Therefore, the next treatment was for less exposure time to oryzalin with 2 and 4 h tested. None of the seed lots were the same for the three Rounds, therefore the underlying difference of genotypes can be an explanatory factor for different responses to treatment.

The shorter exposure time resulted in higher survival: 61.1% for 2 h and 45.8% for 4 h, which was to be expected. An additional trend with increasing time of exposure was an average decrease (of accessions) in the number of tetraploids per number of seedlings treated: 10.1% for 2 h, 8.2% for 4 h, and 1.5% for 6 h. This reduction of converted tetraploids by oryzalin with decreasing survival percentage was also observed by Li and Ruter (2017). Treated seedlings also displayed a "stagnation stage" as observed by Li and Ruter (2017), which is a delay in growth whereby seedlings are recovering from the "chemical shock" of treatment, as compared to the typical growth rate of a *Hibiscus moscheutos* seedling (although no data was taken). The partially tetraploid plants were kept and used for crossing with diploids because at the start of the study it was not known how many fully tetraploid plants were going to be recovered, and the maximum number of possible tetraploid seed parents was desired. However, having the partially tetraploid plants created some complications when the plants grew to the point of flowering because it was difficult to discern whether new shoots had emerged from tissue that was below the originally treated apical meristem and therefore less likely to be converted, or whether there was re-growth from a non-tetraploid stem that had been removed after testing as non-tetraploid. Diploid shoots have been observed to grow faster than polyploid shoots in a number of species; for example, in Japanese quince (Stanys et al., 2006). This can be (at least partially) explained by cell volume increasing with an increase in genomic content, a phenomenon known as of endopolyploidy (Melaragno et al., 1993). The increased amount of nuclear DNA typically requires more time to replicate and divide, as related to the cell cycle (Van't Hof and Sparrow, 1963; Yildiz, 2013), therefore a chemically-induced polyploid plant often displays slower overall growth (Ranney, 2006).

Due to the incidence of partial tetraploid seed parents, the large volume of putative triploid seed collected and limited resources available for propagating, certain seed accessions and a threshold amount of seed had to be selected for sowing in 2019. Following the first set of putative triploid seed sowing in 2018, the approx. 4,620 putative triploid seed for sowing in 2019 was collected, put into separate envelopes by seed accession/cross date/harvest date, and cataloged. Seed was then sorted by most to least likely to be triploid. This was based on how many seed resulted per fruit (from least to most), whether the seed parent was fully or partially tetraploid, and whether the seed parent from seed sown in 2018 resulted in triploid seed. The number of seed per fruit was used with preference for fewer seed since autopolyploids typically are less fertile (Ranney, 2006). There is a further approx. 2,700 putative triploid seed (73 seed lots by accession no./cross date/harvest date) that have not been sown.

The percent germination of putative triploid seed inherently reflects the ideal time to sow *Hibiscus moscheutos* seed, which is typically spring in the southeast U.S. The March sow dates had higher germination percentages at 33% (2018), 27% and 51% (2019), compared to June and July 2018 with 18% and 9%, respectively. Once seedlings germinated, they generally had a high survival percentage: 100%, 54%, and 100% for Mar., June and July 2018, respectively, and 99% for 15 Mar. 2019. Seed sown 1 and 15 Mar. 2019 was prioritized by presumed likelihood to be triploid (as described above), which could have affected the lower germination of 27% for the first seed lots and a 51% germination for the second group of seed lots sown. The seed sown on 1 Mar. likely had more triploid seed, but also had some physiological barriers to germination. Seedlings that germinated from seed sown 1 Mar. 2019 had a survival rate of 20%, which is uncharacteristically low given that the seed were acid scarified. This was a result of improper liquid fertilizer rate application. The fertilizer rate was extremely high and the seedlings died as a

result. The low survival rate is correspondingly reflected in the low % triploid of the seed sown (3.7%) because the seedlings died before being tested for ploidy. Some of the putative triploid seedlings tested for ploidy appeared to be between diploid and triploid or between triploid and tetraploid. This would suggest they are aneuploids, meaning they do not have a complete three-copy set of each chromosome, they either lack a copy or have more than three copies of some chromosomes (Acquaah, 2007). This likely occurred either as a result of improper pairing during fertilization of the chromosomes of the tetraploid by diploid cross or from improper division during meiosis of the tetraploid seed parent.

As mentioned, the main goal of this project was to obtain multiple triploid plants, therefore time and resources were not allocated to taking phenotypic or morphological data on the converted polyploids (4x or 3x). This data could, however, be valuable to further the body of information of induced polyploids, particularly for ornamental taxa and this popular species. Potential traits of interest that could be evaluated in this polyploid population (3x and 4x) are: leaf area, leaf thickness, leaf greenness, flower size, flower color, plant size, duration of flowering, timing of flower initiation, pollen viability, and timing of foliar senescence. Modifications (compared to a diploid plant) of similar morphological traits were observed in the *H. moscheutos* cultivar ‘Luna Red’ by Li and Ruter (2017). A hindrance to taking data on the plants that tested as tetraploids in 2018 is the occurrence of the tissue reverting back to its natural diploid state. Two plants which tested as tetraploid in 2018 were tested Spring 2019 and the results indicated they reverted to diploid. This is not uncommon for chemically-induced autopolyploids to be cytochimeras where not all the tissue layers were doubled in ploidy (Ranney, 2006). Since only leaf tissue was tested in this study, the root tissue could have remained 2x and when shoots emerged Spring 2019 they originated from the diploid root tissue.

Triploid plants of ‘Luna Red’ were found to have a longer duration of flowering, had non-viable pollen, were female infertile and exhibited resistance to aerial phytophthora as compared to diploids (Li and Ruter, 2017). The reproductive status of the triploid plants developed for this study would be an important trait to collect data on, such as pollen viability, fruit set, seed production and viability. A few triploid plants from seed that was sown in March 2018 flowered later in the season of the same year. From these plants a few flowers were noticed to lack pollen (Figure 1.1) and after the flowers were observed to fall off, no fruit formed. Although not likely to have potential as a cultivar, a few small octoploid plants were recovered from oryzalin treatment and had very wrinkled/warped leaf and corolla textures, blistered stems surfaces, reduced flower diameter, compacted stamens on the staminal column and a very asymmetrical form. Figure 1.2 shows an example of an octoploid plant treated for 6 h. These few plants were not kept when plants were moved to the Durham Farm because they did not yield fruit.

Embryo Rescue/Ovule Culture Work

The 11 seedlings received from Star Roses and Plants tested as triploid via flow cytometry when compared to diploid and tetraploid genotypes. The only fruit to have embryos develop was from a cross made 19-20 d prior to separating from the female plant (Table 1.8). At the time of removing the ovules for tissue culture, the fruit was slightly opening along the edges, unlike the other nine fruits which were closed. Since the only fruit out of the ten to develop viable embryos was harvested 19-20 d after pollination, it would seem the ideal time to remove fruit for embryo rescue/ovule culture would be (at least) more than six days. Interestingly, the seed accession number SED2018-96 which resulted in the 11 genotypes from embryo rescue/ovule culture had another fruit from a pollination made the same day (27 Aug. 2018). There were 20 seed from this

second fruit that were sown 15 Mar. 2019 at the Durham Farm but none germinated. This brief study also demonstrates it is possible to recover seedlings from embryo rescue of a tetraploid × diploid cross of *Hibiscus moscheutos* hybrids.

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Table 1.1. Parent plants of seedlings treated for polyploidy induction and their corresponding seed lot name. Parents were intraspecific hybrids of *Hibiscus moscheutos* subsp. *moscheutos*, unless otherwise indicated.

Round used in	**Name of seed lot**	**Female parent**	**Male parent**	**Number of seed**
1	HibSED2016-382	Hib2016-6	Hib2014-113	646
1	HibSED2016-386	Hib2016-8	Hib2014-85[z]	539
1	HibSED2016-388	Hib2016-1	*Hibiscus* 'Robert Fleming'	771
1	HibSED2016-395	Hib2015-11[y]	Hibiscus 1-7[x]	587
1	HibSED2016-410	Hib2015-45[w]	Hib2014-54[z]	700+
2	HibSED2016-334	⊗ Hibiscus 1-5 [x,v]		587
2	HibSED2016-378	Hib2016-16	Hib2015-123[u]	639
2	HibSED2016-379	Hib2016-6	*Hibiscus* 'Robert Fleming'	676
2	HibSED2016-391	Hib2016-9	Hib2014-85[z]	500
2	HibSED2016-396	Hib2015-29[w]	*Hibiscus* 'Robert Fleming'	700+
3	HibSED2016-358	⊗ *Hibiscus* 'Cherry Brandy'		n/a
3	HibSED2016-366	*Hibiscus* 'Robert Fleming'	Hib2016-4	676
3	HibSED2016-392	Hib2016-9	*Hibiscus* 'Cherry Brandy'	465
3	HibSED2016-394	Hib2016-2	*Hibiscus* 'Cherry Brandy'	n/a
3	HibSED2016-405	⊗ Hib2015-45[z]		700+
3	HibSED2016-411	O.P. of Hib2015-45 [z,t]		1,000+
3	HibSED2016-456	⊗ Hib2015-124[u]		n/a

[z] This plant has the cultivar *Hibiscus* ‘Midnight Marvel’ in its genetic background.

[y] This plant has the cultivar *Hibiscus* ‘Summer Storm’ in its genetic background.

[x] This plant originated from a cross between *Hibiscus* ‘Crown Jewels’ and the experimental genotype Hibiscus 13-19.

[w] This plant has *Hibiscus moscheutos* subsp. *lasiocarpos* in its genetic background.

[v] The symbol ⊗ represents selfing a plant, i.e. making a cross of the same genotype.

[u] This plant has *Hibiscus grandiflorus* in its genetic background.

[t] The abbreviation O.P. stands for open pollinated, which refers to the male parent being unknown. Seed was collected from the female parent.

n/a Stands for not available. Seed count was not recorded.

Table 1.2. Data by seed lot of seedlings treated with oryzalin (100 μM) for 2 h of intraspecific hybrids of *Hibiscus moscheutos* subsp. *moscheutos*. Seedlings which were at the cotyledon stage were treated over nine dates (14 and 29 June; 11, 14, 19, and 25 July; 2, 9, and 17 Aug.) and values below are totaled over the nine treatment dates.

Seed lot		Round used in[z]	Germination %[y]	No. seedlings treated	% Survival[x]	No. fully tetraploid[w]	No. partially tetraploid[v]	No. mixoploids[u]	No. diploids	% Tetraploid[t]
Hib. SED16 -	334	2	6%	1	0.0%	-	-	-	-	-
Hib. SED16 -	378	2	8%	15	66.7%	2	2	4	2	26.7%
Hib. SED16 -	379	2	4%	10	70.0%	0	0	1	6	0.0%
Hib. SED16 -	391	2	16%	14	66.7%	3	2	1	2	35.7%
Hib. SED16 -	396	2	4%	2	0.0%	-	-	-	-	-
Hib. SED16 -	358	3	12%	58	58.6%	2	2	9	7	6.9%
Hib. SED16 -	366	3	0%	7	71.4%	2	0	2	1	28.6%
Hib. SED16 -	392	3	8%	14	64.3%	0	2	3	3	14.3%
Hib. SED16 -	394	3	4%	33	75.7%	6	7	2	3	39.4%
Hib. SED16 -	405	3	36%	107	89.7%	1	4	6	12	4.7%
Hib. SED16 -	411	3	32%	208	83.2%	6	7	7	8	6.2%
Hib. SED16 -	456	3	32%	102	87.2%	6	4	3	2	9.8%
Totals and average % tetraploid:				571		28	30	38	46	10.1%

[z] The Round that seedlings were used in refers to seed being sown on two dates: 30 May for Round 2 and 30 June 2017 for Round 3.

[y] Germination % for Round 2 = (no. seedlings with expanded cotyledons at 30 d after sowing / 50) × 100; for Round 3, Germination % = (no. seedlings with expanded cotyledons at 32 d after sowing / 25) × 100.

[x] Percent survival = (no. of seedlings surviving averaged over 8 and 10 weeks after treatment / no. of seedlings treated) × 100.

[w] Fully tetraploid refers to the status of all of the main stems on the plant testing as tetraploid (as compared to a diploid genotype) via flow cytometry.

[v] Partially tetraploid refers to the status of not all of the main stems on the plant testing as tetraploid (as compared to a diploid genotype) via flow cytometry.

[u] Mixoploids refers to plants having cytochimeras where multiple ploidy levels exist within the plant and typically in different histogenic layers (i.e., L-I, II, and III).

[t] Percent tetraploid = [(no. fully tetraploid plants + no. partially tetraploid plants) / no. seedlings treated] × 100.

Table 1.3. Data by seed lot of seedlings treated with oryzalin (100 μM) for 4 h of intraspecific hybrids of *Hibiscus moscheutos* subsp. *moscheutos*. Seedlings which were at the cotyledon stage were treated over nine dates (14 and 29 June; 11, 14, 19, and 25 July; 2, 9, and 17 Aug.) and values below are totaled over the nine treatment dates.

Seed lot		**Round used in[z]**	**Germination % [y]**	**No. seedlings treated**	**% Survival [x]**	**No. fully tetraploid [w]**	**No. partially tetraploid[v]**	**No. mixoploids[u]**	**No. diploids**	**% Tetraploid[t]**
Hib. SED16 -	334	2	6%	1	0.0%	-	-	-	-	-
Hib. SED16 -	378	2	8%	15	53.3%	1	1	3	2	13.3%
Hib. SED16 -	379	2	4%	10	80.0%	1	2	3	2	30%
Hib. SED16 -	391	2	16%	13	61.5%	1	1	1	5	15.4%
Hib. SED16 -	396	2	4%	2	0.0%	-	-	-	-	-
Hib. SED16 -	358	3	12%	54	29.6%	2	3	8	2	9.2%
Hib. SED16 -	366	3	0%	7	85.7%	0	2	2	2	28.6%
Hib. SED16 -	392	3	8%	14	14.3%	1	0	0	1	7.1%
Hib. SED16 -	394	3	4%	33	30.3%	0	2	4	2	6.1%
Hib. SED16 -	405	3	36%	106	63.2%	1	4	7	8	4.7%
Hib. SED16 -	411	3	32%	206	48.1%	4	5	5	4	4.4%
Hib. SED16 -	456	3	32%	102	84.3%	11	4	3	0	14.7%
Totals and average % tetraploid:				563		22	24	36	28	8.2%

[z] The Round that seedlings were used in refers to seed being sown on two dates: 30 May for Round 2 and 30 June 2017 for Round 3.

[y] Germination % for Round 2 = (no. seedlings with expanded cotyledons at 30 d after sowing / 50) × 100; for Round 3, Germination % = (no. seedlings with expanded cotyledons at 32 d after sowing / 25) × 100.

[x] Percent survival = (no. of seedlings surviving averaged over 8 and 10 weeks after treatment / no. of seedlings treated) × 100.

[w] Fully tetraploid refers to the status of all of the main stems on the plant testing as tetraploid (as compared to a diploid genotype) via flow cytometry.

[v] Partially tetraploid refers to the status of not all of the main stems on the plant testing as tetraploid (as compared to a diploid genotype) via flow cytometry.

[u] Mixoploids refers to plants having cytochimeras where multiple ploidy levels exist within the plant and typically in different histogenic layers (i.e., L-I, II, and III).

[t] Percent tetraploid = [(no. fully tetraploid plants + no. partially tetraploid plants) / no. seedlings treated] × 100.

Table 1.4. Data by seed lot of seedlings treated with oryzalin (100 μM) for 6 h of intraspecific hybrids of *Hibiscus moscheutos* subsp. *moscheutos*. Seedlings which were at the cotyledon stage were treated over three dates (21 and 28 Mar. and 4 Apr. 2017) and values below are totaled over the three treatment dates.

Seed lot		**Germination %[z]**	**No. seedlings treated**	**% Survival[y]**	**No. fully tetraploid[x]**	**No. partially tetraploid[w]**	**No. diploids or mixoploids**	**% Tetraploid[v]**
Hib. SED16 -	382	8%	36	5.6%	0	1	1	2.8%
Hib. SED16 -	386	4%	9	22.2%	1	0	1	11.1%
Hib. SED16 -	388	12%	35	0.0%	0	0	0	0.0%
Hib. SED16 -	395	20%	38	10.5%	0	1	2	2.6%
Hib. SED16 -	410	16%	145	3.4%	1	0	1	0.7%
Totals and average % tetraploid:			263		2	2	5	1.5%

[z] Germination % = (no. seedlings with expanded cotyledons at 32 d after sowing / 100) × 100.

[y] Percent survival = (no. of seedlings surviving at 10 weeks after treatment / no. of seedlings treated) × 100.

[x] Fully tetraploid refers to the status of all of the main stems on the plant testing as tetraploid (as compared to a diploid genotype) via flow cytometry.

[w] Partially tetraploid refers to the status of not all of the main stems on the plant testing as tetraploid (as compared to a diploid genotype) via flow cytometry.

[v] Percent tetraploid = [(no. fully tetraploid plants + no. partially tetraploid plants) / no. seedlings treated] × 100.

Table 1.5. Data of putative triploid hybrid *Hibiscus moscheutos* seed in 2018. Seed resulted from crossing a tetraploid seed parent and a diploid pollinator parent. Ploidy levels were verified by flow cytometry with known ploidy-level hibiscus plants.

SED18-	Cross date	Harvest date	Date sowed	No. seed collected	No. seed sowed	% Germ. at 28 d	% Survival at 28 d	No. triploids	% Triploid of no. sowed
1	1/29/2018	2/24/2018	3/28/2018	14	14	0%	-	-	-
2	1/16 & 29/2018	3/3 & 11/2018	3/28/2018	29	29	14%	100%	4	14%
3	2/17/2018	3/27/2018	3/28/2018	29	29	86%	100%	25	86%
6	5/12/2018	5/31/2018	6/7/2018	11	8	0%	-	-	-
7	5/2/2018	5/31/2018	6/7/2018	21	21	43%	78%	7	33%
7	5/16/2018	6/12/2018	6/13/2018	21	21	0%	-	-	-
8	4/17/2018	5/31/2018	6/7/2018	2	2	0%	-	-	-
8	4/27/2018	5/31/2018	6/7/2018	10	9	22%	0%	-	-
8	5/16/2018	6/12/2018	6/13/2018	9	8	0%	-	-	-
9	5/14/2018	6/4/2018	6/7/2018	10	9	0%	-	-	-
10	4/24/2018	6/4/2018	6/7/2018	5	4	0%	-	-	-
12	5/16/2018	6/11/2018	6/13/2018	17	17	65%	64%	0	0%
13	5/10/2018	6/10/2018	6/13/2018	15	15	53%	75%	6	40%
14	6/14/2018	7/2/2018	7/12/2018	11	11	0%	-	-	-
15	6/14/2018	7/2/2018	7/12/2018	3	3	0%	-	-	-
16	6/14/2018	7/9/2018	7/12/2018	53	52	4%	100%	2	4%

SED18-	Cross date	Harvest date	Date sowed	No. seed collected	No. seed sowed	% Germ. at 28 d	% Survival at 28 d	No. triploids	% Triploid of no. sowed
17	6/13/2018	7/9/2018	7/12/2018	22	21	5%	100%	1	5%
18	6/10/2018	7/3/2018	7/12/2018	2	2	0%	-	-	-
18	6/11/2018	7/4/2018	7/12/2018	14	14	14%	100%	2	14%
18	6/20/2018	7/3/2018	7/12/2018	2	2	0%	-	-	-
19	6/27/2018	7/11/2018	7/20/2018	3	3	0%	-	-	-
20	6/18/2018	7/16/2018	7/20/2018	1	1	0%	-	-	-
20	6/20/2018	7/16/2018	7/20/2018	20	20	65%	100%	13	65%
21	6/22/2018	7/16/2018	7/20/2018	16	16	0%	-	-	-
22	6/26/2018	7/17/2018	7/20/2018	35	35	3%	100%	0	0%
23	6/29/2018	7/17/2018	7/20/2018	3	3	0%	-	-	-
24	6/24/2018	7/17/2018	7/20/2018	32	32	28%	100%	8	25%
24	6/25/2018	7/17/2018	7/20/2018	27	24	4%	100%	1	4%
25	6/26/2018	7/18/2018	7/20/2018	32	31	19%	100%	6	19%
Totals and average %'s:				469	456	15%	87%[z]	75	16.4%[y]

[z] The average survival percentage is of accession numbers which had seedlings germinate, it excludes those which had 0% germination.

[y] The average % triploid = total number of triploids / total number of seed sowed.

Table 1.6. Data of putative triploid hybrid *Hibiscus moscheutos* seed sowed 1 Mar. 2019. Seed resulted from crossing a tetraploid seed parent and a diploid pollinator parent. Seed were bulked by accession number/cross date/harvest date. Ploidy levels were verified by flow cytometry with known ploidy-level hibiscus plants.

SED18-	Cross date	Harvest date	No. fruit	No. seed collected	No. seed sowed	% Germ. at 28 d	% Survival at 28 d	No. triploids	% Triploid of no. sowed
82	7/13/2018	7/27/2018	1	1	1	0%	-	-	-
126	7/9/2018	7/24/2018	1	1	1	0%	-	-	-
127	7/20/2018	8/5/2018	1	1	1	0%	-	-	-
129	9/14/2018	10/17/2018	1	1	1	100%	100%	1	100%
60	9/10/2018	10/25/2018	1	2	2	0%	-	-	-
62	7/20/2018	8/7/2018	2	2	2	0%	-	-	-
83	9/14/2018	10/25/2018	2	2	2	50%	0%	-	-
130	7/9/2018	7/29/2018	1	2	2	0%	-	-	-
130	7/9/2018	8/4/2018	1	2	2	100%	50%	1	50%
173	8/29/2018	9/16/2018	1	2	2	0%	-	-	-
30	7/4/2018	7/22/2018	1	3	3	0%	-	-	-
41	9/13/2018	10/12/2018	1	3	3	67%	0%	-	-
61	7/9/2018	7/26/2018	1	3	3	0%	-	-	-
80	7/28/2018	8/27/2018	1	3	3	0%	-	-	-
100	7/28/2018	9/5/2018	1	3	3	33%	0%	-	-

SED18-	Cross date	Harvest date	No. fruit	No. seed collected	No. seed sowed	% Germ. at 28 d	% Survival at 28 d	No. triploids	% Triploid of no. sowed
101	8/29/2018	10/9/2018	1	3	3	0%	-	-	-
129	7/9/2018	7/29/2018	1	3	3	0%	-	-	-
131	7/13/2018	8/7/2018	1	3	3	0%	-	-	-
27	6/28/2018	7/20/2018	1	4	4	75%	33%	1	25%
92	7/20/2018	8/16/2018	1	4	4	0%	-	-	-
132	7/10/2018	7/29/2018	1	4	4	0%	-	-	-
40	9/10/2018	10/12/2018	1	5	5	80%	25%	1	20%
56	9/5/2018	10/3/2018	1	5	5	80%	25%	1	20%
61	7/23/2018	8/9/2018	1	5	5	0%	-	-	-
65	9/13/2018	10/12/2018	1	5	5	80%	0%	-	-
92	7/20/2018	8/4/2018	1	5	5	0%	-	-	-
151	9/14/2018	10/9/2018	1	5	5	40%	100%	2	40%
59	7/28/2018	8/25/2018	1	6	6	0%	-	-	-
80	7/22/2018	8/25/2018	1	7	7	0%	-	-	-
112	7/13/2018	8/7/2018	1	7	7	0%	-	-	-
131	8/14/2018	9/8/2018	1	7	7	14%	0%	-	-
39	9/5/2018	10/3/2018	1	8	8	38%	0%	-	-
161	8/14/2018	9/13/2018	1	8	8	88%	0%	-	-
44	7/22/2018	8/7/2018	1	9	9	0%	-	-	-

SED18-	Cross date	Harvest date	No. fruit	No. seed collected	No. seed sowed	% Germ. at 28 d	% Survival at 28 d	No. triploids	% Triploid of no. sowed
57	7/10/2018	8/4/2018	1	9	9	33%	67%	0	0%
63	7/28/2018	8/25/2018	1	9	9	33%	0%	-	-
66	8/14/2018	9/7/2018	1	9	9	0%	-	-	-
76	9/14/2018	10/17/2018	1	9	9	22%	0%	-	-
97	7/28/2018	8/10/2018	1	9	9	0%	-	-	-
169	9/5/2018	10/2/2018	1	9	9	78%	0%	-	-
171	9/10/2018	10/12/2018	1	9	9	89%	12%	0	0%
81	8/14/2018	9/11/2018	1	10	10	10%	0%	-	-
90	7/10/2018	8/4/2018	1	10	10	0%	-	-	-
162	8/17/2018	9/11/2018	1	10	10	90%	0%	-	-
93	7/22/2018	8/16/2018	1	11	11	0%	-	-	-
95	7/23/2018	8/5/2018	1	11	11	0%	-	-	-
128	7/28/2018	8/25/2018	1	11	11	36%	50%	2	18%
34	7/18/2018	8/14/2018	1	12	12	17%	50%	1	8%
64	8/14/2018	9/11/2018	1	12	12	50%	17%	-	-
73	7/18/2018	8/4/2018	1	13	13	0%	-	-	-
79	8/14/2018	9/8/2018	1	13	13	54%	0%	-	-
95	7/23/2018	8/25/2018	1	13	13	0%	-	-	-
54	8/14/2018	9/8/2018	1	14	14	79%	9%	1	7%

SED18-	Cross date	Harvest date	No. fruit	No. seed collected	No. seed sowed	% Germ. at 28 d	% Survival at 28 d	No. triploids	% Triploid of no. sowed
150	9/10/2018	10/9/2018	1	14	14	64%	22%	4	29%
116	9/14/2018	10/25/2018	2	21	21	19%	0%	-	-
55	8/14/2018	9/8/2018	2	26	26	46%	17%	2	8%
44	9/5/2018	10/9/2018	2	28	28	0%	-	-	-
45	8/22/2018	9/22/2018	3	35	35	3%	0%	-	-
Totals and average %'s:			65	461	461	27%	20%[z]	17	3.7%[y]

[z] The average survival percentage is of accession numbers which had seedlings germinate, it excludes those which had 0% germination.

[y] The average % triploid = total number of triploids / total number of seed sowed.

Table 1.7. Data of putative triploid hybrid *Hibiscus moscheutos* seed sowed 15 Mar. 2019. Seed resulted from crossing a tetraploid seed parent and a diploid pollinator parent. Seed were bulked by accession number/cross date/harvest date. Ploidy levels were verified by flow cytometry with known ploidy-level hibiscus plants.

SED18-	Cross date	Harvest date	No. fruit	No. seed collected	No. seed sowed	% Germ. at 28 d	% Survival at 28 d	No. triploids	% Triploid of no. sowed
38	8/17/2018	9/3/2018	1	15	14	0%	-	-	-
120	8/14/2018	9/7/2018	1	15	14	64%	100%	9	64%
145	8/29/2018	9/22/2018	1	15	15	73%	100%	11	73%
73	7/18/2018	8/14/2018	1	16	16	63%	100%	10	63%
42	7/13/2018	8/4/2018	1	18	18	0%	-	-	-
148	8/29/2018	9/27/2018	1	18	18	67%	100%	12	67%
168	8/22/2018	9/22/2018	1	18	17	94%	100%	0	0%
172	9/5/2018	10/3/2018	1	18	18	89%	100%	0	0%
64	8/14/2018	9/8/2018	1	20	20	80%	100%	16	80%
96[z]	8/27/2018	9/27/2018	1	20	20	0%	-	-	-
115	9/5/2018	10/2/2018	1	20	20	15%	67%	2	10%
115	9/5/2018	10/12/2018	1	20	20	25%	100%	5	25%
143	7/22/2018	8/14/2018	1	20	20	70%	93%	12	60%
91	7/18/2018	8/11/2018	1	21	21	24%	100%	5	24%
113	7/18/2018	8/14/2018	1	21	20	20%	100%	3	15%

SED18-	Cross date	Harvest date	No. fruit	No. seed collected	No. seed sowed	% Germ. at 28 d	% Survival at 28 d	No. triploids	% Triploid of no. sowed
78	7/23/2018	8/16/2018	1	22	22	14%	100%	2	9%
114	7/28/2018	8/22/2018	1	22	22	14%	100%	2	9%
146	9/10/2018	10/9/2018	1	23	23	87%	100%	18	78%
58	7/13/2018	8/7/2018	1	24	24	4%	100%	0	0%
109	8/14/2018	9/3/2018	1	24	24	0%	-	-	-
121	8/22/2018	9/16/2018	1	24	24	58%	100%	14	58%
149	9/5/2018	10/2/2018	1	24	24	50%	100%	11	46%
75	8/14/2018	9/11/2018		50	25	84%	100%	20	80%
77	7/22/2018	8/25/2018	1	26	26	12%	100%	2	8%
78	7/23/2018	8/25/2018	1	26	25	52%	100%	12	48%
144	8/17/2018	9/14/2018	1	26	26	77%	95%	11	42%
94	7/23/2018	8/25/2018	1	27	25	44%	100%	11	44%
147	8/13/2018	9/8/2018	2	54	25	84%	100%	18	72%
52	8/14/2018	9/11/2018	1	28	25	60%	100%	14	56%
74	7/23/2018	7/25/2018	4	112	25	100%	100%	25	100%
98	8/13/2018	9/7/2018	1	29	25	56%	100%	13	52%
144	8/17/2018	9/11/2018	2	59	25	72%	89%	16	64%
99	8/22/2018	9/7/2018	1	30	25	0%	-	-	-
111	8/29/2018	9/27/2018	1	30	26	23%	100%	6	23%

SED18-	Cross date	Harvest date	No. fruit	No. seed collected	No. seed sowed	% Germ. at 28 d	% Survival at 28 d	No. triploids	% Triploid of no. sowed
110	8/17/2018	9/11/2018	1	30	24	21%	100%	5	21%
53	9/14/2018	10/12/2018	1	31	25	12%	100%	3	12%
71	7/9/2018	8/5/2018	1	31	25	84%	100%	21	84%
167	8/17/2018	9/11/2018	1	32	27	93%	100%	0	0%
72	7/13/2018	8/10/2018	1	34	24	54%	100%	12	50%
97	7/28/2018	8/11/2018	1	70	25	0%	-	-	-
144	7/24/2018	8/16/2018	1	36	25	80%	100%	20	80%
174	7/3/2018	7/28/2018	1	37	25	56%	100%	0	0%
170	8/27/2018	9/22/2018	1	44	25	96%	100%	0	0%
28	6/27/2018	7/21/2018	1	46	25	84%	95%	0	0%
133	8/17/2018	9/11/2018	3	147	25	40%	100%	0	0%
61	7/9/2018	8/5/2018	1	58	25	92%	100%	0	0%
160	8/27/2018	9/22/2018	1	58	25	76%	100%	0	0%
159	8/20/2018	9/13/2018	1	69	25	68%	100%	0	0%
Totals and average %'s:			54	1,658	1,087	51%	99%[y]	341	31.4%[x]

[z] This seed accession number had another fruit from the same cross date which was used in embryo rescue and resulted in 11 seedlings.

[y] The average survival percentage is of accession numbers which had seedlings germinate, it excludes those which had 0% germination.

[x] The average % triploid = total number of triploids / total number of seed sowed.

Table 1.8. Data of the immature fruit of hybrid *Hibiscus moscheutos* used in embryo rescue/ovule culture which resulted from crossing a tetraploid seed parent and a diploid pollinator parent. Immature fruit were harvested from the University of Georgia's Trial Gardens greenhouse in Athens, GA. Ovules or embryos were plated 18 Sept. 2018 on Differentiation Media at Star Roses and Plants, West Grove, PA.

SED2018-	**Female (Hib. SED2016-**	**Hours treated**	**Rep.#**	**Date treated**	**Male (Hib. SED2016-**	**Hours treated**	**Rep.#**	**Date treated**	**Cross date**	**Approx. days from cross to harvest**	**No. seedlings recovered**
96	392	4	1	7/11/17	396	2	9	6/14/17	8/27/18	19-20	11
32	358	2	7	7/11/17	440	n/a	111	n/a	9/10/18	5-6	0
175	405	4	2	7/19/17	366	n/a	1	n/a	9/13/18	2-3	0
176	411	2	5	8/2/17	366	n/a	1	n/a	9/13/18	2-3	0
177	366	2	2	7/11/17	392	2	8	7/11/17	9/13/18	2-3	0
178	394	2	4	7/11/17	392	2	8	7/11/17	9/13/18	2-3	0
179	394	2	8	7/11/17	392	2	8	7/11/17	9/13/18	2-3	0
180	396	2	25	6/14/17	366	n/a	1	n/a	9/14/18	3-4	0
181	396	2	11	6/14/17	440	n/a	111	n/a	9/10/18	5-6	0
182	456	2	6	7/14/17	392	2	8	7/11/17	9/14/18	3-4	0

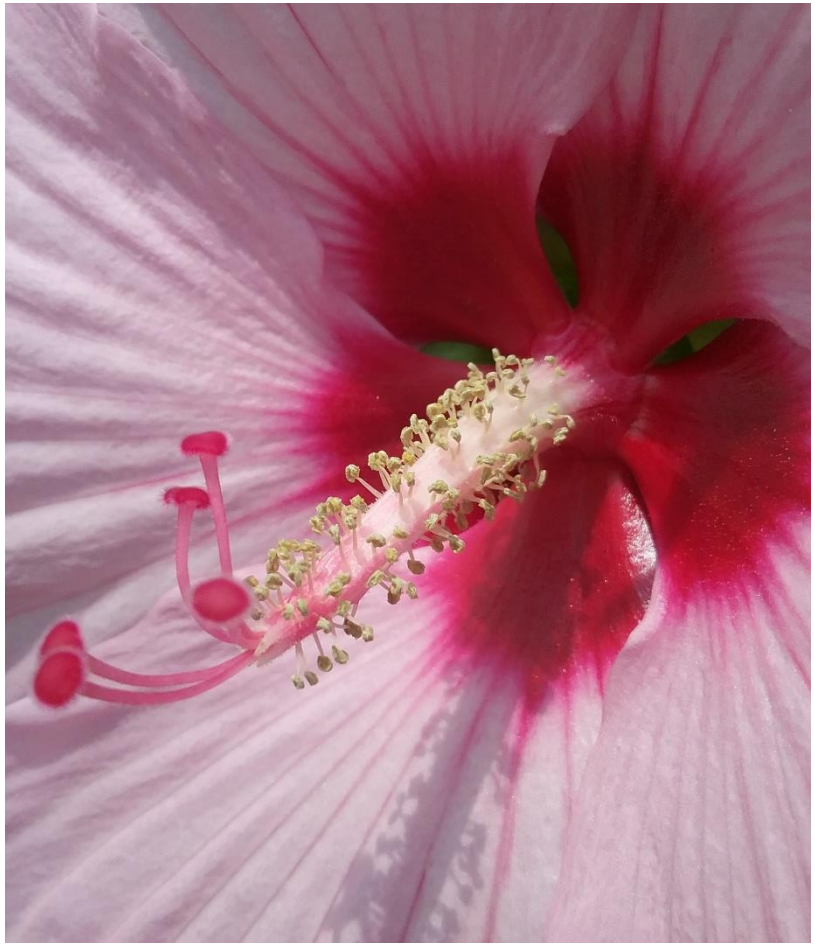

Figure 1.1. Flowers on triploid plants from the seed lot SED18-3. Flower from replicate #15 (left) appears to lack pollen and the corolla from replicate #16 (right) dropped later in the day, leaving the remaining calyx with ovary unfilled. Photos by author.

Figure 1.2. Plant treated at cotyledon stage for 6 h with 100 µM oryzalin on 21 Mar. 2017. Flow cytometry test results showed the plant to be octoploid (8x). Photos taken 5 April 2018 by author.

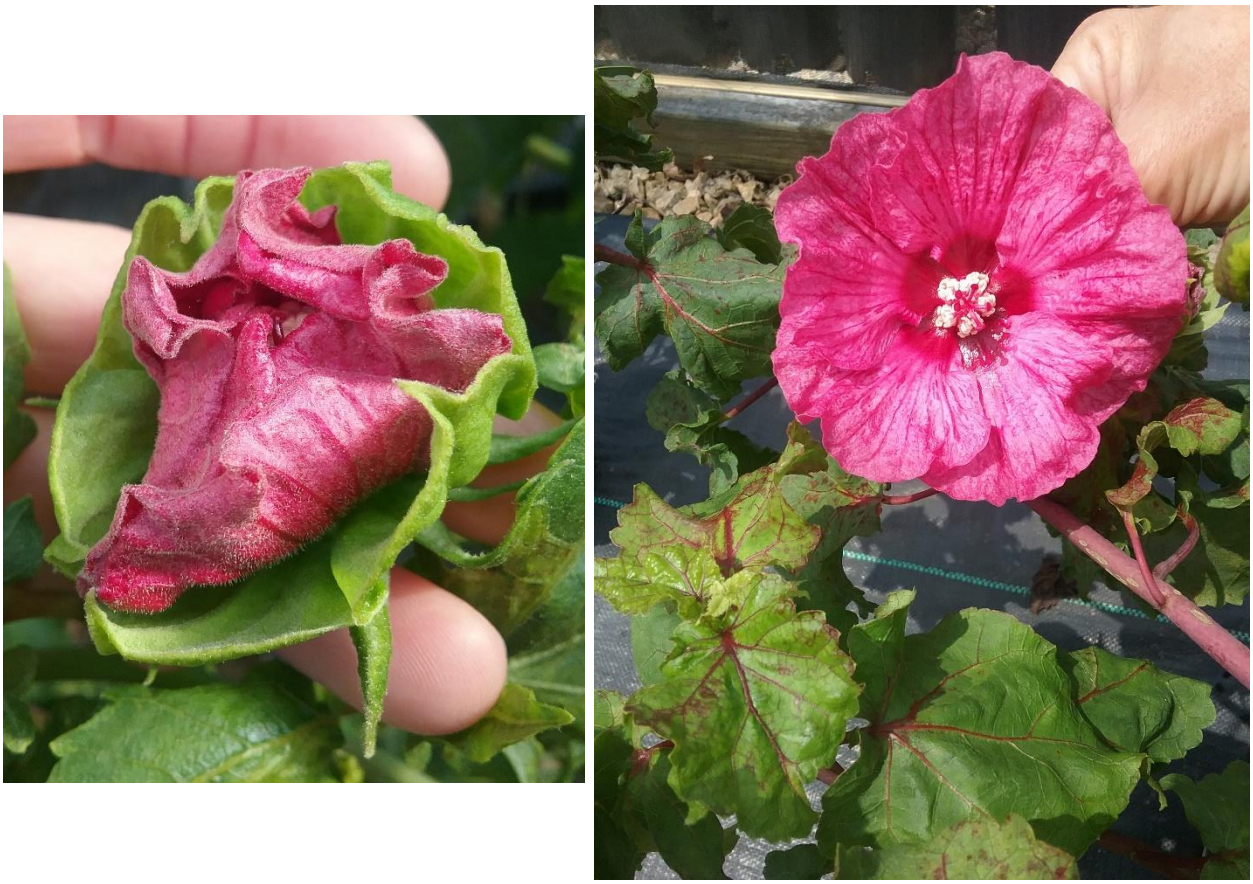

Figure 1.2 (cont'd). Plant treated at cotyledon stage for 6 h with 100 µM oryzalin on 21 Mar. 2017. Flow cytometry test results showed the plant to be octoploid (8x). Photo on left taken 5 April 2018 and photo on right taken 19 Aug. 2018 by author.

CHAPTER 2

Evaluation of Hibiscus Sawfly (*Atomacera decepta*) Damage and Leaf Pubescence of *Hibiscus moscheutos*, *H. grandiflorus*, and *H. moscheutos* hybrids

Introduction

Hardy hibiscus is a popular ornamental shrub due to its attractive and sizeable blooms and cold-hardy nature. The combined subspecies of *H. moscheutos* subsp. *moscheutos* and subsp. *lasiocarpos* are North American-native perennial shrubs with a wide geographic range from the northern regions of Ontario and Indiana, south to Florida and Texas (Blanchard, 2008; FNA, 2019a). The closely related *H. grandiflorus* is also native to North America, specifically to the southeastern region where it can be found growing naturally near fresh or brackish marshes and to some extent in cultivated landscapes for its large light pink blooms, velvety leaves, and grand stature (Christman, 2008; FNA, 2019b). A pest of some *Hibiscus* species is the hibiscus sawfly (*Atomacera decepta*), which if allowed to reach a sufficient population, can defoliate entire shrubs in a matter of days (Hiskes, 2014; Rohwer, 1911; Tippins, 1965). Experimental hibiscus in Blairsville, GA with breeding backgrounds including the two subspecies of *H. moscheutos* and *H. grandiflorus* were severely damaged in 2013 by the hibiscus sawfly, however it was observed that the impact was not even across phenotypes. Plants with more leaf pubescence were noted to have less damage. Increased or abundant foliage pubescence has been attributed to reduced insect damage and/or egg oviposition in other species. Several examples of foliar pubescence correlated with reduced insect damage include: soybean from potato leafhopper (*Empoasca fabae*) (Broersma, 1972), wheat from cereal leaf beetle (*Oulema melanopus*) (Gallun et al., 1966;

Schillinger and Gallun, 1968), domesticated tomato and a wild relative from whitefly (*Bemisia* spp.) (Sánchez-Peña et al., 2006), and cotton from tarnished plant bug (*Lygus lineolaris*) (Wood et al., 2017). Leaf pubescence, comprised of trichomes on the epidermis of a plant, serves as a barrier from insects in several ways inhibiting feeding, attachment, oviposition or movement (Norris and Kogan, 1980). Three historical terms to describe the means (or mechanisms) of a plant to handle arthropod attacks are antixenosis, antibiosis and tolerance (Painter, 1951; Smith, 2005). Antixenosis refers to plant traits affecting herbivore behavior that reduces the acceptance/desirability of a plant as a host. This is also called, 'nonpreference', and could be a morphological or chemical trait of the plant. Antibiosis describes adverse effects of resistant plants on the physiology and life history of an herbivore such as reduced growth, survival and fecundity. Tolerance is defined as the ability of the plant to withstand or recover from insect damage to which susceptible plants are also subjected (Painter, 1951; Smith, 2005). These are not the only means of describing plant host-arthropod interactions, but they were some of the first published and adopted. Certain *Hibiscus* species have been evaluated for sawfly damage (Boyd and Cheatham, 2004), but little focus has been placed on determining the trait which is likely leading to the reduced damage. The objective of this study is to evaluate feeding damage from hibiscus sawfly on plants having different degrees of leaf pubescence ranging from no pubescence to extremely pubescent of hibiscus hybrids and *Hibiscus* spp. in the hybrids' backgrounds at two locations in GA. By evaluating feeding damage in response to pubescence or a lack of pubescence, this trait could prove useful for the hardy hibiscus breeding program.

Materials and Methods

Plants for the study were first chosen, propagated, and then planted in two locations: Blairsville and Watkinsville, GA. Blairsville is located in zone 7a on the USDA Plant Hardiness Map with

average extreme minimum temperatures of -15 to -17.8°C, whereas Watkinsville is in zone 8a with minimum temperatures of -9.4 to -12.2°C (USDA, 2019). The two experimental plots are part of the University of Georgia (UGA): the Georgia Mountain Research and Education Center (Mtn. Station) in Blairsville, GA and the Durham Horticultural Farm (Hort Farm) in Watkinsville, GA. The plot at the Mtn. Station is approx. 599 m above sea level in Union County and located at 34°50' 20.6"N, 83°55' 37.5"W. The soil consists of a fine sandy loam from 0-15 cm (depth) and is considered Clifton-Evard complex (USDA, 2018). The plot at the Hort Farm is ~230 m above sea level in Oconee County and located at 33°53’11.4”N, 83°25’13.2”W. The soil consists of a sandy loam from 0-10 cm (depth) and is considered Cecil, moderately eroded (USDA, 2018). Plants for the study were selected (Table 2.1) to satisfy the objective based on the observed range of leaf pubescence, therefore a rating system was utilized. A rating was assigned to a plant based on the tactile and visual observations of the overall foliage and a subjective scale of 1-5 was used to evaluate the foliage, where 1 = no pubescence/glabrous, 2 = small amount of pubescence, 3 = about 50% pubescent, 4 = mostly pubescent, and 5 = highly pubescent (Figure 2.1). Each of the five ratings was represented by a few genotypes from the ornamental breeding program. These plants were hybrids involving *Hibiscus moscheutos* subsp. *moscheutos* and subsp. *lasiocarpos* and *H. grandiflorus*. Additionally, several genotypes were included as standards of comparison and included: four advanced selections from the hardy hibiscus breeding program with *H. moscheutos* subsp. *moscheutos* prominent in their background, two commercial cultivars, and the species *H. moscheutos* subsp. *lasiocarpos* and *H. grandiflorus*. The experimental design was a randomized complete block with four blocks each containing 25 genotypes and a single plant was considered a replicate. Approximately three to four genotypes of each pubescence rating were included in each block with the remaining being

the standard, or check, genotypes. The experiment was planted in both locations in late Summer 2016 and data was collected 2017 and 2018 during the growing season.

Plant material for the experiments was propagated via cuttings of the chosen genotypes Summer 2016 from in-ground plants at the Hort Farm in Watkinsville, GA. Sub-terminal 4-5 node cuttings were dipped in potassium salt of Indole-3-butyric acid (K-IBA) at 3,000 ppm for approx. 5 s, allowed to air-dry for a few minutes, stuck into propagation mix [2 Jolly Gardener Pro-Line Growing Mix (Old Castle Lawn and Garden, Pageland, SC): 1 Aero-soil perlite (Dicalite, Bala Cynwyd, PA) (by volume)] in 8.8 cm square pots (Kord; The HC Companies, Inc., Twinsburg, OH), and placed under mist (8 s every 5 min from 7:00 $_{AM}$ – 7:00 $_{PM}$) at greenhouse conditions set to 24°C. Cuttings were removed from mist once they were rooted (3-4 weeks after sticking), kept under shaded greenhouse conditions and applied with liquid fertilizer weekly at 200 mg·L^{-1} (20N–4.4P–16.6K, Jack's Professional J.R. Peters, Inc., Allentown, PA). After ~2 weeks they were moved to a non-shaded area of the greenhouse and continued to receive weekly liquid fertilizer. Plants were transplanted to the field at the Mtn. Station in Blairsville, GA on 31 Aug. 2016 and at the Hort Farm on 8 Sept. 2016. Field layout at the Mtn. Research plot consisted of four rows approximately 31 m (100 ft) long separated by turf grass of equal width as a planting row and the entire four-row area was surrounded by turf grass (Figure 2.2). The plot was sited on a slope with rows perpendicular to the slope and Block 1 was the uppermost row, descending in row number and elevation to Block 4. The Hort Farm plot consisted of two rows in an agricultural field with experimental hibiscus hybrids on either side. Blocks 1 and 2 (consecutively) were in one row and Blocks 3 and 4 in the adjacent row. At both locations plants were spaced 1.2 m (4 ft) apart in row. Drip irrigation was installed at both

locations and plants were watered as needed. Hardwood mulch was applied at the Blairsville location around plants covering the tops of the rows.

Several plants did not return in Spring 2017 at both locations, therefore replacement plants from propagation in Summer 2016 were installed on 8 June 2017 in Blairsville and on 7 June 2017 in Watkinsville. Not all replicates could be replaced however, therefore replacement plants were produced in Summer 2017 via propagation. Cuttings were taken 7, 15 and 19 June 2017 and followed the same propagation method as above, except instead of being planted directly to the field from 8.8 cm pots, rooted cuttings were transplanted to 2.8 L pots on 19 and 20 July 2017. At transplanting, 2.8 L pots were filled with potting substrate (pine bark, peat and sand mix; Oldcastle, Shady Dale, GA), top-dressed with a slow-release fertilizer at 10 $g \cdot pot^{-1}$ (16N-2.6P-10.0K Harrell's Polyon, Lakeland, FL) and placed outside on a ground cloth surface under regular overhead irrigation for the remaining growing season. Plants overwintered outside on ground cloth and were used as replacements in Spring 2018 when they were planted 29 May in Watkinsville and 1 June in Blairsville.

Data was collected monthly from June – Oct. 2017 and June – Sept. 2018 in Blairsville and from June – Oct. 2018 in Watkinsville, GA. Each plant was rated on the degree of feeding damage on the overall plant by the hibiscus sawfly using a 0-5 scale where, 0 = no damage at all, 1 = 1-25% damage, 2 = 25-50% damage, 3 = 50-75% damage, 4 = 75 – 99% damage, and 5 = 100% damage, complete defoliation (Figures 2.4-2.9).

The Statistical Consulting Center (SCC) at UGA assisted with analysis of the data and R code (R Core Team, 2016). Each instance that data was collected for 2017 and 2018 at each location was analyzed separately. A Chi-square test was used to determine whether the degree of pubescence affected the feeding damage and each test was conducted on a contingency table. If

the association was significant (at $P = 0.05$), the Goodman and Kruskal's γ was used to measure the association.

Results

The average damage ratings across the months of data collection for each accession, species or cultivar are listed in Table 2.1 with their respective standard error of the mean. For the data in Watkinsville in 2018, see Table 2.5. In general, damage in Blairsville was greater than for Watkinsville for both years. The percentage of plants with each damage rating for the five degrees of pubescence in Blairsville are listed in Table 2.2 for 2017 and for 2018 in Table 2.4. The *P*-value was significant for each instance that data was collected for 2017 and 2018 in Blairsville, so the degree of foliage pubescence had an effect on feeding damage. The G-K γ measurements indicate a negative association between pubescence and hibiscus sawfly feeding damage in Blairsville during July through Oct. 2017 and July through Sept. 2018 (Tables 2.3 and 2.6). In Watkinsville, similar G-K γ measurements for June to 2018 indicate a negative association of pubescence rating with feeding damage, i.e. the higher the pubescence rating, the lower the feeding damage (Table 2.6). *Hibiscus moscheutos* subsp. *lasiocarpos* and *H. grandiflorus* generally incurred the least amount of feeding damage out of the genotypes evaluated compared to species and hybrids means with the two species exhibiting less feeding damage (and more pubescence) than intraspecific hybrids of *H. moscheutos* subsp. *moscheutos* (Table 2.1). The hybrids with these two species generally had more feeding damage than *H. grandiflorus* and *H. moscheutos* subsp. *lasiocarpos*, which is to be expected since the other parent of the hybrids is the glabrous-leaved *H. moscheutos* subsp. *moscheutos*. The two commercial cultivars *Hibiscus* 'Summer Storm' and 'Cherry Brandy' had greater feeding damage both years in Blairsville.

Discussion

Higher feeding pressure from the hibiscus sawfly population in Blairsville is noticeable in the lack of data in 2017 for Watkinsville, and the higher damage ratings in Blairsville as compared to Watkinsville in 2018 (Tables 2.1, 2.4 and 2.5). Plants in Watkinsville were checked for feeding damage in 2017, however none was observed. The resulting negative influence of foliage pubescence on insect feeding damage determined in this study has been found in precedent studies of other species (Broersma, 1972; Gallun et al., 1966; Sánchez-Peña et al., 2006; Schillinger and Gallun, 1968; Wood et al., 2017). It would seem that because the increase in pubescence is associated with a decrease in feeding damage, the pubescence of leaves could be considered a type of antixenosis resistance [as defined by Painter (1951)]. From observations, eggs were not present on the leaves of the highly pubescent foliage, therefore larvae were not produced to commence later generation feeding. It appears that foliar pubescence deters the female sawflies from laying eggs and if they do, the larvae (which do the most feeding damage) have difficulty feeding on plants with a greater amount of pubescence. The results from this study suggest a greater amount of pubescence is not preferred by the hibiscus sawfly when given the options of *Hibiscus* hybrids with varying degrees of pubescence, including a lack of pubescence. The terms constitutive and direct resistance could also be associated with these findings since the highly pubescent plants express this morphology regardless of the hibiscus sawfly presence (at least for the species, i.e. *H. grandiflorus* and *H. moscheutos* subsp. *lasiocarpos*) (Smith, 2005). The resistance would be considered direct as no additional arthropod was observed to deter the hibiscus sawfly from the plants. The results of this study echo those of Boyd and Cheatham (2004), in that *H. grandiflorus* was found to have less feeding damage than other *Hibiscus* spp., such as the hybrids of *H. moscheutos* evaluated in this study. Complete

defoliation from sufficiently large populations of hibiscus sawfly were observed in Blairsville on genotypes having the least amount of pubescence (Figure 2.11). The hibiscus sawfly was even observed to eat the calyxes of flowers when most of the foliage was devoured (Figure 2.12). The Goodman and Kruskal's γ measurements for 2018 show a stronger early association in Watkinsville than in Blairsville. This likely reflects a later emergence of the sawfly in Blairsville due to its different climactic conditions since it is at a higher altitude and further north than Watkinsville. This study demonstrates the value of pubescence on the foliage of *Hibiscus* spp. in relation to the hibiscus sawfly. Incorporating *Hibiscus* species with this added defense, like *H. grandiflorus*, in breeding programs would seem advantageous in regions where the hibiscus sawfly is known to, or likely to, exist thereby reducing the use of insecticides and bolstering the Hibiscus' combat against this destructive pest.

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Table 2.1. List of plants used to evaluate feeding damage from the hibiscus sawfly (*Atomacera decepta*) of hardy hibiscus hybrids at two locations that are part of the University of Georgia: the Georgia Mountain Research and Education Center in Blairsville, GA and the Durham Horticultural Farm in Watkinsville, GA. Means (± standard error of the mean) are of damage ratings per replicate (plant) averaged over months of data collection.

Pubescence rating	**Accession no., species or cultivar name**	**Background species**	**Blairsville 2017** [z]	**Blairsville 2018** [z]	**Watkinsville 2018** [z]
1	Hib2015-25	*H. moscheutos* subsp. *moscheutos* (intraspecific)	3.78 ± 0.40	n/a	2.80 ± 0.26
1	Hib2015-87	*H. moscheutos* subsp. *moscheutos* (intraspecific)	3.65 ± 0.11	3.20 ± 0.22	2.94 ± 0.28
1	Hib2016-1	*H. moscheutos* subsp. *moscheutos* (intraspecific)	3.78 ± 0.12	2.00 ± 0.00[y]	2.30 ± 0.31
1	Hib2015-2	*H. moscheutos* subsp. *moscheutos* (intraspecific)	3.70 ± 0.16	3.60 ± 0.00[x]	3.00 ± 0.35
2	Hib2015-121	*H. moscheutos* subsp. *moscheutos* and *H. grandiflorus*	3.80 ± 0.13	n/a	2.20 ± 0.00[x]
2	Hib2016-2	*H. moscheutos* subsp. *moscheutos* (intraspecific)	3.70 ± 0.15	3.40 ± 0.40	3.15 ± 0.39
2	Hib2015-123	*H. moscheutos* subsp. *moscheutos* and *H. grandiflorus*	3.35 ± 0.15	n/a	1.55 ± 0.22
2	Hib2015-117	*H. moscheutos* subsp. *moscheutos* and *H. grandiflorus*	3.07 ± 0.25	3.00 ± 0.76	1.60 ± 0.00[x]
3	Hib2015-29	*H. moscheutos* subsp. *moscheutos* and subsp. *lasiocarpos*	2.80 ± 0.32	3.17 ± 0.54	0.90 ± 0.06

Pubescence rating	Accession no., species or cultivar name	Background species	Blairsville 2017 [z]	Blairsville 2018 [z]	Watkinsville 2018 [z]
3	Hib2015-28	*H. moscheutos* subsp. *moscheutos* and subsp. *lasiocarpos*	2.45 ± 0.25	1.90 ± 0.32	0.80 ± 0.11
3	Hib2015-56	*H. moscheutos* subsp. *moscheutos* and subsp. *lasiocarpos*	3.50 ± 0.04	3.21 ± 0.52	1.90 ± 0.34
4	Hib2015-36	*H. moscheutos* subsp. *moscheutos* and subsp. *lasiocarpos*	2.10 ± 0.29	1.40 ± 0.51	0.83 ± 0.14
4	Hib2015-53	*H. moscheutos* subsp. *moscheutos* and *H. grandiflorus*	1.60 ± 0.00[x]	1.05 ± 0.26	n/a
4	Hib2015-93	*H. moscheutos* subsp. *moscheutos* and subsp. *lasiocarpos*	1.00 ± 0.20	1.40 ± 0.31	0.65 ± 0.21
5	Hib2015-46	*H. moscheutos* subsp. *moscheutos* and subsp. *lasiocarpos*	1.55 ± 0.25	1.95 ± 0.41	0.60 ± 0.29
5	Hib2015-108	*H. moscheutos* subsp. *moscheutos* and subsp. *lasiocarpos*	1.10 ± 0.10	1.10 ± 0.15	0.75 ± 0.05
5	Hib2015-66	*H. moscheutos* subsp. *moscheutos* and subsp. *lasiocarpos*	1.55 ± 0.15	2.12 ± 0.36	0.65 ± 0.22
5	*Hibiscus grandiflorus*		n/a	0.40 ± 0.16	0.20 ± 0.00
5	*Hibiscus moscheutos* subsp. *lasiocarpos*		0.74 ± 0.10	1.00 ± 0.18	0.30 ± 0.17
1	*Hibiscus* ‘Summer Storm’		3.75 ± 0.20	3.53 ± 0.27	2.00 ± 0.20
1	Hibiscus 1-5[w]	*H. moscheutos* subsp. *moscheutos* (intraspecific)	4.05 ± 0.12	n/a	2.90 ± 0.29

Pubescence rating	Accession no., species or cultivar name	Background species	Blairsville 2017 [z]	Blairsville 2018 [z]	Watkinsville 2018 [z]
1	Hibiscus 1-6[w]	*H. moscheutos* subsp. *moscheutos* (intraspecific)	3.50 ± 0.29	3.15 ± 0.27	2.55 ± 0.30
1	*Hibiscus* 'Cherry Brandy'		3.73 ± 0.07	3.36 ± 0.06[v]	2.93 ± 0.20
1	Hib2014-60	*H. moscheutos* subsp. *moscheutos* (intraspecific)	3.90 ± 0.10	3.29 ± 0.33[v]	3.20 ± 0.20
1	Hib2014-191	*H. moscheutos* subsp. *moscheutos* (intraspecific)	3.89 ± 0.13	3.40 ± 0.49	2.53 ± 0.49

n/a Indicates no plant was available for data collection at any month.

[z] Damage ratings were on a 0-5 scale where, 0 = no damage at all, 1 = 1-25% damage, 2 = 25-50% damage, 3 = 50-75% damage, 4 = 75 – 99% damage, and 5 = 100% damage, complete defoliation.

[y] Average of one replicate plant for two months of data.

[x] Average for one replicate.

[w] This plant originated from a cross between *Hibiscus* 'Crown Jewels' and the experimental genotype Hibiscus 13-19.

[v] This average is of data from 4 replicates in June, 3 replicates from July-Aug., and none in Sept. due to plants dying. Rooted cuttings were planted 1 June 2018, therefore were small and establishing while suffering from defoliation.

Table 2.2. Contingency tables from 2017 data of feeding damage from the hibiscus sawfly (*Atomacera decepta*) of hardy hibiscus plants in Blairsville, GA at the University of Georgia's Georgia Mountain Research and Education Center. Separate chi-square tests were conducted for each data set of the date collected.

Date collected	**Pubescence rating**		**Damage rating**						***P*-value from χ^2 test**
			0	**1**	**2**	**3**	**4**	**5**	
8 June	**1**	% plants	**57.1**	**42.9**					0.019
		No. plants	16	12					
	2	% plants	**50**	**50**					
		No. plants	7	7					
	3	% plants	**58.3**	**41.7**					
		No. plants	7	5					
	4	% plants	**85.7**	**14.3**					
		No. plants	6	1					
	5	% plants	**100**	**0**					
		No. plants	15	0					
11 July	**1**	% plants	**5.7**	**8.6**	**5.7**	**45.7**	**34.3**		0.000
		No. plants	2	3	2	16	12		
	2	% plants	**0**	**0**	**64.3**	**21.4**	**14.3**		
		No. plants	0	0	9	3	2		
	3	% plants	**27.3**	**27.3**	**18.2**	**27.3**	**0**		
		No. plants	3	3	2	3	0		
	4	% plants	**71.4**	**28.6**	**0**	**0**	**0**		
		No. plants	5	2	0	0	0		
	5	% plants	**87.5**	**12.5**	**0**	**0**	**0**		
		No. plants	14	2	0	0	0		
9 Aug.	**1**	% plants		**0**	**0**	**2.9**	**0**	**97.1**	0.000
		No. plants		0	0	1	0	34	
	2	% plants		**0**	**0**	**0**	**21.3**	**78.6**	

Date collected	Pubescence rating		Damage rating						*P*-value from χ^2 test
			0	**1**	**2**	**3**	**4**	**5**	
		No. plants		0	0	0	3	11	
	3	% plants		**0**	**0**	**18.2**	**54.5**	**27.3**	
		No. plants		0	0	2	6	3	
	4	% plants		**57.1**	**14.3**	**28.6**	**0**	**0**	
		No. plants		4	1	2	0	0	
	5	% plants		**62.5**	**25**	**12.5**	**0**	**0**	
		No. plants		10	4	2	0	0	
8 Sept.	**1**	% plants		**0**	**0**	**0**	**17.1**	**82.3**	0.000
		No. plants		0	0	0	6	29	
	2	% plants		**0**	**0**	**0**	**21.4**	**78.6**	
		No. plants		0	0	0	3	11	
	3	% plants		**0**	**0**	**18.2**	**54.5**	**27.3**	
		No. plants		0	0	2	6	3	
	4	% plants		**0**	**14.3**	**28.6**	**57.1**	**0**	
		No. plants		0	1	2	4	0	
	5	% plants		**31.2**	**25**	**37.5**	**6.3**	**0**	
		No. plants		5	4	6	1	0	
12 Oct.	**1**	% plants		**0**	**0**	**0**	**0**	**100**	0.000
		No. plants		0	0	0	0	35	
	2	% plants		**0**	**0**	**0**	**14.3**	**85.7**	
		No. plants		0	0	0	2	12	
	3	% plants		**0**	**0**	**9**	**27.3**	**63.6**	
		No. plants		0	0	1	3	7	
	4	% plants		**14.3**	**14.3**	**28.6**	**42.8**	**0**	
		No. plants		1	1	2	3	0	
	5	% plants		**6.2**	**50**	**43.8**	**0**	**0**	
		No. plants		1	8	7	0	0	

Table 2.3. Goodman and Kruskal's γ measurements for 2017 of feeding damage from the hibiscus sawfly (*Atomacera decepta*) as it relates to the degree of foliage pubescence of hardy hibiscus plants in Blairsville, GA at the University of Georgia's Georgia Mountain Research and Education Center. A negative γ value indicates an inverse relationship between feeding damage and pubescence. Separate tests were conducted for each data collection date.

Date of data collection	**γ estimate**	**Standard error**	**95% confidence limits**	
8 June	-0.482	0.137	-0.751	-0.214
11 July	-0.808	0.057	-0.919	-0.696
9 Aug.	-0.940	0.028	-0.994	-0.886
8 Sept.	-0.882	0.042	-0.964	-0.800
12 Oct.	-0.955	0.021	-0.996	-0.914

Table 2.4. Contingency tables from 2018 data of feeding damage from the hibiscus sawfly (*Atomacera decepta*) of hardy hibiscus plants in Blairsville, GA at the University of Georgia's Georgia Mountain Research and Education Center.

Date collected	**Pubescence rating**		**Damage rating**						***P*-value from χ^2 test**
			0	**1**	**2**	**3**	**4**	**5**	
1 June	**1**	% plants	**48.0**	**40.0**	**8.0**	**4.0**	**0.0**		0.021
		No. plants	12	10	2	1	0		
	2	% plants	**50.0**	**33.3**	**0.0**	**16.7**	**0.0**		
		No. plants	3	2	0	1	0		
	3	% plants	**30.0**	**20.0**	**30.0**	**0.0**	**20.0**		
		No. plants	3	2	3	0	2		
	4	% plants	**63.6**	**0.0**	**27.2**	**9.1**	**0.0**		
		No. plants	7	0	3	1	0		
	5	% plants	**30.0**	**15.0**	**10.0**	**30.0**	**15.0**		
		No. plants	6	3	2	6	3		
2 July	**1**	% plants	**0.0**	**0.0**	**8.0**	**36.0**	**44.0**	**12.0**	0.000
		No. plants	0	0	2	9	11	3	
	2	% plants	**0.0**	**33.3**	**0.0**	**0.0**	**50.0**	**16.7**	
		No. plants	0	2	0	0	3	1	
	3	% plants	**0.0**	**10.0**	**40.0**	**20.0**	**20.0**	**10.0**	
		No. plants	0	1	4	2	2	1	
	4	% plants	**45.5**	**36.4**	**9.1**	**9.1**	**0.0**	**0.0**	
		No. plants	5	4	1	1	0	0	
	5	% plants	**25.0**	**50.0**	**15.0**	**0.0**	**5.0**	**5.0**	
		No. plants	5	10	3	0	1	1	

Date collected	Pubescence rating		Damage rating						*P*-value from χ^2 test
			0	**1**	**2**	**3**	**4**	**5**	
27 July	**1**	% plants		**0.0**	**4.5**	**18.2**	**54.5**	**22.7**	0.000
		No. plants		0	1	4	12	5	
	2	% plants		**0.0**	**0.0**	**33.3**	**50.0**	**16.7**	
		No. plants		0	0	2	3	1	
	3	% plants		**0.0**	**33.3**	**33.3**	**22.2**	**11.1**	
		No. plants		0	3	3	2	1	
	4	% plants		**45.5**	**54.5**	**0.0**	**0.0**	**0.0**	
		No. plants		5	6	0	0	0	
	5	% plants		**84.2**	**10.5**	**5.3**	**0.0**	**0.0**	
		No. plants		16	2	1	0	0	
30 Aug.	**1**	% plants		**0.0**	**0.0**	**4.5**	**45.5**	**50.0**	0.000
		No. plants		0	0	1	10	11	
	2	% plants		**0.0**	**0.0**	**20.0**	**40.0**	**40.0**	
		No. plants		0	0	1	2	2	
	3	% plants		**22.2**	**22.2**	**22.2**	**11.1**	**22.2**	
		No. plants		2	2	2	1	2	
	4	% plants		**81.8**	**18.2**	**0.0**	**0.0**	**0.0**	
		No. plants		9	2	0	0	0	
	5	% plants		**94.7**	**5.3**	**0.0**	**0.0**	**0.0**	
		No. plants		18	1	0	0	0	
30 Sept.	**1**	% plants		**0.0**	**0.0**	**0.0**	**56.3**	**43.7**	0.000
		No. plants		0	0	0	9	7	
	2	% plants		**0.0**	**0.0**	**20.0**	**20.0**	**60.0**	
		No. plants		0	0	1	1	3	

Date collected	Pubescence rating		Damage rating						*P*-value from χ^2 test
			0	**1**	**2**	**3**	**4**	**5**	
	3	% plants		**12.5**	**25.0**	**37.5**	**12.5**	**12.5**	
		No. plants		1	2	3	1	1	
	4	% plants		**9.1**	**81.8**	**9.1**	**0.0**	**0.0**	
		No. plants		1	9	1	0	0	
	5	% plants		**73.7**	**26.3**	**0.0**	**0.0**	**0.0**	
		No. plants		14	5	0	0	0	

Table 2.5. Contingency tables from 2018 data of feeding damage from the hibiscus sawfly (*Atomacera decepta*) of hardy hibiscus plants in Watkinsville, GA at the University of Georgia's Durham Horticultural Farm. Separate chi-square tests were conducted for each data set of the date collected.

Date collected	**Pubescence rating**		**Damage rating**						***P*-value from χ^2 test**
			0	**1**	**2**	**3**	**4**	**5**	
4 June	**1**	% plants	**2.9**	**76.5**	**20.6**				0.000
		No. plants	1	26	7				
	2	% plants	**20.0**	**30.0**	**50.0**				
		No. plants	2	3	5				
	3	% plants	**61.5**	**38.5**	**0.0**				
		No. plants	8	5	0				
	4	% plants	**70.0**	**30.0**	**0.0**				
		No. plants	7	3	0				
	5	% plants	**88.9**	**11.1**	**0.0**				
		No. plants	16	2	0				
5 July	**1**	% plants	**0.0**	**11.8**	**20.6**	**38.2**	**29.4**		0.000
		No. plants	0	4	7	13	10		
	2	% plants	**0.0**	**60.0**	**10.0**	**10.0**	**20.0**		
		No. plants	0	6	1	1	2		
	3	% plants	**0.0**	**76.9**	**15.4**	**7.7**	**0.0**		
		No. plants	0	10	2	1	0		
	4	% plants	**20.0**	**80.0**	**0.0**	**0.0**	**0.0**		
		No. plants	2	8	0	0	0		
	5	% plants	**55.5**	**38.9**	**0.0**	**5.5**	**0.0**		

Date collected	Pubescence rating		Damage rating						*P*-value from χ^2 test
			0	**1**	**2**	**3**	**4**	**5**	
		No. plants	10	7	0	1	0		
28 July	**1**	% plants	**0.0**	**0.0**	**8.8**	**23.5**	**67.6**		0.000
		No. plants	0	0	3	8	23		
	2	% plants	**0.0**	**0.0**	**50.0**	**20.0**	**30.0**		
		No. plants	0	0	5	2	3		
	3	% plants	**0.0**	**53.8**	**38.5**	**0.0**	**7.7**		
		No. plants	0	7	5	0	1		
	4	% plants	**10.0**	**90.0**	**0.0**	**0.0**	**0.0**		
		No. plants	1	9	0	0	0		
	5	% plants	**38.9**	**61.1**	**0.0**	**0.0**	**0.0**		
		No. plants	7	11	0	0	0		
1 Sept.	**1**	% plants	**0.0**	**6.1**	**21.2**	**30.3**	**42.4**		0.000
		No. plants	0	2	7	10	14		
	2	% plants	**0.0**	**0.0**	**50.0**	**30.0**	**20.0**		
		No. plants	0	0	5	3	2		
	3	% plants	**7.7**	**69.2**	**15.4**	**7.7**	**0.0**		
		No. plants	1	9	2	1	0		
	4	% plants	**20.0**	**80.0**	**0.0**	**0.0**	**0.0**		
		No. plants	2	8	0	0	0		
	5	% plants	**44.4**	**55.5**	**0.0**	**0.0**	**0.0**		
		No. plants	8	10	0	0	0		
1 Oct.	**1**	% plants		**0.0**	**24.2**	**63.6**	**12.1**		0.000
		No. plants		0	8	21	4		

Date collected	Pubescence rating		Damage rating						*P*-value from χ^2 test
			0	**1**	**2**	**3**	**4**	**5**	
	2	% plants		**20.0**	**30.0**	**40.0**	**10.0**		
		No. plants		2	3	4	1		
	3	% plants		**76.9**	**15.4**	**7.7**	**0.0**		
		No. plants		10	2	1	0		
	4	% plants		**100.0**	**0.0**	**0.0**	**0.0**		
		No. plants		10	0	0	0		
	5	% plants		**100.0**	**0.0**	**0.0**	**0.0**		
		No. plants		18	0	0	0		

Table 2.6. Goodman and Kruskal's γ measurements for 2018 of feeding damage from the hibiscus sawfly (*Atomacera decepta*) as it relates to the degree of foliage pubescence of hardy hibiscus plants in Blairsville, GA and Durham Horticultural Farm in Watkinsville, GA. A negative γ value indicates an inverse relationship between feeding damage and pubescence. Separate tests were conducted for each data collection date.

Location	**Date of data collection**	**γ estimate**	**Standard error**	**95% confidence limits**	
Blairsville	1 June	0.301	0.120	0.064	0.537
	2 July	-0.645	0.075	-0.791	-0.499
	27 July	-0.866	0.047	-0.958	-0.774
	30 Aug.	-0.896	0.040	-0.975	-0.818
	30 Sept.	-0.896	0.040	-0.974	-0.817
Watkinsville	4 June	-0.762	0.064	-0.887	-0.637
	5 July	-0.853	0.053	-0.956	-0.749
	28 July	-0.940	0.026	-0.992	-0.889
	1 Sept.	-0.880	0.037	-0.953	-0.807
	1 Oct.	-0.920	0.036	-0.991	-0.848

Figure 2.1. Leaves showing foliage pubescence ratings: 1 (left), 3 (middle), and 5 (right). The left and middle leaves are from hybrids of *Hibiscus moscheutos* and the leaf on the right is from *H. grandiflorus*. Photo by author.

Figure 2.2. Experimental plot in Blairsville, GA at the University of Georgia's Georgia Mountain Research and Education Center. Top photo taken 1 June 2018. Bottom photo: Block 1 is the row furthest right; photo taken 27 July 2018. Both by author.

Figure 2.3. Photo of Watkinsville, GA experimental plot at the University of Georgia's Durham Horticultural Farm. The two rows in the center contain the plants for the hibiscus sawfly (*Atomacera decepta*) study. Photo taken 2 June 2019 by author.

Figure 2.4. Example of hardy hibiscus hybrid assigned a rating of 0 for feeding damage (0%) from hibiscus sawfly (*Atomacera decepta*). Photo taken 11 July 2017 at the University of Georgia's Mountain Research Station in Blairsville, GA by author.

Figure 2.5. Example of hardy hibiscus hybrid assigned a rating of 1 for feeding damage (1-25%) from hibiscus sawfly (*Atomacera decepta*). Photo taken at the University of Georgia's Mountain Research Station in Blairsville, GA by author.

Figure 2.6. Example of hardy hibiscus hybrid assigned a rating of 2 for feeding damage (25-50%) from hibiscus sawfly (*Atomacera decepta*). Photo taken 1 Oct. 2018 at the University of Georgia's Mountain Research Station in Blairsville, GA by author.

Figure 2.7. Example of hardy hibiscus hybrid assigned a rating of 3 for feeding damage (50-75%) from hibiscus sawfly (*Atomacera decepta*). Photo taken 1 Oct. 2018 at the University of Georgia's Mountain Research Station in Blairsville, GA by author.

Figure 2.8. Example of hardy hibiscus hybrid assigned a rating of 4 for feeding damage (75-99%) from hibiscus sawfly (*Atomacera decepta*). Photo taken 30 Aug. 2018 at the University of Georgia's Mountain Research Station in Blairsville, GA by author.

Figure 2.9. Example of hardy hibiscus hybrid assigned a rating of 5 for feeding damage (plant on right) from hibiscus sawfly (*Atomacera decepta*). Photo taken 9 Aug. 2017 at the University of Georgia's Mountain Research Station in Blairsville, GA by author.

Figure 2.10. Photos of the adult form of hibiscus sawfly (*Atomacera decepta*) taken 1 June 2017 at the University of Georgia's Mountain Research Station in Blairsville, GA by author.

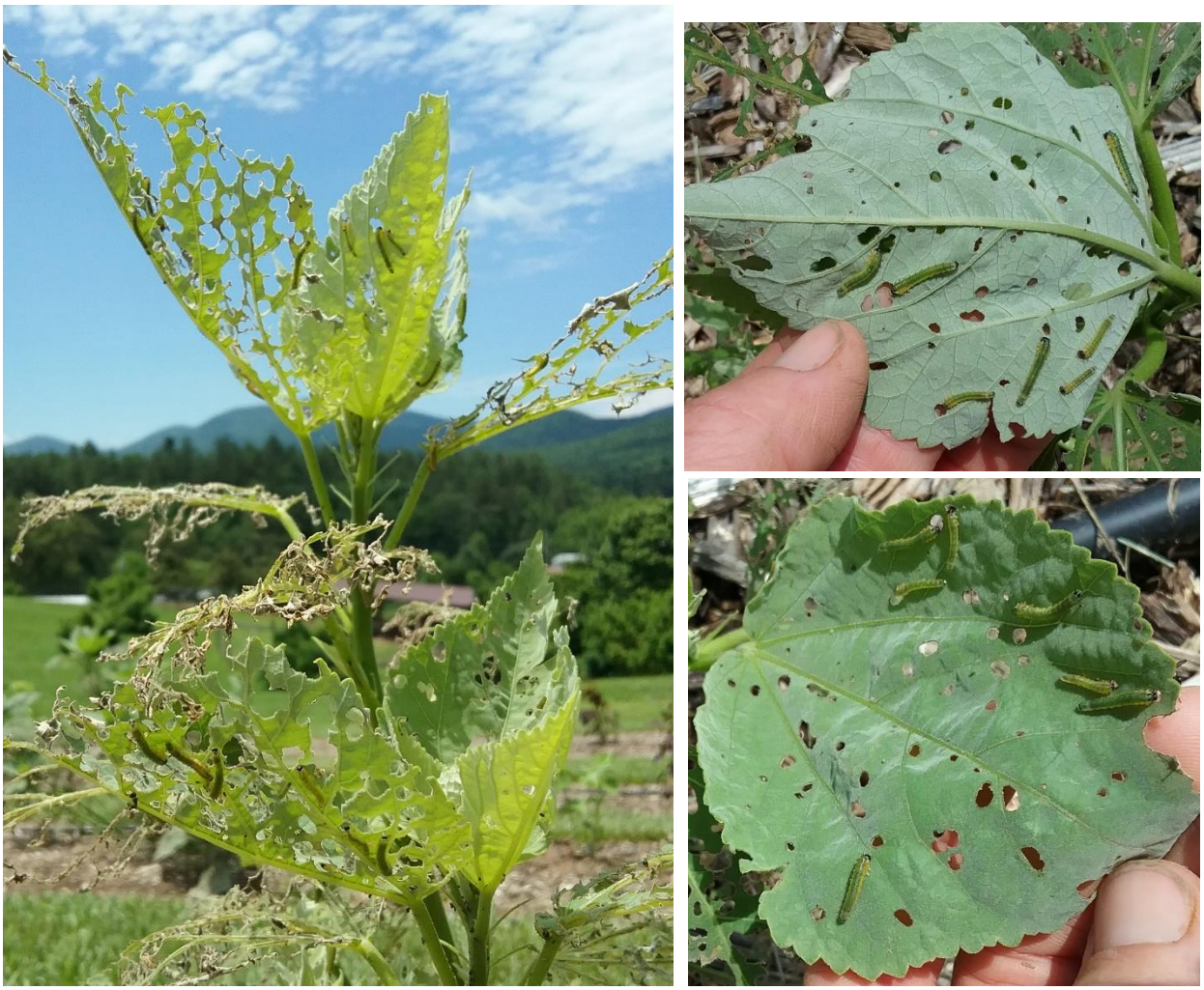

Figure 2.11. Photos of larvae of hibiscus sawfly (*Atomacera decepta*) feeding on foliage of hybrids of hardy hibiscus (*Hibiscus moscheutos*) at the University of Georgia's Mountain Research Station in Blairsville, GA taken 1 June 2018 by author.

Figure 2.12. Photos of larval form of hibiscus sawfly (*Atomacera decepta*) feeding on the calyx of a hybrid of hardy hibiscus (*Hibiscus moscheutos*); taken 9 Aug. 2017 at the University of Georgia's Mountain Research Station in Blairsville, GA by author.

CHAPTER 3

Inheritance of ornamental traits of inter-and intra-specific hybrids of *Hibiscus moschuetos*

Introduction

Four F_2 generations of *Hibiscus* hybrids were evaluated Summer 2015 for several phenotypic traits including foliage color, foliage pubescence, and stem and petiole color. Subjective scales were used to give each plant a rating for the different traits. Ratings of the amount of red color of the foliage, stem and petiole observed on the plant were used for evaluations, and the pubescence of the leaves was evaluated with ratings since there was a range from glabrous, or smooth, to highly pubescent. Three of the four populations originated from crosses between *H. moscheutos* subsp. *moscheutos* and *H. moscheutos* subsp. lasiocarpos and the fourth population originated from a cross between *H. moscheutos* subsp. *moscheutos* and *H. grandiflorus*. Within the data generated from each of the four F_2 families, some of the traits were found to fit certain genetic inheritance models. Therefore, select plants were selfed to test the hypotheses that derived from the observed trends of the F_2 progeny. Additionally, three separate groups of crosses were done to investigate the inheritance of flower color. One group involved reciprocal crosses between a red-flowered cultivar and white- to pale pink-flowered plants, a second group used another red-flowered plant as a pollinator parent with pale to light pink-flowered seed parents, and a third group reciprocally crossed two red-flowered hardy hibiscus hybrids. Presented first is a publication of a portion of the aforementioned work which successfully demonstrated that the proposed hypotheses stemming from F_2 progeny fitting a 3:1 inheritance ratio for red:green

foliage color was supported by additional crossing and selfing. Following the publication is the remainder of the inheritance chapter separated by the trait evaluated.

INHERITANCE OF FOLIAGE COLOR OF COMMON ROSEMALLOW (HIBISCUS MOSCHEUTOS (L.)) SUBSPECIFIC HYBRIDS [1]

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Inheritance of foliage color of common rosemallow (*Hibiscus moscheutos* (L.)) subspecific hybrids

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Abstract

Background: Common rosemallow (*Hibiscus moscheutos* (L.)) is a native wetland perennial taxon that has been widely used as an ornamental landscape plant for several decades. Its showy blooms, attractive form and foliage, and hardiness attracted the attention of plant enthusiasts, leading to extensive hybridization and subsequent selection of superior genotypes. Red foliage color is a desirable trait, therefore we investigated the mode of inheritance of this trait in *H. moscheutos* subsp. *lasiocarpos* (Cavanilles) O. J. Blanchard with intraspecific hybrids of *H. moscheutos* subsp. *moscheutos* (L.).

Results: Two red-foliaged seed parents of *Hibiscus moscheutos* subsp. *moscheutos* (L.) were crossed with green-foliaged paternal parent *H. moscheutos* subsp. *lasiocarpos*. Two F_2 full-sib families (n = 192 and 238) were each found to fit a 3 red: 1 green segregation ratio for foliage color using a Chi square goodness-of-fit analysis. For further evaluation of this segregation pattern, each parent was selfed, as were two red-foliaged F_2 plants. The two red-foliaged parents yielded the expected all-red progeny (n = 53 and 178, 1 red: 0 green) and the green-foliaged parent yielded 244 green and 6 red plants, fitting the expected 0 red: 1 green (P = 0.704) ratio. Additionally, progeny from the two red-foliaged F_2 plants fit the expected 1 red: 0 green ratio (n = 135 and 120).

Conclusions: Results indicate the appearance of red foliage, in any amount, in the two subspecies utilized and our hybrids of hibiscus to be controlled by a single locus with a dominant allele for red foliage. We propose the gene be called “green foliage” where the dominant allele, *G*, yields a

red foliage phenotype. When the recessive allele, *g*, is present in the homozygous form, progeny consist of an all-green foliage phenotype for *Hibiscus moscheutos* (L.). Understanding the mode of inheritance of red-foliage phenotype in hibiscus would prove useful in further ornamental breeding work.

Background

Common rosemallow (*Hibiscus moscheutos* (L.)) is a perennial shrub native to wetland areas of North America and appreciated for its showy white to pink flowers. The abundant, ephemeral and large blooms have garnered this species widespread adoption, and as a result cultivation and breeding since the early 19th century (Winters 1970). Along with four other North American hibiscus species, common rosemallow belongs to section Muenchhusia within the genus in the Malvaceae family, and is diploid (2n = 2x = 38) (Skovsted 1935; Small 2004; Wise and Menzel 1971). Common rosemallow has had several botanical names over time with the currently adopted taxonomy splitting it into two subspecies: *moscheutos* and *lasiocarpos* (Cavanilles) O. J. Blanchard (Blanchard 1977, 2008). The distinctions are based on the presence (subsp. *lasiocarpos*) or absence (subsp. *moscheutos*) of hairs on the adaxial leaf surface, capsules, and bracts of the involucel (epicalyx), as well as their geographic ranges (Flora of North America Editorial Committee 2015). Subspecies *moscheutos* is found in the wild from Ontario to New Hampshire, south to Florida and west to Texas. *Hibiscus moscheutos* subsp. *lasiocarpos* exists naturally from Indiana, south to Alabama, west to Texas, including the mid-western states of Kansas and Oklahoma, and with disjunct populations in Florida, New Mexico, and northern Mexico (Chihuahua) (Blanchard 2008). Although the subspecies' ranges overlap, the Mississippi River serves as a general border with subsp. *moscheutos* found mostly to its east and subsp. *lasiocarpos* to its west (Flora of North America Editorial Committee 2015). Given these native

ranges, *H. moscheutos* is hardy from the United States Department of Agriculture (USDA) zones 4a to 9b, hence its other common name of hardy hibiscus (Winters 1970). Plants typically sprout stems from underground storage structures in Georgia during March with flowers first appearing in May, peaking late June/early July, and blooming sporadically into August and September. Plants set fruit as dehiscent capsules into late summer, senesce in the fall (October/ November), and remain dormant during the winter until emerging in spring. Leaf morphology can vary in shape (broadly lanceolate to triangular-ovate), leaf base (cuneate to cordate), lobing (3-lobed or unlobed), and margins (crenate to serrate) (Flora of North America Editorial Committee 2015). Plants can measure 0.9–2.4 m tall and their form is generally upright to rounded shrub, however, as a wetland native its shape can appear asymmetric, leggy or floppy when planted alone (Godfrey and Wooten 1981). Many cultivars and ornamental hybrids, intra- or interspecific, have improved traits, particularly for form, flower, and foliage color. Inheritance of foliage color has been observed and reported in various genera and is useful information when breeding ornamentals, potentially saving time and resources. Red to purple foliage has been reported to follow monogenic Mendelian 3:1 inheritance as either a dominant or recessive trait. Red foliage is controlled by a dominant monogenic allele in ornamental coleus (Nguyen et al. 2008) and some woody plants, such as beech (Blinkenberg et al. 1958; Heinze and Geburek 1995) and birch (Hattemer et al. 1990). In other plants, red foliage is inherited in a single-locus recessive fashion; for example, with barberry (Cadic 1992), redbud (Roberts et al. 2015), and tutsan (Olsen et al. 2006). In other cases, inheritance of red foliage is reportedly controlled by complementary gene action, as in hazelnut (Smith and Mehlenbacher 1996; Thompson 1985) and flowering dogwood (Wadl et al. 2010); or by a single gene with incomplete allelic dominance as with the bronze foliage allele (Rt) in crabapple (Alston et al. 2000; Sampson and Cameron 1965).

Hibiscus moscheutos subsp. *moscheutos* (L.) exhibits red foliage to varying degrees (Stout 1917) and has been selected for and exploited in several cultivars: 'Crown Jewels' PP11,857, 'Plum Crazy' PP11,854, 'Midnight Marvel' PP24,079, and 'Summer Storm' PP20,443 to name a few (Falstad III 2009; Fleming and Zwetzig 2001a, b; Hurd 2013). *Hibiscus moscheutos* subsp. *lasiocarpos*, on the other hand, displays entirely green foliage. The objective of this study was to identify the mode of gene action and number of loci determining the presence of red foliage in our specimen of *H. moscheutos* subsp. *lasiocarpos* (Cavanilles) O. J. Blanchard and in our *Hibiscus moscheutos* (L.) hybrids.

Methods

Plant material utilized for this study (from 2014 to 2017) was grown at the University of Georgia Durham Horticultural Farm in Watkinsville, GA. Two red-foliaged seed parents (R1 and R2) and one green-foliaged pollen parent (G) were used. R1 and R2 were intraspecific hybrids of *Hibiscus moscheutos* (L.) with red-foliaged cultivars in their background and were selected in 2013 as part of an ornamental breeding program. The pollen parent, G, (*Hibiscus moscheutos* subsp. *lasiocarpos* (Cavanilles) O.J. Blanchard) was obtained from Plant Delights Nursery (Raleigh, NC) in February of 2012. Two F_1 populations (R1 × G and R2 × G) were generated and subsequently, two F_2 populations were obtained from open-pollinations within each F_1 population. The F_2 plants were field-planted in June 2015 and a subjective rating on visual foliage color of the whole plant was given for each seedling in September 2015 using a scale of 1–5, where 1 = no red/entirely green, 2 = small amount of red, 3 = about 50% red, 4 = mostly red, and 5 = completely red (Fig. 3.1). Ratings were assigned based on the visual observation of the overall amount of red of the collective foliage in situ. Based on results from goodness-of-fit (Chi square) tests using observed F_2 segregation ratios within families, testing of hypothesized

gene action was implemented via selfing of P1s (R1 and R2), P2 (G), and 1 red-foliaged seedling from each F_2 family. R1 and R2 had foliage color ratings of 5 and P2 (G) had a rating of 1. F2-R1 (an F_2 seedling from R1 × G) had a rating of 5 and F2-R2 (an F_2 seedling from R2 × G) had a rating of 2. Pollinations were performed in 2016 on plants in the field to obtain the following S_1 populations: ⊗ R1, ⊗ R2, ⊗ G, ⊗ F2-R1, ⊗ F2-R2. Pollinations were done in summer 2016 in the mornings (7–9 a.m.) beginning June 24. A flower from the plant being selfed was removed and its pollen was spread onto flowers of the same plant, fully covering the stigmatic surfaces. Pollination tags were used to identify the fruits that were from controlled pollinations. Fruit were collected following dehiscence and seed were manually removed. In March 2017, seed were scarified by soaking in 95–98% sulfuric acid (Avantor Performance Materials, LLC, Center Valley, PA) for 10 min and sown the following day in Jolly Gardener® Pro-line C/L Growing Mix potting media (Old Castle Lawn and Garden, Pageland, SC) incorporated with Micromax® micronutrients (Everris NA Inc., Dublin, OH) at 594 g m^{-3} in trays (26 × 50 × 6 cm) and kept under greenhouse conditions. Germinated seedlings were individually transplanted to 25-cell-pack tray filled with the same potting media as previous mixed with perlite (2:1, v/v) and were fertilized weekly with a liquid fertilizer at 200 mg L^{-1} (20–10–20, N–P_2O_5–K_2O-Jack's Professional® J.R. Peters, Inc., Allentown, PA). After several weeks in the greenhouse, they were transplanted outside to 2.8 L nursery containers between May 17 and 26, 2017, top-dressed with 11 g pot^{-1} of controlled-release fertilizer (16–6–12, N–P_2O_5–K_2O-Harrell's Polyon™, Lakeland, FL) and placed outside on a ground cloth surface under regular overhead irrigation. Foliage color was rated for each plant once between July 24 and Aug. 2, 2017. Within each S_1 family, the number of plants with a rating from 2 to 5 were pooled to obtain the observed red-foliaged value. Plants with a rating of 1 were considered green, or lacking red, foliaged (Fig.

3.1). Data from F_2 families was used to propose hypotheses of single gene action for red foliage color in hardy hibiscus. Goodness-of-fit, or Chi square (χ^2), tests were used to evaluate the fit of F_2 and selfed populations to the proposed model. Chi square critical value used was $\chi^2_{0.05,1}$ = 3.841 for the two observed phenotypes (Dowdy et al. 2004). P-values were calculated using the R program (R Core Team 2016).

Results

Each of the two full-sib families of the F_2 generation fit the phenotypic ratio of 3:1 for foliage color, where red is dominant to green (Table 3.1). F_2 seedlings from Family 1 (R1 × G) and Family 2 (R2 × G) had Chi square values less than the critical value at 1.361 and 0.050, respectively. All S_1 progeny from each of the red foliaged seed parents (R1 and R2) and a red-foliaged F_2 seedling (F2-R1 and F2-R2) from each of the two families displayed red foliage (rating from 2 to 5), except for 2 plants out of 120 from ⊗F2-R2. These S_1 plants from ⊗ F2-R2, while having two individuals with green foliage, did not significantly differ from the predicted 1 red: 0 green ratio (P = 0.856). S_1 progeny from the green-foliaged parent (G) resulted in 244 green-foliaged plants. Although 6 plants displayed some amount of red among ⊗ G progeny, the observed ratio was not significantly different from the expected 0 red:1 green ratio (P = 0.704).

Discussion

The results of these experiments indicate that red foliage phenotype in our hybrids of *Hibiscus moscheutos* (L.) is determined at a single locus. From Chi square analyses, we concluded that red foliage is completely dominant to the appearance of no red in foliage. When each of the parents with contrasting foliage colors was self-pollinated, the resultant progeny had the same foliage color as the parent from which they were selfed, suggesting homozygosity. Additionally, when a

red-foliaged parent (genotype *GG*) was crossed with the green-foliaged parent (genotype *gg*) of both Family 1 and 2, F_2 progeny displayed a 3 red: 1 green (genotype 1 *GG*: 2 *Gg*: 1 *gg*) segregation ratio for foliage color. The S_1 progeny from the two F_2 red seedlings also fit the 1 red: 0 green ratio, indicating homozygosity (genotype *GG*). We propose that the locus controlling the appearance of red, in any amount (rating 2–5), in the foliage of *Hibiscus moscheutos* (L.) be named "green foliage" with alleles *G* and *g*. The observed deviations from the expected phenotypic ratios in progeny from ⊗ G and ⊗ F2-R2 are due to chance as they are statistically nonsignificant and could be explained by pollen contamination from pollinators or wind during controlled crosses or human error during the many steps of seed and plant handling. Other epistatic models were tested on the observed segregation ratios of F_2 populations, such as dominant gene interaction (9:6:1) and duplicate gene action (15 red: 1 green), but no other model besides 3 red: 1 green was found to fit both F_2 full-sib progeny. F_2 progeny from R1 × P2 did fit dominant suppression gene action (13 red:3 green) (P = 0.355), however the F_2 progeny from R2 × P2 did not, and progeny from selfing a red-foliaged F_2 plant (⊗F2-R1) did not display any progeny with all-green foliage which could be expected with an 13:3 epistatic inheritance model. Investigation into the biochemical pathway leading to red foliage phenotype in common rosemallow is outside the objective of this study, and no investigation identifying anthocyanin production in *Hibiscus moscheutos* (L.) foliage has been found in current literature. In many angiosperms, red to purple appearance of vegetative and reproductive tissue is due to anthocyanins, however, the presence and concentration of other pigments, such as chlorophylls and carotenoids, can have a contributing effect on foliage coloration (Lee 2002; Taiz et al. 2015). Anthocyanins are a diverse class of pigments that can appear red, purple, pink or blue and belong to the flavonoids, a type of secondary metabolite (Taiz et al. 2015). Anthocyanins accumulate in

the vacuole of the cell and have been shown via dissection analyses to reside in ground tissue (layer two; palisade and spongy mesophyll cells) of leaves rather than dermal tissue (layer one; epidermis) (Lee 2002; Lee and Collins 2001). Lightbourn et al. (2008) reported dark red/violet to black foliage of pepper (*Capsicum annuum* L.) had anthocyanins in the vacuoles of palisade mesophyll and spongy mesophyll cells whereas green foliage did not. In addition to the concentration of anthocyanins, the presence of carotenoids and chlorophylls can affect the visible color of foliage. Carotenoids are accessory pigments to chlorophyll during photosynthesis and are located in chloroplasts. There are several forms of carotenoids (β-carotene, lutein, violaxanthin, etc.) that can range in appearance from yellow, orange to red. While uncommon, a few cases of red foliage being attributed to carotenoids have been reported, such as in common box (*Buxus sempervirens*) by Ida et al. (1995), Lee (2002). Foliage shade and hue can vary considerably since pigments, particularly anthocyanins, can be influenced by many abiotic and biotic factors. While colorful flowers and reproductive structures are commonly agreed on as advantageous to survival, the primary function of anthocyanins in foliar tissue is not widely agreed upon. Some theories that researchers have put forth were summarized by Santos-Buelga et al. (2010) and include: photoprotection, antioxidant activity, anti-herbivory, and oxidative signaling. Although the exact role(s) of anthocyanins in common rosemallow is currently undetermined, the occurrence of red foliage has resulted in preference and selection by consumers. The red intensity in University of Georgia hybrid hibiscus lines varies and people typically prefer darker or more intense red color foliage. Consumer personal feedback evaluation panels have consistently chosen plants with red foliage over plants with green foliage which is also reflected in many recent commercial releases. Due to the range in the total amount of red pigmentation in the foliage of hybrids and the potential factors affecting anthocyanin and

secondary pigment concentrations, we pooled all hibiscus with any amount of red pigmentation into one phenotypic grouping. Wadl et al. (2010) grouped hybrids similarly, with *Cornus florida* (L.), and determined red foliage color to be controlled by a completely dominant allele at a single locus. This type of inheritance of monogenic, dominant red over green foliage was also reported in *Betula pendula* var. 'Purpurea' by Hattemer et al. (1990), who observed a gradation (in their case) of purple foliage. Similar findings were reported in *Fagus sylvatica* (L.) by Blinkenberg et al. (1958) and Heinze and Geburek (1995) whereby foliage was described as "copper" color. Similar inheritance of a dominant red–purple foliar phenotype by a single gene was reported in two cultivars of tetraploid coleus (*Solenostemon scutellarioides* (L.) Codd) by Nguyen et al. (2008), however, the recessive trait was described as an orange–yellow phenotype rather than green foliage. This study has not observed any linkage of phenotypic traits with red foliage color. Although the red-foliaged parents R1 and R2 have glabrous foliage compared to the green-foliaged parent, G, which has pubescent leaves, F_2 hybrids display different possible combinations of foliage color and pubescence (data not shown) suggesting independence of gene activity.

Conclusion

Common rosemallow is an attractive native plant known for its showy blooms and newer hybrids have incorporated stunning red–purple foliage. From crossing and selfing parents of contrasting foliage color, as well investigation of red foliage color in F_2 plants, we found the appearance of red foliage, in any amount, to be controlled by a single locus with a dominant allele for red foliage. It follows that the green foliaged parent, a specimen of *Hibiscus moscheutos* subsp. *lasiocarpos* (Cavanilles) O.J. Blanchard, unless crossed with a red-foliaged hibiscus, should only yield all-green foliaged progeny. As red foliaged hibiscus is highly desirable in the nursery trade,

ornamental breeders would benefit from understanding the type of inheritance of red foliage in *Hibiscus moscheutos* (L.) Additionally, an investigation into the genetic control of the range in intensity of red foliage would be worth exploring.

Abbreviations

β -carotene: beta-carotene

F1: first generation from cross of P1 x P2

F2: second generation from cross of P1 x P2

G: green-foliaged *Hibiscus moscheutos* subsp. *lasiocarpos* (Cavanilles) O. J. Blanchard)

G: proposed dominant allele for red foliage phenotype

g: proposed recessive allele for red foliage phenotype

P1: seed/female parent of cross

P2: pollen/male parent of cross

R1 and R2: red-foliaged intraspecific hybrids of *Hibiscus moscheutos* (L.)

S1: first generation from selfing (cross with itself)

USDA: United States Department of Agriculture

⊗: crossing a plant using pollen of the same plant or genotype

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Additional Inheritance Studies

Materials and Methods

Plants utilized in the following studies were treated in the same manner (e.g., substrate, pots, liquid fertilizer, scarification, sowing), maintained at the same greenhouse and outdoor conditions, followed the same schedule (e.g., pollination, seed harvest, sowing, and transplantation dates), and self- or cross- pollinated in the same manner as described in the above publication, unless otherwise stated. Any abbreviations utilized in the above publication are the same in the following text unless otherwise specified. Data presented for investigating the inheritance of leaf pubescence and color and stem and petiole color are of F_2 families which were found to fit certain inheritance ratios. Data from each of the four F_2 families and flower color crosses was tested against several ratios with corresponding inheritance models including, dominant monogenic (3:1), dominant gene interaction (9:6:1), dominant suppression (13:3), recessive epistasis (9:3:4), and complementary gene action (9:7:1). The following presents only the models which fit the F_2 data and further evaluation or validation. The flower color data which fit any of the aforementioned inheritance models is presented.

Leaf pubescence

Parent plant material used to investigate inheritance of leaf pubescence consisted of R1, R2 and G plants with an additional seed parent similar to R1 and R2, given the name R3. Like R1 and R2, R3 is an intraspecific hybrid of *Hibiscus moscheutos* subsp. *moscheutos* with red-foliaged cultivars in its background and was selected for the ornamental breeding program. Red-foliaged seed parent plants have glabrous leaves and the green-foliaged pollinator parent, G, has highly pubescent leaves. F_2 progeny from each of the three crosses: R1 x G, R2 x G, and R3 x G were evaluated for foliage pubescence using the rating scale of 1–5, where 1 = no pubescence/

glabrous, 2 = small amount of pubescence, 3 = about 50% pubescent, 4 = mostly pubescent, and 5 = highly pubescent (Figure 3.2). A rating for each plant was assigned based on the tactile and visual observations of the overall foliage in situ. The F_2 progeny from Family 1 (R1 x G), Family 2 (R2 x G) and Family 3 (R3 x G) were each found to fit a 15:1 duplicate gene epistatic ratio, where pubescence is dominant to a lack of pubescence. Pubescence ratings of 2-5 were grouped collectively and a goodness-of-fit (Chi-square) test was used to evaluate the observed F_2 segregation ratios with the expected 15:1 values within each of the three families. To further test the fit of this inheritance ratio, each parent was selfed and two F_2 seedlings from Family 1 and one F_2 seedling from Family 3 were selfed. No F_2 seedling from Family 2 was available for selfing. Parents R1, R2, and R3 had pubescence ratings of 1 and parent G had a rating of 5. The two F_2 seedlings from Family 1 had contrasting pubescence ratings of 1 and 5, and the F_2 seedling from Family 3 had a rating of 1. Populations from selfing R1, R2, R3, G, F2-R1 (F_2 seedling from Family 1 with rating of 1), F2-R1-2 (F_2 seedling from Family 1 with rating of 5), and F2-R3-1 (F_2 seedling from Family 3 with rating of 1) were generated (S_1 populations) and each seedling was rated for leaf pubescence in Summer 2017. Plants assigned ratings of 2-5 were pooled for the number of observed plants with a pubescent foliage phenotype and plants with a rating of 1 were considered to have no pubescence. Chi-square tests were used to evaluate the fit of populations from selfing to the proposed model. Chi-square critical value used was $\chi^2_{0.05,1} = 3.841$ for the two observed phenotypes (Dowdy et al., 2004). *P*-values were calculated using the R program (R Core Team, 2016).

Stem and Petiole Color

Parent plant material used to investigate inheritance of the color of stems and petioles on *Hibiscus* hybrids consisted of the same R2 and G plants as above with an additional pollinator

parent *Hibiscus grandiflorus* (Figure 3.3), given the name G2. Like parent G, G2 has green foliage, stem and petioles (and pubescent foliage) and was selected for the ornamental breeding program. The seed parent has red stems and petioles. F_2 progeny from each of the two crosses: R2 x G and R2 x G2 were evaluated for stem and petiole color using the rating scale of 1–5, where 1 = no red/ all green, 2 = small amount of red, 3 = about 50% red, 4 = mostly red, and 5 = totally red (Figure 3.4). A rating for each plant was assigned based on the visual observations of the overall stems and petioles in situ. The F_2 progeny from Family 2 (R2 x G), Family 4 (R2 x G2) were each found to fit a 15:1 duplicate gene epistatic ratio, where red is dominant to green stem and petiole color. Red stem and petiole ratings of 2-5 were grouped collectively and a goodness-of-fit (chi-square) test was used to evaluate the observed F_2 segregation ratios with the expected 15:1 values for each of the two families. To further test the fit of this inheritance ratio, parents R2 and G were selfed and two F_2 seedlings each from Family 2 and Family 4 were selfed. Parent R2 had a stem and petiole rating of 5, parent G had a rating of 1, the two F_2 seedlings from Family 2 had ratings of 2, and the F_2 seedlings from Family 4 had contrasting ratings of 1 and 5. Populations from selfing R2, G, F2-R2 (F_2 seedling from Family 2 with rating of 2, and same as in publication), F2-R2-2 (F_2 seedling from Family 2 with rating of 2), F2-R4-1 (F_2 seedling from Family 4 with rating of 1), and F2-R4-2 (F_2 seedling from Family 4 with rating of 5) were generated (S_1 populations) and each seedling was rated for stem and petiole color in Summer 2017. Plants assigned ratings of 2-5 were pooled for the number of observed plants with a red stem and petiole phenotype (Figure 3.4) and plants with a rating of 1 were considered to have no red in the stems or petioles. Chi-square tests and *P*-values were conducted in the same manner as for other traits.

Flower Color

Reciprocal crosses with 'Robert Fleming'

The cultivar 'Robert Fleming' has a dark red flower and was used as both a pollinator and seed parent crossed with three plants having white, blush or light pink flowers that were also used as pollinator and seed parents. The non-red-flowered plants were chosen for their contrasting flower colors to 'Robert Fleming' (RF) and were developed from the ornamental breeding program of intraspecific crosses of *Hibiscus moscheutos* subsp. *moscheutos*. They will specifically be known as plant A (white-flowered), plant B (white to blush-flowered), and plant C (blush to light pink-flowered). Many pollinations were made during July and Aug. 2016 in a reciprocal manner between RF and plants A, B, and C. Self-pollinations were also performed on RF. Progeny were processed by the same methods as the plants in above experiments and the flower color of each individual plant was assigned from observations. Determination of flower color was a subjective evaluation in situ between 30 July and 13 Sept. 2017 and consisted of three groups: red/dark pink, white, and intermediate between red/dark pink and white.

Red-flowered pollinator on white- to light pink-seed parents

The red-flowered plant, 2014-54 (Figure 3.6), from the ornamental breeding program was selected in 2014 from progeny of a cross between the intraspecific *H. moscheutos* subsp. *moscheutos* line, R2, and the dark red-foliaged and -flowered commercial cultivar 'Midnight Marvel'. Four plants from the breeding program were chosen for having pale to light pink flowers and were used as seed parents in crosses with 2014-54. The four seed parents comprised of plant D (pink flowers), plant E (pale pink flowers), plant F (white/blush flowers), and F2-R2 (pink flowers), which was also used in other inheritance studies (above). Of note is that plants E and F were also F_2 plants from Family 2 in the other trait inheritance studies. Progeny from crosses between the four seed parents and 2014-54 were processed by the same methods as the

plants in above experiments and the flower color of each individual plant was assigned from observations. Determination of flower color was a subjective evaluation in situ between 23 July and 29 Aug. 2017 and consisted of three phenotypic groupings: red/dark pink, white, and intermediate between red/dark pink and white.

Red by red reciprocal

Two red-flowered plants were reciprocally crossed to investigate the inheritance of red flower color. The two parent plants, 2014-80 and 2014-82 (Figure 3.7), are siblings from a cross of R3 and 'Midnight Marvel' and were selected as part of the breeding program in 2014. Progeny from the two reciprocal crosses between 2014-80 and 2014-82 were generated and processed by the same methods as experiments above, and the flower color of each individual plant was assigned by observation. Determination of flower color was a subjective evaluation in situ between 4 and 24 Aug. 2017 and consisted of three phenotypic groups: red/dark pink, white, and intermediate (between red/dark pink and white). Different gene models were tested for the three phenotypes (plus two phenotype models by pooling the red and intermediate flower color) using chi-square tests. Chi-square tests and *P*-values were conducted in the same manner as for other traits.

Results

Leaf pubescence

From selfing each of the parents from the three crosses, the progeny that fit the expected segregation ratios were from selfing R3, which was 0 pubescent: 1 glabrous, and from selfing G, which was 1 pubescent: 0 glabrous (Table 3.2). From selfing the three F_2 plants, progeny from F2-R1-2, a plant from Family 1 with a pubescent rating of 5, fit the expected 1 pubescent: 0 glabrous.

Stem and Petiole Color

Progeny from selfing the seed parent, R2, fit the expected all red and no green stem and petiole color segregation ratio (Table 3.3). From selfing the select F_2 plants, progeny from F2-R2, which had a rating of 2, fit the expected 1 red: 0 green segregation ratio; from F2-R2-2, which had a rating of 2, fit the expected 1 red: 0 green segregation ratio; and from F2-R4-2 fit the expected segregation ratio of 1 red: 0 green.

Flower Color

Reciprocal crosses with 'Robert Fleming'

The three crosses with *Hibiscus* 'Robert Fleming' as the seed parent had plants with all red flowers and no white nor intermediate colored flowers (Table 3.4). The three crosses with 'Robert Fleming' as the pollinator parent had progeny with flowers ranging from red to intermediate and white (Figure 3.5). By pooling the red/pink number of progeny as one phenotype (pigmented) and a white flower phenotype (lack of pigment) as another phenotype, crosses of Plant A x RF (chi-square: 0.305, *P*-value: 0.581), Plant B x RF (chi-square: 0.085, *P*-value: 0.771), and Plant C x RF (chi-square: 0.065, *P*-value: 0.799) fit an expected 1 red: 0 white ratio for heterozygosity at a single gene.

Red-flowered pollinator on white- to light pink-seed parents

The crosses involving the red-flowered pollinator parent, 2014-54 (Figure 3.6), with four white/light pink-flowered seed parents had progeny with mostly red to pink flowers (Table 3.5). When the red/dark pink and intermediate groups for flower color were pooled into one phenotype for each cross, the crosses fit an expected 1 red: 0 white segregation ratio of heterozygosity for flower color.

Red by red reciprocal

The two groups of progeny from reciprocally crossing 2014-80 with 2014-82 fit the ratio for dominant gene interaction (9 red: 6 intermediate: 1 white) (Table 3.6). If the red/dark pink and intermediate flower color groups are pooled, the observed ratios fit the duplicate gene action model of 15 red-intermediate (or pigmented): 1 white (or non-pigmented) (Table 3.6).

Discussion

Leaf Pubescence

The inheritance of foliar pubescence was expected to follow a duplicate gene action epistatic model (15:1) whereby the pubescent phenotype would be observed when at least one copy of the dominant allele is present in either gene and the glabrous phenotype is observed when both genes are homozygous recessive. This proposed model was based on observations from three F_2 families that fit the 15:1 segregation ratio and that had P_1 and P_2 (original parents) with contrasting pubescent ratings (highly pubescent and glabrous) (Table 3.2). The observation that all three F_2 families fit the same segregation ratio also made sense because they resulted from the same pollinator parent (G, aka *Hibiscus moscheutos* subsp. *lasiocarpos*) and the seed parents (R1, R2, and R3) are from similar backgrounds. This model hypothesis could be considered rejected due to the lack of supporting evidence resulting from selfing R1, R2, F2-R1 and F2-R3-1. Family 2 could not be investigated at the F_2 level due to the lack of plants available from that generation. These results suggest the parents R1 and R2 are not homozygous recessive as expected. It is interesting that progeny from selfing the pollinator parent, *H. moscheutos* subsp. *lasiocarpos,* fit the expected all-pubescent ratio completely, i.e. had no glabrous progeny. Similarly, progeny fully-fitting the expected ratio was observed from selfing F2-R1-2 (from Family 1), which also had a pubescence rating of 5. Because G is a separate species, it is possible that the genes regulating pubescence in *H. moschuetos* subsp. *lasiocarpos* are different than in

the species *Hibiscus moscheutos* subsp. *moscheutos*. Additionally, the seed parents of the crosses studied were not 100% *Hibiscus moscheutos* subsp. *moscheutos*, but rather intraspecific hybrids and potentially contain the species *H. coccineus* in their background (from introgressed cultivars). Pubescence in the seed parents and resulting F_2 progeny could be regulated by multiple genes (more than two) given their somewhat mixed pedigrees and therefore difficult to determine and predict by traditional methods. A cross from the breeding program of an F_2 seedling from Family 3 with its seed parent, R3, resulted in 32 seedlings all with a pubescence rating between 5 and 2 (i.e. had some amount of pubescence). The outcome of backcrossing an F_2 seedling with a pubescence rating of 5 to its P_1 did fit the expected 1:0 ratio where pubescence is dominant to glabrous foliage. Other possible affects on the mixed results for evaluating leaf pubescence could be environmental differences; the F2 progeny were evaluated in the field vs. progeny from selfing were evaluated in pots on ground cloth, and the subjective method of evaluating pubescence could have impacted the ratings, as well.

Stem and Petiole Color

From the F_2 progeny, stem and petiole color appeared to be regulated by two genes in duplicate gene action (15:1) whereby a single copy of the dominant allele is required at either gene to obtain a phenotype having some amount of red/pigment in the stem and petiole of the plant. The green, or lack of red/pigment, phenotype is only observed when both genes are homozygous recessive with this model. The results of this experiment are somewhat mixed because while the selfed progeny from the seed parent for both Family 3 and 4 displayed 100% red stem and petiole color (Figure 3.4), the pollinator parent, G, when selfed did not display the expected all-green stem and petiole color phenotype (Table 3.3). The pollinator parent for Family 4, G2, was not selfed and therefore no data was available to evaluate for this study. Two F_2 plants from

Family 3 having ratings of 2 to 3 were selfed and resulting progeny fit the expected red/pigmented phenotype. A cross was made as part of the breeding program of an F_2 seedling (F2-R2-2) with a rating of 2 from Family 2 with R2 (P_1) and the resulting progeny had an all-red phenotype (data not shown; chi-square value= 0.045, *P*-value= 0.832) which would be expected since both R2 has shown to likely have at least one copy of the dominant allele and the F_2 seedling also has the red phenotype. The F_2 seedlings from Family 4 used in this study had contrasting ratings and gave contrasting results when selfed. Plant F2-R4-1 had a rating of 1 and its selfed progeny did not fit the expected all-green stem and petiole color (0:1). Plant F2-R4-2 had a rating of 5 and its selfed progeny fit the all-red ratio (1 red: 0 green) completely (*P*-value= 1). Interestingly, the progeny which did not fit the expected ratio were from selfing G and F2-R4-1 each of which was supposed to have all-green stem and petiole color. This lack of fit could be due to environmental effects or possibly that a small amount of pigmentation might be inherent and controlled separately from the pathway regulating the amount of pigmentation observed. Additionally, the majority of plants from selfing G and F2-R4-1 did not have strong red/pigmentation phenotypes and the majority had ratings of 2: ⊗G had 60% of progeny with a 2 rating, 24% with a 3 rating and 14% with a 1 rating; and ⊗F2-R4-1 had 63% with a 2 rating, 21% with a 3 rating, and 11% with a 1 rating. Another cross from the breeding program was coincidentally a backcross within Family 4. An F_2 seedling with a rating of 5 from Family 4 was crossed with R2 resulting in 149 plants with a red rating and no all-green plants for stem and petiole color (data not shown). These results would be expected given both parents of the cross had red ratings of 5 for stem and petiole color. Considering the results from all the crosses and selfing to investigate inheritance of stem and petiole color, the red/pigmented phenotype appears to be dominant (Figure 3.4) and could be controlled by more than two genes. Further studies are

needed to conclusively determine the mode of inheritance as well as investigate the degree of environmental effects on stem and petiole color.

Flower Color

Reciprocal crosses with 'Robert Fleming'

From crossing the red-flowered cultivar, 'Robert Fleming,' with white to pale pink-flowered hybrids of intraspecific crosses of *Hibiscus moscheutos* subspecies *moscheutos* different outcomes were observed (Figure 3.5). When RF was the pollinator, progeny were mostly red, with some intermediate and fewer white flowers, but when RF was the seed parent the progeny were all red for each of the three crosses. These results strongly suggest an effect of maternal cytoplasmic inheritance on flower color with red, or the production of anthocyanins, to be dominant (Campanella, 2019). The observation of all red-flowered progeny from the selfing of RF further supported the effect of cytoplasmic inheritance of red-flower phenotype. The influence of extranuclear inheritance has been reported in other traits such as variegated foliage in the *Cercis* hybrid 'Floating Clouds' (Roberts et al., 2015) and multiple characteristics of interspecific hybrids of *Brassica* (Chang et al., 2007). Crossing a strong red-flowered plant (RF) with a white- to pale pink-flowered plant was expected to produce all-red flowered progeny since the two phenotypes are distinctly contrasting and are likely due to a production or lack of production of anthocyanins in the flower. Therefore the F_1 generation was expected to show one uniform phenotype. If we consider the red/dark pink and intermediate flowers as one phenotype (production of anthocyanins) and the lack of pigment (white) as the other phenotype, then the progeny statistically fit an expected ratio of 1 red: 0 white. Progeny from crosses with RF as the pollinator parent displayed the expected pigmented flowers over the lack of pigmented (white) flowers. This would suggest that RF is homozygous dominant (when it is the pollinator) and

Plants A, B, and C are sufficiently homozygous recessive for the flower color trait. Further selfing and backcrossing could be conducted to evaluate these observations and hypotheses. Two additional plants from the breeding program with similar background and flower color to Plants A, B, and C were crossed reciprocally with RF and the resulting seed numbered between 60 to 676 per cross but were not sown due to shortage of resources.

Red-flowered pollinator on white- to light pink-seed parents

The results from the four crosses involving the same red-flowered pollinator parent (2014-54) (Figure 3.6) with four different white- to light pink-flowered seed parents suggest the progeny are heterozygous for the gene controlling flower color. (No images of flowers of the seed parents are shown, but they are similar to Plants A, B, and C crossed with RF in Figure 3.5.) If pigmented flowers with any amount of red are considered to be one phenotype and white, or non-pigmented, flowers are a second phenotype, then the parents could be presumed to be homozygous in their respective ways due to their contrasting phenotypes. Further crossing and/or selfing could potentially verify if the pollinator parent 2014-54 is homozygous dominant and/or the seed parents are homozygous recessive for a single locus controlling flower pigmentation.

Red by red reciprocal

The models which were evaluated were based on the assumption that the two siblings (2014-80 and -82) (Figure 3.7) were heterozygous since progeny were observed to segregate into different and contrasting phenotypes. The two models which the progeny for both crosses fit involve two genes which are epistatic to each other. Dominant gene interaction (9:6:1) describes the outcome of three phenotypes: the majority of F_2 progeny display one phenotype due to the dominant allele being present in both genes, a second phenotype is observed in 6/16 of the progeny because only one of the two genes has a dominant allele and the other gene is homozygous recessive, and a

third phenotype is observed in a few of the progeny that are homozygous recessive for both genes. The model of duplicate gene action (15:1) defines activity of two genes that when either one has the dominant allele (either in one (heterozygous) or copies (homozygous)), one phenotype is observed, and only when both genes are homozygous recessive is the recessive (or mutant) phenotype observed. In this study, the pigmented phenotype is observed when a dominant allele is present in either of two genes likely regulating anthocyanin biosynthesis, and the white/non-pigmented phenotype is only observed when the two genes are homozygous recessive resulting in a lack of anthocyanin production. Since two models were found to fit and each seems plausible, additional studies need to be conducted to investigate which is the more appropriate predictor.

Additional Traits from F_2 Progeny

A few other traits were observed of the F_2 families, and different genetic models via their respective segregation ratios were evaluated. The models tested on the traits of flower openness, petal overlap, and compactness were monogenic dominance (Mendelian 3:1) and several epistatic models: duplicate gene action (15:1), complementary gene interaction (9:7), dominant suppression gene action (13:3), and dominant gene interaction (9:6:1). Studies to evaluate the above-mentioned traits which fit certain ratios were not pursued due to limited resources and time.

The degree that flowers were open was estimated by a subjective rating scale of 1-5, where 1 = the least open/ less than 45°, 2 = partially open/ about 45°, 3 = about 90° open, 4 = between 90° and fully flat (180°), and 5 = fully open and flat (like a dinner plate). The F_2 progeny from Families 2 and 4 fit Mendelian monogenic inheritance where the ratings 1-4 were grouped into one dominant phenotype of less than fully open and a 5 rating (fully open) was the

recessive phenotype (Family 2: chi-square = 1.246, *P*-value= 0.264; Family 4: chi-square = 1.673, *P*-value= 0.196). Interestingly, Families 2 and 4 share the same seed parent (R2) but have different pollinator parents (*Hibiscus moscheutos* subsp. *lasiocarpos* and *H. grandiflorus*, respectively) (Figure 3.3). For flower openness, all families were found to fit dominant suppression gene action with the same two phenotypes as above (1-4:5).

There is variation to some degree in the amount that petals overlap ranging from fully separate with space between the petals, to overlapping by almost 50%. In the F_2 progeny of Families 1, 2, 3 and 4, this trait was evaluated via subjective visual ratings on a scale of 1-5, where 1 = no overlap at all/petals barely touching, 2 = < 15% overlap/slight overlap, 3 = 15-30% overlap, 4 = 30-50% overlap, and 5 = ≥ 50% overlap of petals. Of the models tested, Families 1, 2, and 3 were found to fit duplicate gene action (15:1) where ratings of 2-5 were pooled into one phenotype of any amount of petal overlap and the second phenotype being no petal overlap (rating = 1). Chi-square and *P*-values for testing petal overlap against the 15:1 model are as follows: Family 1: 0.047, 0.828; Family 2: 0.058, 0.810; Family 3: 0.023, 0.879; and Family 4: 237.223, 0.000, respectively. The crosses resulting in Families 1, 2 and 3 had the same pollinator parent and the seed parents are siblings, whereas Family 4 had a pollinator parent of a different species (*H. grandiflorus*) but a similar seed parent. These results suggest the mode of inheritance of petal overlap could be regulated by more than two genes when *H. grandiflorus* is the male parent, and that some amount of petal overlap in *H. moscheutos* hybrids is much more prevalent than no overlap. None of the other models evaluated was found to fit any of the families.

Compactness of a plant is not easily predicted as compared to a trait like foliage color. Plant compactness of the *Hibiscus* hybrids of the F_2 generation for Families 1, 2, 3 and 4 was evaluated by a rating scale of 1-5, where 1 = not compact/very open, 2 = slightly compact, 3 =

moderate compactness, 4 = compact, and 5 = very or extremely compact. Genetic models of two phenotypes were tested by pooling ratings of 2-5 into one phenotype with a 1 rating as the second phenotype. Family 1 was found to fit the complementary gene interaction model (9:7) (chi-square = 0.529, *P*-value = 0.467) and Family 3 fit dominant suppression action (13:3) (chi-square = 0.002, *P*-value = 0.964). These results are not too clear because similar families did not result in similar chi-square values. Further studies would need to be conducted to investigate these findings.

Hibiscus grandiflorus

The species *Hibiscus grandiflorus* was used as pollinator parent in a cross with an intraspecific cross of *H. moscheutos* subsp. *moscheutos* resulting in the F_2 seedlings comprising Family 4. The data and chi-square test statistics evaluating the traits for which no genetic inheritance model was found to fit the phenotypic data of F_2 progeny of Family 4 is shown in Appendix C; these traits are: foliage color, leaf pubescence, petal overlap and compactness.

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Table 3.1. Number of observed and expected *Hibiscus* plants for foliage color with corresponding chi-square and *P* values, where $\chi^2_{0.05, 1} = 3.84$. Plants were evaluated subjectively using a rating scale for the amount of red (2-5) or green (1) foliage.

Parent(s)	**Generation**	**Plants Observed (no.)**		**Expected Ratio[z]**	**Plants Expected (no.)**		χ^2	***P***
		Red (rating: 2-5)	**Green (rating: 1)**	**Red:Green (rating: 2-5:1)**	**Red (rating: 2-5)**	**Green (rating: 1)**		
R1 x G[y]	F_2 [x]	151	41	3:1	144	48	1.361	0.243
R2 x G	F_2	180	58	3:1	179	60	0.050	0.823
⊗ R1[w]	S_1 from P ♀ [v]	53	0	1:0	53	0	-	-
⊗ R2	S_1 from P ♀	178	0	1:0	178	0	-	-
⊗ G	S_1 from P ♂	6	244	0:1	0	250	0.144	0.704
⊗ F2-R1[u]	S_1 from F_2	135	0	1:0	135	0	-	-
⊗ F2-R2	S_1 from F_2	118	2	1:0	120	0	0.033	0.856

[z] The expected ratio is single gene action where the dominant allele leads to a red-foliage phenotype and the completely green-foliage (or lack of red) phenotype is observed when a plant is homozygous recessive.

[y] The symbols R1 and R2 represent two intraspecific hybrid genotypes of *Hibiscus moscheutos* subsp. *moscheutos* having a red-foliage phenotype. The symbol G represents *H. moscheutos* subsp. *lasiocarpos*, which has a green-foliage phenotype.

[x] F_2 denotes the second generation of plants from the cross in the Parent(s) column. Foliage color was rated once for each F_2 plant during Sept. 2015.

[w] The symbol ⊗ represents selfing a plant, i.e. making a cross of the same genotype.

[v] S_1 denotes the first generation of plants from selfing. Foliage color was rated once for each S_1 plant between July 24 and Aug. 2, 2017.

[u] The symbols F2-R1 and F2-R2 represent seedlings from F_2 generations of R1 x G and R2 x G, respectively, which had the red-foliage phenotype.

Table 3.2. Number of observed and expected *Hibiscus* plants for leaf pubescence with corresponding chi-square and *P* values, where $\chi^2_{0.05, 1} = 3.84$. Plants were evaluated subjectively using a rating scale for the amount of pubescent (2-5) or glabrous (1) foliage.

Parent(s)	**Generation**	**Pubescence Ratings of Parent(s)**	**Plants Observed (no.)**		**Expected Ratio[z]**	**Plants Expected (no.)**		χ^2	***P***
			Pubescent (rating: 2-5)	Glabrous (rating: 1)	Pubescent: Glabrous (rating: 2-5:1)	Pubescent (rating: 2-5)	Glabrous (rating: 1)		
R1 x G[y]	F_2 [x]	1 x 5	180	12	15:1	180	12	-	-
R2 x G	F_2	1 x 5	227	11	15:1	223	15	1.077	0.299
R3 x G	F_2	1 x 5	190	6	15:1	184	12	3.401	0.065
⊗ R1[w]	S_1 from P ♀[v]	1	20	33	0:1	0	53	7.547	0.006
⊗ R2	S_1 from P ♀	1	61	117	0:1	0	178	20.904	0.000
⊗ R3	S_1 from P ♀	1	22	129	0:1	0	151	3.205	0.073
⊗ G	S_1 from P ♂	5	250	0	1:0	250	0	-	-
⊗ F2-R1[u]	S_1 from F_2	1	72	63	0:1	0	135	38.400	0.000
⊗ F2-R1-2	S_1 from F_2	5	249	0	1:0	249	0	-	-
⊗ F2-R3-1[t]	S_1 from F_2	1	36	28	0:1	0	64	20.250	0.000

[z] The expected ratio is of duplicate gene action where a dominant allele of either of two genes leads to the pubescent phenotype; the glabrous phenotype is only observed when a plant is homozygous recessive for both genes.

[y] The symbols R1, R2 and R3 represent three intraspecific hybrid genotypes of *Hibiscus moscheutos* subsp. *moscheutos* having glabrous foliage. The symbol G represents *H. moscheutos* subsp. *lasiocarpos*, which has highly pubescent foliage.

[x] The symbol F_2 denotes the second generation of plants from the cross in the Parent(s) column. Leaf pubescence was rated once for each F_2 plant during Sept. 2015.

[w] The symbol S_1 denotes the first generation of plants from selfing. Leaf pubescence was rated once for each S_1 plant during Summer 2017.

[v] The symbol ⊗ represents selfing a plant, i.e. making a cross of the same genotype.

[u] The symbols F2-R1 and F2-R1-2 represent seedlings from an F_2 population of R1 x G.

[t] The symbol F2-R3-1 represents a seedling from an F_2 population of R3 x G.

Table 3.3. Number of observed and expected *Hibiscus* plants for stem and petiole color with corresponding chi-square and *P* values, where $\chi^2_{0.05,\,1} = 3.84$. Plants were evaluated subjectively using a rating scale for the amount of red (2-5) or green (1) foliage.

Parent(s)	**Generation**	**Red Rating(s) of Parent(s)**	**Plants Observed (no.)**		**Expected Ratio[z]**	**Plants Expected (no.)**		χ^2	***P***
			Red (rating: 2-5)	Green (rating: 1)	Red:Green (rating: 2-5:1)	Red (rating: 2-5)	Green (rating: 1)		
R2 x G[y]	F_2 [x]	5 x 1	230	8	15:1	223	15	3.390	0.066
R2 x G2	F_2	5 x 1	285	11	15:1	278	18	3.243	0.072
⊗ R2 [w]	S_1 from P ♀ [v]	5	178	0	1:0	178	0	-	-
⊗ G	S_1 from P ♂	1	214	36	0:1	0	250	183.184	0.000
⊗ F2-R2 [u]	S_1 from F_2	2	120	0	1:0	120	0	-	-
⊗ F2-R2-2	S_1 from F_2	2	239	12	1:0	251	0	0.574	0.449
⊗ F2-R4-1[t]	S_1 from F_2	1	122	15	0:1	0	137	108.642	0.000
⊗ F2-R4-2	S_1 from F_2	5	94	0	1:0	94	0	-	-

[z] The expected ratio is of duplicate gene action where a dominant allele of either of two genes leads to the red phenotype; the green phenotype is only observed when a plant is homozygous recessive for both genes.

[y] The symbol R2 represents an intraspecific hybrid genotype of *Hibiscus moscheutos* subsp. *moscheutos* having a red-foliage phenotype. The symbol G represents *H. moscheutos* subsp. *lasiocarpos* and G2 represents *H. grandiflorus*, both having green-foliage phenotype.

[x] The symbol F_2 denotes the second generation of plants from the cross in the Parent(s) column. Stem and petiole color was rated once for each F_2 plant during Sept. 2015.

[w] The symbol S_1 denotes the first generation of plants from selfing. Stem and petiole color was rated once for each S_1 plant during Summer 2017.

[v] The symbol ⊗ represents selfing a plant, i.e. making a cross of the same genotype.

[u] The symbols F2-R2 and F2-R2-2 represent seedlings from an F_2 population of R2 x G.

[t] The symbols F2-R4-1 and F2-R4-2 represent seedlings from an F_2 population of R2 x G2.

Table 3.4. Investigation of the inheritance of flower color with reciprocal crosses of a red-flowered cultivar of *Hibiscus* and white/blush to light pink-flowered hybrids of *H. moscheutos* subsp. *moscheutos*.

Cross	**Flower Colors of Cross**	**Total Plants**	**Total Plants in Flower**	**Number of Plants with Observed Flower Colors[z]**		
				Red/Dark Pink	Intermediate	White
RF[y] x Plant A[x]	red x white	22	20	20	0	0
RF x Plant B	red x white/blush	12	10	10	0	0
RF x Plant C	red x blush/light pink	31	30	30	0	0
Plant A x RF	white x red	100	82	62	15	5
Plant B x RF	white/blush x red	58	47	40	5	2
Plant C x RF	blush/light pink x red	150	139	123	13	3
⊗ RF[w]	selfed red	105	76	76	0	0

[z] The determination of flower color was a subjective evaluation in situ between 30 July and 13 Sept. 2017.

[y] The symbol RF represents the commercial cultivar *Hibiscus* ‘Robert Fleming’.

[x] Plants A, B, and C are intraspecific hybrid genotypes of *Hibiscus moscheutos* subsp. *moscheutos*.

[w] The symbol ⊗ represents selfing a plant, i.e. making a cross of the same genotype.

Table 3.5. Investigation of the inheritance of *Hibiscus* flower color with four crosses each having a red-flowered pollinator parent and a seed parent having white/blush to pink flowers.

Cross	**Flower Colors of Cross**	**Total Plants**	**Total Plants in Flower**	**Number of Plants with Observed Flower Colors[z]**			**Number of Plants with Expected Flower Colors[y]**		**$\chi^{2,x}$**	***P***
				Red/Dark Pink	Intermediate	White	Red-Intermediate	White		
Plant D[w] x 2014-54[v]	pink x red	21	18	9	8	1	18	0	0.056	0.813
Plant E[u] x 2014-54	pale pink x red	100	21	11	8	2	21	0	0.190	0.663
Plant F x 2014-54	white/blush x red	249	147	55	69	23	147	0	3.599	0.058
F2-R2 x 2014-54	pink x red	125	7	6	1	0	7	0	0.000	1

[z] Determination of flower color was a subjective evaluation in situ between 23 July and 29 Aug. 2017.

[y] The expected values are based on an F_1 (first generation) ratio of 1:0, where pigmented (red-intermediate) is dominant to non-pigmented (white) flower.

[x] The chi-square critical value for two phenotypes is 3.84 ($\chi^2_{0.05,\,1}$).

[w] Plant D is an experimental breeding line from intraspecific crosses of *Hibiscus moscheutos* subsp. *moscheutos*.

[v] Plant 2014-54 was selected as an experimental breeding line from a cross between an intraspecific hybrid of *Hibiscus moscheutos* subsp. *moscheutos* and *Hibiscus* ‘Midnight Marvel’.

[u] Plants E and F are F_2 (second generation) progeny from a cross between an intraspecific hybrid of *Hibiscus moscheutos* subsp. *moscheutos* and *H. moscheutos* subsp. *lasiocarpos*.

Table 3.6. Investigation of the inheritance of *Hibiscus* flower color of reciprocal crosses between two sibling genotypes. The expected values are based on two gene epistatic models.

Cross	**Flower Colors of Cross**	**Total Plants**	**Total Plants in Flower**	**Number of Plants with Observed Flower Colors[z]**			**Number of Plants with Expected Flower Colors**			$\chi^{2,y}$	***P***
				Red/Dark Pink	Intermediate	White	Red/Dark Pink	Intermediate	White		
2014-80[x] x 2014-82	red x red	150	130	79	43	8	73[w]	49	8	1.152	0.562
2014-82 x 2014-80	red x red	144	130	77	46	7	73	49	8	0.516	0.773
2014-80 x 2014-82				122		8	122[v]		8	0.000	1
2014-82 x 2014-80				123		7	122		8	0.166	0.684

[z] Determination of flower color was a subjective evaluation in situ between 4 and 24 Aug. 2017.

[y] The chi-square critical value for three phenotypes is 5.999 ($\chi^2_{0.05, 2}$) and for 2 phenotypes is 3.84 ($\chi^2_{0.05, 1}$).

[x] Plants 2014-80 and 2014-82 were selected as experimental breeding lines from an intraspecific cross of *Hibiscus moscheutos* subsp. *moscheutos* and *Hibiscus* ‘Midnight Marvel’.

[w] The expected values are based on a two gene epistasis model of dominant gene action (9 red: 6 intermediate: 1 white).

[v] The expected values are based on a two gene epistasis model of duplicate gene action (15 red/pigmented: 1 white/non-pigmented).

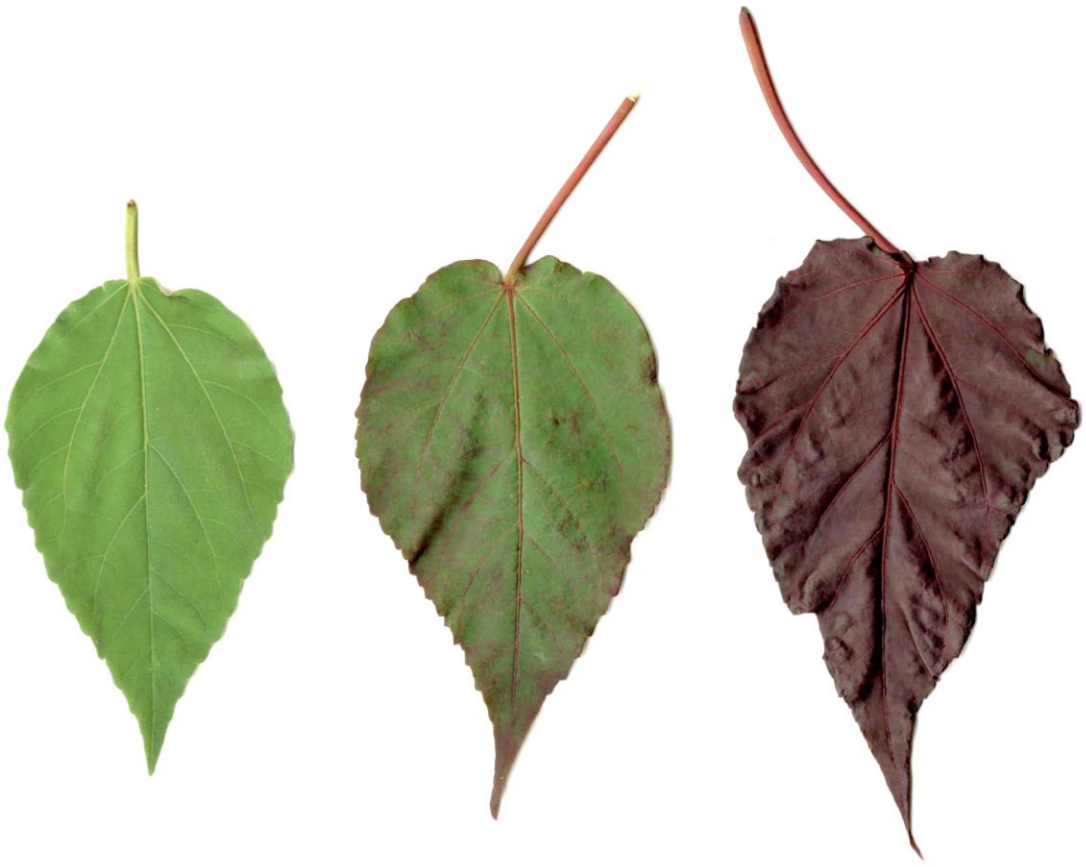

Figure 3.1. Leaves of hybrids of *Hibiscus moscheutos* L. showing foliage color ratings: 1 (left), 3 (middle), and 5 (right).

Figure 3.2. Leaves of hybrids of *Hibiscus moscheutos* L. showing foliage pubescence ratings: 1 (left), 3 (middle), and 5 (right). Photo by author.

Figure 3.3. Plants used as pollinators in crosses investigating the inheritance of leaf color, pubescence and stem and petiole color: *Hibiscus moscheutos* subsps. *lasiocarpos* (top) and *H. grandiflorus* (bottom two photos). Taken July 2016 at the University of Georgia's Durham Research Farm in Watkinsville, GA by the author.

Figure 3.4. Examples of *Hibiscus moscheutos* intraspecific hybrids with strong red stem and petiole phenotype. Rating of 5 on 1-5 scale, where 1 = no red/ all green, 2 = small amount of red, 3 = about 50% red, 4 = mostly red, and 5 = totally red. Taken July 2016 at the University of Georgia's Durham Research Farm in Watkinsville, GA by the author.

Seed Parent	Pollinator Parent	Red/Dark Pink	Intermediate	White
‘Robert Fleming’	 Plant A		-	-
	 Plant B		-	-
	 Plant C		-	-
Plant A	‘Robert Fleming’			
Plant C				

Figure 3.5. Flower colors of the red-flowered *Hibiscus* ‘Robert Fleming’, white-to-light pink intraspecific hybrids of *Hibiscus moscheutos* subsp. *moscheutos* parents and progeny from their crossing which demonstrate three flower-color phenotypic groups.

Figure 3.6. The red-flowered pollinator parent (2014-54) used in crosses with white to pink seed parents to investigate the inheritance of flower color. The accession 2014-54 was selected in 2014 and originated from crossing an intraspecific hybrid of *Hibiscus moscheutos* subsp. *moscheutos* and *Hibiscus* 'Midnight Marvel'. Taken July 2016 at the University of Georgia's Durham Research Farm in Watkinsville, GA by the author.

Figure 3.7. The red-flowered parents used in reciprocal crosses to investigate the inheritance of flower color in *Hibiscus* hybrids. Plants were selected in 2014 as part of the hardy hibiscus breeding program: 2014-80 (top) and 2014-82 (bottom). Taken July 2016 at the University of Georgia's Durham Research Farm in Watkinsville, GA by the author.

CHAPTER 4

Propagation of *Liquidambar formosana* ‘Formosan Gold’ Sweetgum via Somatic Embyrogenesis

Introduction

A brilliant-yellow, variegated form of Formosan sweetgum (*Liquidambar formosana* Hance) exists within the University of Georgia’s ornamental plant research collection. The genotype was obtained from seed of Formosan sweetgum received by Dr. John M. Ruter from Lawyer Nursery, Inc. in Plains, MT in 1994. A single unique seedling was found that displayed light green to yellow, chartreuse colored foliage. This noteworthy feature has bestowed potential for the introduction of this clone as an ornamental tree. Clonal propagation in large numbers is necessary if the selection is to be released as a commercial product. Asexual propagation via stem cuttings of this specimen located in Tifton, GA has been conducted in the past. However, very few rooted cuttings successfully overwintered, mortality often being attributed to root rot. The occurrence of cutting mortality associated with overwintering of certain deciduous taxa has been documented and reviewed previously by Wilson and Struve (2004). Literature reports various methods of propagating Formosan, and the related American (*L. styraciflua* L.), sweetgum with mixed success over the past 55+ years. A rooting study conducted on *L. styraciflua*, concluded that it is a “difficult-to-root” species (Bilan, 1974). Some ex vitro methods include rooting of stem, tip and root cuttings, shoots from girdled mother plant, suckers, and mini-cuttings from stumps (Bilan, 1974; Brown and McAlpine, 1964; Farmer, Jr., 1966; Hare, 1976; Rieckermann et al., 1999; Wendling et al., 2010). Alternative methods, i.e. in vitro, to propagate Formosan sweetgum are rare in available literature (Durkovic et al., 2005; Xu et al.,

2007). To our knowledge, there are no reports of Formosan sweetgum plants being generated via somatic embryogenesis. Somatic embryogenesis is also often utilized with plants that are difficult to clonally propagate (Hartmann et al., 2002). There has been extensive research on somatic embryogenesis to produce plants of American sweetgum, as well as of hybrids of American × Formosan sweetgum (*L. styraciflua* x *L. formosana*) (Dai et al., 2004; Merkle and Battle, 2000; Merkle et al., 1998; Merkle et al., 2003; Merkle et al., 2010; Vendrame et al., 2001). Evaluating somatic embryogenesis as a successful propagation method for this variegated Formosan sweetgum genotype could prove useful for furthering its potential as a new marketable ornamental tree.

Some factors examined with previous somatic embryogenesis research include: utilization of different plant parts, plant growth regulators (PGRs), collection dates of source material, pre-germination treatments, and containers in which to germinate and grow developing seedlings (Merkle and Battle, 2000; Merkle et al., 1998; Merkle et al., 2010). In most of the studies, clone or source plant had a significant effect on variables tested (Merkle and Battle, 2000; Merkle et al., 1998; Merkle et al., 2003; Merkle et al., 2010). Prior research has found explant type (plant organ), to significantly affect induction of embryogenesis and other dependent variables. Studies using inflorescences from dormant buds and seed from immature fruit have generated embryogenic cultures from *Liquidambar* (Merkle and Battle, 2000; Merkle et al., 1998); leaves from dormant buds have also been tested but no embryogenic cultures were produced. In addition to explant type, age of tissue is of interest for its potential effect on somatic embryogenesis induction. Previous research with American sweetgum in the southeastern U.S. found that collecting samples of fully dormant buds yielded more successful induction of somatic embryogenic cultures than using buds at the point of or which had already opened

(Merkle et al., 1998). Additionally, earlier research has shown initial induction media type to have a significant effect on somatic embryogenic culture of sweetgum (Dai et al., 2004; Merkle et al., 1998; Merkle et al., 2010; Vendrame et al., 2001). Therefore, to find the optimal procedure we hypothesized that plant growth regulators (PGRs) incorporated in induction media, utilization of dormant buds and immature fruits affect somatic embryogenesis and subsequent embryo germination in our genotype. The goal for this project was to determine if it is possible to obtain seedlings via somatic embryogenesis and subsequent micropropagation of this unique genotype of *Liquidambar formosana*. To accomplish this, we proposed to culture different explant material, specifically leaves and pistillate and staminate inflorescences from dormant buds and seeds from immature fruit, from our tree on medium with different PGRs and rates, as well as from material collected from the tree over a few successive dates. Tissue was then observed for effects of said treatments and evaluated for any growth of callus, proembryogenic masses, and somatic embryos. Evaluating this propagation technique as an avenue for multiplying this variegated Formosan sweetgum will prove advantageous to it becoming a popular cultivar.

Materials and Methods

Dormant buds experiment. The experiment was a three-way factorial (3x5x3) testing the effects of explant type (leaf, staminate inflorescence, and pistillate inflorescence), plant growth regulator (PGR) (TDZ at low and high rate, NAA at low and high rate, and no-PGR Control), and time of collection on induction of somatic embryogenesis. Dormant mixed buds (containing leaves and inflorescences) were used as source of explant material and were collected from a clone of a variegated Formosan sweetgum specimen planted in 2001 on the University of Georgia's ornamental research plot in Tifton, GA (31.4766°N, 83.5202°W) on three dates in 2016: 7 and 29 Jan. and 22 Feb. Buds were placed in sterile bags on ice in a cooler for approx. three hours,

transferred to refrigerator at 4°C overnight and then surface-disinfested, dissected and cultured the following day. Surface-disinfestation and dissection was done as described in Merkle et al. (1998), except that individual heads from the staminate inflorescence axis were severed aseptically with a scalpel and sliced in half longitudinally and laterally. The four segments were then placed cut-side down on semisolid medium in a single 60x15mm plastic Petri dish (VWR International, Radnor, PA) plate along with pieces from some, but not all, of other staminate heads from the same bud. Pistillate heads, when present, were singular and observed close to the base of inflorescence axis, as noted in Merkle and Battle (2000). Pistillate inflorescences were excised from the bud interior, sliced in half and pieces placed cut-side down in the same plate. Unexpanded leaves were cut at the base where they connect to the petiole, opened, cut longitudinally, then laterally, and the four leaf pieces were plated together along with other leaf pieces from the same bud. Most leaf pieces were plated with either abaxial or adaxial surface down and the number of pieces per plate varied depending on number of leaves present in each bud (but not all leaves were used). Approximately seven plates were used for staminate inflorescences, seven for leaf tissue, and five or six plates for pistillate inflorescences per PGR medium/collection date combination. Number of plates of pistillate inflorescence varied due to the occasional absence of female flowers from the mixed buds. Each plate represented a single replicate and the five PGR treatment levels consisted of: control (basal medium + no PGR), TDZ low (basal + 0.01mg·L^{-1} TDZ), TDZ high (basal + 0.1mg·L^{-1} TDZ), NAA low (basal + 1mg·L^{-1} NAA), and NAA high (basal + 5mg·L^{-1} NAA). Basal medium consisted of a modified Blaydes' medium (Witham et al., 1971) with Brown's minor salts (Sommer and Brown, 1980), iron according to Murashige and Skoog (1962), vitamins of Gresshoff and Doy (1972), 1g·L^{-1} of casein hydrolysate (CH), and 40 g·L^{-1} sucrose. The medium was gelled using 7 g·L^{-1} agar-

substitute gelling agent (Phytagel, Sigma-Aldrich Co., St. Louis, MO) and poured into petri dishes to solidify and cool for approx. 30 mins. Once explant pieces were cultured, they were maintained in the dark at 25°C and transferred aseptically to fresh medium after four weeks. Plates were checked 5 d after initiation, followed by every 2 weeks (approx.) for first month until fresh medium transfer, then at varying weekly intervals for 10 weeks. Checks included observation and count of cultures contaminated and any growth (e.g. callus, embryogenic induction), as well as photographs taken of noteworthy growth.

Immature fruits experiment. Experiment was a two-way factorial (5x2) to evaluate the effect of PGRs and collection date on somatic embryogenic induction of tissue excised from unfertilized ovules of immature fruits. Approx. 40 young, green fruits were bagged 13 May 2016 on the same specimen used for the dormant buds experiment. A paper pollination bag was placed over the gumball, cotton was placed inside the bag at the opening, and a zip-tie was used to seal the bag opening and hold it on the peduncle to minimize pest and pathogen infestation. Approximately half of the bagged gumballs were collected 26 June and the remainder on 12 July 2016 by cutting peduncle near bag opening. Fruits were left in bags until returning to Athens, GA where they were placed in refrigerator at 4°C and de-bagged indoors. A few days following collection, fruits were surface-disinfested, dissected and cultured over a two day period. Surface-disinfestation followed the same protocol as for buds, except the first wash with Roccal (Pfizer, Inc., Groton, CT) was eliminated, 50% bleach (sodium hypochlorite, The Clorox Company, Oakland, CA) wash was reduced to 20% and lasted 5 mins instead of 15, and Captan (48.9% N-Trichloromethylthio-4-cyclohexene-1,2-dicarboximide, Southern Agricultural Insecticides, Inc., Palmetto, FL) rinse and final three 3 min water rinses were omitted. Fruits were dissected similar to Merkle et al. (1998), including nicking with a scalpel and placing three immature seeds per

plate. Three fruits were used for each treatment level at each of the two collection dates. Seed from each fruit were used to fill three Petri plates with three seeds per plate. PGR treatment levels consisted of: control (basal medium + no PGR), low 2,4-D without BAP (benzylaminopurine) (basal + 0.5mg·L^{-1} 2,4-D), low 2,4-D with BAP (basal + 0.5mg·L^{-1} 2,4-D + 0.25mg·L^{-1} BAP), high 2,4-D without BAP (basal + 2mg·L^{-1} 2,4-D), and high 2,4-D with BAP (basal + 2mg·L^{-1} 2,4-D + 0.25mg·L^{-1} BAP). Basal medium had the same recipe used in bud experiment. Cultures were maintained in the dark at 25°C and checked 2, 8 and 13 weeks after initiation (WAI) for first collection date and 6 and 11 WAI for second collection date for contamination and any growth (e.g. callus, embryogenesis induction), and photographs taken of noteworthy culture growth on 28 Aug. 2016. Cultures were not transferred to fresh medium due to lack of callus development and widespread observed necrosis of tissues.

Data collection and analysis. On dates cultures were checked, counts were taken on number of contaminated tissues, as well as notes on appearance of cultures. As both experiments progressed, necrosis was observed and documented per tissue culture. Due to lack of mature callus or embryogenic growth on all cultures observed, no statistical analysis was conducted. Means are presented of amount of contamination or callus observed of cultures per Petri plate.

Results

Dormant buds experiment. For bud tissue initiated at the first date, contamination was relatively low with the exception of NAA high and TDZ low and high of staminate inflorescence cultures (Table 4.1). Most contamination was observed within the first month after experiment initiation for the first treatment date. For cultures initiated on the second date, contamination was relatively low and more staggered over experiment duration, the majority within the first 2 weeks (Table 4.2). Cultures initiated on the third date had relatively low contamination, with the majority

observed within the first two weeks, and 14 weeks later the cumulative contamination rates were highest for staminate inflorescence tissue from basal and NAA high treatments (Table 4.3). After transferring to fresh media, tissue that had not developed callus appeared mostly necrotic 10 and 7 weeks later for the first and second collection dates, respectively (Figure 4.1). Approx. 17 and 14 weeks after transferring (WAT) to fresh media for the first set and second set, respectively, most plates were discarded due to necrosis. However, some plates had live callus or callus-like tissue and were kept: three plates of NAA low and four plates of NAA high from staminate inflorescences (Figure 4.2), two plates of pistillate inflorescences and eight plates of leaf tissue of NAA high, one plate of TDZ low and ten plates of TDZ high of staminate inflorescence tissue. No plates from the controls were kept from the first or second dates due to lack of callus. At that time, a few plates had cultures transferred to fresh media due to promising callus growth appearance: two plates of NAA high and six plates of TDZ high, all from staminate inflorescence tissue. For the third set of buds initiated, about 10 WAT many of the cultures were discarded due to prominence of necrotic tissue. Only three plates of low NAA and one of high TDZ of staminate inflorescence tissue were observed to have callus or callus-like growth and were transferred to fresh basal medium. Although a number of callus and callus-like growths were observed among the plates (Tables 4.4, 4.5 & 4.6), no embryonic structures were observed to develop. There was one exception observed at 4 WAI on a culture from staminate tissue on high NAA initiated 8 Jan. 2016 (Figure 4.3). However, it was not seen after transferring the explant tissue sample to fresh medium.

Averaging over phytohormone treatments and dates of collection/initiation, staminate inflorescence tissue had the most callus growth as well as contamination (Table 4.7). Pistillate tissue had the least amount of contamination and staminate tissue had the most callus and callus-

like growth averaged over collection dates and PGR treatments (Table 4.8, Figure 4.4). Callus and callus-like growth averaged over tissue type and collection date was lowest for the PGR control, mid-range for TDZ, and highest for NAA treatments (Table 4.9, Figure 4.5). Additionally, low NAA had the least contamination with the other treatments in increasing order were: high TDZ, low TDZ, basal, and high NAA having the most contamination averaged over collection dates and tissue types (Table 4.10).

Immature fruits experiment. Contamination for the first initiation date was relatively low 2 weeks after plating, with the exception of low 2,4-D with BA, and similar contamination was observed 6 weeks later (Table 4.11). When plates were checked for the final time at 13 WAI, much of the tissue was necrotic and contamination had not increased. For the second collection date contamination was low for all treatments 6 WAI, with none exceeding 15%. At 11 WAI, most cultures were becoming necrotic and contamination had not increased. No immature seed culture resulted in callus nor embryonic-like growth, with the exceptions of one culture each from low 2,4-D without BA treatment from the first and second initiation dates and one culture from low 2,4-D with BA from the second initiation date which were observed to have a small amount of callus at 6 (for first collection date) or 8 (for second collection date) WAI. One culture from the second collection date at high 2,4-D with BA had a root hair at 6 WAI (Figure 4.6).

Discussion

Somatic embryos can either arise directly (no callus formed) or indirectly (callus formed) from the explant tissue when in amended medium in vitro. While having the ability to produce mass quantities of new plants, somatic embryogenesis also restores juvenility or reestablishes the plant to the juvenile phase of its cycle. For woody plants this reversion to juvenility can be advantageous, enabling the tissue to be used for micropropagation or other forms of propagation.

Somatic embryos can develop from a cluster of cells or from a single cell (Hartmann et al., 2002; Williams and Maheswaran, 1986). To coerce explant material to undergo induction, forms of auxin, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and NAA, and cytokinins are often used. For the bud tissue experiment, the auxin NAA and the cytokinin TDZ were applied at low and high rates. Averaging over tissue type and collection date, NAA seemed to generate a higher frequency of callus than did TDZ (Table 4.10). Also, both phytohormones had higher rates of callus induction than medium without auxin or cytokinin. This would suggest phytohormones had an effect on callus generation. However, there was no observation of callus producing embryos or pro-embryogenic masses (PEMs). Collection date with the highest frequency of callus was 30 Jan., which had a mean more than 2x and 7x greater that of the first and third collection dates, respectively. This would suggest late Jan. is the optimal time to initiate inflorescence cultures. From these results, future experiments should utilize similar rates of NAA in medium, with additional rates around the range used here, collect and initiate dormant bud cultures around 30 Jan. from the Tifton, GA area. Additional future research could focus on and explore alternative methods to develop mature callus and production of somatic embryos for this variegated form of Formosan sweetgum.

For the immature fruits experiment, contamination rates were low for the seed tissue cultures, however very little callus growth was observed. The second collection date had less contamination, which is somewhat unexpected given that more time on the tree would provide more opportunity for pathogens to enter the fruit. However, it could also have been that the first collection date had a higher contamination percent due to contaminants encountered during the surface disinfestation or dissection stages. Medium from fruit cultures, especially from the second collection date, appeared progressively darker in color over time with an orange/brown

hue, possibly due to tannins or other secondary compounds seeping out of the immature seed tissue. The observation of callus development from a few cultures on 2,4-D with and without BA would suggest these two treatments to be the most worthwhile to apply, were a related experiment to be carried out. Low generation of callus or embryogenic cultures was observed from a similar study in 2001 which used hybrid seed from nine controlled crosses of American and Formosan sweetgum trees to obtain clonal somatic embryos (Vendrame et al., 2001). Immature seed was collected at two dates in Summer 1999 and plated on two induction media containing 2,4-D. Neither media nor cross had an effect on induction frequency of embryogenic cultures, however collection date did. From the seeds cultured, only 2% resulted in repetitive embryogenic cultures that produced embryos that germinated into somatic seedlings. A later study was carried out to improve on this work of generating somatic embryos and seedlings from *Liquidambar* hybrids (*L. styraciflua* x *formosana*) by Merkle et al. (2010). Trees displaying ornamental potential were chosen for cloning and it was found that applying cold temperatures to cultures for at least 8 weeks prior to germination raised average germination and conversion of the embryos as compared to those given only 4 weeks of cold or none. An additional related study using immature seed of hybrid sweetgum evaluated the production of somatic embryos yielding seedlings when proembryogenic masses (PEMs) were grown in liquid induction-maintenance medium (IMM). Suspension culture can aid in streamlining the formation of distinct somatic embryos (SEs) which are synchronous and of high quality. The liquid IMM was also supplemented with amino acids to enhance embryo development (Dai et al., 2004). Many publications present advancements of somatic embryogenic work with *Liquidambar styraciflua* and have proved useful in designing this experiment (Merkle and Battle, 2000; Merkle et al., 2003; Merkle et al., 1998). This study had initial plans of using liquid medium and applying cold

temperatures to aid embryo development and germination, however, due to the lack of mature callus development, those steps were not possible.

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Table 4.1. Number of new cultures from *Liquidambar formosana* 'Formosan Gold' observed contaminated during study initiated 8 Jan. 2017 (1st set of dormant buds).

1st set				# cultures newly contaminated						
Treatment	**Explant part**	**# plates**	**# cultures**	**5 DAT[z]**	**17 DAT**	**28 DAT**	**1 wk after transfer**	**3 wk(s) after transfer**	**10 wk(s) after transfer**	**% contamination**
Basal	**Staminate**	7	30	4	2	2	4	0	1	43
	Pistillate	6	12	0	0	0	0	0	0	0
	Leaf	7	37	0	0	1	0	0	0	3
NAA low	**Staminate**	7	28	4	0	1	0	0	0	18
	Pistillate	6	12	0	0	0	0	0	0	0
	Leaf	7	35	0	0	0	0	0	0	0
NAA high	**Staminate**	7	30	12	6	0	1	3	0	73
	Pistillate	6	12	0	0	0	0	0	0	0
	Leaf	7	38	5	1	1	0	0	0	18
TDZ low	**Staminate**	7	28	4	9	4	0	0	0	61
	Pistillate	6	12	0	0	0	0	0	0	0
	Leaf	7	50	0	0	2	0	0	0	4
TDZ high	**Staminate**	7	28	0	1	9	0	2	0	43
	Pistillate	6	12	0	2	0	0	0	0	17
	Leaf	7	55	0	0	1	0	0	0	2

[z]Days after treatment/initiation of experiment.

Table 4.1. Number of new cultures from *Liquidambar formosana* 'Formosan Gold' observed contaminated during study initiated 31 Jan. 2017 (2nd set of dormant buds).

2nd set			# of plates newly contaminated						
Treatment	**Explant part**	**# plates**	**5 DAT[z]**	**10 DAT**	**14 DAT**	**28 DAT**	**10 d(s) after transfer**	**7 wk(s) after transfer**	**% contamination**
Basal	**Staminate**	7	2.5	1	0.5	0	0	0	57.1
	Pistillate	6	0	0	0	0	0.5	0	8.3
	Leaf	7	0	0.5	0	0	0	0.5	14.3
NAA low	**Staminate**	6	2	0	0	0	0	0	33.3
	Pistillate	6	0	0	0	0	0	0	0
	Leaf	6	1	1	0	0.5	0	0	41.7
NAA high	**Staminate**	7	3	0	0	0.5	0	1	64.3
	Pistillate	5	0	0	0	0	0	0	0
	Leaf	7	0	1	0	0.5	0	0	21.4
TDZ low	**Staminate**	7	0	1	0.5	0	0.5	1	42.8
	Pistillate	6	0	0	0	0	0	0	0
	Leaf	7	0	2	0	0	0	0	28.6
TDZ high	**Staminate**	7	1	0	0	0.5	0	0	21.4
	Pistillate	6	0	0	0	0	0	0	0
	Leaf	7	0	0	1	0	0	0	14.3

[z]Days after treatment/initiation of experiment.

Table 4.3. Number of new cultures from *Liquidambar formosana* 'Formosan Gold' observed contaminated during study initiated 23 Feb. 2017 (3rd set of dormant buds).

3rd set			# plates newly contaminated					
Treatment	**Explant part**	**# plates**	**5 DAT[z]**	**14 DAT**	**28 DAT**	**4 wk(s) after transfer**	**10 wk(s) after transfer**	**% contamination**
Basal	**Staminate**	7	2	1	0	0	0	42.86
	Pistillate	6	0	0	0	0	0	0
	Leaf	7	0	0.25	0.25	0	0.5	14.28
NAA low	**Staminate**	7	1	1.25	0	0	0	3.21
	Pistillate	6	0	0.25	0	0	0	4.17
	Leaf	7	0	0.25	0.25	0	0	7.14
NAA high	**Staminate**	7	2	0.25	0	1.25	0	50
	Pistillate	5	1	0.5	0	0	0	30
	Leaf	7	1	0.25	0.25	0	0	21.43
TDZ low	**Staminate**	7	0.5	1.5	0	0.25	0	32.14
	Pistillate	5	0	0	0	0	0	0
	Leaf	7	0	0.25	0	0	0	3.57
TDZ high	**Staminate**	7	0.25	0	0	0	0	3.57
	Pistillate	6	0	0	0	0	0	0
	Leaf	7	1	1.25	0.25	0	0	35.71

[z]Days after treatment/initiation of experiment.

Table 4.4. Number of cultures from *Liquidambar formosana* 'Formosan Gold' with new callus or callus-like growth observed during study initiated 8 Jan. 2017 (1st set of dormant buds).

1st set				# cultures with new callus or callus-like growth				
Treatment	**Explant part**	**# plates**	**# cultures**	**28 DAT[z]**	**1 wk after transfer**	**3 wk(s) after transfer**	**10 wk(s) after transfer**	**% callus observed**
Basal	**Staminate**	7	30	2	0	2	1	17
	Pistillate	6	12	0	0	1	0	8
	Leaf	7	37	0	0	1	0	3
NAA low	**Staminate**	7	28	4	0	4	1	32
	Pistillate	6	12	2	0	1	0	25
	Leaf	7	35	10	0	4	0	40
NAA high	**Staminate**	7	30	8	0	4	0	40
	Pistillate	6	12	1	0	1	0	17
	Leaf	7	38	17	0	0	0	45
TDZ low	**Staminate**	7	28	5	0	2	0	25
	Pistillate	6	12	3	0	0	0	25
	Leaf	7	50	3	0	0	1	13
TDZ high	**Staminate**	7	28	7	0	3	2	43
	Pistillate	6	12	3	0	1	0	33
	Leaf	7	55	2	0	0	0	4

[z]Days after treatment/initiation of experiment.

Table 4.5. Number of cultures from *Liquidambar formosana* 'Formosan Gold' with new callus or callus-like growth observed during study initiated 31 Jan. 2017 (2nd set of dormant buds).

2nd set			**# plates with new callus or callus-like growth**				
Treatment	**Explant part**	**# plates**	**14 DAT[z]**	**28 DAT**	**10 d(s) after transfer**	**7 wk(s) after transfer**	**% callus observed**
Basal	**Staminate**	7	1	1	0	0	28.6
	Pistillate	6	0	3	0	0	50.0
	Leaf	7	0	1.5	0	0	21.4
NAA low	**Staminate**	6	3.5	1.5	0	0	83.3
	Pistillate	6	3	0	0	0	50.0
	Leaf	6	2	2	0	0	66.7
NAA high	**Staminate**	7	2	2.5	0.5	0	71.4
	Pistillate	5	2	2	0	0	80.0
	Leaf	7	3	2.5	0	0	78.6
TDZ low	**Staminate**	7	1	2.5	0	0	50.0
	Pistillate	6	2.5	2	0	0	75.0
	Leaf	7	0	1	0	0	14.3
TDZ high	**Staminate**	7	2.5	1.5	0	0	57.1
	Pistillate	6	0	2.5	0	0	41.7
	Leaf	7	0	3.5	0	0	50.0

[z]Days after treatment/initiation of experiment.

Table 4.6. Number of cultures from *Liquidambar formosana* 'Formosan Gold' with new callus or callus-like growth observed during study initiated 23 Feb. 2017 (3rd set of dormant buds).

3rd set			# plates with new callus or callus-like growth			
Treatment	**Explant part**	**# plates**	**28 DAT[z]**	**4 wk(s) after transfer**	**10 wk(s) after transfer**	**% callus observed**
Basal	**Staminate**	7	0	0	0	0
	Pistillate	6	0.5	0	0	8.3
	Leaf	7	0	0	0	0
NAA low	**Staminate**	7	0	0.25	0.5	10.7
	Pistillate	6	0.5	0.5	0	16.7
	Leaf	7	3.25	0	0	46.4
NAA high	**Staminate**	7	0	0	0	0
	Pistillate	5	0.5	0	0	10
	Leaf	7	1	0	0	14.3
TDZ low	**Staminate**	7	0	0	0	0
	Pistillate	5	0	0	0	0
	Leaf	7	0	0	0	0
TDZ high	**Staminate**	7	0	0.5	0	7.1
	Pistillate	6	0	0	0	0
	Leaf	7	0	0	0	0

[z]Days after treatment/initiation of experiment.

Table 4.7. Average contamination of cultures of *Liquidambar formosana* ‘Formosan Gold’ by phytohormone treatment, collection date, and dormant bud tissue type.

Collection:	**1**	**2**	**3**	
Initiation date:	**8 Jan. 2016**	**30 Jan. 2016**	**23 Feb. 2016**	**Means**
Staminate:	47.60	43.78	26.36	39.25
Pistillate:	3.40	1.66	6.83	3.96
Leaf:	5.40	24.06	16.43	15.30
Means:	18.80	23.17	16.54	19.50

Table 4.8. Average callus and callus-like growth of cultures of *Liquidambar formosana* ‘Formosan Gold’ by phytohormone treatment, collection, date and dormant bud tissue type.

Collection:	**1**	**2**	**3**	
Initiation date:	**8 Jan. 2016**	**30 Jan. 2016**	**23 Feb. 2016**	**Means**
Staminate:	31.40	58.08	3.56	31.01
Pistillate:	21.60	59.34	7.00	29.31
Leaf:	21.00	46.20	12.14	26.45
Means:	24.67	54.54	7.57	28.92

Table 4.9. Average callus and callus-like growth of cultures of *Liquidambar formosana* ‘Formosan Gold’ by dormant bud tissue type, collection date, and phytohormone treatment.

Collection:	**1**	**2**	**3**	
Initiation date:	**8 Jan. 2016**	**30 Jan. 2016**	**23 Feb. 2016**	**Means**
Basal:	9.33	33.33	2.77	15.14
Low NAA:	32.33	66.67	24.60	41.20
High NAA:	34.00	76.67	8.10	39.59
Low TDZ:	21.00	46.43	0.00	22.48
High TDZ:	26.67	49.60	2.37	26.21
Means:	24.67	54.54	7.57	

Table 4.10. Average contamination of cultures of *Liquidambar formosana* ‘Formosan Gold’ by dormant bud tissue type, collection date, and phytohormone treatment.

Collection:	**1**	**2**	**3**	
Initiation date:	**8 Jan. 2016**	**30 Jan. 2016**	**23 Feb. 2016**	**Means**
Basal:	15.33	26.57	19.05	20.32
Low NAA:	6.00	25.00	4.84	11.95
High NAA:	30.33	28.57	33.81	30.90
Low TDZ:	21.67	23.80	11.90	19.12
High TDZ:	20.67	11.90	13.09	15.22
Means:	18.80	23.17	16.54	

Table 4.11. Average contamination of immature seed tissue cultured of *Liquidambar formosana* 'Formosan Gold' by phytohormone treatment.

Collection:	**1**		**2**
Initiation date:	**29 June 2016**		**14 July 2016**
	2 WAI[z]	**8 WAI**	**6 WAI**
Basal:	20.7%	29.2%	3.7%
Low 2,4-D with no BA:	22.2%	34.5%	14.8%
High 2,4-D with no BA:	21.4%	21.4%	0%
Low 2,4-D with BA:	44.4%	55.5%	0%
High 2,4-D with BA:	25.9%	25.9%	0%
Means:	26.9%	33.3%	3.7%

[z]WAI: weeks after initiation

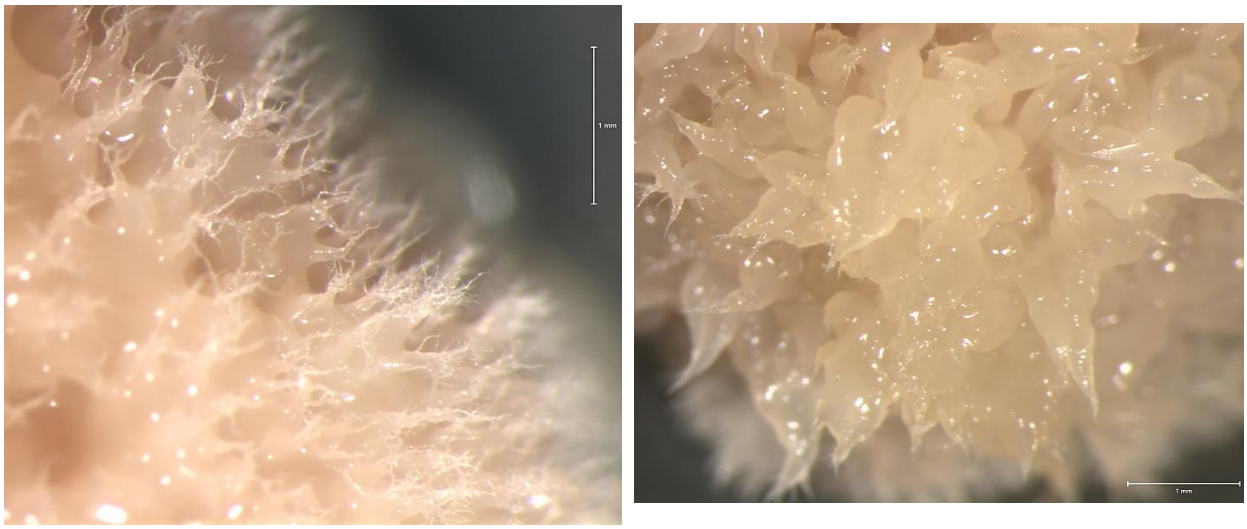

Figure 4.1. Growth (either fungal growth or callus) from staminate inflorescence tissue from *Liquidambar formosana* ‘Formosan Gold’ on basal medium without plant growth regulators initiated 8 Jan. 2016. Photos taken 28 Feb. 2016 (approx. 7 weeks after initiation).

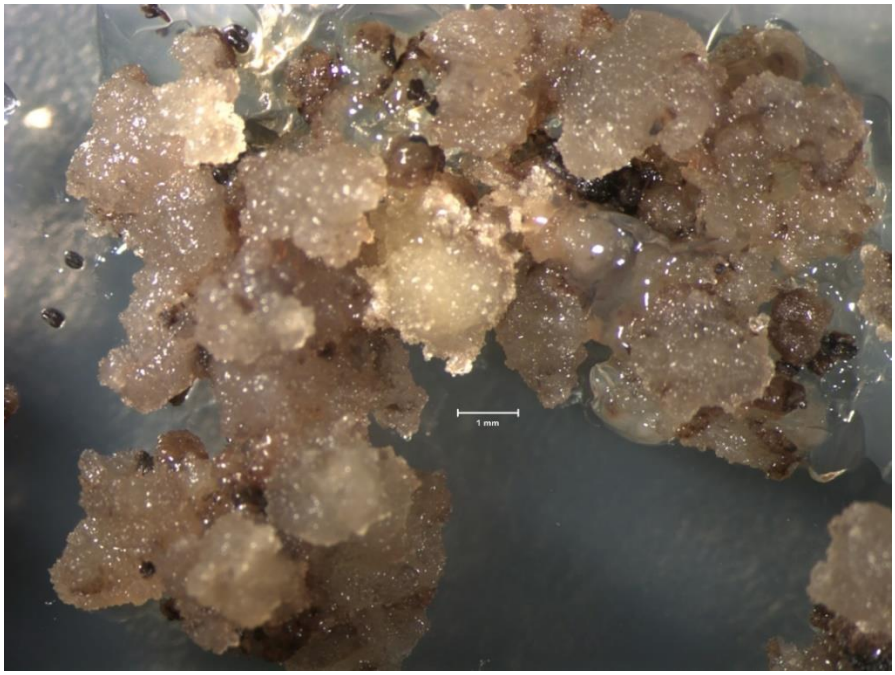

Figure 4.2. Callus from staminate inflorescence tissue from *Liquidambar formosana* ‘Formosan Gold’ cultured on basal medium with high NAA treatment initiated 8 Jan. 2016. Photo taken 19 April 2016 (approx. 14 weeks after initiation).

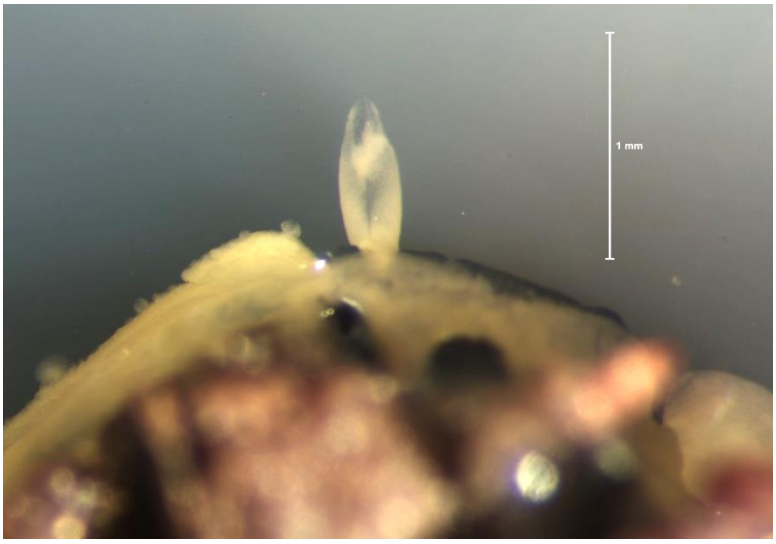

Figure 4.3. Possible somatic embryo growth observed on staminate inflorescence tissue from *Liquidambar formosana* ‘Formosan Gold’ cultured on medium with high NAA treatment initiated 8 Jan. 2016. Photo taken 6 Feb. 2016 (4 weeks after initiation).

Figure 4.4. Growth from staminate inflorescence tissue from *Liquidambar formosana* ‘Formosan Gold’ cultured on basal medium with low NAA treatment initiated 30 Jan. 2016. Photo taken 26 Feb. 2016 (4 weeks after initiation).

Figure 4.5. Callus and root growth from leaf tissue of *Liquidambar formosana* 'Formosan Gold' on basal medium with high NAA treatment initiated 30 Jan. 2016. Photos taken 26 Feb. 2016 (4 weeks after initiation).

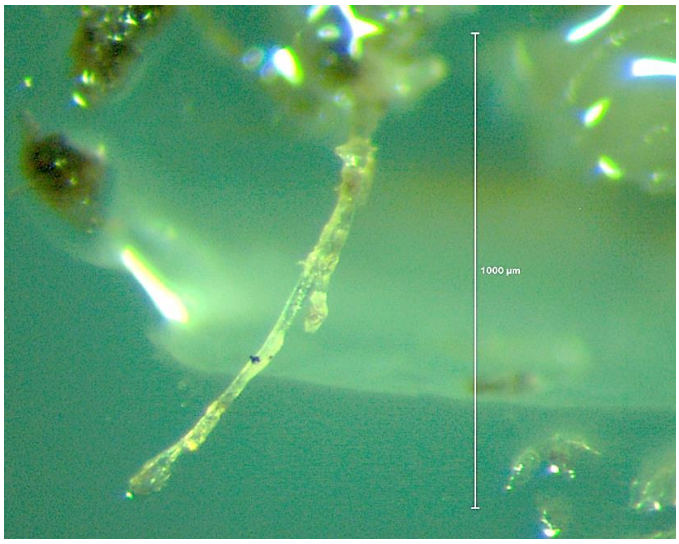

Figure 4.6. Root hair growth from seed tissue from *Liquidanbar formosana* 'Formosan Gold' initiated 14 July 2016 on basal medium with high 2,4-D and BA. Photo taken 26 Aug. 2016 (6 weeks after initiation).

CHAPTER 5

LIQUIDAMBAR FORMOSANA ‘FORMOSAN GOLD’ [2]

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Liquidambar formosana ‘Formosan Gold’

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Introduction

Formosan sweetgum (*Liquidambar formosana* Hance) is a handsome landscape tree with unique three-lobed leaves, brilliant yellow to red fall color, and tall stately form. It is native to China and distributed in temperate, montane forests across the southern and eastern range of the country, as well as in South Korea and Taiwan (Dirr, 1998; Hoey and Parks, 1994; Hong et al., 2003). The species was introduced to North America in 1884 and is considered to grow rapidly and perform well in U.S. Department of Agriculture (USDA) hardiness zones 7–9 (USDA, 2012). Formosan sweetgum belongs to the Altingiaceae family and is closely related to the American sweetgum *L. styraciflua* L.), a native of the eastern United States and the cloud forests of eastern Mexico and Central America. It is closely related to *L. acalycina* H. T. Chang, another taxon native to China with similar three-lobed leaves but which differs in the number of capsules per fruit (Grimshaw and Bayton, 2009; Hoey and Parks, 1994; Hong et al., 2003). In addition to being an excellent ornamental tree, the wood of *L. formosana* is used for the production of timber and resin, while stems, leaves and fruit are used for medicine (Dirr, 1998; Durkovic et al., 2005; Gilman and Watson, 1993; Hong et al., 2003; Zomlefer, 1994). *Liquidambar formosana* is not widely planted as an ornamental in the United States and has a limited presence in American botanical gardens. Despite its sparse use, it deserves more recognition for its unique leaf shape and excellent fall foliage color. ‘Formosan Gold’ sweetgum offers a unique cultivar of the species with attractive yellow to chartreuse foliage during the growing season.

Origin

The original plant of ‘Formosan Gold’ was from seed received from the Taiwan Forestry Research Institute in 1998. A golden-foliaged seedling was found among the green-foliaged seedlings at the University of Georgia Tifton Campus in the summer of 1998. At planting on Mar. 2, 2001, the tree was 1.53 m in height and in 2010 had reached 10.2 m. Clonal trees from rooted cuttings have been planted at the University of Georgia Tifton Campus, the University of Georgia Durham Horticulture Farm in Watkinsville, GA, Cox Arboretum in Canton, GA, Leu Gardens in Orlando, FL, Jackson Nursery in Belvidere, TN and the Atlanta Botanical Garden location in Gainesville, GA. To date, all clonally propagated trees display the same foliar phenotype.

Description

Liquidambar formosana ‘Formosan Gold’ is a deciduous tree with observed heights estimated to be 9–13 m at about 18 years of age. The original tree in Tifton, GA is estimated to be in excess of 12 m tall by 8 m wide at 16 years after planting. Two other clones planted in 2003 in Belvidere, TN and in 2006 in Orlando, FL have approximate heights of 9 m and 12 m, respectively, in 2018. The cultivar has an upright and oval to rounded habit with age (Fig. 5.1). Leaves are alternate, simple and 3-lobed (rarely 5-lobed, with bottom lobes appearing as a subset of bottom-most lobes) with palmate venation (Fig. 5.2). Mature leaves are generally wider (about 15.3 cm) than long (≈ 13.2 cm). Margins are serrate to finely-serrate and lobe apices are acuminate to caudate. The base of the leaf is typically cordate with a few leaves truncate or sagittate (5-lobed leaves). Adaxial leaf color varies with age or location on the branch. Newly emerged leaves have an adaxial surface color of red-purple (red-purple group 59A; Royal Horticultural Society (RHS), 1995) and are pubescent on both surfaces. Fully expanded foliage

near the distal end of the branch displays a light yellow adaxial surface color (green-yellow group 1B; RHS, 1995) (Fig. 5.2). As leaves age, color ranges from chartreuse yellow (yellow-green group 145A; RHS, 1995) to a light green (yellow-green 144A; RHS, 1995) further into the canopy, or the proximal end of the branch (Fig. 5.2). Once foliage fully expands, leaves are glabrous on the adaxial surface. The abaxial surface has very little pubescence with a sparse amount at the base of the prominent, rounded central vein. Stipules are linear, ≈1.0–1.6 cm long with a slight curve or curl along their length, and are adnate to the petiole base. Grayed-red (180B&C; RHS, 1995) petioles of mature leaves average 4.3 cm long with a slight widening at their base and lack pubescence. Foliage changes color in the fall and ranges on the plant from dark yellow (yellow group 7A; RHS, 2001), orange (orange group 26A; RHS, 2001), to dark orange-red (grayed-orange group 169A; RHS, 2001) (Fig. 5.3).

Bark on mature tree trunks in Tifton is gray (201C; RHS, 1995) and moderately flaky with shallow furrows. Branches and less mature trunks have smoother bark and stems are lenticellate. Dormant buds have several overlapping scales: the lowest are brown (200C; RHS, 1995) with no pubescence, fading to brown (200B; RHS, 1995), and at the very tip, scales are brown (200A; RHS, 1995) with pubescence. Buds can contain leaves only or mixed leaves and reproductive structures. Bud break occurs from the last week of February until the first week of March in Tifton, GA and the second to third week of March in Watkinsville, GA. Trees are monoecious with separate male and female inflorescences. The male inflorescence is similar to that of the species, being a raceme with many stamens on each head. On reproductive trees male and female inflorescences emerge with new leaves and male flowers quickly senesce after 1 to 2 weeks. The female inflorescence is a globose head which blends in with the foliage color and consists of many ovaries with extended and recurved styles that persist through maturity. Each

ovary has a pair of beak-like calyxes and as the fruit matures in late summer, either a filled, winged seed or more often an aborted small seed is released. Fruit is a syncarp of dehiscent, woody capsules that have an average of 38 per fruit and are brown (200C; RHS, 1995). Mature fruit peduncle length averages 8.3 cm and fruit width averages 3.2 cm. The parent tree first produced fruit at 13 years of age. Fruit is produced in limited quantities but remain relatively hidden among the vibrant foliage and persist throughout the summer and often through the winter after leaf drop. Holotype: field grown plant, Ornamental Horticulture Research Area, University of Georgia, Tifton Campus, Tifton, GA (lat. 31°47'66"N, long. 83°52'02" W). A specimen has been deposited at the Valdosta State University Herbarium, Ruter (VSC).

Cultural Notes

Three to four node semihardwood terminal cuttings collected in Tifton, GA in the third week of May (2002 and subsequent years) have had rooting success of about 75%. Cuttings were dipped in a 1:2 dilution of rooting hormone to water (IAA + NAA; Dip 'N Grow, Inc., Clackamas, OR), planted in a substrate of pine bark and perlite (2:1, v/v) and placed under intermittent mist for several weeks. Cuttings were also taken from a clone in Watkinsville, GA in mid to late Summer 2017, treated similarly and yielded comparable rooting percentages. Although rooting has been high during the growing season, survival through the subsequent winter has been low. 'Formosan Gold' has been successfully side-veneer grafted onto *L. styraciflua* understock. Propagation via somatic embryogenesis has been attempted using dormant mixed buds and immature fruits but resulted in no embryogenic cultures or somatic seedlings. Root suckers produce foliage true-to-type, so root cuttings may be another propagation method worthy of experimentation. No seed collected from 'Formosan Gold' in Tifton has germinated. Hybrids between *L. styraciflua* and *L.*

formosana have been produced via crossing (Santamour, 1972; Vendrame et al., 2001), yet natural hybridization with *L. styraciflua* in South Georgia is unlikely as the native species often flowers 2–4 weeks later than 'Formosan Gold', which could account for the lack of observed seedlings surrounding the tree. Additionally, research indicates *Liquidambar* species to be largely selfsterile (Santamour, 1972; Schmitt, 1964). In late summer, foliage can develop fungal leaf spots (Colletotrichum spp.), but infection does not appear to affect the overall health or growth of this cultivar (Little, 2016). The tree has performed well for several years in Belvidere, TN (USDA hardiness zone 7b), Canton, GA (zone 7b), Gainesville, GA (zone 7b), Watkinsville, GA (zone 8a), Tifton, GA (zone 8b), and Orlando, FL (zone 9b). Across these locations there have been no reports of major pest problems, winter injury or invasiveness. The tree growing in Tennessee survived winter low temperatures of –17.2 °C with no damage (Ray Jackson, personal communication). Only one cultivar of *Liquidambar formosana*, 'Afterglow', is known to exist in the United States. It originated from the Saratoga Horticultural Foundation of California and is rarely sold in nurseries in the United States. 'Afterglow' differs from the species by displaying purplish-red new growth and rose-red fall color (Jacobson, 1996; Thomas, 1961) with an asymmetrical form (Santamour and McArdle, 1984). In addition to 'Afterglow', a few other cultivars are listed on the Botanic Gardens Conservation International database (BGCI, 2018), however, they appear to have little horticultural significance or do not exist in the trade. 'Monticola' is listed and described in previous reports, but scientists doubt its validity as a distinct cultivar (Jacobson, 1996; Santamour and McArdle, 1984; Wilson, 1913). 'Formosan Gold' differs from *L. formosana* and 'Afterglow' by having bright yellow to yellow-green foliage all spring and summer, and a minor distinction is shorter petiole length (Table 5.1). This cultivar can be used similarly to Formosan sweetgum: as a shade tree in a park, lawn or wide

street with the bonus of bright chartreuse foliage (Gilman and Watson, 1993). The unique foliage color displays 'Formosan Gold' sweetgum can bring to public or residential landscapes make it a valuable addition to the commercial ornamental market.

Availability

Contact the University of Georgia Research Foundation, Inc. (UGARF – research.uga.edu) or Georgia Seed Development (www.gsdc.org).

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Table 5.1. Comparison of morphological traits of *Liquidambar. formosana* 'Formosan Gold' to familiar species.

	'Formosan Gold'	***L. formosana*[y,w,v]**	***L. styraciflua*[y,v,u]**	***L. acalycina*[y,x,w]**
Height (m)	9 – 13[z]	15-30	18 – 23	25
Leaf lobe no.	3 (5)	3 (5)	5	3
Leaf dimensions (cm), width x length	15.3 x 13.2	17 x 13	14.5 x 14.5	11.5 x 10.5
Petiole length (cm)	3.9 - 5.0	8.0 – 12.0	6.3 – 10.2	4.0 – 8.0
No. of capsules/fruit	29 - 46	24 – 43		15 - 26
Fruit width (cm)	2.5 – 3.5	3 – 4	2.5 – 3.8	
Spring-summer foliage color	Yellow-green to yellow	Green to dark green	Green to dark green	Green to dark green
Fall foliage color	Dark yellow to orange-red	Yellow-orange-purple-red-brown; variable	Yellow-purple-red; variable.	Orange-purple-burgundy

[z]Trees used for measurement were ≤ 18 years old.

[y]Dirr (1998).

[x]Grimshaw and Bayton (2009).

[w]Hong et al. (2003).

[v]Jacobson (1996).

[u]Thomas (1961).

Figure 5.1. *Liquidambar formosana* ‘Formosan Gold’ in Orlando, FL on 28 Feb. 2018. The tree was planted 2006.

Figure 5.2. Terminal leaves of *Liquidambar formosana* ‘Formosan Gold’ in Tifton, GA on 6 Mar. 2017.

Figure 5.3. Fall foliage of *Liquidambar formosana* ‘Formosan Gold’ in Tifton, GA on 23 Dec. 2002.

CHAPTER 6

SUBSTRATE DRENCH APPLICATIONS OF FLURPRIMIDOL AND PACLOBUTRAZOL INFLUENCE GROWTH OF SWAMP SUNFLOWER (*HELIANTHUS SIMULANS*)[3]

[3] Barrios, K. and J.M. Ruter. Accepted for publication on 31 May 2019 by *HortTechnology* pending revisions.

Substrate Drench Applications of Flurprimidol and Paclobutrazol Influence Growth of Swamp Sunflower (*Helianthus simulans*)

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Abstract. Swamp sunflower (*Helianthus simulans* E. Watson) is an underused perennial plant native to the southeast U.S. that produces an abundance of golden yellow inflorescences in the fall. It is a vigorous grower and tolerates a wide variety of soil conditions, growing in wetland or non-wetland habitats. Swamp sunflower warrants wider use in perennial beds and landscapes, and research on nursery and shipping protocols could promote its production. This study evaluated the application of plant growth regulators (PGRs). Treatments were applied to rooted cuttings in 2.8 L pots as a 120-mL (4 fl. oz.) substrate drench of 1.0, 2.0, 4.0, or 6.0 mg active ingredient (a.i.) paclobutrazol, 0.5, 1.0, 2.0, or 4.0 mg a.i. flurprimidol, or water (control)/pot for experiment 1 (Expt. 1). A second experiment (Expt. 2) examined 4.0, 6.0, or 8.0 mg a.i. paclobutrazol, 2.0, 4.0, or 6.0 mg a.i. flurprimidol, or water (control)/pot. Six weeks after treatment (WAT) for Expt. 1, paclobutrazol applied at 4.0 and 6.0 and flurprimidol at 2.0 and 4.0 mg a.i./pot resulted in smaller plants (as reflected by growth index) by 29%, 34%, 22%, and 48%, respectively, compared to the control. Furthermore, at the termination (6 WAT) of Expt. 1, flurprimidol at 4.0 mg a.i./pot produced smaller plants (by a minimum of 27%) with less dry weight than all other PGR treatments, with the exception of paclobutrazol at 6 mg a.i./pot. By the end of Expt. 1, plants treated with paclobutrazol at 6.0 and flurprimidol at 4.0 mg a.i./pot had lower dry weights and higher chlorophyll measurements than untreated plants. All PGR treatments for Expt. 2 resulted in smaller plants than the control by 27-36% at 4 WAT and 23-

41% at 6 WAT. Differences for internode length and flower diameter were observed for Expts. 1 and 2, respectively. Results from these experiments suggest a substrate drench application of paclobutrazol at 6.0 or flurprimidol at 4.0 mg a.i./pot can be used for producing smaller plants compared to non-treated plants for swamp sunflower under greenhouse conditions.

Introduction

Swamp sunflower is an underused, fall-blooming southern U.S. native perennial plant producing a swath of eye-catching inflorescences. The numerous flower heads reside above the foliage in a corymbose or racemose arrangement, each with a medium sized disk of 1.3-2 cm diameter. A member of the Asteraceae, it has golden-yellow ray florets, numbering 12-23, that surround the central disk florets with dark purplish-red corollas (FNA, 2006; Heiser et al., 1969; Watson, 1929). The plant typically grows about 1-1.8 m tall but can reach 2.5 m in height. Swamp sunflower's native range is from Georgia west to Texas and Arkansas southward to the Gulf Coast into southern Florida. Other common names, like muck sunflower, suggest it is typically found in moist or saturated soils near ponds, riparian areas, and drainage ways. As a facultative wetland plant, this species can be found in non-wetland habitats (TWC, 2008; Wunderlin et al., 2019). The plant grows best in full sun to part shade and tolerates a variety of growing conditions, reportedly staying more compact in denser, saturated soils (TWC, 2008). In addition to aesthetics, the flowers and seeds are valuable to wildlife such as native bees and birds (TWC, 2008). With the growing interest in natives as ornamentals, swamp sunflower is a strong candidate for wider use in perennial beds and landscapes, and research on nursery and shipping protocols could result in wider production and landscape use.

To feasibly and economically ship and sell plants, their size and shape need to be kept within certain dimensions while retaining aesthetic characteristics. This can be accomplished by

pruning or pinching plants, yet this is labor intensive and costly, particularly for vigorous plants. A less labor-intensive and widely used method to control growth and branching in commercial environments is the use of plant growth regulators (PGRs) (Whipker, 2013). Plant growth retardants, a subgroup of PGRs, commonly inhibit biosynthesis of the plant hormone gibberellin, thereby restricting plant growth (Davis et al., 1988; Rademacher, 2000). The most widely used PGR in the U.S. to control excessive growth in greenhouse-grown floriculture is paclobutrazol [(2RS,3RS)-1-(4-Chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl) pentan-3-ol], a plant growth retardant that inhibits the biosynthesis of gibberellin (Rademacher, 2000; Whipker, 2013). Restrictions in height of annual sunflower (*Helianthus annuus* L.) in response to paclobutrazol and other PGRs have been demonstrated (Ahmad et al., 2015; Barbosa et al. 2008; Dasoju et al., 1998; Koutroubas et al., 2014; Vernieri et al., 2003; Whipker and Latimer, 2016; Whipker and McCall, 2000). The rate of paclobutrazol has been evaluated on potted sunflower in previous studies with Dasoju et al. (1998) finding paclobutrazol at 2 mg active ingredient (a.i.)/pot as a substrate drench on 'Pacino' reduced height by 17-25%. Similar responses from a similar application of paclobutrazol were obtained by Whipker and McCall (2000) on five cultivars of annual sunflower. Whipker and McCall (2000) and Ahmad et al. (2015) determined that 4 mg a.i./pot effectively reduced growth of 'Pacino Gold'. Barbosa et al. (2008) observed reduced plant height with increased paclobutrazol concentration and recommended 6 mg a.i./pot for the annual sunflower 'Golden'. Dasoju et al. (1998) tested a wide range of rates with reductions in plant height and diameter up to 16 mg a.i./pot, but observed phytotoxicity at 16 and 32 mg a.i./pot. Insufficient height reduction by paclobutrazol on pot sunflower was reported by Whipker and Dasoju (1998) when applied as a foliar spray and suggested spraying at even higher concentrations (80+ mg a.i./pot) or using a substrate drench application. A 2-3 ppm (≈ 0.2-0.3

mg/ 4 fl. oz.) substrate drench application of paclobutrazol 1-2 weeks after pinching during greenhouse production is suggested for an interspecific hybrid of sunflower (*Helianthus hybrida*) 'Sunfinity' (Syngenta Flowers, 2017). The application method of paclobutrazol in precedent literature has largely been by foliar spray or substrate drench with more favorable results from the use of substrate drenches (Hawkins et al., 2015; Keever et al., 1990; Whipker and Dasoju, 1998). Recommendations for greenhouse floriculture growers advise the application of paclobutrazol as a high-rate substrate drench for enduring effects throughout the growing season. For sunflower the recommended application is a 2-4 mg a.i. substrate drench of 4 fl. oz. (≈120 mL) per 6-in pot (≈2.8L) (Whipker, 2015).

Flurprimidol [Isopropyl-(p-(trifluoromethoxy)phenyl)-5-pyrimidinemethanol] is another popular plant growth retardant for ornamentals that inhibits the biosynthesis of gibberellin and has restricted plant height and diameter of potted sunflower 'Pacino' when applied as a substrate drench or foliar spray (Whipker, 2013; Whipker et al., 2004). Whipker et al. (2004) compared two PGRs to flurprimidol and found results from a 2 mg a.i./pot substrate drench application of flurprimidol to be similar to those of paclobutrazol. Another comparison by Vernieri et al. (2003) found flurprimidol 60 mg a.i./L applied as a foliar spray on sunflower was less effective at height reduction than a low dose of paclobutrazol (2 mg a.i./pot) applied as a substrate drench. Application of flurprimidol as a drench rather than spray has been shown to produce more ideal plant effects, including reduction in plant size while not affecting flowering time or flower size, in other species (Krug et al., 2005a; Krug et al., 2005b; Rezazadeh and Harkess, 2015; Whipker et al., 2006).

The objective of this study was to evaluate the effects of two common plant growth retardants, paclobutrazol and flurprimidol, applied as substrate drenches on the growth and flowering of swamp sunflower.

Materials and Methods

Experiment 1. Sub-terminal cuttings of three to four nodes were taken 10 Apr. 2018 from clonal material of swamp sunflower from a stock plant at the University of Georgia's (UGA) Durham Horticulture Farm in Watkinsville, GA. Cuttings were dipped in a fungicide solution of 15.58 $g \cdot L^{-1}$ azoxystrobin [methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate] (Abound; Syngenta Crop Protection, Greensboro, NC) for 10-15 s before sticking in propagation substrate in 1020 trays (21.2 × 10.8 × 2.3 in) (Landmark Plastic, Akron, Ohio). Propagation substrate was wet 1-2 d prior to sticking cuttings and consisted of 2 potting mix : 1 perlite (by volume). Potting mix used was Jolly Gardener Pro-line C/L Growing Mix (Oldcastle, Shady Dale, GA) and was amended with micronutrients (Micromax; Everris NA Inc., Dublin, OH) at 594 $g \cdot m^{-3}$. After cuttings were stuck, a humidity dome was placed over the tray, placed under mist (8 s every 5 min from 7:00 $_{AM}$-7:00 $_{PM}$), and covered by shade cloth (50% exclusion) under greenhouse conditions. After 30 d rooted cuttings were removed from trays and placed in 4 inch (height) square pots (Kord; The HC Companies, Inc., Twinsburg, OH) filled with potting substrate (Pro-mix BX Mycorrhizae; Premier Tech Horticulture, Rivière-du-Loup, QC, Canada) amended with same micronutrients as above. Rooted cuttings were placed in a shaded area in greenhouse, watered as needed, and fertilized weekly with a 20N-4.37P-16.6K water-soluble liquid fertilizer at 200 $mg \cdot L^{-1}$ nitrogen (Jack's Professional; J.R. Peters, Inc., Allentown, PA). After ≈16 d, rooted cuttings were moved to an unshaded area of polyethylene greenhouse and continued to grow for about 1 week. Rooted cuttings were then transplanted to

2.8 L pots filled with potting substrate (pine bark, peat and sand mix; Oldcastle, Shady Dale, GA) and top-dressed with a 16N-2.6P-10.0K controlled-release fertilizer at 10 g/pot (Harrell's Polyon, Lakeland, FL). Plants were transferred on the same day to a polycarbonate greenhouse at the UGA Trial Gardens in Athens, GA and allowed to grow before initiating the experiment. Plants were cut back for uniformity (≈15-20 cm from the substrate) on 21 May 2018. Fans in the greenhouse were programmed to vent when internal temperature ≥ 27 °C day and night, however, daytime temperatures in the summer often exceeded 32 °C within the greenhouse. The experiment was a completely randomized design with 12 replicates per treatment level, where a replicate was a single plant in a 2.8 L pot and pots were spaced approximately 46 cm apart.

Four days prior to application, plants were cut back to 2 nodes/stem. The evening prior (6:00 PM) to the application, plants were watered to container capacity. Treatments were applied 25 June 2018 beginning at 8:15 AM as a 120-mL substrate drench of paclobutrazol (Piccolo 10 XC; Fine Americas, Walnut Creek, CA), flurprimidol (Topflor; SePRO, Carmel, IN) or municipal water (control). PGR treatment was delivered as 1.0, 2.0, 4.0, or 6.0 mg a.i. paclobutrazol or 0.5, 1.0, 2.0, or 4.0 mg a.i. flurprimidol/pot. Plants were hand watered as needed and fertigated once weekly with liquid fertilizer at 200 $mg \cdot L^{-1}$ nitrogen (20N-4.4P-16.6K, Jack's Professional; J.R. Peters, Allentown, PA). The experiment terminated at 6 WAT (6 Aug. 2018).

Plant height and width measurements were taken the day following treatment (Day 1) and 2, 4 and 6 WAT. Plant height was measured from the surface of the substrate to the highest point of the plant. Width was the average of the two widest perpendicular dimensions. Height and width increases were calculated by subtracting the initial plant height or width measured on Day 1 from the height or width at the week of data collection. Growth index (GI) was calculated as height × width1 × width2 to reflect overall plant volume in cm^3 and increases were calculated as

differences from Day 1 to a specific week of data collection. Chlorophyll meter (SPAD-502; Minolta Camera Comp., Osaka, Japan) measurements were taken between the mid-vein and margin of three random leaves and were averaged per plant to evaluate leaf greenness with a SPAD reading. Dry weight was obtained by cutting stems at the top of the substrate and oven-drying for ≈3 d at 60 °C. Dry weight, SPAD and internode length were measured just before the termination of the experiment. Internode length was taken by averaging the lengths between the second and third node from the apex of three random stems per plant.

Experiment 2. Plants were obtained using the method outlined above and the experiment was conducted in the same location as described in Expt. 1. Cuttings were taken 3 June 2018, allowed to root in 1020 trays (21.2 × 10.8 × 2.3 in) (Landmark Plastic, Akron, Ohio), and transplanted to 4 inch square pots (Kord; The HC Companies, Inc., Twinsburg, OH) 28 d after sticking. Rooted cuttings were then transplanted to 2.8 L pots after 24 d and brought to the UGA Trial Gardens in Athens, GA. Plants were cut back for uniformity 26 d before treatment (12 Aug. 2018). Greenhouse temperatures were maintained similar to Expt. 1, and beginning 22 Oct., heat was provided to maintain internal greenhouse temperatures at ≥ 24 °C day/ ≈ 18 °C night. Experimental design and setup were the same as Expt. 1 with one less treatment level for each PGR. Rates of PGRs for Expt. 2 were adjusted based on results from Expt. 1 and treatments were applied 7 Sept. 2018 using the same method as Expt. 1. Treatment was 4.0, 6.0, or 8.0 mg a.i. paclobutrazol or 2.0, 4.0, or 6.0 mg a.i. flurprimidol/pot. The experiment was terminated at 8 WAT (2 Nov. 2018), 2 weeks longer than Expt. 1 to allow for flowering observations.

Height and width measurements were taken identical to Expt. 1 with an additional measurement of height at 8 WAT. Chlorophyll meter readings and dry weight were also

measured identically to Expt. 1. Internode length for Expt. 2 was not measured due to the development of inflorescences.

Floral data was collected for Expt. 2 (but not Expt. 1), since it blooms in the fall (Aug.-Nov.) (FNA, 2006; TWC, 2008). At termination (8 WAT), the number of flowers and buds was counted to obtain the total flower count. Flower diameter (to nearest 0.25 cm) was measured and the number of ray florets was counted on three random mature flowers for each plant; a mature flower was one with disc florets at anthesis. Eleven plants were used per treatment for flower diameter and ray floret number per flower since not all plants had three mature flowers. Flower timing data was taken for the date of first visible yellow ray floret, date of the first opened flower and the date of full flower. An opened flower had ray florets at ≈ 60° from the center of the head, and a plant was in full flower when at least 80% of flowers were opened.

Data analysis. Data for both experiments were analyzed by a one-way ANOVA for each week of collection to obtain significance, and means comparisons of treatment levels were analyzed with the Bonferroni adjustment ($P \leq 0.05$) in the R program (R Core Team, 2016).

Results

Experiment 1. Application of paclobutrazol and flurprimidol as substrate drenches at the specified rates affected the plant height and width, dry weight, and SPAD readings of swamp sunflower (Table 6.1 and Figs. 6.1 and 6.3). Height was less than the control for plants treated with the two highest rates of paclobutrazol and flurprimidol at 2, 4, and 6 WAT (Table 6.1 and Fig. 6.1). Height reductions of the aforementioned PGR rates compared to the control were 21-45% at 4 WAT and 19-46% at 6 WAT. For flurprimidol, plants receiving 1.0 mg a.i./pot application were shorter than the control at 2 WAT (Fig. 6.1). Among PGR rates, the two highest rates of paclobutrazol and the highest rate of flurprimidol restricted plant height at 6 WAT

compared to other PGR rates by at least 20%, with the exception of flurprimidol at 2.0 mg a.i./pot. Similar height differences among PGR applications at 6 WAT were observed at 4 WAT with the highest rate of flurprimidol having a minimum of 22% height restriction.

Plant width was reduced from the control by application of the highest rate of paclobutrazol and the two highest rates of flurprimidol at 2, 4 and 6 WAT (Table 6.1 and Fig. 6.2). At the termination of the study (6 WAT), the two highest PGR rates, paclobutrazol at 6.0 and flurprimidol at 4.0 mg a.i./pot, restricted width growth compared to flurprimidol at 1.0 mg a.i./pot by 10% and 18%, respectively. Similar differences were observed between the lowest and highest rates of PGRs at 2 and 4 WAT (Fig. 6.2). At 4 WAT, application of the highest rate of flurprimidol restricted width growth by 18-23% from the two lowest rates of both PGRs. Among paclobutrazol rates, plants treated with the highest paclobutrazol rate were narrower at 4 WAT than the lowest paclobutrazol rate by 13%.

Growth index was affected by PGR application at 2 and 4 WAT. Plants treated with the two highest rates of paclobutrazol and all but the lowest rate of flurprimidol were smaller than the control by 18-50% at 2 and 4 WAT (data not shown). After 2 weeks of growth, application of paclobutrazol at the two highest rates restricted growth compared to the lowest pacloubtrazol rate, and the application of the highest rate of flurprimidol restricted growth compared to the two lowest flurprimidol rates. After 4 weeks, the highest rate of flurprimidol produced smaller plants than other PGR treatments, with the exception of the highest rate of paclobutrazol. At termination (6 WAT), the two highest rates of each PGR yielded smaller plants than the control by 22-48% (Table 6.2). Furthermore, plants treated with the two highest rates of paclobutrazol were smaller than those treated with the lowest rate of paclobutrazol by 28% and 33%, and the

highest rate of flurprimidol resulted in smaller plants than all PGR treatments by at least 27%, with the exception of the highest rate of paclobutrazol.

Dry weight of plants treated with the two highest rates of paclobutrazol and all but the lowest rate of flurprimidol was restricted by 12-30% compared to the control (Table 6.1). The highest rate of flurprimidol resulted in plants with the lowest dry weight of all treatments, apart from the highest rate of paclobutrazol, and the two highest concentrations of paclobutrazol resulted in plants with lower dry weights than the lowest concentration of the same PGR by 16% and 24%. Similarly, the highest concentration of flurprimidol resulted in plants with 17-25% lower dry weights than other rates of flurprimidol.

The highest rate of paclobutrazol had the highest SPAD reading of all treatments (with a 17% increase from the control), apart from the highest rate of flurprimidol that had a 15% increase from the control (Table 6.1).

While not different from the control, the highest rate of paclobutrazol and flurprimidol had shorter internode lengths by 1.2 and 1.4 cm, respectively, at 6 WAT ($P \leq 0.01$) compared to the lowest rate of flurprimidol (data not shown).

Experiment 2. Application of paclobutrazol and flurprimidol as substrate drenches at the specified rates affected plant height and width and flower diameter of swamp sunflower (Table 6.3 and Fig. 6.3). All PGR application rates resulted in shorter plants than the control at each week of data collection, with the greatest reductions occurring 4 WAT (34-40%) (Fig. 6.3). After 8 weeks of growth, plants treated with the highest concentration of paclobutrazol (8.0 mg a.i./pot) were shorter than plants treated with half that concentration (4.0 mg a.i./pot), but no other differences of height increase among PGR treatments were observed (Table 6.3 and Fig. 6.3).

The application of paclobutrazol at 6.0 and 8.0 mg a.i./pot restricted plant width at 6 WAT compared to the control by 29% and 26%, respectively (Table 6.3). Plant width at 2 and 4 WAT was not influenced by PGR treatment (data not shown).

Growth index was affected by PGR treatment at each week of data collection (6 WAT growth index shown in Table 6.2). The highest rate of flurprimidol and the two highest rates of paclobutrazol yielded smaller plants than the control by 35%-40% at 2 WAT (data not shown). All PGR treatments yielded smaller plants than the control by 27-36% at 4 WAT and 23-41% at 6 WAT (Table 6.2, data not shown for 4 WAT).

Treatment effect on dry weight was not found to be significant (data not shown). SPAD meter readings were not different among treatments when measured at 8 WAT (data not shown).

The diameter of mature flowers (Table 6.3) and the number of flowers and flower buds per plant ($P = 0.041$), when measured or counted 8 WAT, were affected by PGR treatments. The diameter of flowers on plants treated with flurprimidol at 6.0 mg a.i./pot was 7% wider than plants treated with paclobutrazol at the same rate (Table 6.3). The number of flowers and flower buds per plant ranged from 95 to 115, and, although treatment was found to have an effect on this parameter, no differences between treatment levels were found using pairwise comparison tests (data not shown). The number of ray florets per mature flower, which ranged from 13 to 15, was not affected by treatment (data not shown). The number of days to first visible yellow of ray florets, first fully open flower and full flower from the day of treatment application were not affected by treatment (data not shown). The means of the number of days to first visible yellow florets were: 33.9 ± 1.1 for the control; 36.6, 36.4, and 35.9 ± 1.1 in ascending order by the rate for paclobutrazol; and 35.8, 36.7, and 37.2 ± 1.1 in ascending order by rate for flurprimidol. The means of the number of days to first full flower were: 37.8 ± 1.3 for the control; 40.6, 40.0, and

39.7 ± 1.3 in ascending order by rate for paclobutrazol; and 39.4, 40.4, and 40.9 ± 1.3 in ascending order by rate for flurprimidol. The means of the number of days to full flower were: 50.8 ± 2.2 for the control; 57.2, 55.3, and 53.7 ± 2.2 in ascending order by rate for paclobutrazol; and 54.8, 55.0, and 55.8 ± 2.2 in ascending order by rate for flurprimidol.

Discussion

Restrictions of plant height from the control that were observed in Expts. 1 and 2 for paclobutrazol and flurprimidol are similar to previous studies. After 6 weeks for Expt. 1 and 4 WAT for Expt. 2, plants treated with paclobutrazol at 4.0 mg a.i./pot were 27% and 36% shorter, respectively, than the control. Similar results with a substrate drench application of 4 mg a.i./pot paclobutrazol were observed as 26-36% shorter plants than the control by Dasoju et al. (1998), Whipker and McCall (2000), and Ahmad et al. (2015) for potted annual sunflower. Although previous studies observed 20% to 27% shorter plants with a 2 mg a.i./pot drench of paclobutrazol on potted sunflower (Ahmad et al., 2015; Dasoju et al., 1998; Whipker and McCall, 2000; Whipker et al., 2004), the same application method and concentration in this study on swamp sunflower was not different from the control. Expts. 1 and 2 had further height restrictions with plants treated at paclobutrazol rates higher than 4.0 mg a.i./pot (Figs. 6.1 and 6.2) which was similarly observed in potted sunflower by Barbosa et al. (2008), who recommended 6 mg a.i./pot for ‘Golden,’ and Vernieri et al. (2003) with a reported 50% reduction in height at 16 mg a.i./pot (but with variation among cultivars). Dasoju et al. (1998) reported severe retardation in the growth of potted sunflower at 16 and 32 mg a.i./pot. Substrate drench application of flurprimidol at 2 mg a.i./pot by Whipker et al. (2004) restricted the height of potted sunflower by 22%. Similar results were observed in this study at the same PGR rate with a 21% and 34% height restriction of plants from the control for Expt. 1 and 2, respectively, 4 WAT. In contrast to

Whipker et al. (2004), this study found further height restrictions from the control with an increasing rate of flurprimidol (Figs. 6.1 and 6.3).

SPAD meter readings for paclobutrazol 6.0 and flurprimidol 4.0 mg a.i./pot during Expt. 1 were higher than the control. These results are consistent with those of Ahmad et al. (2015) who observed increased darker green foliage for 'Pacino Gold' potted sunflower treated with 2-4 mg a.i./pot paclobutrazol substrate drenches and for Barbosa et al. (2008) who reported increased SPAD values with increased paclobutrazol substrate drench rates, peaking between 4 to 6 mg a.i./pot for potted sunflower 'Golden.' Increased foliar chlorophyll content with increasing paclobutrazol rate has been reported in additional plant genera to *Helianthus* (Bañón et al., 2001; Dahab et al., 2015; França et al., 2017). Increase in relative chlorophyll with the application of anti-gibberellin compounds is likely explained by secondary effects of reduced leaf expansion leading to an increased density of chloroplasts, as well as an increase of chlorophyll biosynthesis (Davis 1988). The lack of increased SPAD meter readings with an increase of PGR rate for Expt. 2 could have been due to the use of foliar resources for inflorescence development (Kitonyo et al., 2018; Leopold, 1961). Interestingly, SPAD meter readings for Expt. 1 were lower (range: 32.5 - 39.4) than for Expt. 2 (range: 40.6 - 43.9) for all treatment levels.

Flowering dates for the control and PGR-treated plants occurred between 4 and 8 WAT when treatment was applied 7 Sept. 2018, and no effect on flowering date was observed due to PGR application. There was no delay to anthesis for potted sunflower which was similarly observed by Whipker et al. (2004) with the application of paclobutrazol (2 mg a.i./pot drench) or flurprimidol (10-50 mg/L spray or 0.5-4 mg a.i./pot drench) and by Whipker and Dasoju (1998) with paclobutrazol application of 5-40 mg/L as foliar spray. However, delays in flowering of 4-6 d for potted sunflower by PGRs have been reported by Dasoju et al. (1998) with paclobutrazol

substrate drench at 2-32 mg during winter production and by Whipker and Dasoju (1998) with a foliar spray of paclobutrazol 80 mg/L. Flowering delays resulting in less impact to the plants' marketability have been reported for paclobutrazol substrate drenches by Whikper and McCall (2000) (2 and 4 mg a.i./1.2 L pot) and by Vernieri et al. (2003) (2-16 mg a.i./pot), and for flurprimidol (7.5-60 mg a.i./L foliar spray) (Vernieri et al., 2003) on potted sunflower. Plant growth regulator effect on flowering can vary among species with no effect observed by paclobutrazol treatment on *Dissotis rotundifolia* (Hawkins et al., 2015) or on 'Anna Marie' hyacinth (*Hyacinthus orientalis*) bulbs (Krug et al. 2005b), but delays observed from paclobutrazol on *Osteospermum ecklonis* (Barnes et al. 2009) and by flurprimidol on 'Star Gazer' oriental lily (*Lilium* hybrids) (Krug et al., 2005a) and 'Anna Marie' hyacinth bulbs (Krug et al., 2005b).

Flower diameter results from Expt. 2 were interesting, in that no differences from the control were observed for different PGR rates, but flurprimidol 6.0 mg a.i./pot had a 7% wider flower than paclobutrazol 6.0 mg a.i./pot. Precedent studies with potted annual sunflower found no differences of flower diameter from control when treated with paclobutrazol substrate drench at 2 or 4 mg a.i./pot (Whipker and McCall, 2000) or as a foliar spray at 5-80 mg/L (Whipker and Dasoju, 1998). On the other hand, reductions of flower diameter by application of higher paclobutrazol substrate drench rates (4-32 mg a.i./pot) and flurprimidol spray (60 mg a.i./L) have been observed (Dasoju et al., 1998; Vernieri et al., 2003).

In summary, paclobutrazol 4.0 and 6.0 mg a.i./pot and flurprimidol 1.0, 2.0, and 4.0 mg a.i./pot applied 25 June 2018 resulted in smaller plants (by GI) at 2 and 4 WAT and less biomass at 6 WAT than the control. After 6 weeks, paclobutrazol 4.0 and 6.0 and flurprimidol 2.0 and 4.0 mg a.i./pot yielded smaller plants than the control. Furthermore, plants treated with paclobutrazol

at 4.0 and 6.0 mg a.i./pot were smaller and had less dry weight than 1.0 mg a.i./pot of the same PGR at 6 WAT. Plants treated with paclobutrazol 6.0 mg a.i./pot and flurprimidol 4.0 mg a.i./pot had higher SPAD readings than the control and shorter internodes than the lowest rate of flurprimidol at 6 WAT.

For experiment 2, initiated 7 Sept. 2018, all PGR treatments were smaller (by GI) than the control by 4 and 6 WAT. At termination (8 WAT), plants treated with paclobutrazol 8.0 mg a.i./pot were shorter than plants receiving half that amount. Since the statistical analyses showed no difference in restrictions of plant GI among PGR rates at 4 and 6 WAT in Expt. 2 (Fall 2018), applying the least amount of PGR (i.e. flurprimidol at 0.5 mg a.i./pot) would result in a similar smaller size (compared to no PGR) of plants as applying the most amount of PGR (i.e. paclobtrazol at 8 mg a.i./pot). Expt. 2 results also suggest an application of flurprimidol 6.0 mg a.i./pot over paclobutrazol at the same rate would result in plants with a 7% wider flower.

In conclusion, optimal PGR substrate drench application rates for reducing overall plant growth of swamp sunflower are paclobutrazol at 4.0 or 6.0 mg a.i./pot or flurprimidol at 2.0 or 4.0 mg a.i./pot. Application of paclobutrazol at 4.0 or 6.0 mg a.i./pot would result in plant sizes 27-29% or 34-35% less, respectively, compared to no PGR application for about 2 to 6 weeks. For flurprimidol, application of 2.0 or 4.0 mg a.i./pot would result in plant sizes 30% or 50% less, respectively, compared to no PGR application after 4 weeks.

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Table 6.1. Experiment 1 data of final height, width, dry weight, and SPAD meter reading of swamp sunflower plants treated with plant growth regulator (PGR) or control on 25 June 2018.

Treatment (PGR mg a.i./pot) [z]		**Final height increase (cm)** [y, x]	**Final height as percent decrease from control** [x, v]	**Final width increase (cm^2)** [y, x, w]	**Dry weight (g)** [z, x, v]	**SPAD meter reading** [x, v]
Control		40.4 a		43.9 a	66.3 a	32.6 c
Paclobutrazol	1	39.2 ab	2.8% c	43.3 abc	65.4 ab	32.8 c
	2	38.9 ab	3.8% c	40.6 abc	61.1 abc	34.0 c
	4	29.5 c	27.0% b	40.4 abc	54.7 cd	35.6 bc
	6	26.0 cd	35.6% ab	38.9 cd	49.4 de	39.4 a
Flurprimidol	0.5	37.9 ab	6.1% c	42.9 abc	61.2 abc	32.5 c
	1	36.7 ab	9.1% c	43.4 ab	58.5 bc	33.9 c
	2	32.5 bc	19.5% bc	39.1 bcd	55.7 c	34.5 c
	4	21.6 d	46.5% a	35.7 d	46.1 e	38.2 ab
Significance		***	***	***	***	***

*** indicates significance at $P \leq 0.001$ ($n = 12$).

[z] 1 mg = 3.5274×10^{-5} oz.

[y] Final increase = measurement at week 6 – measurement on day immediately after treatment application; 1 cm = 0.3937 inch.

[x] Means separations (in columns) by pairwise comparison tests with Bonferroni adjustment $P \leq 0.05$ (lowercase letters). Treatments levels within column having the same letter are not significantly different.

[w] Width = (width1 × width2)/2.

[v] Measured at end of the experiment (6 weeks after treatment).

Table 6.2. Volumetric growth increases for plants of swamp sunflower treated with plant growth regulator (PGR) or control on 25 June 2018 (Expt. 1) or 7 Sept. 2018 (Expt. 2).

Treatment (mg a.i./pot) [z]		Percent decrease from control of final volumetric growth [y, x]		Final volumetric growth (m^3) [y, x]	
		Expt. 1	Expt. 2	Expt. 1	Expt. 2
Control				0.22 a	0.38 a
Paclobutrazol	**1**	2.7% d	n/a [w]	0.21 a	n/a [w]
	2	12.1% cd	n/a	0.19 abc	n/a
	4	28.6% bc	22.9% a	0.15 cd	0.29 b
	6	34.5% ab	38.5% a	0.14 de	0.23 b
	8	n/a	41.2% a	n/a	0.22 b
Flurprimidol	**0.5**	8.5% d	n/a	0.20 ab	n/a
	1	8.1% d	n/a	0.20 ab	n/a
	2	22.0% bc	23.9% a	0.17 bcd	0.29 b
	4	48.1% a	27.2% a	0.11 e	0.28 b
	6	n/a	30.1% a	n/a	0.26 b
Significance		***	*	***	***

***, * indicate significance at $P \leq 0.001$ or 0.05, respectively ($n = 12$).

[z] 1 mg = 3.5274×10^{-5} oz.

[y] Final volumetric growth = volume at week 6 - volume the day immediately following treatment application, where volume = (height × width × width); 1 m = 3.28 feet.

[x] Means separations (in columns) by pairwise comparison tests with Bonferroni adjustment $P \leq 0.05$ (lowercase letters). Treatment levels within column having the same letter are not significantly different.

[w] No PGR was applied at a specific rate for a particular experiment.

Table 6.3. Experiment 2 data of final height, width, and flower diameter of swamp sunflower plants treated with plant growth regulator (PGR) or control on 7 Sept. 2018.

Treatment (PGR mg a.i./pot) [z]		**Final height increase (cm)** [y, x, v]	**Final height as percent decrease from control** [v]	**Final width increase (cm^2)** [y, w, v]	**Flower diameter (cm)** [y, v, u]
Control		82.4 a		29.8 a	9.8 ab
Paclobutrazol	4	69.5 b	15.7%	27.5 ab	10.3 ab
	6	63.8 bc	22.6%	21.0 b	9.7 b
	8	60.9 c	26.5%	21.9 b	9.9 ab
Flurprimidol	2	65.8 bc	20.2%	26.7 ab	10.1 ab
	4	63.9 bc	22.5%	27.4 ab	10.2 ab
	6	65.1 bc	21.0%	25.1 ab	10.4 a
Significance		***	NS	**	*

NS, ***, **, * indicate nonsignificance or significance at $P \leq 0.001$, 0.01, or 0.05, respectively ($n = 12$).

[z] 1 mg = 3.5274×10^{-5} oz.

[y] 1 cm = 0.3937 inch.

[x] Final increase = measurement at week 8 – measurement on day immediately after treatment application.

[w] Final increase = measurement at week 6 – measurement on day immediately after treatment application; width = (width1 × width2)/2.

[v] Means separations (in columns) by pairwise comparison tests with Bonferroni adjustment $P \leq 0.05$ (lowercase letters). Treatment levels within column having the same letter are not significantly different.

[u] Flower diameter was measured at week 8 and is the average of three mature flowers per plant. A mature flower was considered to have disc florets that have reached anthesis.

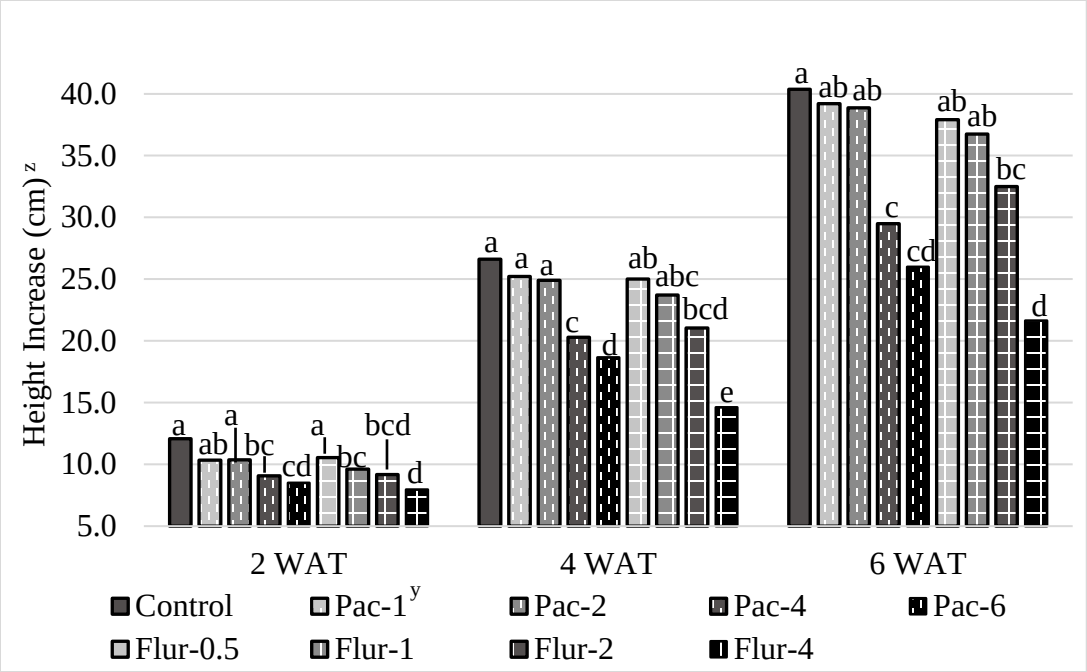

Figure 6.1. Effects of flurprimidol (Flur) and paclobutrazol (Pac) substrate drenches (mg active ingredient (a.i.)/pot) on the height increases of container-grown swamp sunflower from the day following application to 2, 4, and 6 weeks after treatment (WAT) for Experiment 1. ANOVA was conducted for each week of data collection ($P \leq 0.05$, $n = 12$). Means separation by pairwise comparison tests with Bonferroni adjustment $P \leq 0.05$ (lowercase letters) at each week of data collection. Treatment levels within week having the same letter are not significantly different. [z] Height increase = height at week 2, 4, or 6 – height the day after treatment; 1 cm = 0.3937 inch. [y] Indicates PGR (Pac or Flur) and rate (e.g., 1 = 1 mg a.i./pot); 1 mg = 3.5274×10^{-5} oz.

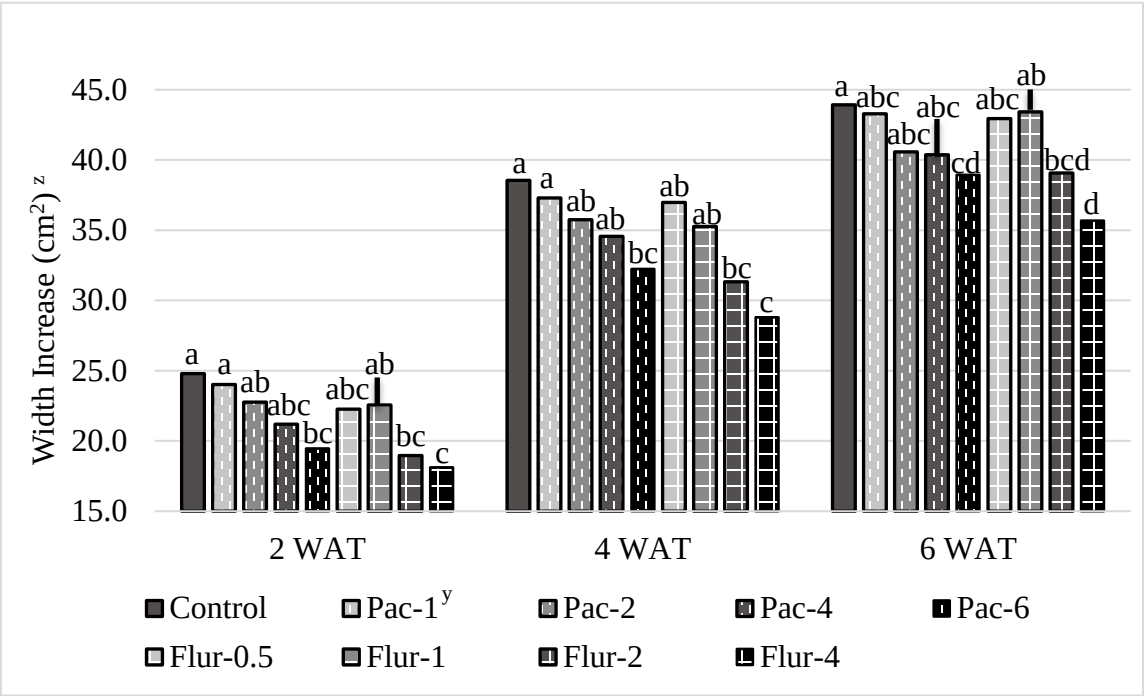

Figure 6.2. Effects of flurprimidol (Flur) and paclobutrazol (Pac) substrate drenches (mg active ingredient (a.i.)/pot) on the width (width1 × width2) increases of container-grown swamp sunflower from the day following application to 2, 4, and 6 weeks after treatment (WAT) for Experiment 1 (initiated 25 June 2018). ANOVA was conducted for each week of data collection ($P \leq 0.05$, $n = 12$). Means separation by pairwise comparison tests with Bonferroni adjustment $P \leq 0.05$ (lowercase letters) at each week of data collection. Treatment levels within week having the same letter are not significantly different. [z] Width increase = width at week 2, 4, or 6 – width the day after treatment; 1 cm = 0.3937 inch. [y] Indicates PGR (Pac or Flur) and rate (e.g., 1= 1 mg a.i./pot); 1 mg = 3.5274×10^{-5} oz.

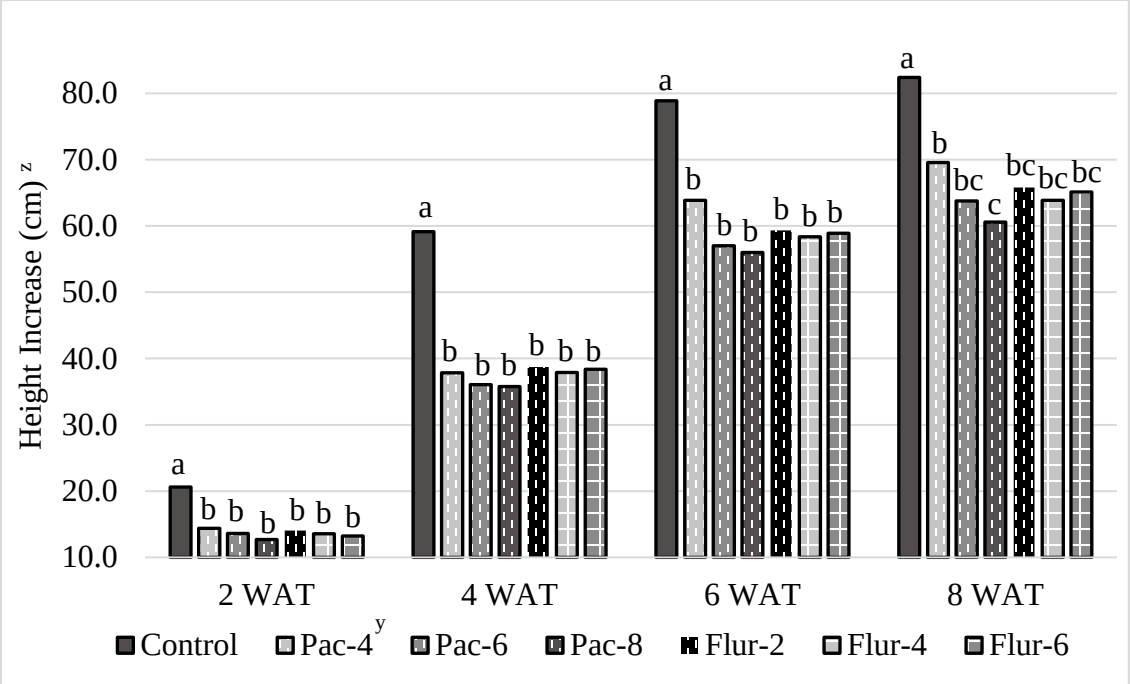

Figure 6.3. Effects of flurprimidol (Flur) and paclobutrazol (Pac) substrate drenches (mg active ingredient (a.i.)/pot) on the height increases of container-grown swamp sunflower from the day following application to 2, 4, 6, or 8 weeks after treatment (WAT) for Experiment 2 (initiated 7 Sept. 2018). ANOVA was conducted for each week of data collection ($P \leq 0.05$, $n = 12$). Means separation by pairwise comparison tests with Bonferroni adjustment $P \leq 0.05$ (lowercase letters) at each week of data collection. Treatment levels within week having the same letter are not significantly different. [z] Height increase = height at week 2, 4, 6 or 8 – height the day after treatment; 1 cm = 0.3937 inch. [y] Indicates PGR (Pac or Flur) and rate (e.g., 4= 4 mg a.i./pot); 1 mg = 3.5274×10^{-5} oz.

CHAPTER 7

Inducing Phenotypic Variation of *Illicium parviflorum* via Gamma Radiation

Introduction

Small anise tree (*Illicium parviflorum* Michx.) is a prevalent landscape shrub for a myriad of reasons: its adaptability, fast growth, ability to form a dense mass, and lack of pests. Small anise, or yellow-anise, tree provides an evergreen mass of green to olive-green color and is reliably hardy in USDA hardiness zones 6-9 (Dirr, 1986, 1998). Although it is popular in Georgia and its native state of Florida for hedges and beds, very few cultivars exist. Much of the nursery stock of *Illicium parviflorum* is clonal from very few wild sources (Hardin, 1972; Newell and Morris, 2010). This inherent survival risk attributed to mass reproduction of the same genotype, plus the phenotypic uniformity among plantings, begs for the introduction of distinct cultivars for homeowners and landscapers. Due to its uniform appearance and the shortage of cultivars, there is much room for improvement in market options. Traditional breeding is a common avenue for developing novel plants, however, because of deterring factors like low fruit and seed set, small flowers, and reported self-incompatibility (White and Thien, 1985; Zomlefer, 1994), mutation induction was selected as an avenue for cultivar development by the author of this dissertation. Mutation breeding has led to many ornamental cultivars, and gamma radiation is relatively quick, with minimal waste, and capable of changing one to a few traits in plants (Micke et al., 1990; Van Harten, 1998). The objectives of this project were to observe and select plants with phenotypic variations induced by gamma irradiation of stem cuttings, determine the

optimal dose for rooting cuttings, as well as evaluate the interaction of the effects of stage of tissue growth with those of gamma radiation.

Materials and Methods

The cultivar 'Forest Green' which is described as having lustrous, darker green foliage (than the species) was utilized for this study and cutting material was obtained from the State Botanical Garden of Georgia in Athens, GA. Shoot tip cuttings were collected 2016 and 2017 in Feb., May and July/Aug. for hard-, soft- and semi-hardwood tissue types, respectively. Cuttings had four to six nodes and during radiation the bottom ~2.5 cm of cuttings was protected by a lead ring. Radiation was administered with a ^{60}Co source and was conducted at the Center for Applied Isotope Studies on the UGA Athens campus. Dose rate (gray, or Gy) levels varied for tissue type and year (see Table 7.1) and always included a non-irradiated control. Dose rate is quantified as the absorbed radiation by an object with the SI unit of 1 Gy, which is equivalent to 1 joule of energy per kg (van Harten, 1998). Following irradiation treatment were dipped in potassium salt of indole-3-butyric acid (K-IBA) at 3,000 ppm for approximately 5 s at the Univeristy of Georgia's (UGA) Durham Horticultural Farm (Hort Farm) in Watkinsville, GA. After allowing to air-dry for a few minutes, cuttings were stuck into propagation mix [2 Jolly Gardener Pro-Line Growing Mix : 1 Aero-soil perlite (by volume)] in 8.8 cm (3.5 inch) square pots (Kord; The HC Companies, Inc., Twinsburg, OH), randomized by irradiation treatment level, and placed under mist (8 s every 5 mins from 7:00 $_{AM}$-7:00 $_{PM}$) for 9-12 weeks (see Table 7.1). Replicate, or experimental unit, was a single stem cutting and there were 10 replicates per irradiation level for each tissue type each year.

Stem cuttings were taken out of mist and evaluated for rooting after 9-12 weeks from sticking. A successfully rooted cutting was scored as 1 and an unrooted cutting was scored as a

0. Rooting data for 2017 was analyzed as a randomized complete block design (RCBD) where each tissue type, or collection date, was treated as a block and radiation as the main effect. For 2016 rooting data, each tissue type, or collection date, was treated as a distinct analysis of a completely randomized design (CRD) and radiation was treated as the main effect. Rooting data for 2017 was analyzed as a two-way ANOVA and for 2016 as a one-way ANOVA with transformation of the binomial data in R (R Core Team, 2016). Survival of the irradiated rooted cuttings for each tissue type was recorded at approximately 2 and 6 months after removing from mist and expressed as a percentage of the number of surviving plants/10 original replicates.

Results

Stem cuttings of *Illicium parviflorum* were affected by both exposure to gamma radiation and type of tissue when evaluated for rooting success. Data from each year was analyzed separately due to differences in radiation levels. For 2017, there was a 60 Gy treatment for hardwood and softwood but not semi-hardwood. Additionally, neither tissue type had any successful rooting at that level, therefore it was omitted in a two-way ANOVA for sake of balancing data (Figure 7.1). Since the data was still unbalanced from an additional radiation treatment level of 3 Gy for softwood and semi-hardwood for 2017 data, Type II Sums of Squares was used with the 'car' package in R. The analysis of rooting data found radiation to have an effect ($p < 0.001$), as well as tissue type ($p < 0.01$) with a non-significant interaction ($P = 0.30$). The highest number of rooted cuttings was for the untreated control using hardwood tissue (Figure 7.1). Within each of the three tissue types, there was no irradiation treatment that had higher rooting than the non-irradiated control. Each of the three tissues had zero rooting when irradiation was greater than 10 Gy, with the exception of semi-hardwood with one rooted cutting at 20 Gy. The level of radiation for which cuttings had peak rooting was 5 Gy for hardwood, 3

Gy for softwood, and 10 Gy for semi-hardwood tissue during 2017. Survival percentages of rooted cuttings several months after removal from mist were similar to rooting percentages (Table 7.2 and Figure 7.1). Hardwood cuttings had higher survival percentages than other tissue types and no irradiated cuttings had equal or greater survival percentages than the control among tissue types.

Data from 2016 could not be analyzed as a two-way ANOVA due to substantial unbalanced data from differences in radiation treatment levels. Therefore, data for each collection date, or tissue type, was analyzed separately and radiation had a significant effect for each tissue type ($p < 0.001$). Rooting of hardwood and softwood cuttings was influenced by irradiation and even the control had a relatively moderate rooting percentage of 60% (Figure 7.2). Semi-hardwood had the highest rooting for 2016 with all cuttings rooting for the control and just below that were cuttings treated at 5 Gy with 90% rooting and 3 Gy with 70% rooting. This observation of such a high rooting percentage in the summer for *Illicium parviflorum* was expected based on propagation suggestions in Dirr and Heuser (2006).

Discussion

Rooting was similarly high for 3 and 5 Gy-treated cuttings in both years, suggesting these are the optimal levels for irradiating *Illicium parviflorum* to obtain successfully rooted cuttings. Cuttings readily root at < 3 Gy, however at such a low rate of irradiation, it is likely that the incidence of mutations would be insufficiently low to recover any changes in plant phenotype. Due to the high number of plants required for inducing mutations via irradiation (Van Harten, 1998), it is likely that the time and resources spent on evaluating and maintaining propagules would outweigh the rare event of a mutated plant. At radiation levels higher than 5 or 10 Gy, rooting percentage dropped drastically for cuttings of all three tissue types for the 2 years the

experiment was conducted. A threshold of rooting and survival of cuttings was expected as radiation increased, since gamma (and other ionizing) radiation disrupts tissue at the cellular and chromosomal level. During ionizing radiation, free electrons and radical ions are produced and resultant free radicals in solution are trapped by the cellular membrane and interact with deoxyribonucleic acid (DNA). This is likely the source of most damage to DNA with ionizing radiation, however the DNA molecules can also be damaged by directly absorbed radiation (Ahnström, 1977; Britt, 1996; Ward, 1975). Consequently, the higher radiation rates (approx. >10 Gy) detrimentally affected the tissue of the cuttings which was beyond repair and lead to death of the tissue. Around 2 weeks after treatment, some cuttings were observed to have brown lesions and these later turned completely brown/black. A few cuttings did not have lesions and did not form roots. We speculate that these cuttings survived the radiation but did not have enough endogenous auxin or carbohydrates to initiate adeventitious root formation, nor was the exogenous application of 3,000 ppm K-IBA sufficient (Hartmann et al., 2002). Another possibility is the irradiation detrimentally affected a biochemical process related to auxin or another phytohormone. We would have expected adventitious root initiation from actively growing cuttings because endogenous indole-3-acetic acid (IAA) is synthesized at the apical meristem and in young leaves and moves (slowly) down towards the base of the plant (basipetally) in a polar gradient thereby suppressing lateral bud growth; this is known as apical dominance (Acquaah, 2002; Hartmann et al., 2002). When the shoot tips were cut from the plants, the IAA would be moving down the shoot away from the apical meristem and likely collect near the cut site. Mutagenic treatments are known to affect rooting capacity of plants ex vitro (Suprasanna et al., 2011). In tissue culture medium, antioxidants may be incorporated to potentially reduce the intial negative effects of irradiation such as tissue browning, necrosis or

chlorosis (Suprasanna et al., 2011). It is also possible mutations did result in the cuttings but the DNA was able to be repaired unbeknownst to the observing scientist (Britt, 1996). Suggested irradiation rates for vegetative plant material range from 20-80 Gy by Van Harten (1998) and 15-30 Gy by Suprasanna et al. (2011), therefore *Illicium parviflorum* cuttings were expected to withstand higher irradiation rates than 5 or 10 Gy. Reports of LD_{50} rates (in Gy) (the rate at which 50% of the treated material dies) for gamma irradiated shoots or shoot tips of some agricultural crops include: 20-40 for grapevine, 20-30 for banana, and 30 for Japanese plum (Suprasanna et al., 2011). Furthermore, a study treating hardwood cuttings of grapevine (*Vitis vinifera*) with gamma radiation found the LD_{50} for cultivars 'Red Globe' and 'Muscat' were 15-20 Gy and 15-25 Gy, respectively, with a complete loss of live tissue at rates greater than 35 Gy (Surakshitha et al., 2017). Semi-hardwood cuttings of three ornamental species of *Jasminum* were irradiated at eight dose rates from 5-40 Gy (Ghosh et al., 2018). Radiosensitity of the cuttings was evaluated by LD_{50} of percent survival and growth rate and ranged from 17.8-28 Gy depending on the species (Ghosh et al., 2018). From these results, it could be inferred that *Illicium parviflorum* cuttings are generally more sensitive to gamma radiation than other taxa. Although, hardwood cuttings of *Bougainvillea spectabilis* cv. Lalbagh were irradiated at 5, 7.5 and 10 Gy with the LD_{50} occuring about 10 Gy based on survival after 60 days (Anitha et al., 2017). Clearly, variability exists among genera, species and cultivars as to the optimal dose rate. Several factors can affect the radiosensitivity of the treated tissue, including water content "since the most frequent primary target of ionizing radiation is the water molecule" (Suprasanna et al., 2011).

Cuttings irradiated at 5 Gy that rooted in 2017 had differing survival percentages after 6 months, depending on tissue type. Hardwood cuttings had 70%, whereas softwood and semi-

hardwood cuttings had 10% and 30% survival, respectively. This difference could be due to the fact that actively dividing cells (as would be the case with soft- and semi-hard-wood) are more radiosensitive to gamma radiation than non-dividing, or resting, cells (like hardwood). Van Harten (1998) report that actively dividing cells can withstand 10% of the radiation dose rate that non-dividing cells can. It could be that hardwood cuttings had higher survival because the tissue was not actively dividing and therefore less sensitive to the gamma radiation.

The high rooting percentage in 2016 (particularly of the untreated control) for semi-hardwood cuttings was expected based on reports in the literature. Dirr and Heuser (2006) suggest taking terminal cuttings June/July through Nov. in GA of *Illicium parviflorum*, dipping in 3,000 ppm IBA and keeping under mist in peat:perlite for 4-6 weeks for 100% rooting. Caution is emphasized to not use soft, new growth (Dirr and Heuser, 2006) and an earlier article noted cuttings taken in Mar. in GA were dipped in 5,000 ppm IBA for successful rooting (Dirr, 1986). Cuttings taken 3 June 1986, dipped in a 5,000 ppm IBA or phenyl indole-3-thiolobutyrate (P-ITB) solution and kept for about a year had 97% rooting (Dirr, 1990). It is curious that such a high concentration of externally applied auxin would be suggested for cuttings taken in Mar. and early June (of likely softwood), since softwood typically has a high amount of endogenous auxin (Hartmann et al., 2002). A similar protocol to that of *I. parviflorum* is recommended for *I. anisatum* and *I. floridanum* with similar rooting percentages expected (Dirr and Heuser, 2006). Hartmann et al. (2002) state vegetative propagation of most *Illicium* spp. is of semi-hardwood cuttings with poor to good rooting varying by species. A study of propagation techniques of *I. anisatum* cuttings in the Shanghai area of China found 92.6% of cuttings rooted when semi-lignified tissue was used and dipped in ~500 ppm naphthalene acetic acid (NAA); rates evaluated were 100-800 ppm NAA and IBA (Sun et al., 2014). The effect of exogenous auxin application

was not tested in this study, however it could be a factor on rooting, particularly for the different stages of tissue that were treated. It could prove valuable to evaluate different levels of IBA and/or different types of auxins on the different stages of tissue (i.e., soft-, semihard-, and hard-wood).

From phenotypic observations thus far, a few plants have started to display differences compared to controls. Two replicates (cuttings) treated at 5 Gy in the summer of 2016 have shoots with unique foliage morphology (Figures 7.3 and 7.4). The leaf shapes appear curved, rather than the uniformly linear shape typical of the species, and the leaf surface has a warped, wrinkly texture that is very different from the smooth untreated plants. These findings suggest that a dose of 5 Gy for semi-hardwood cuttings is optimal for obtaining phenotypic mutations in rooted cuttings of *Illicium parviflorum*. Another type of source material that could be treated with gamma radiation is in vitro culture, to maximize meristematic tissue and potentially induce mutations at a single- or few-cell level, thereby reducing the ocurrence of chimeras (Suprasanna and Nakagawa, 2011; Van Harten, 1998).

Surviving plants were maintained at the Hort Farm and checked for fruit from July to Nov. 2018. Five fruit were collected 8 and 27 Nov. 2018 from four plants and the 11 recovered seed were placed in cold, moist stratification at 4°C for 90 d, as suggested in Olsen and Ruter (2001). The four plants that produced seed were: 2 plants from cuttings treated at 5 Gy on 4 Aug. 2016 (replicates 3 and 8) and 2 untreated control plants from cuttings taken 28 July 2016 (replicates 3 and 4). Following cold stratification, seed were sown 11 Mar. 2019 in potting mix in a 72-cell tray on a heating mat (21°C) in a greenhouse set to ~22°C at the Hort Farm. After 10 weeks, none of the seed had germinated and they were discarded. The surviving 38 plants from irradiated cuttings were planted 29 Mar. 2019 at UGA's Durham Research Farm in Watkinsville,

GA to collect any seed that may develop to evaluate second generation plants (M_2 seed). The M_2 seed would be useful to observe recessive phenotypes because most mutations are recessive, meaning the dominant allele of a gene for which the organism is heterozygous becomes mutated to a recessive allele leading to a homozygous recessive geno- and pheno-type (Acquaah, 2012) or perhaps mutates to a novel allele with the resultant phenotype potentially observed in subsequently segregating progeny.

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Table 7.1. Details of gamma irradiation treatment levels by tissue type for 2016 and 2017 of *Illicium parviflorum* 'Forest Green' cuttings.

Year	Tissue type	Initiation date	Weeks under mist	Radiation rates applied in Grays (Gy)
2016	Hardwood	16 Feb.	9	0, 20, 40, 60
	Softwood	20 May	12	0, 5, 10, 20, 40, 60
	Semi-hardwood	4 Aug.	12	0, 3, 5, 10, 20, 40
2017	Hardwood	17 Feb.	10	0, 5, 10, 20, 40, 60
	Softwood	16 May	10	0, 3, 5, 10, 20, 40, 60
	Semi-hardwood	27 July	10	0, 3, 5, 10, 20, 40

Table 7.2. Survival percentages for rooted cuttings of *Illicium parviflorum* ‘Forest Green’ by tissue type and dose rate of gamma irradiation in 2017.

Tissue type:	**Hardwood**		**Softwood**		**Semi-hardwood**	
	Months after removing from mist					
Dose rate (Gy)	**2**	**6**	**2**	**6**	**2**	**6**
0	90%	90%	70%	70%	80%	70%
3	-	-	40%	10%	30%	30%
5	70%	70%	20%	10%	30%	30%
10	20%	0%	0%	0%	0%	0%
20	0%	0%	0%	0%	0%	0%
40	0%	0%	0%	0%	0%	0%
60	0%	0%	0%	0%	-	-

Table 7.3. Survival percentages for rooted cuttings of *Illicium parviflorum* ‘Forest Green’ by tissue type and dose rate of gamma irradiation in 2016.

Tissue type:	**Hardwood**		**Softwood**		**Semi-hardwood**	
	Months after removing from mist					
Dose rate (Gy)	**1**	**6**	**2**	**6**	**2**	**10**
0	40%	0%	30%	30%	90%	70%
3	-	-	-	-	70%	70%
5	-	-	0%	0%	90%	70%
10	-	-	0%	0%	30%	0%
20	0%	0%	0%	0%	0%	0%
40	0%	0%	0%	0%	0%	0%
60	0%	0%	0%	0%	-	-

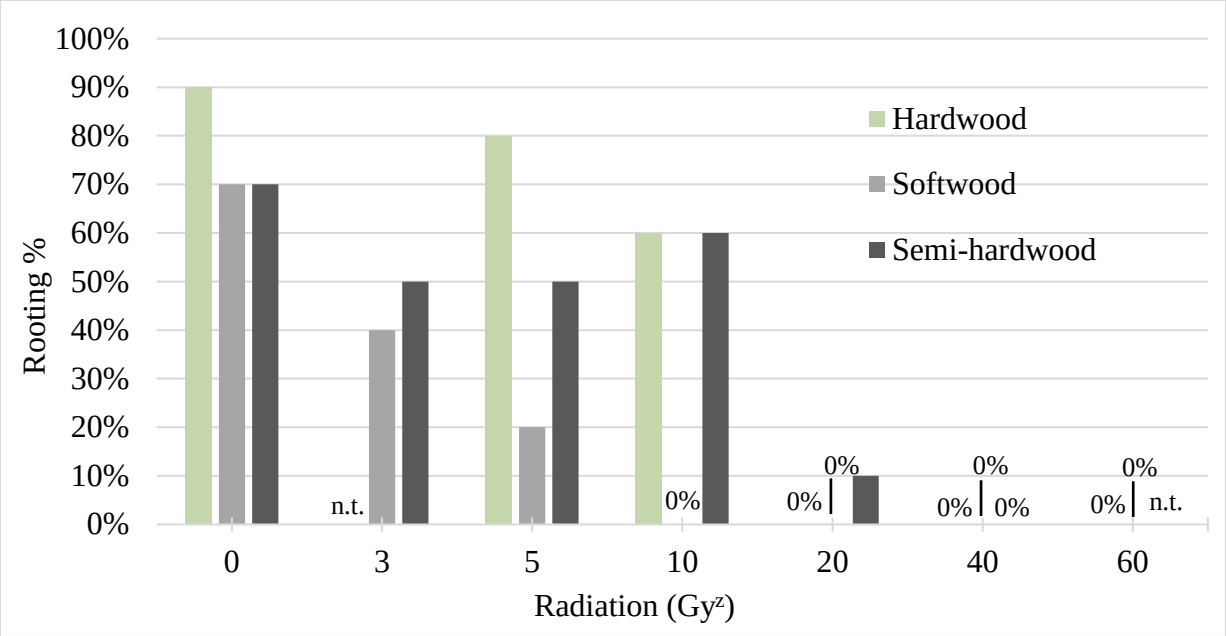

Figure 7.1. Rooting percentages for 2017 of *Illicium parviflorum* 'Forest Green' cuttings based on tissue type and gamma irradiation dose rate. Percentages are the number of rooted cuttings for each radiation level where *n* = 10. 'n.t.' indicates there was no treatment for tissue type at that irradiation level. [z] Indicates dose rate received in grays (Gy).

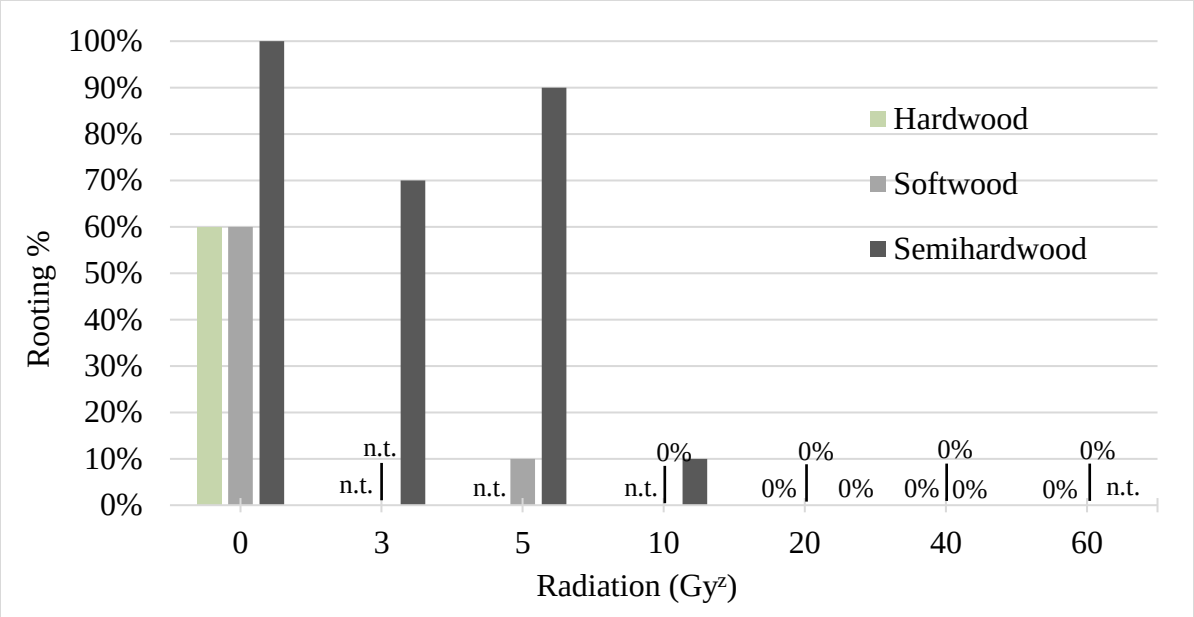

Figure 7.2. Rooting percentages for 2016 of *Illicium parviflorum* 'Forest Green' cuttings based on tissue type and gamma irradiation dose rate. Percentages are the number of rooted cuttings for each radiation level where *n* = 10. 'n.t.' indicates there was no treatment for tissue type at that irradiation level. [z] Indicates dose rate received in grays (Gy).

Figure 7.3. Shoots (top) and leaves (below) from *Illicium parviflorum* plants obtained from rooted cuttings. On left is from an untreated control and on right is from a cutting that was irradiated at 5 Gy via ^{60}Co on 28 July 2016 (replicate no. 7) at the University of Georgia's Center for Applied Isotope Studies. Photos by author.

Figure 7.4. Shoots (top) and leaves (below) from *Illicium parviflorum* plants obtained from rooted cuttings. On left is from an untreated control and on right is from a cutting that was irradiated at 5 Gy via ^{60}Co on 4 Aug. 2016 (replicate no. 5) at the University of Georgia's Center for Applied Isotope Studies. Photos by author.

APPENDICES

Appendix A. List of the seed lots (SED2018-) of putative triploid progeny collected Summer 2018. The parental information of the crosses, when the cross was made, when fruit bearing seed were collected (harvest date), how many seed were collected and when or if the seed was sown.

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
1	396	2	2	6/14/17	410	n/a	4	4/4/17	1/29/18	2/24/18	14	3/28/18
2	391	2	6	6/14/17	410	n/a	4	4/4/17	1/16& 29/2018	3/3& 11/2018	29	3/28/18
3	391	2	6	6/14/17	Hib. 1-6	n/a	n/a	n/a	2/17/18	3/27/18	29	3/28/18
4[z]	396	2	9	6/14/17	346	n/a	49	n/a	4/17/18	5/17/18	92	5/24/18
5[z]	391	4	5	6/14/17	346	n/a	49	n/a	5/3/18	5/27/18	39	6/7/18
6	391	2	1	6/14/17	401	n/a	108	n/a	5/12/18	5/31/18	11	6/7/18
7	391	2	4	6/14/17	346	n/a	49	n/a	5/2/18	5/31/18	21	6/7/18

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
7	391	2	4	6/14/17	346	n/a	49	n/a	5/16/18	6/12/18	21	6/13/18
8	378	2	6	6/14/17	346	n/a	49	n/a	4/17/18	5/31/18	2	6/7/18
8	378	2	6	6/14/17	346	n/a	49	n/a	4/27/18	5/31/18	10	6/7/18
8	378	2	6	6/14/17	346	n/a	49	n/a	5/16/18	6/12/18	9	6/13/18
9	379	4	1	6/29/17	346	n/a	49	n/a	5/14/18	6/4/18	10	6/7/18
10	391	4	1	6/14/17	346	n/a	49	n/a	4/24/18	6/4/18	5	6/7/18
11 [z]	396	2	9	6/14/17	401	n/a	106	n/a	5/10/18	6/6/18	92	6/11/18
12	379	4	2	6/14/17	346	n/a	49	n/a	5/16/18	6/11/18	17	6/13/18
13	391	2	4	6/14/17	401	n/a	106	n/a	5/10/18	6/10/18	15	6/13/18
14	396	2	7	6/14/17	434	n/a	2	n/a	6/14/18	7/2/18	11	7/12/18
15	396	2	26	6/14/17	434	n/a	2	n/a	6/14/18	7/2/18	3	7/12/18
16	378	2	6	6/14/17	434	n/a	2	n/a	6/14/18	7/9/18	53	7/12/18
17	410	n/a	1	4/4/17	394	2	6	7/11/17	6/13/18	7/9/18	22	7/12/18
18	396	2	29	6/14/17	402	n/a	25	n/a	6/10/18	7/3/18	2	7/12/18

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
18	396	2	29	6/14/17	402	n/a	25	n/a	6/11/18	7/4/18	14	7/12/18
18	396	2	29	6/14/17	402	n/a	25	n/a	6/20/18	7/3/18	2	7/12/18
19	379	4	2	6/14/17	395	n/a	1	3/28/17	6/27/18	7/11/18	3	7/20/18
20	396	2	8	6/14/17	402	n/a	25	n/a	6/18/18	7/16/18	1	7/20/18
20	396	2	8	6/14/17	402	n/a	25	n/a	6/20/18	7/16/18	20	7/20/18
21	378	2	6	6/14/17	391	n/a	1	6/14/17	6/22/18	7/16/18	16	7/20/18
22	378	2	6	6/14/17	366	n/a	1	7/19/17	6/26/18	7/17/18	35	7/20/18
23	396	2	7	6/14/17	391	n/a	2	6/14/17	6/29/18	7/17/18	3	7/20/18
24	378	2	6	6/14/17	410	n/a	4	n/a	6/24/18	7/17/18	32	7/20/18
24	378	2	6	6/14/17	410	n/a	4	n/a	6/25/18	7/17/18	27	7/20/18
25	378	2	6	6/14/17	391	n/a	2	6/14/17	6/26/18	7/18/18	32	7/20/18
26	391	2	4	6/14/17	366	n/a	1	7/19/17	6/26/18	7/19/18	27	
27	396	2	7	6/14/17	391	n/a	3	6/14/17	6/28/18	7/20/18	4	3/1/19
28	379	4	2	6/14/17	382	n/a	2	3/21/17	6/27/18	7/21/18	46	3/15/19

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
29	378	2	6	6/14/17	410	n/a	5	4/4/17	6/29/18	7/22/18	18	
29	378	2	6	6/14/17	410	n/a	5	4/4/17	7/3/18	7/24/18	28	
29	378	2	6	6/14/17	410	n/a	5	4/4/17	7/3/18	7/26/18	96	
30	378	2	10	6/14/17	391	4	5	6/14/17	7/4/18	7/22/18	3	3/1/19
31	391	2	4	6/14/17	391	n/a	3	6/14/17	6/28/18	7/22/18	34	
32	358	2	7	7/11/17	440	n/a	111	n/a	9/10/18	10/12/18	21	
33	358	2	7	7/11/17	392	2	8	7/11/17	9/13/18	10/17/18	44	
34	358	2	9	7/11/17	440	n/a	111	n/a	7/18/18	8/14/18	12	3/1/19
35	358	4	9	7/11/17	402	n/a	25	n/a	7/18/18	8/14/18	65	
36	358	4	9	7/11/17	394	2	1	8/2/17	7/20/18	8/16/18	58	
37	358	4	9	7/11/17	392	2	7	7/11/17	7/24/18	8/25/18	18	
38	366	2	2	7/11/17	405	2	2	7/19/18	8/17/18	9/3/18	15	3/15/19
39	366	2	2	7/19/18	401	n/a	106	n/a	9/5/18	10/3/18	8	3/1/19
40	366	2	2	7/19/18	392	2	8	7/11/17	9/10/18	10/12/18	5	3/1/19

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
41	366	2	2	7/19/18	394	4	3	7/11/17	9/13/18	10/12/18	3	3/1/19
42	366	4	1	7/11/17	394		1	7/26/18	7/13/18	8/4/18	18	3/15/19
43	366	4	1	7/11/17	402	n/a	25	n/a	8/22/18	9/16/18	150+	
44	366	4	1	7/26/17	440	n/a	111	n/a	7/22/18	8/7/18	9	3/1/19
44	366	4	1	7/26/17	440	n/a	111	n/a	9/5/18	10/9/18	28	3/1/19
45	366	4	1	7/26/17	402	n/a	25	n/a	8/22/18	9/22/18	35	3/1/19
46	378	2	6	6/14/17	391	n/a	2	6/14/17	6/27/18	7/19/18	33	
47	378	2	6	6/14/17	396	n/a	1	6/14/17	7/12/18	8/4/18	32	
48	378	2	6	6/14/17	392		3	6/30/17	7/13/18	8/5/18	26	
49	378	2	6	6/14/17	402	n/a	25	n/a	8/14/18	9/8/18	38	
50	378	2	6	6/14/17	401	n/a	106	n/a	9/5/18	10/3/18	46	
51	378	2	6	6/14/17	366	n/a	1	7/19/18	9/10/18	10/9/18	42	
52	378	2	8	6/14/17	394	4	7	7/11/17	8/14/18	9/11/18	28	3/15/19
53	378	2	8	6/14/17	410	n/a	5	4/4/17	9/14/18	10/12/18	31	3/15/19

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
54	378	2	10	6/14/17	394	2	1	6/14/17	8/14/18	9/8/18	14	3/1/19
55	378	2	10	6/14/17	402	n/a	25	n/a	8/14/18	9/8/18	26	3/1/19
56	378	2	10	6/14/17	366	n/a	1	8/9/17	9/5/18	10/3/18	5	3/1/19
57	379	4	1	6/29/17	392		3	6/30/17	7/10/18	8/4/18	9	3/1/19
58	379	4	1	6/29/17	394	n/a	1	7/26/17	7/13/18	8/7/18	24	3/15/19
59	379	4	1	6/29/17	392	n/a	4	7/11/17	7/28/18	8/25/18	6	3/1/19
60	379	4	1	6/29/17	44	n/a	111	n/a	9/10/18	10/25/18	2	3/1/19
61	379	4	2	6/14/17	440	n/a	111	n/a	7/9/18	7/26/18	3	3/1/19
61	379	4	2	6/14/17	440	n/a	111	n/a	7/9/18	8/5/18	58	3/15/19
61	379	4	2	6/14/17	440	n/a	111	n/a	7/23/18	8/9/18	5	3/1/19
62	379	4	2	6/14/17	379	4	2	6/14/17	7/20/18	8/7/18	2	3/1/19
63	386	6	2	3/21/17	392	n/a	4	7/11/17	7/28/18	8/25/18	9	3/1/19
64	386	6	2	3/21/17	394	2	1	8/2/17	8/14/18	9/8/18	20	3/15/19
64	386	6	2	3/21/17	394	2	1	8/2/17	8/14/18	9/11/18	12	3/1/19

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
65	386	6	2	3/21/17	366	n/a	1	8/9/17	9/13/18	10/12/18	5	3/1/19
66	391	2	3	6/14/17	402	n/a	25	n/a	8/14/18	9/7/18	9	3/1/19
67	391	2	4	6/14/17	401	n/a	106	n/a	7/9/18	7/26/18	19	
67	391	2	4	6/14/17	401	n/a	106	n/a	7/9/18	8/4/18	46	
68	391	2	4	6/14/17	440	n/a	111	n/a	7/18/18	8/11/18	62	
69	391	2	4	6/14/17	391	n/a	1	6/14/17	7/20/18	8/14/18	47	
70	391	2	4	6/14/17	366	4	2	7/11/17	8/14/18	9/8/18	133	
71	391	2	6	6/14/17	410	n/a	5	4/4/17	7/9/18	8/5/18	31	3/15/19
72	391	2	6	6/14/17	392		3	6/30/17	7/13/18	8/10/18	34	3/15/19
73	391	2	6	6/14/17	396	n/a	1	6/14/17	7/18/18	8/4/18	13	3/1/19
73	391	2	6	6/14/17	396	n/a	1	6/14/17	7/18/18	8/14/18	16	3/15/19
74	391	2	6	6/14/17	392	2	8	7/11/17	7/23/18	7/25/18	112	3/15/19
75	391	2	6	6/14/17	366	4	2	7/11/17	8/14/18	9/11/18	50	3/15/19
76	391	2	6	6/14/17	366	n/a	1	7/19/17	9/14/18	10/17/18	9	3/1/19

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
77	391	2	8	6/14/17	392	n/a	4	7/11/17	7/22/18	8/25/18	26	3/15/19
78	391	2	8	6/14/17	402	n/a	4	n/a	7/23/18	8/16/18	22	3/15/19
78	391	2	8	6/14/17	402	n/a	4	n/a	7/23/18	8/25/18	26	3/15/19
79	391	2	8	6/14/17	366	4	2	7/11/17	8/14/18	9/8/18	13	3/1/19
80	391	4	1	6/14/17	392	n/a	4	7/11/17	7/22/18	8/25/18	7	3/1/19
80	391	4	1	6/14/17	392	n/a	4	7/11/17	7/28/18	8/27/18	3	3/1/19
81	391	4	1	6/14/17	392	2	8	7/11/17	8/14/18	9/11/18	10	3/1/19
82	391	4	6	6/14/17	394	n/a	1	7/26/17	7/13/18	7/27/18	1	3/1/19
83	391	4	6	6/14/17	410	n/a	5	4/4/17	9/14/18	10/25/18	2	3/1/19
84	392	2	2	7/11/17	410	n/a	5	4/4/17	7/24/18	8/20/18	22	
85	392	2	2	7/11/17	402	n/a	29	n/a	7/28/18	8/25/18	23	
86	392	2	2	7/11/17	346	n/a	141	n/a	8/13/18	9/10/18	23	
87	392	2	2	7/11/17	401	n/a	106	n/a	8/17/18	9/13/18	32	
88	392	2	2	7/11/17	401	n/a	106	n/a	8/17/18	9/13/18	38	

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
89	392	2	2	7/11/17	366	4	1	7/19/17	8/20/18	9/16/18	18	
90	392	4	1	7/11/17	391	n/a	2	6/14/17	7/10/18	8/4/18	10	3/1/19
91	392	4	1	7/11/17	391	4	5	6/14/17	7/18/18	8/11/18	21	3/15/19
92	392	4	1	7/11/17	396	n/a	1	6/14/17	7/20/18	8/4/18	5	3/1/19
92	392	4	1	7/11/17	396	n/a	1	6/14/17	7/20/18	8/16/18	4	3/1/19
93	392	4	1	7/11/17	392	n/a	4	7/11/17	7/22/18	8/16/18	11	3/1/19
94	392	4	1	7/11/17	402	n/a	4	n/a	7/23/18	8/25/18	27	3/15/19
95	392	4	1	7/11/17	440	n/a	111	n/a	7/23/18	8/5/18	11	3/1/19
95	392	4	1	7/11/17	440	n/a	111	n/a	7/23/18	8/25/18	13	3/1/19
96	392	4	1	7/11/17	396	2	9	6/14/17	8/27/18	9/27/18	20	3/15/19
97	394	2	1	7/11/17	410	n/a	5	4/4/17	7/28/18	8/10/18	9	3/1/19
97	394	2	1	7/11/17	410	n/a	5	4/4/17	7/28/18	8/11/18	70	3/15/19
98	394	2	1	7/11/17	346	n/a	141	n/a	8/13/18	9/7/18	29	3/15/19
99	394	2	1	7/11/17	392	2	7	7/11/17	8/22/18	9/7/18	30	3/15/19

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
100	394	2	5	7/11/17	394	2	1	8/2/17	7/28/18	9/5/18	3	3/1/19
101	394	2	5	7/11/17	392 or 402	2 or n/a	5 or 25	7/11/17 or n/a	8/29/18	10/9/18	3	3/1/19
102	394	2	9	7/11/17	402	n/a	25	n/a	7/18/18	8/10/18	32	
102	394	2	9	7/11/17	402	n/a	25	n/a	7/18/18	8/11/18	24	
102	394	2	9	7/11/17	402	n/a	25	n/a	7/18/18	8/13/18	37	
103	394	2	9	7/11/17	410	n/a	5	4/4/17	7/20/18	8/7/18	56	
103	394	2	9	7/11/17	410	n/a	5	4/4/17	7/22/18	8/7/18	25	
104	394	2	9	7/11/17	391	n/a	3	6/14/17	7/22/18	8/7/18	11	
105	394	2	9	7/11/17	410	n/a	5	4/4/17	7/22/18	9/3/18	31	
106	394	2	9	7/11/17	402	n/a	29	n/a	7/28/18	8/11/18	30	
107	394	2	9	7/11/17	405	2	2	7/19/17	8/17/18	9/3/18	44	
108	394	2	9	7/11/17	392	2	7	7/11/17	8/22/18	9/11/18	18	
108	394	2	9	7/11/17	392	2	7	7/11/17	8/22/18	9/16/18	23	
109	394	2	10	7/11/17	391	n/a	3	6/14/17	8/14/18	9/3/18	24	3/15/19

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
110	394	2	10	7/11/17	405	2	2	7/19/17	8/17/18	9/11/18	30	3/15/19
111	394	2	10	7/11/17	394	n/a	1	7/26/17	8/29/18	9/27/18	30	3/15/19
112	394	2	13	7/11/17	394	n/a	1	7/26/17	7/13/18	8/7/18	7	3/1/19
113	394	2	13	7/11/17	440	n/a	111	n/a	7/18/18	8/14/18	21	3/15/19
114	394	2	13	7/11/17	392	n/a	4	7/11/17	7/28/18	8/22/18	22	3/15/19
115	394	2	13	7/11/17	401	n/a	106	n/a	9/5/18	10/2/18	20	3/15/19
115	394	2	13	7/11/17	401	n/a	106	n/a	9/5/18	10/12/18	20	3/15/19
116	394	2	13	7/11/17	410	n/a	5	4/4/17	9/14/18	10/25/18	21	3/1/19
117	394	2	14	7/11/17	440	n/a	111	n/a	7/23/18	9/3/18	18	
118	394	2	14	7/11/17	394	2	6	7/11/17	8/14/18	9/11/18	22	
118	394	2	14	7/11/17	394	2	6	7/11/17	8/14/18	9/16/18	8	
118	394	2	14	7/11/17	394	2	6	7/11/17	8/14/18	9/22/18	6	
119	394	2	14	7/11/17	394	4	3	7/11/17	9/13/18	10/12/18	15	
120	394	2	16	7/11/17	394	4	7	7/11/17	8/14/18	9/7/18	15	3/15/19

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
121	394	2	16	7/11/17	402	n/a	25	n/a	8/22/18	9/16/18	24	3/15/19
122	394	4	1	7/11/17	402	n/a	25	n/a	7/22/18	8/9/18	25	
123	394	4	1	7/11/17	394	2	1	8/2/17	7/28/18	8/16/18	22	
124	394	4	1	7/11/17	346	n/a	141	n/a	8/13/18	9/8/18	22	
125	394	4	1	7/11/17	392	2	7	7/11/17	8/17/18	9/14/18	13	
126	396	2	2	6/14/17	401	n/a	106	n/a	7/9/18	7/24/18	1	3/1/19
127	396	2	7	6/14/17	395	n/a	1	3/21/17	7/20/18	8/5/18	1	3/1/19
128	396	2	7	6/14/17	392	n/a	4	7/11/17	7/28/18	8/25/18	11	3/1/19
129	396	2	26	6/14/17	410	n/a	5	4/4/17	7/9/18	7/29/18	3	3/1/19
129	396	2	26	6/14/17	410	n/a	5	4/4/17	9/14/18	10/17/18	1	3/1/19
130	396	2	26	6/14/17	440	n/a	111	n/a	7/9/18	7/29/18	2	3/1/19
130	396	2	26	6/14/17	440	n/a	111	n/a	7/9/18	8/4/18	2	3/1/19
131	396	2	26	6/14/17	394	n/a	1	7/26/17	7/13/18	8/7/18	3	3/1/19
131	396	2	26	6/14/17	394	n/a	1	7/26/17	8/14/18	9/8/18	7	3/1/19

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
132	396	2	29	6/14/17	396	n/a	1	6/14/17	7/10/18	7/29/18	4	3/1/19
133	405	2	1	7/19/17	366	4	2	7/11/17	8/17/18	9/11/18	147	3/15/19
134	405	2	1	8/2/17	402	n/a	25	n/a	7/18/18	8/10/18	19	
134	405	2	1	8/2/17	402	n/a	25	n/a	7/18/18	8/11/18	16	
135	405	2	1	8/2/17	392	n/a	3	6/30/17	7/18/18	8/11/18	17	
136	405	2	1	8/2/17	394	2	1	8/2/17	7/20/18	8/13/18	32	
137	405	2	1	8/2/17	410	n/a	4	4/4/17	7/20/18	8/13/18	33	
138	405	2	1	8/2/17	392	2	7	7/11/17	7/22/18	8/14/18	13	
138	405	2	1	8/2/17	392	2	7	7/11/17	7/22/18	8/16/18	29	
139	405	2	1	8/2/17	392	n/a	4	7/11/17	7/23/18	8/16/18	22	
140	405	2	1	8/2/17	346	n/a	141	n/a	8/13/18	9/8/18	25	
141	405	2	1	8/2/17	366	4	1	7/19/17	8/17/18	9/11/18	20	
141	405	2	1	8/2/17	366	4	1	7/19/17	8/22/18	9/16/18	15	
142	405	2	1	8/2/17	392	2	8	7/11/17	9/14/18	10/9/18	6	

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
143	411	2	1	8/9/17	366	4	1	7/19/17	7/22/18	8/14/18	20	3/15/19
144	411	2	1	8/9/17	392	2	7	7/11/17	7/24/18	8/16/18	36	3/15/19
144	411	2	1	8/9/17	392	2	7	7/11/17	8/17/18	9/11/18	59	3/15/19
144	411	2	1	8/9/17	392	2	7	7/11/17	8/17/18	9/14/18	26	3/15/19
145	411	2	1	8/9/17	392	2	5	7/11/17	8/29/18	9/22/18	15	3/15/19
146	411	2	1	8/9/17	366	4	2	7/11/17	9/10/18	10/9/18	23	3/15/19
147	411	2	3	7/14/17	346	n/a	141	n/a	8/13/18	9/8/18	54	3/15/19
148	411	2	3	7/14/17	402	n/a	25	n/a	8/29/18	9/27/18	18	3/15/19
149	411	2	3	7/14/17	366	n/a	1	7/11/17	9/5/18	10/2/18	24	3/15/19
150	411	2	3	7/14/17	392	2	8	7/11/17	9/10/18	10/9/18	14	3/1/19
151	411	2	3	7/14/17	402	n/a	4	n/a	9/14/18	10/9/18	5	3/1/19
152	411	2	4	7/14/17	440	n/a	111	n/a	7/18/18	8/11/18	20	
153	411	2	4	7/14/17	395	n/a	1	3/21/17	7/20/18	8/14/18	22	
154	411	2	4	7/14/17	410	n/a	5	4/4/17	7/24/18	8/20/18	135	

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
154	411	2	4	7/14/17	410	n/a	5	4/4/17	7/24/18	9/3/18	53	
155	411	2	4	7/14/17	402	n/a	29	n/a	7/28/18	8/22/18	33	
156	411	2	4	7/14/17	402	n/a	29	n/a	7/28/18	8/25/18	36	
157	411	2	4	7/14/17	394	2	1	8/2/17	8/17/18	9/11/18	16	
158	411	2	7	7/14/17	394	2	1	8/2/17	7/28/18	8/25/18	100+	
159	411	2	7	7/14/17	366	4	1	7/19/17	8/20/18	9/13/18	69	3/15/19
160	411	2	7	7/14/17	392	2	5	7/11/17	8/27/18	9/22/18	58	3/15/19
161	411	4	1	7/26/17	394	4	7	7/11/17	8/14/18	9/13/18	8	3/1/19
162	411	4	1	7/26/17	396	n/a	1	6/14/17	8/17/18	9/11/18	10	3/1/19
163	456	2	10	7/14/17	410	n/a	5	4/4/17	8/13/18	9/8/18	156	
164	456	2	10	7/14/17	366	4	1	7/19/17	8/17/18	9/11/18	50	
164	456	2	10	7/14/17	366	4	1	7/19/17	8/22/18	9/16/18	82	
165	456	2	10	7/14/17	391	n/a	3	6/14/17	8/20/18	9/14/18	33	
166	456	2	10	7/14/17	392	2	5	7/11/17	8/27/18	9/22/18	44	

SED2018-	Female (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Male (Hib. SED2016-	Hours treated	Rep. no.	Date treated	Cross date	Harvest date	No. seed collected	Date sown
167	456	4	1	7/14/17	410	n/a	5	4/4/17	8/17/18	9/11/18	32	3/15/19
168	456	4	1	7/14/17	366	4	2	7/11/17	8/22/18	9/22/18	18	3/15/19
169	456	4	1	7/14/17	366	n/a	1	7/11/17	9/5/18	10/2/18	9	3/1/19
170	456	2	8	7/14/17	396	2	9	6/14/17	8/27/18	9/22/18	44	3/15/19
171	456	4	3	7/11/17	392	2	8	7/11/17	9/10/18	10/12/18	9	3/1/19
172	456	4	8	7/14/17	392	n/a	4	7/11/17	9/5/18	10/3/18	18	3/15/19
173	379	4	2	6/14/17	394	n/a	1	7/26/17	8/29/18	9/16/18	2	3/1/19
174	379	4	2	6/14/17	396	n/a	1	6/14/17	7/3/18	7/28/18	37	3/15/19

[z] Seed from cross was sown but then the female parent was re-tested using flow cytometry and found to be diploid. Therefore the seed were abandoned and eliminated.

Appendix B. Details of tissue culture medium for ovule culture/embryo rescue of immature seed of *Hibiscus moscheutos* hybrids from crossing tetraploid with diploid plants.

Media Name:	Differentiation Medium (WPM)
Label as:	DM WPM
COMPONENTS	
Lloyd & McCown Woody Plant Mixture (WPM)	2.30g
Vitamin Mixture 1 (1000x)	1ml
Myo-inositol	19.9g
Gibberellic acid (GA3) solution (1mg/ml)	1ml
Abscisic acid (ABA) (1mg/ml stock)	2ml
Sucrose	20g
Plant Preservative Mixture (PPM)	1ml
Final Volume	1L
pH	5.5
Activated charcoal	1g
Gelrite	2g

Per Liter

Media Name:	Maturation Medium (WPM)
Label as:	MM WPM
COMPONENTS	
Lloyd & McCown Woody Plant Basal Medium (WPM)	2.30g
Vitamin Mixture 1 (1000x)	1ml
Gibberellic acid (GA3) solution (1mg/ml)	1ml
Abscisic acid (ABA) (1mg/ml stock)	200µl

Sucrose	20g
Plant Preservative Mixture (PPM)	1ml
Final Volume	890ml
pH	5.5
Activated charcoal	1g
Gelrite	2g
Add after autoclaving	
Coconut water (from Phytotechnology Labs)	100ml
Kao & Michayluk Vitamin solution (100x)	10ml

Per Liter

Appendix C. Genetic inheritance models and associated ratios tested on F_2 progeny (Family 4) of the cross R4 (an intraspecific hybrid of *Hibiscus moscheutos* subsp. *moscheutos*) × *H. grandiflorus* for pertinent ornamental traits. Phenotypic evaluations were made Summer 2015.

Trait (and rating scale)	**Genetic model**	**Genetic model ratio**	**Phenotypic Ratio by Ratings**	**Observed Phenotypic Ratio[z]**	**Expected Phenotypic Ratio[z]**	χ^2
Foliage color (1 = no red/entirely green 2 = small amount of red 3 = about 50% red 4 = mostly red 5 = completely red)	Single gene/Mendelian inheritance	3:1	(2-5):1	262:34	222:74	28.829 [y]
	Dominant gene interaction	9:6:1	(2-4):1:5	255:34:7	167:111:19	107.604 [x]
	Duplicate gene action	15:1	(2-5):1	262:34	278:19	13.852
	Dominant suppression	13:3	(2-5):1	262:34	241:56	10.251
	Complementary gene interaction	9:7	(2-5):1	262:34	167:130	125.203
Leaf pubescence (1 = no pubescence/ glabrous 2 = small amount of pubescence 3 = about 50% pubescent 4 = mostly pubescent 5 = highly pubescent)	Single gene/Mendelian inheritance	3:1	(2-5):1	256:40	222:74	20.829
	Dominant gene interaction	9:6:1	(2-4):1:5	235:40:21	167:111:19	73.934
	Duplicate gene action	15:1	(2-5):1	256:40	278:19	26.652
	Dominant suppression	13:3	(2-5):1	256:40	241:56	5.328

	Complementary gene interaction	9:7	(2-5):1	256:40	167:130	109.965
Petal overlap (1 = no overlap at all/petals barely touching 2 = < 15% overlap/slight overlap 3 = 15-30% overlap 4 = 30-50% overlap 5 = ≥ 50% overlap of petals)	Single gene/Mendelian inheritance	3:1	(2-5):1	138:66	153:51	5.882
	Dominant gene interaction	9:6:1	(2-4):1:5	138:66:0	115:77:13	18.902
	Duplicate gene action	15:1	(2-5):1	138:66	191:13	237.224
	Dominant suppression	13:3	(2-5):1	138:66	166:38	24.778
	Complementary gene interaction	9:7	(2-5):1	138:66	115:89	10.768
Compactness (1 = not compact/very open 2 = slightly compact 3 = moderate compactness 4 = compact 5 = very or extremely compact)	Single gene/Mendelian inheritance	3:1	(2-5):1	134:162	222:74	139.532
	Dominant gene interaction	9:6:1	(2-4):1:5	134:162:0	167:111:19	48.276
	Duplicate gene action	15:1	(2-5):1	134:162	278:19	1187.301
	Dominant suppression	13:3	(2-5):1	134:162	241:56	251.526
	Complementary gene interaction	9:7	(2-5):1	134:162	167:130	14.500

[z] The phenotype ratings in the column titled “Phenotypic Ratio by Ratings” were used for the totals of the observed and expected number of plants for the ratios in each row. The total number of plants used for observed values was 296 for the traits of foliage

color, pubescence and compactness, whereas the total number of plants for petal overlap was 204 because not all plants were flowering at data collection.

[y] The chi-square critical value for two phenotypes is 3.84 ($\chi^2_{0.05, 1}$).

[x] The chi-square critical value for three phenotypes is 5.999 ($\chi^2_{0.05, 2}$).