HYBRIDIZATION OF ASCLEPIAS SPECIES FOR THE CREATION OF NOVEL CULTIVARS

by

MARY LEWIS

(Under the Direction of JOHN RUTER)

ABSTRACT

Milkweed (*Asclepias* sp.) is a vital pollinator genus across North America and is a host plant for many butterfly species, notably the monarch butterfly (*Danaus plexippus*). However, commercial production of *Asclepias* is limited because most species lack commercial traits, having minimal branching habits, excessive height, and minimal color variation. Using a commercially viable *Asclepias* species, *Asclepias tuberosa,* as a maternal parent, three different pollination methods were trialed to create interspecific hybrids. Pollination methods included a traditional method, a pollen-solution-based method, and a novel inverted pollinia method. Using the inverted pollinia method increased pollination success rates fourfold among intraspecific crosses of *A. tuberosa*. Once pollination methods were optimized, *A. tuberosa* was used as the maternal parent, and one-way crosses were made to seven other *Asclepias* species. Four species successfully developed hybrid seeds of the seven species used as pollen donors; *Asclepias hirtella, Asclepias purpurascens, Asclepias speciosa,* and *Asclepias syriaca*. This is the first documented case of a controlled interspecific hybridization event among these species. Three germination techniques were then trialed: direct seeding, embryo rescue, and stratification. Of the three methods, stratification for 30 days had the highest germination rates across the four

hybrid groups compared to the other treatments. After germinating seeds to grow out the F_1 hybrids, inheritance of commercially desirable traits was also investigated. Pubescence was found to be a recessively inherited trait across all hybrid groups, although specific inheritance ratios did vary depending on the pollen parent used. White sap color was found to be dominantly inherited across all hybrid groups at a ubiquitous 1:0 ratio. Flower color inheritance varied depending on the parents used but generally trended towards the dominant inheritance of a pink coloration across the hybrid groups. Quantitative traits such as height, leaf length, leaf width, branching, and flower height were inherited at values intermediate of the parents, typical of an F_1 hybrid cross. Thus, interspecific hybridization of *Asclepias* is possible, and commercially desirable traits can be observed in the hybrid progeny. To our knowledge, this is the first documentation of intentional hybridizations between *Asclepias* species.

INDEX WORDS: Plant breeding, ornamental hybrids, Asclepiadoidea, milkweed, butterfly weed, native plant

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Asclepias is a member of the Apocynaceae family, but more specifically, the Asclepiadaceae subfamily found almost exclusively in North America. *Asclepias* includes 90 species found throughout the U.S., Canada, and some parts of Central America. *Asclepias* is an important ecologic host and food source for many butterfly species that utilize nectar as a food source for adults and oviposition of egg masses, which after developing into caterpillars, consume *Asclepias* foliage almost exclusively. *Asclepias* is known for its attractive floral structures and performance in landscape environments with minimal fertilizer and irrigation inputs, particularly in native landscapes and habitat restoration projects. When appropriately sited, many species are extremely drought tolerant. Despite having ornamentally and ecologically valuable traits, cultivars of *Asclepias* spp. are not commonly found in the ornamental market. Reasons include *Asclepias* spp. does not tolerate currently employed commercial growing protocols that incorporate high fertility and irrigation rates, and as a result, grow to heights deemed too large for efficient racking and shipping or suffer from pathogens. Once installed in a landscape, plants are highly susceptible to root and crown rots if sited in high irrigation/fertility environments or sites prone to high soil moisture. Little breeding work has been done to overcome these negative traits, especially given the market potential of the genus, with the majority of work focusing on ecology and floral biology of the genus. The primary goal of this study is to attempt interspecific hybridizations for the creation of novel hybrids. Research conducted on the natural barriers to interspecific hybridization included making controlled interspecific hybrids. These resulted in no released cultivars. One of the significant challenges in

developing interspecific hybrid cultivars is that fertilized embryos often undergo a late-term abortion. Such challenges have deterred many ornamental breeding programs from attempting interspecific crosses within the genus. This project will attempt to overcome this barrier by developing an embryo rescue protocol to increase the success rates of interspecific crosses. Embryo rescue has not been attempted within the genus and, based upon parental selection, could lead to new interspecific *Asclepias* hybrids with enhanced production/landscape tolerances and ornamental value. Such hybrids would have beneficial traits such as shorter stature, better branching, improved floral display, and tolerance to consistently moist soils (and associated pathogen pressure). The ornamental plant industry also lacks published protocols on vegetatively propagating *Asclepias*; via stem cuttings, root cuttings, or tissue culture. Currently, only growers specializing in native plants attempt to propagate and commercially produce this genus for live-plant sales. This study will also attempt to establish a working protocol for vegetative propagation, allowing the industry a possible avenue to increase production and distribution of *Asclepias* to the market.

Additionally, determining the genome size and ploidy of *Asclepias* is essential, given that little work has been conducted on the topic. Potential barriers to hybridization could arise from ploidy differences or genome size differences, which we would be unaware of; neither possibility has been adequately researched to date. Finally, if hybridizations are possible, the final goal of this study is to begin determining inheritance patterns for simply inherited and quantitative traits. Knowing anything about inheritance across different interspecific hybrids would be groundbreaking and the first of its kind. In summary, this project will study hybridization between different species of *Asclepias,* delve into a vegetative propagation protocol, determine a

working embryo rescue method, discover total genomic content for an array of *Asclepias* species, and deduce the inheritance patterns of phenotypic traits in interspecific hybrid groups.

Asclepias is a member of the Apocynaceae family, dicot counterparts to the Orchidaceae family (Wyatt and Broyles, 1994). Both families transmit pollen via pollinia, or sac-like structures containing large amounts of pollen. The pollinia of Asclepiadaceae also boasts one of the most florally complex structures known, only rivaled by the complexity of the Orchidaceae family. Pollinia are part of a "lock and key" pollination method (Stebbins, 1970) where pollinia are removed from the corpusculum of the pollen parent, usually by the pollinator's hair or legs, dried for a short period of time (typically during flight), rotated 90 degrees and then inserted into the stigmatic slit of another plant correctly for fertilization to initiate (Figure 1.1). Pollinia and the stigmatic slit will vary significantly in size depending on the species, and these differences will impact successful hybridization if carried out naturally by pollinators (Wyatt and Broyles, 1994). *Asclepias* have two separate ovaries, and if pollinium are inserted correctly, all seeds will share a single pollen parent (genotype) (Wyatt and Broyles, 1994). Pollen viability of the pollinia packet is viable for over four days once removed from the corpusculum. However, its fertilization ability does decline by half after the first 24 hours (Kephart, 1981). *Asclepias* spp. seedling dormancy is classified as primary dormancy and is broken with 30 days of cold-moist stratification, removal of the seed coat, or Gibberellic Acid treatments (Evetts and Burnside, 1972). *Asclepias* is a facultative long-day plant that can be forced to flower by exposing plants to 15-hour days, night interruption of 5 minutes every hour, and 25°C day/ 14°C night temperatures (Albrecht and Lehmann, 1991). *Asclepias* spp. also has a juvenile period that ends after one season of vegetative growth following seedling germination (Shannon and Wyatt, 1986). During

the second year of growth, flower production is initiated, and plants will perennially produce flowers from that point forward (Shannon and Wyatt, 1986).

The complex floral structure of *Asclepias* encourages outcrossing and carries an S-locus gene that promotes self-incompatibility, which can either be gametophytic or sporophytic (Lipow and Wyatt, 2000). The gene is late acting in the ovary and hinders endosperm maturation (Lipow and Wyatt, 2000). This postzygotic self-incompatibility (SI) is highly polymorphic and can be overcome if favorable genetic recombination occurs (Lipow and Wyatt, 2000). Interestingly, a knowledge gap exists in overcoming self-incompatibility, despite published work on its mechanisms and heritability. Self-incompatibility not only reduces the number of fertile seeds developed overall, but the seed pod will also go through an abortion if less than half the seeds (embryos) are fertilized at pollination (Bookman, 1984).

Hybridization of *Asclepias* (both intra- and interspecific) has had little success in published reports. Recent genetic diversity work has shown that this may be influenced by the highly polymorphic sites that *Asclepias* shares among the 90 species and cultivars of the genus and differing chromosome numbers (Weitemier *et al*., 2015). Analysis of polymorphic sites is a way to determine the amount of genetic variance at the same chromosomal point across many different genotypes (or species in this case) (Weitemier *et al*., 2015). In theory, the more polymorphic a genetic region is across multiple species, the more genetic variation a breeder can draw from. Among *Asclepias* spp., the fact that there are so many highly polymorphic sites indicates there is little inbreeding depression. Genetic variation can mean the ability to overcome hybridization barriers or the potential for natural hybridizations to occur at a low rate, and controlled hybridization could still be possible (Weitemier *et al.*). This makes the hybridization process both problematic and promising. Difficult in that to obtain a hybrid individual, hand

pollinations will be necessary as some of the crosses will require pollinia of differing sizes that under natural circumstances would not lead to successful pollination. Genome size and chromosomal numbers are also not known for many *Asclepias* species. This creates uncertainty when selecting species to utilize as parents in an interspecific breeding program. Despite the overall lack of genetic information, recent AFLP diversity estimates of species within the genera (Weitemier *et al*., 2015) should provide a starting point whereby *Asclepias* spp. sharing clades (indicating higher genetic similarity) can be tested for interspecific compatibility. The process is also promising as the amount of diversity in this species should be extensive enough to create and find many novel phenotypes that could be commercially viable. Novel traits would express some form of hybrid vigor; be it as pest resistance, improved branching, improved ecologic adaptability, improved flower color, greater environmental tolerances, and possibly sterility.

The chances of widespread novel gene escape into native populations of *Asclepias* is extremely low, given the multiple barriers to fertilization, including self-incompatibility genes, differences in genome sizes across the species, hybrid sterility, and pollinator traveling distances (Kephart, 1981; Lipow and Wyatt, 2000; Broyles, 2002; Bookman, 1984, Wyatt and Broyles, 1994; Wyatt, 1976; and Khan and Morse, 1991). Interspecific hybrids, when outcrossing, face late-term abortion via endosperm fertilization failure (Bookman, 1984). Any surviving hybrids (between hybrid cultivars and wild-type plants) face potential sterility and low flower or fruit set in the next generation (Kephart, 1981). Another effective barrier to gene escape in wild plants is the pollinia packets. Varied in sizes, pollinators may visit several species, but depositing the pollinia into another species stigmatic slit would be greatly reduced by differing pollinia and stigmatic slits.

Studies have reported successful natural interspecific hybridizations, with researchers indicating that success was due to habitat overlap (Wyatt and Broyles, 1992). Some species have been successfully hybridized under controlled conditions (Kephart and Heiser, 1980). Successful crosses under controlled conditions have been between *A. speciosa* x *A. syriaca*, (Stevens, 1945 *A. exaltata* x *A. syriaca* (Wyatt and Hunt, 1991), *A. exaltata* x *A. quadrifolia*, and *A. purpurascens* x *A. syriaca* (Kephart *et al*., 1988). These successful crosses occurred from species in the same phylogenetic clade (Weitemier *et al*., 2015). For this reason, the species selected as parents for this study were also from closely related clades, except for *A. incarnata* and *A. hirtella.*

There are specific characteristics present in *Asclepias* spp. selected as pollen parents in this study that would be beneficial if introgressed into *A. tuberosa*, and that that could result in a superior ornamental crop (Table 1). For example, *A. incarnata* and *A. hirtella* thrive under wet soil conditions. These are the only two species native to sites with consistently moist soil conditions, similar to production environments and irrigated landscapes. *A. fascicularis* and *A. viridis* (in addition to *A. tuberosa*) are the only freely branching species, an unusual trait for the genus, and a valuable trait to the industry. *A. viridis* has the shortest height at 0.3-0.76 meters, differing drastically from most species that range from 1.2 to 1.8 meters. As detailed in Table 1.1, the flower color of species used in this study is far different from A. tuberosa, ranging from white to green to pinks and purples (Wilbur, 1976), which could broaden the floral color palette of hybrids.

In this project, seven species will be utilized as parents, with *A. tuberosa* serving as the female parent and all others as pollen parents in a series of one-way crosses. *Asclepias tuberosa* is native to most regions of the U.S., excluding the upper northwest. Hardy from USDA Zones 3-

9, with lanceolate foliage, it is the only species that does not ooze white latex sap (cardenolides) when the stem is broken. The species prefers dry soil conditions and full sun, has a taproot system, floral display orange to orangey-yellow, and is the shortest species in the genus (Cullina, 2000). *Asclepias speciosa* is native from the Midwest to the West coast and is hardy from USDA Zones 3-9. The leaves are usually grey-green at maturity, ovate in shape, and pubescent. Usually preferring full sun conditions, their floral coloration is light pink and has the characteristic taproot system but will vegetatively propagate using rhizomes (Cullina, 2000). *Asclepias syriaca* is native to most regions of the U.S. east of the Rocky Mountains and is hardy from USDA Zones 4-9. Floral coloration can be a mauve to bleached purple white, producing copious amounts of nectar depending on age. Producing sturdy stems with oblong and reddish-veined leaves, this is an extremely drought tolerant plant and thrives in full sun conditions (Cullina, 2000). *Asclepias incarnata* is native to all parts of the U.S., excluding CA, OR, WA, AZ, and MS. Cold hardy from USDA Zones 3-6 is a species that prefers marsh or pond-edges as its preferred habitat (Wilbur, 1976). Leaves are lanceolate in shape and will tolerate full sun conditions if enough water is present. Floral coloration is of dark pink, although white coloration has been observed on rare occasions. Exposure to full sun conditions does bleach out the coloration on the florets faster, meaning *A. incarnata* prefers partial shade conditions. One of the few species with natural branching patterns boasts a less extensive taproot system and spreads vegetatively via rhizomes (Cullina, 2000). *Asclepias purpurascens*, having purple floral coloration, is native to the Eastern U.S., extending west to TX and SD, with a cold hardiness range from USDA Zones 3-9. Leaves are elliptic to oblong, and much like *A. incarnata*, the species prefers partial shade over full sun conditions but does not tolerate wet soil conditions. *A. purpurascens* is one of the few species that tolerates and even requires high nutrition to maintain

growth (Cullina, 2000). *Asclepias fascicularis* has a smaller native range, covering the states of CA, NV, UT, OR, WA, and ID. Hardy from USDA Zones 7-10, leaves are arranged in whorls that are narrowly lanceolate. The species prefers dry soil and full sun conditions and has a taproot characteristic of *Asclepias* spp. The flower color is white, although some slight pink shading can be visible depending on the location and age of the flower (Anderson, 2001). *Asclepias hirtella* is native to the east-central portion of the U.S., from SC and PA to TX and WI. Hardy from USDA Zones 4-9 possesses very narrow lanceolate leaves. Preferring both dry and moist soil conditions, *A. hirtella* thrives under full sun conditions, has no natural branching tendencies, and has a taproot system. The flower color is an unusual green, with tiny dots of purple pigmentation on the reflexed petal tips when the floret is young (Cullina, 2000).

Looking at the basics of vegetative propagation, there are two main methods by which *Asclepias* spp. undergoes reproduction in the wild, seed, and rhizomal tubers (Luna and Dumroese, 2013). While seed production can be helpful, it does not allow for the maintenance of cultivars. Vegetative propagation through the stem or root tissue holds promise in commercial propagation, as cuttings allow plants to grow rapidly and with more uniformity than if relying strictly on seed production (Ecker and Barzilay, 1993). However, very few studies have addressed vegetative propagation of *Asclepias* spp. Of the few studies that have been published, what was discussed focused on optimum environmental conditions to initiate callus and root development (Ecker and Barzilay, 1993). Across species, *Asclepias* root cuttings perform best under long-day conditions (15-17 hours), temperatures ranging from 25-16 °C, and well-drained soils (Ecker, 1993).

One of the challenges of vegetatively propagating *Asclepias* is susceptibility to root pathogens that are exacerbated by high moisture levels in propagation environments (Luna and

Dumroese, 2013). To mitigate this problem, this study will utilize perlite as a rooting media. Increased drainage provided by perlite should reduce the chances that cuttings will succumb to root diseases or damping off. Another step that the project will take in staving off disease is the implementation of a soil drench fungicide rotation and adding micronutrients to low dose fertigation that will be applied to the cuttings. A study in 2013 found that the application of micronutrients to cuttings increased adventitious root formation and increased both rooting percentage and eventual plant health (Martin *et. all.*, 2016).

Vegetative propagation may prove to be a viable propagation protocol since some *Asclepias* spp. naturally propagate via vegetative means. For example, *Asclepias syriaca* forms adventitious buds on roots that propagate outward, relying on seed propagation as a secondary form of dispersal (Luna and Dumroese, 2013). When roots are employed to propagate the species vegetatively, cuttings taken from the upper root system develop faster and with greater success than those taken from the bottom root portions (Ecker and Barzilay, 1993). In the same study, IBA (Indole-3-butyric acid) was not applied to the roots to induce initiation (Ecker and Barzilay, 1993). In general, no rooting hormones are utilized in the vegetative propagation of root sections.

Vegetative propagation using stem cuttings is a widespread propagation protocol in ornamental production but rarely used to propagate Asclepias, as no clonally propagated cultivars exist in the marketplace. One study was conducted to evaluate hormone concentration effects on *Asclepias* rooting. Stem cuttings received low or medium concentrations of IBA (10% and 40% concentrations) and resulted in a meager success rate for the cuttings (Bowles et. al., 1991). Our study will be using IBA in addition to NAA (1-Naphthaleneacetic acid), another auxin hormone commonly used to promote root initiation. In addition to ideal environmental and nutritional conditions, the time cuttings are taken can impact cutting success. Phillips (1985)

indicated the best time to collect stem cuttings was about 3-5 weeks before flower initiation or just after dormancy break.

Asclepias tuberosa will be used as a model species within the genus to determine optimal vegetative propagation protocols. *Asclepias tuberosa* is native to most regions of the U.S., excluding the upper northwest portion of the US. Hardy from USDA Zones 3-9, and having lanceolate foliage, it is the only *Asclepias* species that does not ooze white latex sap when the stem is broken. *A. tuberosa* prefers dry soils and full sun conditions and has a taproot (Cullina, 2000). *A. tuberosa* is also one species that naturally propagates via rhizomes and is used most commonly in the industry because of the species diminutive height and floral display. If both a meristem and root propagation protocol can be optimized through this study, this would allow growers greater success and profitability when growing this important native plant.

Assuming that any potential hybrids resulting from surviving crosses will not perform similarly to *A. tuberosa*, a methodology for embryo rescue was examined. Embryo rescue, or the isolation and growth of immature embryos under sterile conditions to obtain viable plants, has been practiced for over half a century (Bridgen, 1994). The first recorded success was Charles Bonnet, late in the 18th century and improved upon by the work done by Hannig in 1904 (Bridgen, 1994). These researchers were responsible for setting the groundwork for future scientists to bypass seed dormancy, shorten breeding cycles, test seed viability, and develop hybrids from previously incompatible crosses. The ability of embryo rescue to save wide inter- and intra-specific crosses that ordinarily would not be capable of surviving has proven to be a valuable tool for breeders (Dunwell, 1986).

The principal reason why hybridization fails in *Asclepias* spp. is because of embryo abortion. While fertilization is successful, a pollen sperm cell fails to fertilize the endosperm,

causing the embryo to starve before seed maturation or germination is possible (Sparrow and Pearson, 1948). This same condition can be seen in many other ornamental crops such as *Abelia* spp.¸ *Rhododendron* spp.*, Freesia, Lotus corniculatus,* and *Medicago sativa* (Seavey and Bawa, 1986). By aseptically culturing the young embryo on nutrient media, a lack of nutrients naturally provided by the endosperm needed to initiate germination would no longer be a barrier and enable hybrids to exist that ordinarily would be unable to survive through natural seed development and germination (Bridgen, 1994).

There are typically two stages of embryo development; the pro-embryo stage (heterotrophic phase) and the autotrophic phase. The heterotrophic phase is classified as embryos one-third of their mature size and requires the presence of growth regulators to complete development and be successful hybrids (Sharma *et. al.*, 1996). Autotrophic embryos are metabolically capable of providing their own nutrition and only require media to supply salts and sugars to complete development and become a hybrid (Bridgen, 1994). *Asclepias* species undergo late-term abortion, so theoretically, embryos rescued later (in the autotrophic stage) would be viable and not require the extra time and combinations of complex media with growth regulators to yield successful regeneration of hybrid explants (Lipow and Wyatt, 2000). However, suppose a successful embryo rescue methodology is to be developed for commercial production. In that case, this process needs to be both cost-effective and quick to ensure hybrids can quickly be germinated and moved into evaluation.

Embryo rescue requires specific environmental factors that must be considered and managed to ensure success. Upon rescuing embryos, a seven-day dark period is required for many species to allow chlorophyll formation and break dormancy. Temperatures should also be maintained at 25-30°C for the germination of embryos (Narayanaswamy and Norstog, 1964).

When considering media, the age of the embryo dictates the complexity and needs of the embryo to facilitate regeneration. For this study, autotrophic embryos will be utilized, and the use of plant growth regulators is unnecessary (Bridgen, 1994). Autotrophic embryos require salts and sugars at much lower concentrations than younger embryos (2-3% sucrose vs. 8-12%), and exogenous auxin application is unnecessary for embryo growth if the embryo is mature (Norstog, 1979). Cytokinin applications are ineffective for growth promotion, and both auxin and cytokinins are not used unless callus induction is needed (Bridgen, 1994). Gibberellins, however, have been shown to stimulate germination and overcome dormancy and is the primary hormone used in this project for that reason.

No published embryo rescue protocols are available for *Asclepias spp.* A few studies vaguely document embryogenesis protocols in Asclepias spp. However, as genetic recombination happens before the embryo's death, the goal is to find the most efficient and fastest way to obtain hybridized seedlings, embryogenesis protocols do not apply to developing embryo rescue protocols. Embryogenesis, when compared to embryo rescue, takes considerably longer, as an embryo is dedifferentiated, multiplied via callus formation, and then redifferentiated with the aid of plant growth regulators (Kim *et. al.*, 2004). In *Asclepias*, only one study (Groet and Kidd, 1981) attempted to develop a methodology for the somatic embryogenesis of milkweed. However, the study was written so that replication or determination of correct methodology was complicated to determine. The only conclusion that can be drawn was that indole-3-acetic acid (IAA), in combination with kinetin (KN) and sodium phosphate, resulted in successful callus growth and subsequent plantlet development (Groet and Kidd, 1981). What concentrations of these chemicals, applied at which times and suspended in what suspension culture for how long is unknown. More detailed studies in closely related species of

Catharanthus roseus and Tylophora indica indicated that both 2,4-D and BA could successfully induce and regenerate plantlets (Kim et. al, 2003; Sahai et. al, 2010). These two species are members of *Apocynaceae* and could yield similar results to *Asclepias*. While neither study provides information in developing an embryo rescue protocol, results from this project indicate that rescue of the embryos to be used in embryogenesis needs to occur at a younger age. Such results are expected when harvesting embryos in the *heterotrophic* phase of development. Although large differences in embryo rescue versus embryogenesis protocols, the embryogenesis of *Tylophora indica*, in the same family as *Asclepias*, preferred a less nutrient-heavy media. A half-strength media increased the success rate of explants by over 20% when applied at embryo maturation (Sahai *et al.*, 2010). Our study will apply this finding and test to see if the same success can be observed in embryo rescue protocols.

Embryo rescue should be a viable mechanism to overcome late-term abortion, and if the embryo is excised from the seed at the proper time, only minimal nutrition would be needed to enable subsequent growth and development. In addition, rescuing viable embryos would enable the survival of hybrids, allowing breeders to access previously unattainable genetic traits. Going forward, a working protocol would allow the production of future hybrid lines in a shorter timeframe and be useful from a commercial standpoint.

If embryo rescue was successful, but hybridization still was not possible, another possibility to investigate is the genome size of the species used. Flow cytometry can estimate genome level, general ploidy, nuclear replication, and endopolyploidy (Doležel et al., 2007). For the Asclepiadoideae family (a subfamily of Apocynaceae) and *Asclepias spp.*, FCM research has been used to determine the ploidy and total genomic content for only a few species. Some *Asclepias* species with a known ploidy are *A. curassavica* L., *A. fascicularis* Decne., *A.*

incarnata L., *A. latifolia* Torr., *A. salicifolia* Lodd., *A. speciosa* Torr., *A. syriaca* L., *A. verticillata* L., and *A. tuberosa* L., all being diploids (Darling and Wylie, 1956 and Moyer, 1936). In addition, it appears that 22 of the over 130 *Asclepias* species have a base chromosome count of 11 (2n=2x=22) (Darling and Wylie, 1956; Gadella et al., 1969; Heiser and Whitaker, 1948; CCDB database, 2021). The 2001 study by Albers and Maeve found in their analysis of over 650 species within the Apocynaceae family, only 3% deviated from the x=11 chromosome count. Thus, within the Asclepiadoideae subfamily, polyploidy was not observed. With only a small number of the species in the family documented, there is still work to be done to ensure that ploidy levels are consistent across the family and the species and where geographically, those ploidy deviations occur. Hybridization and natural ploidy variations could be detected and utilized for improved commercial production by further exploring the ploidy levels across a more extensive range of species.

The C-value usually characterizes total genomic content, measured ordinarily using propidium iodide (PI). C-value is the number of base pairs (picograms) of DNA present in a whole chromosome complement (Greilhuber et al., 2005; Swift, 1950). While DAPI is another stain that binds to DNA and can measure genome size, DAPI only binds to the AT base pairs and is generally used to measure ploidy. In contrast, PI will bind to both AT and GC regions, providing estimations of DNA content with greater accuracy (Doležel et al., 2007). However, finding literature on total genomic content for *Asclepias* yields sparse results. Previous studies that include the genomic content of *Asclepias* included one to three species. The intention was to cover a wide range and variety of species not confined to the *Asclepias* genus. The few *Asclepias* species with their total genomic content measured are *A. curassavica* L., *A. incarnata, A. syriaca, A. tuberosa, and A. verticillata* (Bai et al., 2012). Based on the available literature, what

is interesting is the relatively small genome size *Asclepias* has. In angiosperms, species with a genome size 1.4 pg – 3.5 pg (2C value) have very small to small genome sizes (Soltis et al., 2003). *Asclepias* genome size (2C values) ranges from 0.7 – 1.1 pg, with *A. verticillata* recorded as the smallest and *A. tuberosa* the largest (Bai et al., 2012). Given that total DNA content between species can vary from 40 – 70 % (Verloove et al., 2017), to what degree the range of genome size *Asclepias* could extend to has yet to be investigated at length. Greater differences in DNA content usually result in difficulty forming hybrids between species, which explains why there are few naturally occurring hybrids (Kephart et al., 1988; Zonneveld, 2019).

As intentional interspecific hybridizations have never been documented to this point, inheritance of any traits are unknown. As all species of *Asclepias* are outcrossing by nature, it would be reasonable to assume that any parents used in the hybridization efforts associated with this study would not be completely homozygous for any trait (Lipow and Wyatt, 2000). A standard hypothesis based on Mendel's law of the inheritance of simply inherited traits would indicate that for every phenotype, the dominant form of the gene will be expressed in the next generation. For a true F_1 population, this would mean all progeny would express that dominant gene. Simply inherited traits can mean progeny are an intermediate of the two parents (Lipow and Wyatt, 2000). However, research indicates that this simple inheritance model does not apply to all traits in *Asclepias*. For example, comparing observations from other taxa with those of Asclepias, a study by Agrawal et al. in 2009 looked at the phylogeny of pubescence in 43 *Asclepias* species, and approximately half (20) were non-pubescent. While that is a small sample of the *Asclepias* genus, it does indicate that pubescence in *Asclepias* F_1 interspecific hybrids may not be a dominant phenotype.

Asclepias is unique in that the genus falls within the 10% of flowering plant species that produce latex sap (Agrawal and Konno, 2009). Produced in specialized cells called laticifers running from root meristems of the plant to the apical meristems, the latex not only coagulates when exposed to air, but it also carries varying levels of cardiac glycosides (Agrawal and Konno, 2009). These cardenolides, or cardiac glycosides, are astringent at a minimum and toxic at high consumption levels to a wide array of insects and mammals. As a result of consuming foliage containing cardenolides, insects such as butterflies confer protection from predation (Malcom, 1991). While it is a protective mechanism, studies have shown that excess latex consumption can increase monarch butterfly caterpillar mortality by up to 33% when feeding upon *Asclepias* spp. (Zalucki et al. 2001; Zalucki & Malcolm 1999). It has also been observed that this latex production is phenotypically plastic or responsive to environmental conditions or inputs. Specifically, increased latex production was documented after adding fertilizer (under greenhouse growing conditions) or due to herbivory damage (Agrawal and Konno, 2009). What is unusual is that one *Asclepias* spp., *A. tuberosa*, does not produce this latex sap but still produces high levels of cardenolides. From both a commercial and ecological standpoint, it is interesting to determine the inheritance of latex sap between the pollen parents that produce it and A. tuberosa that does not (Züst et al., 2019). Latex sap inheritance is significant from the consumer perspective as latex sap is both sticky and can stain surfaces, two factors that could reduce consumer demand. While only being two phenotypic traits of interest, previous research indicates that inheritance of other traits, some quantitatively inherited, may not follow traditional F_1 generation inheritance patterns. Besides never being achieved and published to this point, the mechanics of inheritance, growth, and development, and best practices are vital to industry

integration. If *Asclepias* hybrids are possible, they would be welcome into the commercial market, and knowing inheritance, propagation, and genome size help facilitate integration.

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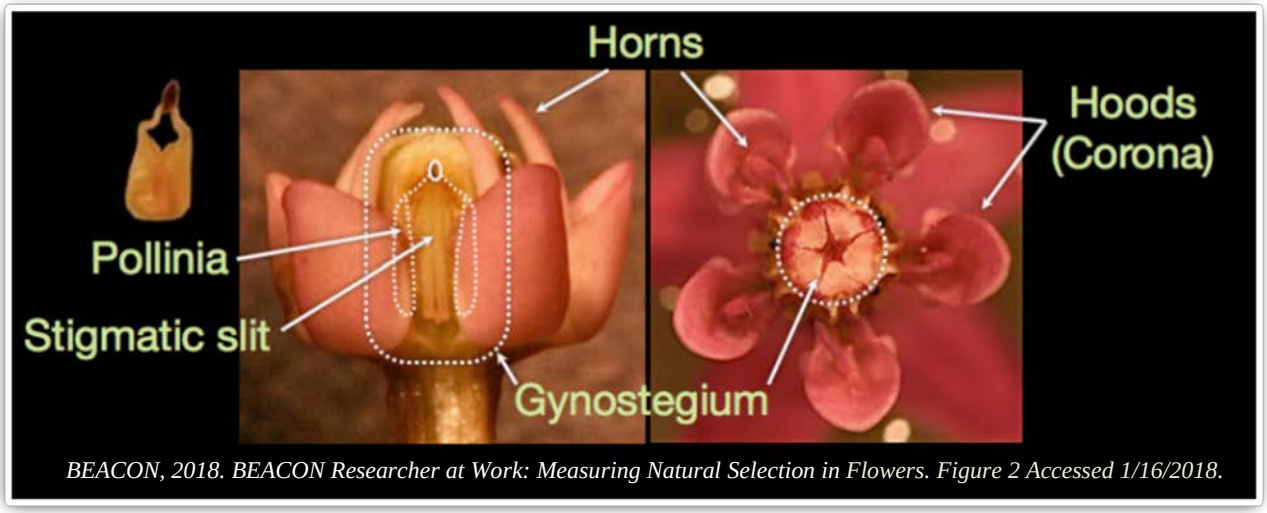
Table 1.1: Individual characteristics of all the species used during this experiment. Bloom length is not given in numerical length of bloom as that information is not consistently recorded for all species. Generally, flowering spans a length of about 2-3 months, but the length in which a flower remains open and viable for pollination has not explicitly been recorded or measured for all species.

Species	**Height (M)**	**Soil Moisture Preference**	**Flower Color**	**Bloom Length**	**Fragrance**	**Branching**
Asclepias speciosa	0.6-1.8	Dry	White/Pink	Long	Yes	None
Asclepias syriaca	1.2-1.8	Dry	Pink	Short	Yes	None
Asclepias incarnata	1.2-1.5	Wet	White, Pink, Mauve	Long	Yes	None
Asclepias purpurascens	0.6-.9	Dry	Purple Pink	Long	Yes	None
Asclepias viridis	0.3-0.76	Dry	Green	Short	No	Some
Asclepias fascicularis	0.3-0.9	Dry	White/Pink	Long	Yes	Some
Asclepias hirtella	0.9-1.2	Wet	Green	Medium	No	None

Figure 1.1. Pollen grains are encapsulated in packets known as pollinia. To initiate pollination and fertilization, pollinia rotated 90 degrees can be inserted into the stigmatic slit (upside down). The Gynostegium is the fused form of anther and stigma and refers to the entire floral structure as a whole. The Horn is simply an appendage of the hood.



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CHAPTER 2

Development and Verification of an Interspecific Hybridization Protocol for *Asclepias*[1]

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Introduction

Asclepias L. is a member of the Apocynaceae (milkweed family) that is comprised of 106 species native to North America (Woodson, 1954; Wyatt and Broyles, 1994). Many species are important nectar sources for indigenous butterfly species (Luna and Dumroese, 2013). Additionally, adult butterflies use many species for oviposition sites, with larvae using foliage as a food source (Brower, 1969; Hutchings, 1923). Several *Asclepias* species are known for attractive floral structures (including fragrant blooms) and exceptional performance in landscape environments despite minimal fertilizer and irrigation inputs. However, some species possess traits that make them difficult to produce commercially. Excessive height, particularly in high fertility soils indicative of commercial production environments, limits commercial viability. Poor branching, aversion to moist soil conditions, and poor germination rates have also dissuaded many commercial producers from adopting *Asclepias* species into production. Outside of seed-produced varieties developed for the cut-flower industry, little genetic improvement has taken place in the genus, with minimal clonally produced cultivars available to ornamental producers.

A major reason for the lack of breeding and selection progress is that *Asclepias* boasts one of the most complex reproductive structures known in the plant kingdom (Wyatt, 1976). Much like the Orchidaceae family, pollen is transmitted via pollinia, or sac-like structures containing pollen.

Pollinia are part of a “lock and key” pollination method (Stebbins, 1970) whereby successful pollination requires removal of pollinia from the corpusculum of the pollen parent, usually via a pollinator’s rake and comb (on legs), dried for a short period (typically during flight), rotated 90 degrees and then inserted into the stigmatic slit of the maternal parent to facilitate fertilization. Pollinia and stigmatic slit size vary depending upon species, and these differences are purported to limit successful naturally occurring interspecific hybridization via pollinator activity (Wyatt and Broyles, 1994). *Asclepias* is also unusual in that they have two independent ovaries and, if pollinia are inserted perfectly, all subsequent seed will share a single pollen parent (genotype) (Wyatt and Broyles, 1994). Once removed from the corpusculum, pollen is viable within the pollinia packet for approximately four days. However, the fertilization rate declines by 50 percent after the first 24 h (Kephart, 1981).

The complex floral structure of *Asclepias* encourages outcrossing and carries an S-locus gene that fosters either gametophytic or sporophytic self-incompatibility (Lipow and Wyatt, 2000). The gene is late acting in the ovary and hinders endosperm maturation (Lipow and Wyatt, 2000). This postzygotic self-incompatibility (SI) is highly polymorphic, and as such, can be overcome if favorable genetic recombination occurs (Lipow and Wyatt, 2000). Despite knowledge of the mechanisms of self-incompatibility, overcoming all aspects of self-incompatibility has not been thoroughly investigated in the genus. Levels of SI can vary from species to species (even within species), and additional factors can impact success of self-fertilization. For example, even when successful pollinations are made, it remains unknown as to why the seed pods abort if less than half the seeds (embryos) are fertilized (Bookman, 1984; Kahn and Morse, 1991).

Given the major physiological and ecological barriers presented, hybridization of *Asclepias* (both intra- and interspecific) has had relatively little success in published reports. Recent genetic diversity work has shown a lack of success in interspecific hybridizations may be influenced by high levels of genetic diversity among *Asclepias* species, as well as potentially differing chromosome numbers (Weitemier et al., 2015). Despite potential genetic barriers that would inhibit the creation of interspecific hybrids within *Asclepias*, successful interspecific hybrids could yield segregating populations with greater phenotypic variability to draw upon when selecting F_1 individuals for potential commercial release (Wyatt and Hunt, 1991).

Among *Asclepias* species, there is also wide variability in geographic range and specific habitat requirements. Therefore, many *Asclepias* species have evolved in relative physiographic isolation. This could have contributed to greater genetic diversity among species. Habitat (e.g., cultural) variability among species, while limiting natural interspecific hybridization, suggests that controlled crosses could result in progeny with wider environmental tolerance due to hybrid vigor (Ellstrand and Schierenbeck, 2000). Hybridization is also promising in that the amount of diversity in this genus should be extensive enough to identify novel phenotypes that could be commercially viable. These commercially viable phenotypes may express hybrid vigor and/or intermediate phenotypes that yield improved pest/pathogen tolerance, improved plant form, novel flower color(s), improved growth and flowering rates, and/or sterility.

High genetic diversity and habitat variability among *Asclepias* species have contributed to relatively few documented interspecific hybridizations, both naturally occurring and in controlled environments. Studies investigating natural interspecific hybridizations have

suggested that success was a function of habitat overlap on the margins of the species range(s) and may be more common than previously thought (Broyles, 2002; Klips and Culley, 2004; Wyatt and Broyles, 1992; Wyatt and Hunt, 1991). Some species have also been successfully hybridized under controlled conditions (Kephart and Heiser, 1980). Successful crosses under controlled conditions have been between *A. speciosa* × *A. syriaca* (Stevens, 1945), poke milkweed (*A. exaltata* L.) × *A. syriaca* (Broyles, 2002; Wyatt and Hunt, 1991), *A. exaltata* × whorled milkweed (*A. quadrifolia* Jacq.), and *A. purpurascens* × *A. syriaca* (Kephart et al., 1988). All of these successful crosses occurred from species in the same phylogenetic clade based upon a recent genetic diversity study (Weitemier et al., 2015).

Genome size, ploidy, and chromosome number are unknown for many *Asclepias* species. This creates uncertainty when selecting species to utilize in controlled hybridizations. Despite a lack of comprehensive genetic information available for all *Asclepias* species, recent AFLP diversity estimates (Weitemier et al., 2015) provide a starting point whereby species sharing clades (indicating higher genetic similarity) could be tested for interspecific compatibility. Based on previously documented successful interspecific hybridization efforts, (Kephart and Heiser, 1980; Kephart et al., 1988; Stevens, 1945; Wyatt and Hunt, 1991), it would be logical to use species from the same clade as a starting point for breeding efforts.

Variability in seed dormancy also presents a challenge when developing interspecific *Asclepias* hybrids, with many species having no defined stratification or seed treatment protocol. *A. tuberosa* seedling dormancy is classified as primary dormancy and is overcome with 30 days of cold-moist stratification, removal of the seed coat, or gibberellic acid treatment (Evetts and

Burnside, 1972). However, *A. syriaca* and *A. purpurascens* require cold stratification for one year after maturity before their germination rate reaches 50% (Baskin and Baskin, 1977; Groh, 1943). *Asclepias* species also have a juvenile period that ends after one season of vegetative growth following seedling germination (Shannon and Wyatt, 1986). During the second year of growth, flower production is initiated, and plants will perennially produce flowers from that point forward (Shannon and Wyatt, 1986). Published accounts indicate that all *Asclepias* species are facultative long-day plants that can be forced to flower by exposing plants to 15-h days, night interruption of 5 m every h, and 25 °C day/ 14 °C night temperatures (Albrecht and Lehmann, 1991).

The objectives of this project were threefold. The first was to determine an optimal pollination protocol for *A. tuberosa*. The second was to utilize the developed pollination protocol to attempt interspecific hybridizations using *A. tuberosa* as the model maternal parent and seven other species as pollen parents. These seven species were chosen based on their relative relatedness (and non- relatedness) to *A. tuberosa* genetically, diversity in growing conditions, flower color, fragrance, height, branching, and current prevalence in the commercial market. The third objective was to determine if a seed treatment protocol could be identified to maximize germination of hybrid seed.

Materials and Methods:

Parent Production. Seven species were initially grown from seed to serve as pollen parents, with *A. tuberosa* serving as the maternal parent (Table 2.1, Figure 2.1). Upon receiving seed from their differing locations (Table 2.1), all seed lots were divided into their respective species,

wrapped in a moist paper towel, then wrapped in aluminum foil and placed in 0.004 m^3 (1-gallon) Ziploc bags. Seeds were then cold-moist stratified in a refrigerator held at 3-4 °C for 30 days. Upon removal from stratification, seeds were germinated in Oct. 2018 at the University of Georgia Athens campus, College Station Greenhouse Complex (lat. 33.9480°N, long. 83.3773°W) in 804 inserts (T.O. Plastics, Minneapolis, MN) containing 100% perlite (Carolina Perlite Co. Inc., Gold Hill, NC) with a topdressing of vermiculite (TX401, BWI, Greer, SC) to a 0.635 cm depth. Supplemental light was provided by light-emitting diode (LED) arrays (Fluence Spyder with PhysioSpec, Fluence Technologies Inc., Austin, TX), providing a *PPFD* (photosynthetic photon flux density) of 250μmol·m^{-2}·s^{-1} and 14-h daylength. Seedlings were placed on a mist bench with a misting cycle applying municipal water (pH 6.2 and alkalinity of 11 ppm) at 6 s every 10 m. Greenhouse temperatures were maintained at 25 °C day and 18 °C night. Upon germination and expansion of the first set of true leaves, seedlings were transplanted into 804 inserts (T.O. Plastics) held by 1020 greenhouse trays (T.O. Plastics) filled with 80% milled peat (Sungro Peat Moss Grower Grade Orange, Agwam, MA) and 20% perlite (Carolina Perlite Co. Inc.). Once established, seedlings were transplanted into 0.004 m^3 (1-gallon) containers (Classic 400, Nursery Supply, Agwam, MA) containing an 80% bark (3/8” particle size) and 20% milled peat (Foothills Compost, Gainesville, GA). Throughout seedling germination, establishment, and transplanting stages, seedlings were irrigated with municipal water and fertilized twice a week with Peter’s 20N- 4.4P-16.6K liquid soluble fertilizer (Scotts Co., Marysville, OH) at 100 ppm. For all species, 130 plants in 0.004 m^3 containers were produced. Once plants were established in containers, the plants to be used in interspecific breeding objectives were placed into cold storage with environmental conditions consisting of 96% relative humidity and 3-4 °C. Chilling hour requirements to induce flowering of the various

species were unknown, so fifteen plants of each species were removed from cold storage at 2-week intervals beginning at 8 weeks and terminating at 34 weeks. Once plants were removed from cold storage, they were placed in a greenhouse with temperatures at 25 °C day and 18 °C night. Fluence Spyder LED arrays with PgysioSpec spectrum were used as supplemental lighting, maintaining a PPF of 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 14-h daylength. *Asclepias* species are long-day plants and typically initiate flowering under 14-h days (starting 18 May in Athens, GA). New growth was pinched back to the fourth node at week 5 after dormancy break. Pinching was performed to promote greater branching and thus greater number of flowers produced.

Hybridization Protocol. All interspecific crosses attempted were one-way crosses using *A. tuberosa* as the female parent. The species used as pollen donors were *A. curassavica* L., *A. fascicularis* Decne., *A. hirtella, A. incarnata* L., *A. purpurascens, A. speciosa,* and *A. syriaca*. Based upon previous work (Wyatt, 1981) and preliminary floral observations (data not shown), the inner 9-10 flowers of *A. tuberosa* open concurrently with outer florets opening sporadically over 7-10 days. To reduce unintentional selfing, outer florets were removed, leaving only the inner 9-10 florets. Preliminary data indicated that stigmatic receptivity of *A. tuberosa* occurs 48 h after flowers open, yet 24 h before pollen dehiscence. Additionally, *A. tuberosa* selfing rates are extremely low (< 1%) (Wyatt, 1981). For these reasons, emasculation of flowers was deemed unnecessary. Crosses were made in the morning and finished before noon, and pollinated florets were bagged (A&S Creavention, El Monte, CA) to mitigate unintentional crosses by insects.

Pollination techniques. In a preliminary study that employed *A. tuberosa* as both pollen parent and pollen donor, three different pollination techniques were trialed to determine highest

pollination success. Removal of all pollinia from pollen donors was performed with forceps (ESD-10,14, and 16, PIXNOR, Amazon) for all three methods. The first method used (termed "traditional method") was described by Wyatt (1976) where pollinia were removed from the paternal genotype (as it sits parallel to the stigmatic slit), rotated 90 degrees (inverted to pollinia edge), and inserted into the stigmatic chamber of *A. tuberosa* maternal genotype parallel to the stigmatic slit (Figure 2). One to two pollinium (two pollinium making one pollinia) from the same pollen parent were inserted into one stigmatic chamber of each floret of the maternal parent, and the same pollen donor parent was utilized for the entire floral structure (9-10 florets). Approximately 550 pollinations were completed utilizing this method. The second method was a solution-based pollination method. Pollen inside pollinia were determined to germinate optimally when suspended in a 30% sucrose solution (Wyatt and Shannon, 1986). Using 1 mL of a 30% sucrose solution, five different numbers of pollinia (p) (100p, 200p, 250p, 350p, and 450p) were combined and crushed using a mortar and pestle (FB970J, Fisher Scientific, Lenexa, KS). After suspending pollen in 1 mL sucrose solution, a hypodermic needle (1 mL, Terumo Medical Corp., Elkton, MD) was used to insert the pollen solution through the stigmatic slit and into the gynostegium (main structure of the flower made from the fused filaments and stigma) (Figure 3b) until excess solution exuded from the stigmatic slit. For each pollinia concentration, between 95-900 crosses were made. The third pollination method was a variation of the first described method (Wyatt, 1976). Rather than inserting pollinia parallel to the stigmatic slit, pollinia were inserted perpendicular to the stigmatic slit, best described as inserting a key into a lock (Figure 4) as opposed to sliding a credit card in a reader. In each case, the convex end of the pollinia pointed down, to be closer to the stigma after insertion into the stigmatic slit. Ideally this would increase the chance at successful pollinations due to closer proximity and orientation of the

pollinia and germination pore to the internal pistil (Galil and Zeroni, 1969). Approximately 400 pollinations were initially conducted using this method, and after data comparison between the other pollination techniques (Figure 3), a total of 7,000 additional crosses among additional pollen parent species (*A. curassavica, A. fascicularis, A. hirtella, A. incarnata, A. purpurascens, A. speciosa,* and *A. syriaca*) and *A. tuberosa* (maternal parent) were carried out. Reciprocal crosses were not attempted due to space limitations. After pollination, flowers were tagged with the date the cross was made, paternal and maternal parent species, and the number of florets pollinated. Flowers were bagged and resulting seed pods were allowed to mature until dehiscence initiated or seed pods were brown.

Germination. Upon harvest of mature seed pods from individual crosses, seeds were removed from the seed pod and the length of the seed pod was measured. Seeds from each individual pod were then counted and divided equally and randomly into three groups. Seeds from the three groups were used as three treatment methods to determine optimal stratification method. One-third of the seeds were immediately (upon harvest) sown in 100% perlite with a top dressing of vermiculite at 0.635 cm depth (direct-seeded; DS). Another third of seeds were immediately placed into cold-moist stratification for 30 days at 3-4 °C in a washed builder's sand media (stratified). After stratification, seed were sown by filling 1020 greenhouse trays with 100% perlite, placing seed on the surface of the perlite, and covering with 0.635 cm of vermiculite. Germination for DS and stratified seed was initiated by placing flats on a mist bench using municipal water applied at a rate of 6 s of mist every 10 m. After seedlings germinated and the cotyledons expanded, seedlings were transferred to 804 inserts (32-cell trays) held by 1020 greenhouse trays (with holes) filled with 80% composted bark, 10% milled peat and 10% perlite

mix, and irrigated using municipal water as needed. Thirty days after transplanting seedlings into flats, flats were placed in cold storage for 12 weeks at 96% relative humidity and 3-4 °C. The final third of seeds underwent embryo rescue (ER), as described by Lewis et al. (2020). Resulting ER plantlets were allowed to mature in culture for 60 days before removing from culture and hardening off in the greenhouse. Plantlets arising from ER were rinsed in municipal tap water and transferred to 804 inserts (32-cell trays) held by 1020 greenhouse trays (with holes) filled with 80% composted bark, 10% milled peat, and 10% perlite mix before being placed in a humidity chamber receiving 6 s mist every 10 s. Plants were incrementally exposed to lower humidity levels over three weeks and then moved to mist benches with the same environmental conditions as DS and stratified seeds before being transferred to cold storage.

Upon removal from cold storage, all hybrid seedlings (DS, stratified, and ER) were placed in a greenhouse with temperatures at 24 °C daytime and 20 °C nighttime. Fluence Spyder LED arrays with PgysioSpec spectrum were used as supplemental lighting, maintaining a PPF of 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 15-h daylength. When seedlings broke dormancy, plants were transferred to 0.004 m^3 containers filled with 80% bark (0.98 cm particle size) and 20% milled peat mix, and topdressed with Harrell's 20N-6.6P-16K controlled-release fertilizer. Twice weekly, additional fertility was provided via a Peter's 20N- 4.4P-16.6K liquid soluble fertilizer at 100 ppm. Otherwise, all plants were hand-watered as needed using municipal water.

Data Collection and Analysis. Preliminary data on parents was collected for individual parental genotypes and included time to first flower (in days after dormancy break). To determine optimal pollination methodology among *A. tuberosa* genotypes, the total number of crosses were

recorded using each pollination technique, with the number of pods resulting from each method recorded. After obtaining preliminary hybridization technique results, the optimal pollination method (inverted pollination) was used for interspecific hybridizations using *A. tuberosa* (maternal parent) and the seven paternal species. Data collected from these hybridizations included the total number of florets hybridized per parental combination, number of successful pollinations as measured by seed pod formation (by parent combination), and days from pollination to seed maturity for each seed pod. At seed harvest, data collected included seed pod length for each successful hybrid seed pod and the number of seeds in each pod. Upon seeding (using DS or stratification techniques), germination percentage for all seed by cross (individual seed pods) and within species combinations was recorded.

All data collection occurred in a single controlled environment greenhouse structure with uniform environmental conditions. Regulating temperature, light, and production inputs (e.g., soil and fertility) was performed to minimize environmental effects on the production of F_1 phenotypes. Parental plants and F_1 genotypes were arranged in a completely randomized design within the greenhouse. Statistical analysis was performed using JMP (version 13.0; SAS Institute, Cary, NC). Data was analyzed to determine normality and homogeneity, with one-way analysis of variance (ANOVA) and separation of treatment means using Tukey's HSD (honestly significant difference) to analyze differences among pollination methods, percentage of pod set depending on pollen donors, average length of time until pod harvest, direct-seeded germination percent, stratified seed germination percent, and ER germination percent. Only data showing significant differences ($\alpha = 0.05$, $P \leq 0.05$) among treatments are reported.

Results and Discussion

Optimizing Pollination Methods

The extremely low rates of pollination success using traditional pollination methods (Wyatt, 1976) served as a stimulus for experimenting with differing pollination methods. During preliminary trials that involved attempts to make successful *A. tuberosa* intraspecific hybrids among genotypes of *A. tuberosa* in this study, approximately 100 crosses were needed to obtain one seed pod using traditional pollination methods. In this preliminary work, each cross took one minute, or 100 m for each successful hybrid seed pod. Alternative pollination methods were sought out due to the ineffectiveness and inefficiency of traditional methods. In pistachio (*Pistacia vera* L.), kiwi (*Actinidia chinensis* Planch.), and Japanese pear (*Pyrus pyrifolia* L.), increased levels of pollination success were observed when pollen grains were suspended in sucrose and/or agar solution and sprayed on flowers (Sakamoto et al., 2009; Yano et al., 2006; Zeraatkar et al., 2013). Wyatt and Shannon (1986) indicated germination of pollinia was optimized when suspended in a 30% sucrose solution. Based on these results, we hypothesized that suspending pollen in a sucrose solution and injecting the solution through the stigmatic slit and into the gynostegium may increase seed pod set. However, when compared to a concurrent trial of traditional pollination, the solution-based method resulting in no pod formation (Table 2.2). Traditional pollination method (Wyatt, 1976) had a final pollination success rate of 2.28 ± 1.00% (Table 2.2). During the process of making traditional pollinations, researchers noticed that pollinia could be inserted perpendicular to the stigmatic slit (at the bottom of the slit) and doing so could enable direct contact of pollinia with the stigmatic surface, located in the stigmatic chamber. This technique was refined and is termed the inverted pollination method, which resulted in an 11.72 ± 1.14% success rate (Table 2.2) when attempting intraspecific

hybridizations. With a four-fold increase in pod set compared to traditional methods, the inverted pollination method was applied to all interspecific hybridizations attempted in this study. We postulated that the inverted pollination method yielded superior results because the pollinia are placed in direct contact with the stigmatic surface, when compared to the traditional pollination method (Figure 3C, 4C). The solution-based pollination method was likely unsuccessful due to inconsistent solution deposition inside the gynostegium. Controlling the release of solution and not rupturing the gynostegium with the pressure difference, while making the pollinations, was challenging.

Interspecific Hybridization

Seven *Asclepias* species were used as pollen parents, with *A. tuberosa* serving as the maternal parent for all hybridizations (Table 2.1, Figure 2.1). Approximately 1,000 inverted pollination attempts were made per parent combination between March and October 2018, resulting in 253 hybrid pods. Four paternal species produced successful interspecific hybridizations with *A. tuberosa* (maternal parent). These included *A. hirtella, A. purpurascens, A. speciosa,* and *A. syriaca* (Table 2.3). While no paternal species resulted in a pod set rate higher than *A. tuberosa* (inverted), *A. speciosa* had a success rate of 8.58 ± 0.70%; similar to intraspecific *A. tuberosa* using the same pollination method (Table 2.3). *A. syriaca* had the lowest success rate (3.71 ± 0.63%) that was similar to *A. tuberosa* using traditional pollination methods. Of *Asclepias* species that have ploidy documented, *A. curassavica, A. fascicularis, A. incarnata, A. speciosa, A. syriaca,* and *A. tuberosa* are all diploids with a chromosome count of 11 (2n = 2x = 22) (Chromosome Counts Database, 2019; Darlington and Wylie, 1956; Heiser and Whitaker, 1948). This indicated ploidy difference was likely not a contributing factor to incompatibility between *A. tuberosa* and *A. curassavica, A. fascicularis,* or *A. incarnata* in this study. A trend was

observed when viewing phylogenetic relationships among *A. tuberosa* and those species that did not result in hybrid progeny (*A. curassavica, A. incarnata,* and *A. fascicularis*) (Fishbein et al., 2011). *A. curassavica, A. incarnata,* and *A. fascicularis* were more distantly related to *A. tuberosa* than the four species that produced successful hybrid seed (Fishbein et al., 2011). Concurrently, a study by Weitemier et al. (2015) confirmed Fishbein et al.'s (2011) conclusion as to the relatedness of these species. Together, these previous genetic studies indicate that those species that resulted in successful hybrids (*A. hirtella, A. purpurascens, A. speciosa,* and *A. syriaca*) were more genetically similar than the species that failed to form hybrid seed.

Very little information exists on seed pod maturity (measured in days after pollination; DAP) of interspecific hybrid seed pods versus hybrid parents in ornamental crops. It should be noted that seed (embryo and endosperm) often matures earlier than seed pods, and thus drawing a correlation between seed maturity and the date that the seed pod matures could lead to confounding principles. Because seed pod maturity (measured in DAP) differs among *Asclepias* species in native environments, the length of time needed for pods to reach maturity based on their pollen parent was recorded in this study. *A. tuberosa* intraspecific pollinated data was used as the control, as this could serve as a test of cytoplasmic or nuclear inheritance of seed pod maturity. Overall, *Asclepias* pod maturity was achieved in 93.38 – 126.64 DAP, with differences noted in pod maturity depending upon the pollen parent. *A. speciosa* (pollen parent) had the quickest pod maturity of 93.38 ± 2.78 d (Table 2.4). *A. tuberosa* (control) had a maturation time of 110.32 ± 3.87 d, similar to *A. syriaca* pods (114.14 ± 3.84 d) (Table 2.4). *A. purpurascens* and *A. hirtella* hybrid pods took the longest to mature, taking 124.64 ± 3.55 d for *A. purpurascens* hybrid pods to mature, and 126.64 ± 3.52 d for *A. hirtella* hybrid pods to mature. This indicates

that seed pod maturity differs based upon the paternal parent, and therefore is not a maternally inherited (cytoplasmic) trait.

Little published work exists on seed pod growth parameters among populations of interspecific hybrids. For this reason, seed pod length and seeds per pod of hybrid crosses were compared to a control of intraspecific *A. tuberosa* genotypes. The average pod length of intraspecific pollinated *A. tuberosa* genotypes was 10.69 ± 0.28 cm and did not differ from other hybrid populations having varied paternal parents (Table 2.4). Only *A. hirtella* hybrids (11.12 ± 0.26 cm) produced longer pods when compared to *A. speciosa* hybrids (9.99 ± 0.21 cm) and *A. syriaca* hybrids (9.92 ± 0.29 cm) (Table 2.4). *A. tuberosa* had a pod length ranging from 8.4-15.5 cm, *A. hirtella* from 7.0- 11.8 cm, *A. purpurascens* from 9.0- 15.0 cm, and *A. syriaca* from 6.5- 13.6 cm (Betz and Lamp, 1992; Wilbur, 1976). The number of seeds per pod did not vary among intraspecific *A. tuberosa* and *A. hirtella, A. speciosa,* and *A. syriaca* hybrid populations. *A. purpurascens* hybrids produced the fewest seed per pod (32.92 ± 2.99) (Table 2.4). In a study on *Asclepias* species, Benz and Lamp (1992) determined that *A. tuberosa* on average produced 73.8 ± 14.7 seeds, which is higher than the seed counts observed in this study. *A. hirtella* was documented to produce 51.2 ± 7.2 seeds per pod in the wild (Betz and Lamp, 1992), which was similar to hybrid *A. hirtella* results observed in this study. However, wild seed production in *A. purpurascens* (180.7 ± 31.5) and *A. syriaca* (244.3 ± 31.2) (Betz and Lamp, 1992) was much higher when compared to observed seed set of hybrids in this study (Table 2.4).

Germination Methods

Germination methods for *Asclepias* species vary depending on their native range. *A. curassavica* does not require a germination treatment to break seed dormancy, while *A. syriaca* requires an

extended seed dormancy period (of a year or more) combined with cold stratification before a germination rate of 50% or greater can be achieved (Baskin and Baskin, 1977; Groh, 1943). *A. fascicularis* requires four to six weeks of stratification to break dormancy, and *A. speciosa* requires on average two to four weeks of stratification (Kaye et al., 2018) As hybrid parents varied in germination requirements, the two most common germination methods of direct seeding and stratification were utilized to determine the optimal germination method. Embryo rescue, as previously described by Lewis et al. (2020), was also compared to DS and stratification in an attempt to ensure some hybrids were recovered. Within the DS treated hybrid populations, only *A. hirtella* hybrids germinated at a lower rate (3.82 ± 2.2%) than the control, intraspecific *A. tuberosa* (22.7 ± 3.18%) (Figure 2.5). Within the stratified seed treatment, *A. hirtella* hybrids and *A. purpurascens* hybrids had similar germination rates (75.94 ± 3.7% and 68.88 ± 4.1%) compared to *A. tuberosa* (71.37 ± 5.6%), while *A. speciosa* hybrid seed and *A. syriaca* hybrid seed had lower germination rates (50.71 ± 2.99% and 47.60 ± 4.17%) (Figure 2.5). In the ER treatment, only *A. purpurascens* (65.53 ± 5.12%) had a higher germination rate than *A. tuberosa* (17.35 ± 13.19%) (Figure 2.5). Overall, the DS germination method was the least successful, followed by ER, with the most effective germination method being stratification (Figure 2.5). As all species used in successful hybridizations have native ranges from zones 3-8, with large concentrations of the population in northern climates having a distinct (dormant) winter season, a necessity for stratification is understandable.

Conclusion

This study documents the first controlled interspecific hybridizations between *A. tuberosa* and *A. hirtella, A. purpurascens, A. speciosa,* and *A. syriaca*. Previous manuscripts predominantly

reported naturally occurring hybrids or intraspecific hybrids. Development of an effective pollination method increased pollination success from 2% to 11% and increased the efficiency and effectiveness of crosses. Of seven species selected to be pollen donors, 7,000 crosses were made, which yielded over 250 viable hybrid pods, maturing in 93.38 – 126.64 days. *A. speciosa* hybrid pods matured the quickest, while *A. purpurascens* and *A. hirtella* hybrid pods took the longest. Of the 15,000 seeds collected from all hybridizations, approximately 5,500 genotypes successfully germinated. Three different germination techniques were assessed; direct seeding, stratification, and embryo rescue. It was determined that cold-moist stratification is necessary to achieve maximum germination. While embryo rescue was the second most effective method of germination, cost could be a barrier to its use in commercial plant breeding efforts. The goal of these studies was to determine if interspecific hybridizations were possible, and if they were, what would be the most efficient method to produce viable germplasm for breeding purposes. It was found that interspecific hybridization is possible, and that progeny could successfully germinate utilizing stratification techniques identified as part of this study. These findings provide a starting point for future breeding efforts of *Asclepias* and showcase the potential of introducing novel or improved cultivars of *Asclepias* to the commercial market.

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Table 2.1: *Asclepias* species used with selected phenotypic traits of interest to commercial producers.

Species	Height (cm)	Soil [z]	Flower color	Bloom[y]	Fragrant	Branching	Seed source [w]
A. tuberosa [x]	30-100	Dry	Yellow/Orange	Medium	No	Yes	WCGa
A. curassavica	60-91	Medium	Yellow/Red	Long	No	None	Ball
A. fascicularis	30-90	Dry	White/Pink	Long	Yes	Some	USDA
A. hirtella	90-120	Wet	Green	Medium	No	None	EF, PMN
A. incarnata	120-150	Wet	Pink/Mauve	Long	Yes	Some	EF, PMN, USDA
A. purpurascens	60-90	Dry	Purple/Pink	Long	Yes	None	EF, WC
A. speciosa	60-180	Dry	White/Pink	Long	Yes	None	PMN, USDA
A. syriaca	120-180	Dry	Pink	Short	Yes	None	PMN, USDA

[z] Soil represents arbitrary soil moisture preference based on textbooks.

[y] Bloom length (Bloom) is not given in numerical length as information is not available for all species. Generally, flowering continues for 2-3 months, yet flowering longevity and flower viability is not documented in all species.

[x] *A. tuberosa* represents both the maternal parent of all crosses in addition to the species used to develop the optimal pollination method.

[w] WCGa: wild collected from Oglethorpe County, GA, Ball: Ball horticulture Co., Chicago IL, USDA: United States Department of Agriculture, Columbus, OH and Parlier, Ca germplasm centers. National germplasm centers (GRIN) in CA, Wyoming, Ohio, and Iowa. EF: Everwilde Farms, PMN: Prairie Moon Nursery, WC: wild collected in Idiantown and Lebanon County, PA.

Table 2.2: Results from three pollination methods tested to determine seed set optimization for future hybrid crosses in intraspecific *A. tuberosa* crosses; traditional pollinations, solution, and inverted pollinia pollination. Solution pollination occurred at four different concentrations of pollinia (p) to one mL of 30% sucrose solution.

Method	Crosses (n)	Pods (n)	Percent Success
Traditional	570	13	2.28 ± 0.84 **b**
Solution 200p: 1mL	185	0	0 **b**
Solution 250p: 1mL	113	0	0 **b**
Solution 350p: 1mL	915	0	0 **b**
Solution 450p: 1mL	95	0	0 **b**
Inverted	401	47	11.72 ± 1.00 **a** [z]

[z] Letters by mean represent significant difference based upon Tukey's HSD at $P \leq 0.0001$.

Table 2.3: Successful seed pod formation for four *Asclepias* species hybridized with *A. tuberosa* and their corresponding success rates. The traditional pollination method used on *A. tuberosa* is included (and documented) as the control.

Pollen donor (father) [z]	**Crosses (n)**	**Pods (n)**	**Percent Success** [y]
A. tuberosa (Control)	570	13	2.28 ± 0.96 **b**
A. tuberosa (Inverted)	401	47	11.72 ± 1.14 **a**
A. hirtella	1159	58	5.00 ± 0.67 **b**
A. purpurascens	1142	56	4.90 ± 0.68 **b**
A. speciosa	1071	91	8.50 ± 0.70 **a**
A. syriaca	1294	48	3.71 ± 0.64 **b**

[z] All crosses apart from *A. tuberosa* (Control) were made using the inverted pollination method.

[y] Letters by means represent groupings based upon Tukey's HSD at $P \leq 0.001$.

Table 2.4. A comparison of the average length of time to harvest (in days after pollination) among different hybrid crosses. *A. tuberosa* intraspecific hybrid means served as the control. Maternal parent for all hybrid crosses was *A. tuberosa*.

Pollen donor	Days to pod harvest (av.) [z]	Average pod length (cm.) [y]	Seeds per pod (av.) [z]
A. tuberosa	110.32 ± 3.87 **b**	10.69 ± 0.28 **ab**	50.67 ± 3.13 **ab**
A. hirtella	126.64 ± 3.52 **a**	11.12 ± 0.26 **a**	61.33 ± 2.94 **a**
A. purpurascens	124.64 ± 3.55 **a**	10.15 ± 0.27 **ab**	32.93 ± 2.99 **c**
A. speciosa	93.38 ± 2.78 **c**	9.99 ± 0.21 **b**	49.60 ± 2.34 **b**
A. syriaca	114.14 ± 3.84 **ab**	9.92 ± 0.29 **b**	45.65 ± 3.23 **b**

[z] Letters by means represent similarity (within columns) based upon Tukey's HSD at $P \leq 0.001$.

[y] Letters by means represent similarity (within columns) based upon Tukey's HSD at $P \leq 0.004$.

Figure 2.1. Species used to develop interspecific hybrid populations. (A.) *A. tuberosa* (maternal parent) (B.) *A. curassavica*, (C.) *A. fascicularis*, (D.) *A. incarnata*, (E.) *A. hirtella*, (F.) *A. purpurascens*, (G.) *A. syriaca*, and (H.) *A. speciosa.*

Figure 2.2. Depicted using the largest pollen parent *A. speciosa*, the traditional pollination method involves removing pollinia packets from inside the gynostegium, as outlined in (A). Individual pollinium are rotated 90 degrees from how they sit in the gynostegium and inserted parallel into the stigmatic slit to initiate pollination and fertilization (B). Where the pollinia rests once inserted in relation to the pistil located in the gynostegium is seen in (C).

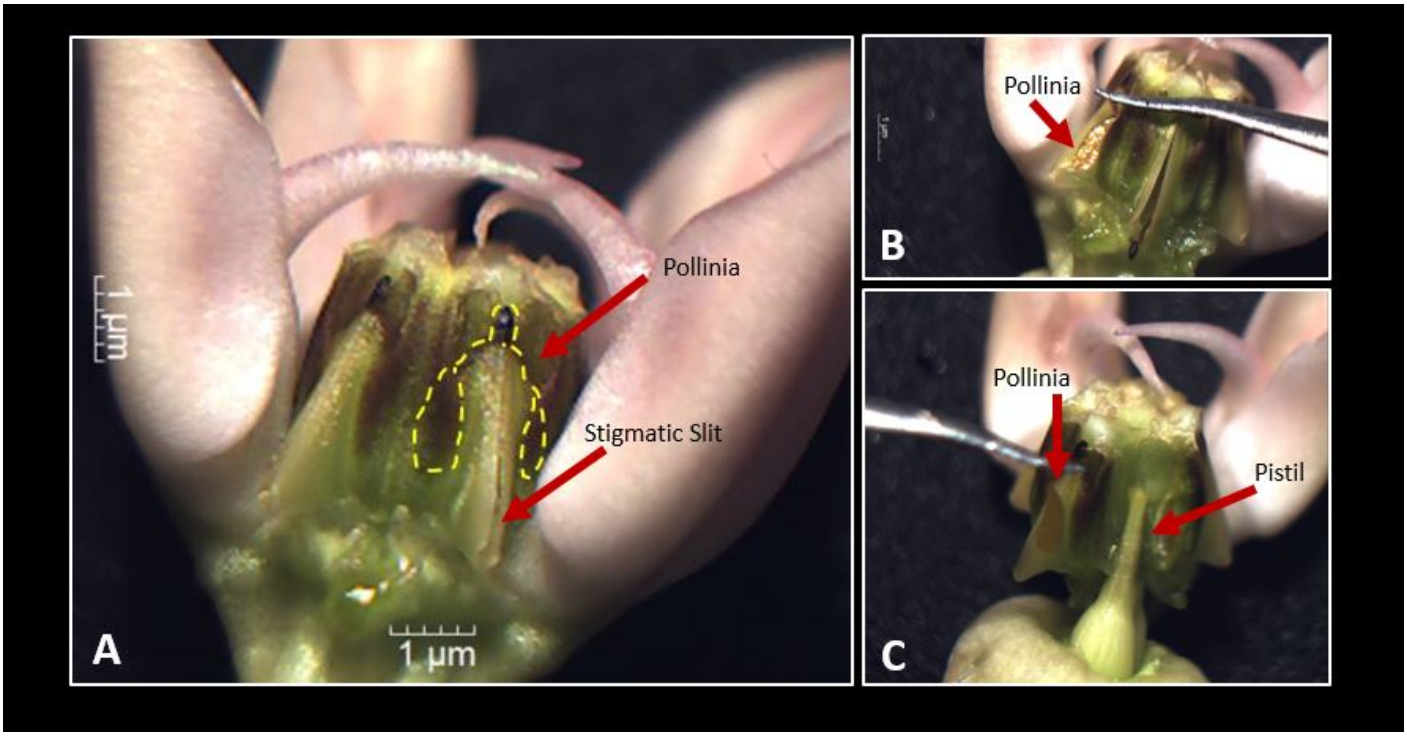


Figure 2.3. Three methods of pollination were trialed; traditional, solution-based, and inverted. The traditional method (A), where individual pollinium is rotated 90 degrees and inserted parallel into the stigmatic slit to initiate pollination and fertilization. Solution-based pollination (B) with differing numbers of pollinia were removed from paternal flowers, crushed in 1 mL of a 30% sucrose solution, and dispensed inside the stigmatic slits until solution exuded from the stigmatic slit. The inverted pollination method (C) is where individual pollinium were rotated 90 degrees and inserted perpendicular to the stigmatic slit to initiate pollination and fertilization.

Figure 2.4. The inverted pollination method involves removing pollinia packets from inside the gynostegium, as outlined in (A). The individual pollinium is rotated 90 degrees from how it sits in the gynostegium and inserted perpendicular to the stigmatic slit to initiate pollination and fertilization (B). Where the pollinia rests once inserted in relation to the pistil in the gynostegium is seen in (C).

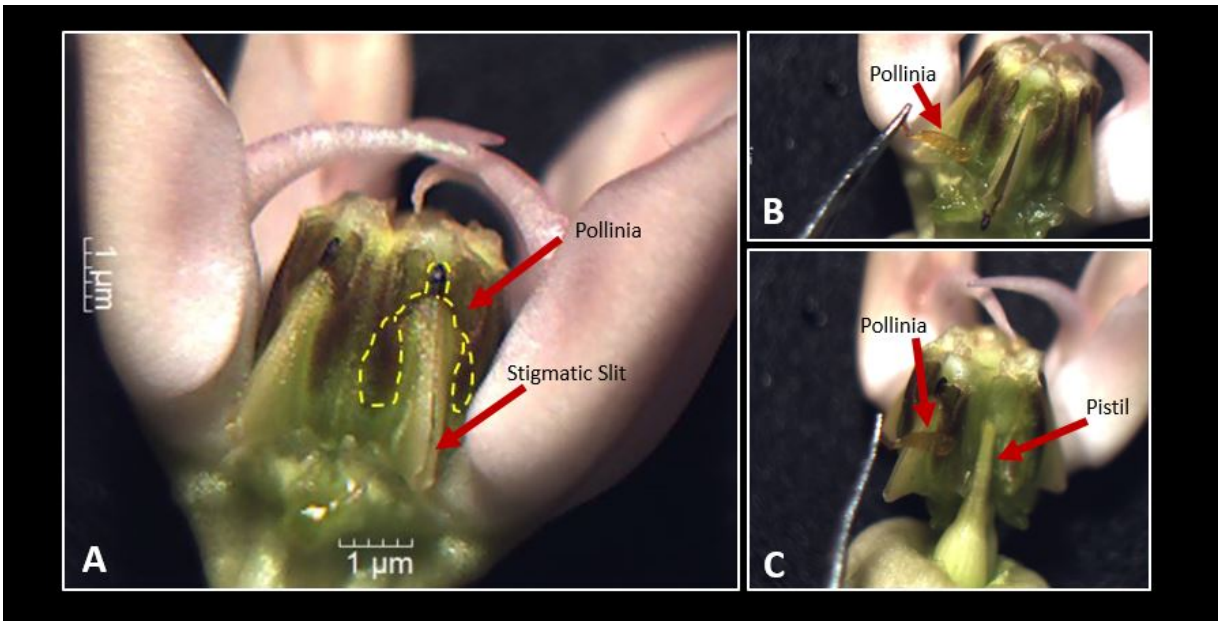
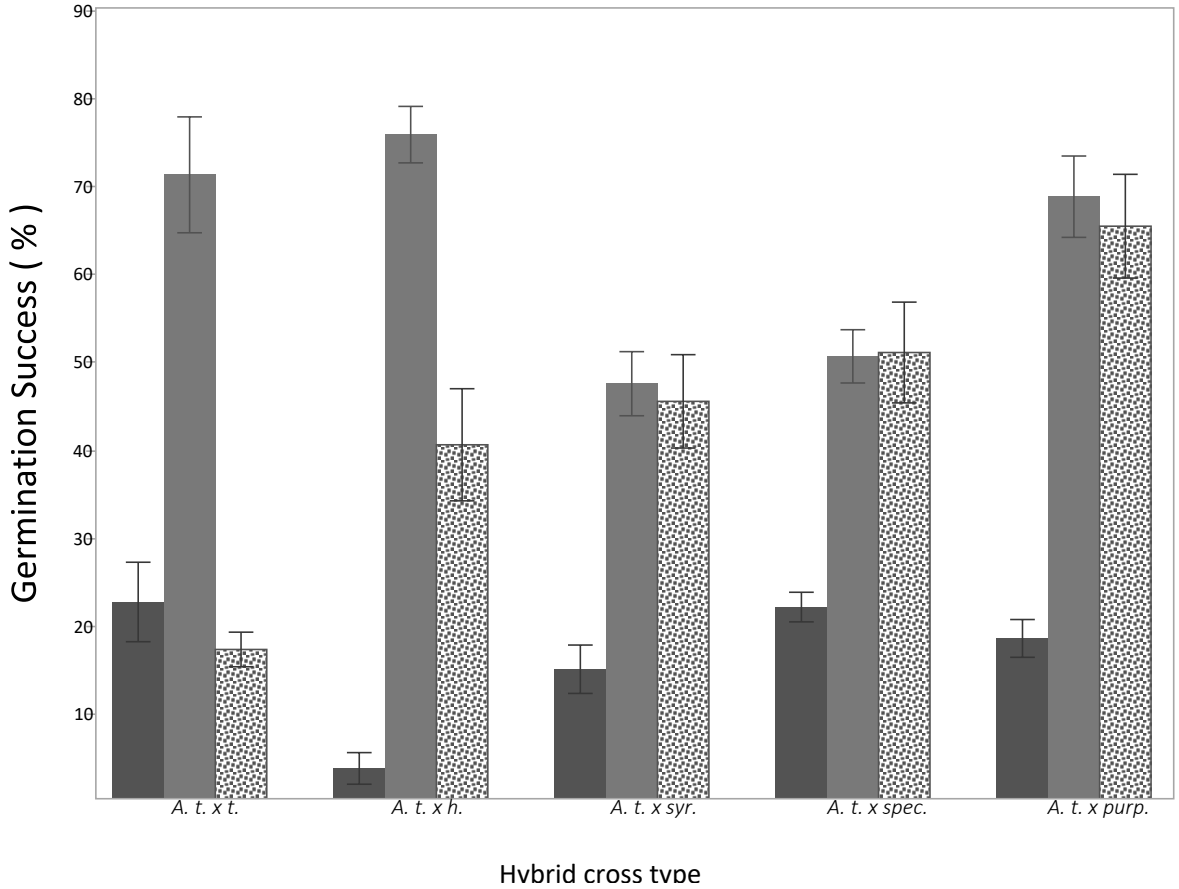


Figure 2.5. Comparison of three methods of germination based on successful pod set among *Asclepias* hybrid populations with varying paternal parents. Direct seeded (DS) (dark gray), stratified (gray), and embryo rescue (ER) (speckled). Bars represent means with error bars displaying one standard error from the mean at $P < 0.001$.



Each error bar is constructed using one standard error from the mean.

CHAPTER 3

Development of a Vegetative Propagation Protocol for *Asclepias tuberosa*[2]

[2] Lewis, M.E., Chappell, M., Thomas, P., Zhang, D., Greyvenstein, O. 2020. *Native Plants Journal*, 21(1): 27-34.

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Development of a Vegetative Propagation Protocol for *Asclepias tuberosa*

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Introduction

Commercial production of ornamental herbaceous perennial crops relies on three methods of plant propagation: seed, vegetative (stem) cuttings, or tissue culture. Of these methods, vegetative propagation ensures the preservation of a particular genotype (cultivar) over many production cycles. Vegetative propagation also affords growers a shorter production cycle and more uniform crop growth compared to seed production (Ecker and Barzilay 1993). A majority of clonal propagules are derived from cuttings due to minimal infrastructure required, compared to tissue culture, to achieve a high rate of success once a protocol is available for the species and/or cultivar.

Milkweed (*Asclepias* L.), a member of the Apocynaceae family, contains 108 species that are found exclusively in North America (Woodson 1954). The genus is recognized as an important pollinator taxa to many butterfly species, as well as forage source for butterfly larvae, particularly the monarch butterfly (*Danaus plexippus*) (Hutchings 1923; Brower 1969). Many *Asclepias* spp. also possess landscape value, having extended flowering periods and considerable drought tolerance relative to other summer-flowering herbaceous perennials.

There are two avenues whereby *Asclepias* spp. undergo reproduction in the wild; seed and rhizomatous tubers (Luna and Dumroese 2013). Propagation via seed does not allow for maintenance of cultivars, as seedling embryos undergo genetic recombination. Furthermore, many *Asclepias* spp., such as common milkweed (*Asclepias syriaca* L.), require an extended seed dormancy period (of a year or more) combined with cold stratification before a germination rate of 50% or greater can be achieved (Groh 1943; Baskin and Baskin 1977). Such requirements, in addition to seedling phenotypic variability and uneven germination timing, have likely contributed in limited commercial production of *Asclepias* spp.

Much of what is known about *Asclepias* biology and propagation is based on studies of *Asclepias syriaca* (Bhowmik and Bandeen 1976) in natural environments or other studies with vague or incomplete methodology. Propagation protocols have been developed to facilitate production of other *Asclepias* spp., including the federally threatened Mead's milkweed (*Asclepias meadii* Torr.) (Bowles and others 1991; Bhowmik and Bandeen 1970; Bhowmik and Bandeen 1976) and *Asclepias syriaca* (Luna and Dumroese 2013; Bandeen and Bhowmilk 1973); yet these protocols focus on tuber and seed propagation. Of the few detailed studies that have been published on vegetative propagation protocols for *Asclepias*, development of optimum environmental conditions to initiate callus and root development during propagation were the focal areas (Ecker and Barzilay 1993). Across species, *Asclepias* propagules were found to root and achieve optimal growth in well-drained soils (to mitigate pathogen pressure) and when exposed to 15-17 hours of daylength with temperatures ranging from 16-25°C (60.8-77.0°F) night/day (Ecker and Barzilay 1993; Luna and Dumroese 2013).

Vegetative propagation has historically been fostered by employing various (synthetic) auxin hormones to expedite the formation of callus and root initials that improve rooting

uniformity. A study by Bowles and others (1991) treated stem cuttings of two *Asclepias* spp. with 1,000 ppm and 4,000 ppm concentrations of Indole-3-butyric acid (IBA). Both treatments resulted in approximately 40% rooting success (Bowles and others 1991), an unacceptable rate for commercial production. The time at which cuttings are taken can impact cutting survivability. Phillips (1985) indicated the best time to collect *Asclepias* spp. stem cuttings was 3-5 weeks prior to flower initiation, or 3-4 weeks after dormancy break.

Asclepias tuberosa L. was employed as the model species in this study as it is the most widely produced species in commercial floriculture. An optimized vegetative propagation protocol would allow commercial growers to increase the production of *A. tuberosa*. A study conducted by Bowles and others (1991) failed to develop a propagation protocol for *A. tuberosa* when utilizing IBA concentrations at or below 4,000 ppm. This study hypothesized that stronger concentrations of rooting compounds would lead to increased plant regeneration and greater rooting success.

Materials and Methods

Container stock plant production. Seed was initially collected in 2015 from a single maternal parent randomly selected in a naturally occurring population in Oglethorpe Co., GA (33.875460° N, -83.211567° W) and germinated in July 2016. From these seedlings, one hundred *A. tuberosa* stock plants were generated as rooted cuttings in July 2017 and transplanted into 1-gallon (3.79L) containers (Classic 400, Nursery Supply, Agawam, MA) in September 2017. Plants were maintained and overwintered outdoors at the University of Georgia Athens campus (33.9480° N, 83.3773° W; USDA Zone 8a) on an outdoor gravel pad. On March 3, 2018, plants were transferred to a climate-controlled chamber held at 2°C (35.6°F). After 13 weeks in cold storage,

plants were moved into a controlled environment greenhouse with temperature set to 24-29°C (75.2-84.2°F) night/day with 14-hour daylength. These 1-gallon (3.79L) plants were used as the source of cuttings harvested from mature stock plants. Six weeks after stock plants had broken dormancy (June 18, 2018), yet prior to floral initiation, vegetative cuttings were harvested.

Seedling stock plant production. Seeds were collected from a controlled one-way cross (of two genotypes) of *A. tuberosa.* The parent plants originated from the same wild-collected population in Oglethorpe County, GA used to generate stock plants from which mature cuttings were harvested. This was done to minimize confounding results that could be the result of genotype by environment interaction, although we do recognize it is impossible to completely mitigate these interactions when working with a seedling population that has undergone genetic recombination. Immediately after harvesting, seed were cold stratified for 30 days at 2°C (35.6°F), and planted into seedling trays (STI-804, T.O. Plastics, Minneapolis, MN) on May 1, 2018. Seeds germinating between May 23 and May 30, 2018 were selected and used as stock material for cuttings from juvenile plants at 14 weeks after germination.

Experimental Design. Cuttings were harvested from plants at two different maturities, which represent a treatment effect. The first of these were propagules collected from mature containerized stock plants at six weeks after exiting dormancy (mature cutting) and the second was cuttings harvested from juvenile plants 14 weeks after germination (juvenile cutting). All cuttings were taken from leafy apical shoot tips, approximately 5 cm (2.0 in.) in length. Cuttings contained three nodes, and were propagated with 2.0 cm (0.79 in.) of stem (containing 1 node) below the soil line and 3 cm (1.18 in.) of stem (and 2 nodes) above the soil line (Figure 3.1). This study utilized 90% sand (Lowe's All-purpose Builder's Sand, Charlotte, NC) and 10% peat (Sungro Peat Moss Grower Grade Orange, Agwam, MA) as a rooting media to enhance porosity

and reduce soil borne diseases (Ecker and Barzilay 1993; Luna and Dumroese 2013). Cuttings were taken between 7:00 - 8:00 AM and immediately placed into cold water mixed with 10% hydrogen peroxide to maintain turgor and provide surface sterilization. Two rooting compounds; K-IBA (Hortus USA, New York, NY) and laboratory-grade NAA (Sigma-Aldrich, St. Louis, MO) at differing concentrations represented the second treatment effect. K-IBA was applied as a liquid dip at 0 ppm, 50 ppm basal soak of 24 hours, and quick (5-second) dips at 1,000 ppm, 3,000 ppm, 5,000 ppm, and 8,000 ppm. NAA was applied as a liquid (5-second) dip at 500 ppm, 1,000 ppm, 1,500 ppm, and 2,000 ppm. Both hormones were dissolved in deionized water. Each treatment combination was replicated four times, with 25 cuttings per replication. Immediately after all cuttings were excised from stock plants, cuttings were re-cut to the appropriate length, treated with hormone, and struck in 804 inserts each having 32-cells (BWI, Greer SC) held inside 1020 greenhouse trays (BWI, Greer SC) (Figure 3.2). Once cuttings were struck in 32-cell trays, replications were placed in a randomized complete block design on a mist bench covered with 60% shade cloth. Misting source for all treatments was municipal water (pH 6.8, alkalinity 9 mg/l) applied via NetaFim Vibromist nozzles (NetaFim USA, Fresno, CA) every six min for 10 sec. The misting system also delivered 20-10-20 (Harrell's, Lakeland, FL) + STEM (Peters, Dublin, OH) fertigation mixture to the cuttings at 50 ppm N every 5 days for a 2-hour window for the duration of the study. Misting was only applied from sunrise to sunset. While under mist, greenhouse conditions were kept between 24-29°C (75.2-84.2°F) day/night with 13-14 hours of natural daylength. To prevent root pathogens a soil drench fungicide rotation of Subdue MAXX (mefenoxam, Syngenta Crop Production, Greensboro, NC; FRAC Code 4) and Heritage (azoxystrobin, Syngenta Crop Production, Greensboro, NC; FRAC Code 11) was used in rotation and applied every 14 days.

Data collection and statistical analysis. Destructive harvest occurred 8 weeks after cuttings were struck (Figure 3.3) and data immediately collected. Measurements included rooting (success/fail), plant height from the soil line (cm), number of roots, and length of the longest root (cm). Statistical analysis was performed using JMP (v. 13.0, SAS Institute, Inc., Cary, NC). Data from juvenile and mature cuttings were analyzed separately, to determine treatment significance between hormones and among concentrations. One-way Analysis of Variance (ANOVA) and separation of treatment means with Tukey's HSD were used to analyze plant height, root number, and root length. Rooting success data from juvenile and mature cuttings were analyzed together using one-way Analysis of Variance (ANOVA) analyzing maturity and concentration as treatment effects. Separation of treatment means with Tukey's HSD and pairwise comparisons by hormone type were used to determine significance level. Only data showing significant difference among treatment combinations were reported.

Results and Discussion

Methodology for this study was based on commercially accepted protocols, including the use of intermittent misting and exogenous (K-IBA and NAA) hormone application. Informally surveying 10 floriculture growers indicated that the maximum time window allotted in the propagation of a herbaceous crop was 6-8 weeks and all growers perform propagation under 50-70% shade with intermittent mist (data not shown). The use of intermittent misting in *A. tuberosa* was also consistent with results from Phillips (1985) and Grabowski (1996) that stressed the necessity of high humidity levels throughout the propagation cycle of *Asclepias* spp. to maximize rooting success.

Results of studies by Phillips (1985) and Grabowski (1996) indicated cuttings had increased success when taken before floral initiation. Preliminary experimentation as part of this study indicated that 6 weeks after breaking dormancy was the optimal time to harvest cuttings from mature stock plants prior to floral initiation (data not shown). Successful propagation using juvenile seedlings of *A. tuberosa*, has yet to be reported, yet in many perennial taxa, cuttings from juvenile tissue afforded higher success (Borchert 1975). Data from the two trials utilizing cuttings from stock plants of differing maturities was combined to assess if age of the propagule had any influence on rooting success. Stock plant age affected rooting success, regardless of the hormone applied (Figures 3.3 and 3.4; $P < 0.0001$). Propagules harvested from mature stock plants treated with K-IBA had greater overall success (85-100%) than those collected from juvenile seedlings (25-60%) and treated with K-IBA (Figure 3.4; $P < 0.001$). Our results contradict those of Bowles and others (1991) who found no difference in success rate when comparing stem cuttings from mature *A. syriaca* and juvenile green comet milkweed (*A. viridiflora* Raf.) treated with two IBA concentrations. Bowels and others (1991) utilized IBA dissolved in alcohol solution (1,000 ppm and 4,000 ppm). Alcohol based solutions can damage plant tissue and therefore reduce success. The current study employed commercial production protocols using a larger range of hormone concentrations as well as K-IBA dissolved in deionized water, not alcohol. This single factor could explain contradictory results between Bowels and others (1991) results and those of this study.

Trends observed among different NAA concentrations paralleled those observed in K-IBA treatments. Success was enhanced in cuttings obtained from mature stock (80-100%) when compared to juvenile cuttings. The exception was juvenile cuttings treated with 1,500 ppm NAA (85%) (Figure 3.5; $P < 0.001$). Differences within cutting age groups to differing auxin treatment

was not surprising. NAA has a similar mode of uptake, translocation, and metabolic activity as K-IBA, despite having a lower binding affinity with auxin receptors than K-IBA and a longer (yet slower response) efficacy period (Blythe and others 2004; Pacurar and others 2014). For this reason, many commercial operations use a combination of K-IBA and NAA to maximize the period of uptake potential. Propagules obtained from mature plants also had greater stem diameter and larger leaves compared to seedling-derived propagules (data not shown). As a result, it is assumed these propagules had greater carbohydrate reserves and photosynthetic capacity compared to cuttings obtained from seedlings. Both stored and cutting-produced carbohydrates have been shown to directly influence rooting success of leafy stem cuttings in other taxa (Leakey 2004) and was likely the cause for reduced success in propagules obtained from juvenile seedlings in the present study.

Despite observing differences in rooting success rates of mature versus juvenile cuttings, hormone concentration surprisingly had little effect on rooting success. Compared to the control treatment, there was no difference in rooting success of propagules treated with varying hormone concentrations that originated from mature stock plants ($P < 0.001$) (Figures 3.4 and 3.5). This trend was also true for juvenile cuttings ($P < 0.001$), with the exception of the NAA at 1,500 ppm treatment, that had a success rate of 85% compared to the control at 55% ($P < 0.001$) (Figure 3.5). Studies on a number of taxa have shown that exogenous auxins increase the speed of adventitious root formation (Leakey 2004) as well as rooting uniformity (De Klerk and others 1999; Ramtin and others 2011); yet that did not seem to be the case for *A. tuberosa*. While the use of rooting hormones is common, there are many herbaceous taxa that have been documented as not requiring rooting compounds to achieve commercially acceptable levels of success (Ford and others 2002; Hawkins and others 2013).

Greater root number, root length, and final plant height are typically linked to vegetative propagule quality (Hawkins and others 2013). When assessing juvenile cuttings, no differences were observed in the mean number of roots ($P = 0.8$), root length ($P = 0.1$), or plant height ($P = 0.1$) regardless of hormone or concentration on juvenile cuttings (data not shown). Additionally, mean values of number of roots, root length, and plant height were universally lower than the mature cuttings (data not shown). When assessing mature cuttings, no differences were observed in mean root number ($P = 0.02$) or root length ($P = 0.05$) regardless of hormone type (Table 3.1). K-IBA at 3,000 ppm produced the greatest number of roots (11.94 cm ± 1.35; 4.70 in ± 0.53), but that value did not differ from the control (9.19 cm ± 1.32; 3.61 in ± 0.52) (Table 3.1). These results concur with Davies and others (2011) that generalized *A. tuberosa* is an easy to root species. Differences were observed in mean plant height, with 1,000 ppm K-IBA (6.20 cm ± 0.41; 2.44 in ± 0.16 in) and 3,000 ppm K-IBA (7.94 cm ± 0.42; 3.12 in ± 0.16) having the greatest observed plant height six weeks after striking (Table 3.1; $P < 0.001$). Similarly, Parađiković and others (2013) reported that the application of K-IBA increased plant height in kitchen sage (*Salvia officinalis* L.) and rosemary (*Rosemarinus officinalis* L.) despite not enhancing root elongation. Considering that K-IBA at 1,000 ppm and 3,000 ppm only positively influenced plant height in this study (not root number or length), these concentrations would only be recommended to propagators wishing to produce taller liners (Table 3.1). When assessing all three growth parameters, it should be noted that NAA did not outperform the control and would thus not be recommended for the propagation of *A. tuberosa* from mature stock plants (Table 3.1). Equivalent cutting responses in NAA-treated and non-treated cuttings have been reported in a variety of herbaceous and woody taxa by Salas and others (2013), Tchinda and others (2013), Yan and others (2017), and Nagalakshmi and others (2018).

This report is a novel documentation of a detailed (and commercially viable) cutting propagation protocol for *A. tuberosa*. To date, several research reports have addressed cutting propagation protocols for an *Asclepias* spp., yet no study has provided a detailed methodology for successful regeneration at a commercially viable level. The goal of our study was to examine two water-based rooting hormones (K-IBA and NAA) applied to both mature (2-year old) and juvenile (seedling) stem cuttings of *A. tuberosa*. Results indicated that cuttings taken from mature plants resulted in commercially acceptable rooting success. This was likely due to greater carbohydrate reserves and photosynthetic ability of cuttings obtained from mature stock plants. It was also observed that regardless of hormone used (K-IBA or NAA) or concentrations applied, there was no overall improvement in cutting survival or root growth parameters (root number or length) compared to the control with no hormone applied. The only growth parameter positively influenced by an exogenous auxin application was cutting height, which was greatest at 1,000 or 3,000 ppm K-IBA. Translating our findings into practical application, results of this study indicated that no rooting hormone is needed when sourcing cuttings from mature stock plants that are taken between emergence from dormancy and floral initiation. These findings should afford commercial and conservation-based producers of *A. tuberosa* a more rigid, science-based, simplified, and cost-effective propagation protocol. As Asclepias contains 108 recognized species, this study should also afford propagators and researchers of *Asclepias* spp. a platform to base future studies upon. With a successful vegetative propagation protocol established, future propagation studies of *A. tuberosa* that focus on development of tissue culture protocols could further enhance commercial production of elite genotypes and enhance conservation efforts.

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Table 3.1. Effect of hormone type (K-IBA or NAA) and concentration on the mean number of roots, root length, and plant height at harvest (8 wks after striking).

	Mature Cuttings		
TREATMENT	**Mean Number of**	**Mean Root**	**Mean Plant**
CONTROL	9.19±1.32 ab	9.43±0.82 a	3.71±0.37 c
K-IBA-1000 PPM	7.5±1.35 ab	8.5±0.89 a	6.2±0.41 ab
K-IBA-3000 PPM	**11.94±1.35 a**	8.34±0.91 a	**7.94±0.42 a**
K-IBA-5000 PPM	11.1±1.35 ab	7.79±0.86 a	3.46±0.39 c
K-IBA-8000 PPM	9.52±1.35 ab	8.15±0.86 a	4.4±0.39 bc
K-IBA-50 PPM	----	---	----
NAA-500 PPM	5.75±1.35 b	8.18±0.84 a	4.74±0.38 bc
NAA-1000 PPM	8.52±1.35 ab	5.75±0.91 a	3.28±0.42 c
NAA-1500 PPM	9.87±1.35 ab	6.5±0.94 a	4.11±0.43 c
NAA-2000 PPM	10.35±1.35 ab	8.11±0.84 a	4.23±0.38 c
SIGNIFICANCE[A]	0.0212	0.05	<0.001

Notes: Results depicted apply only to propagules collected from mature (2-y-old) stock plants collected and struck 6 wk after breaking dormancy yet prior to floral initiation. All values are reported in cm: 1 cm = 0.4 in. Standard error of the means are reported in addition to means. K-IBA = indole-3-butyric acid; NAA = 1-naphthaleneacetic acid.

[A] Significance of treatment effects (P > F; ns = not significant at the 0.05 level). Means in same treatment group (columns) not sharing a letter are significantly different at P < 0.05 level based on adjusted P values using Tukey HSD and a standard one-way ANOVA

Figure 3.1. Photo of a propagule at the midpoint of the study (4 weeks after striking). Cuttings were initially three nodes in length, and frequently resumed growth via axillary bud break or re-initiating growth from apical meristem while in the propagation environment.

Figure 3.2. Photo of cutting block taken 3 weeks after cuttings were struck.

Figure 3.3. Photo of cutting block taken 8 weeks after cuttings were struck, at the time destructive harvesting occurred.

Figure 3.4. Effect of cutting maturity on rooting success. With 25 successful cuttings possible per rep, comparisons among hormone concentrations and maturity are displayed. Lighter bars (left) indicate all juvenile cuttings at differing K-IBA (Potassium salt of Indole-3-butyric acid) concentrations. Dark bars (right) indicate mature cuttings at differing K-IBA concentrations. Cutting age is defined as propagules harvested from 2 year old stock plants 6 weeks after breaking dormancy (M; mature) and cuttings harvested from seedlings 14-weeks after germination (J; juvenile). Bars represent means with standard error based on standard one-way ANOVA. Treatment groups (columns) not sharing a letter are significantly different at P=0.05 level based on adjusted p-values using Tukey HSD.

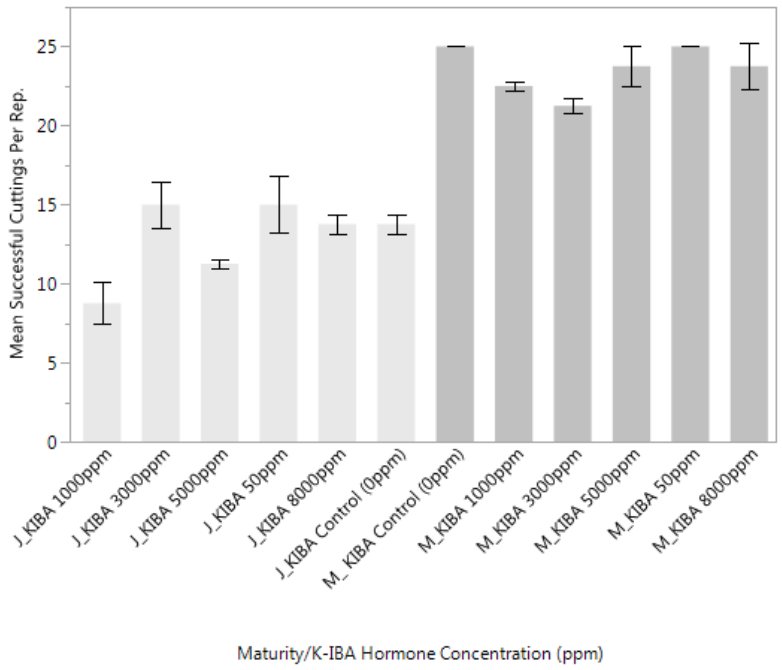
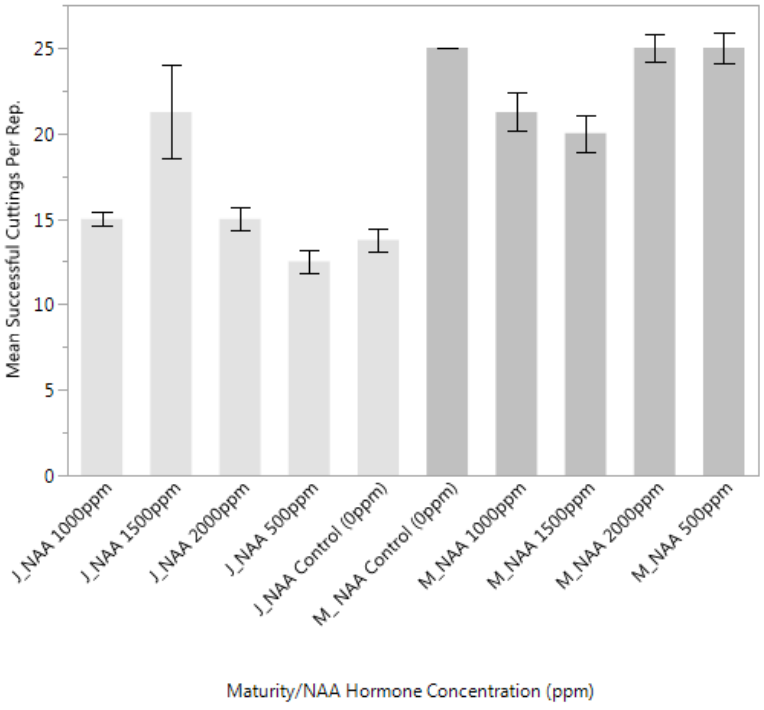


Figure 3.5. Effect of cutting maturity on rooting success. With 25 successful cuttings possible per rep, comparisons among hormone concentrations and maturity are displayed. Lighter bars (left) indicate all juvenile cuttings at differing NAA (laboratory grade, 1-Naphthaleneacetic acid) concentrations. Dark bars (right) indicate mature cuttings at differing NAA concentrations. Cutting age is defined as propagules harvested from 2 year old stock plants 6 weeks after breaking dormancy (M; mature) and cuttings harvested from seedlings 14-weeks after germination (J; juvenile). Bars represent means with standard error based on standard one-way ANOVA. Treatment groups (columns) not sharing a letter are significantly different at P=0.05 level based on adjusted p-values using Tukey HSD.



CHAPTER 4

Development of an Embryo Rescue Protocol in Butterfly Weed[3]

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Development of an Embryo Rescue Protocol for Butterfly Weed

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Introduction

Embryo Rescue, or the excision and culturing of immature zygotic embryos from developing seeds, is conducted under aseptic conditions to obtain viable and pathogen-free plantlets (Bhojwani and Razdan, 1986; Morel, 1960). The technique was first documented by Charles Bonnet in the late 18th century and has been commercially practiced for more than half a century (Bridgen, 1994). Further research by Hannig (1904) refined the technique and laid the groundwork that would allow future scientists to bypass physical and/or late onset chemical seed dormancy, shorten breeding cycles, examine seed/embryo viability, and develop hybrids from previously incompatible crosses (Bridgen, 1994). In plant breeding programs, ER is particularly critical as it can circumvent seed abortion of wide crosses, resulting in retention of novel genotypes (Conger, 1981; Dunwell, 1986). Embryo rescue has also been widely used to efficiently propagate threatened and/or endangered species, as it is noted to improve germination rates (Lakshmi et al., 2010; Stephenson and Fahey, 2004).

Butterfly weed is one of 106 species that are indigenous to North America (Stevens, 1945; Woodson, 1954). While milkweed species have many ornamental and ecologically-important traits, there is little commercial production outside of niche native plant growers. Limited commercial production is thought to result from limited seed set, as milkweed species frequently display late-term seed and pod abortion, possibly due to complex reproductive structures

(Broyles, 2002; Kephart, 1981; Shannon and Wyatt, 1986). The predominant theory for failure of intra- and interspecific seed development in milkweed species is late-term embryo abortion attributed to post-fertilization rejection, where fertilization of the endosperm is successful, yet the egg (precursor to the zygote) remains unfertilized. Gametophytic and sporotphytic SI systems typically experience abortion anywhere between a few hours to a few days after pollination, but in milkweed species, late-term abortion can occur several weeks to 2 months after fertilization (Lipow and Wyatt, 2000; Seavey and Bawa, 1986; Sparrow and Pearson, 1948; Whiting, 1943). Embryo abortion is difficult to overcome, but by prematurely harvesting seeds, removing the embryo, and aseptically culturing the embryo on nutrient media, pod abortion and developmental failure could be circumvented.

There are no published ER protocols available for milkweed species. Several studies document embryogenesis protocols in milkweed species from cell cultures, leaf tissue, or nodal explants (Groet and Kidd, 1981; Kim et. al, 2004; Pramanik and Datta, 1986; Sahai et. al, 2010). Embryogenesis, compared to ER, takes longer as embryonic tissue must first be dedifferentiated, multiplied via callus formation, and then redifferentiated with the aid of plant growth regulators (PGRs) (Kim et al., 2004). Although large nutritional differences in ER protocols versus embryogenesis protocols exist, a study on embryogenesis of indian ipecac (*Tylophora indicia*) found that this species, also in the milkweed family, preferred relatively lower nutrient content compared to other taxa (Sahai et al., 2010). A half strength MS media increased explant regeneration of indian ipecac by over 20% when applied at embryo maturation, compared to other commonly used media (Sahai et al., 2010).

Embryo Rescue may be a commercially viable mechanism to overcome seed pod abortion, if the embryo is excised from the seed at a point when it has reached the autotrophic (self-sufficient) stage but prior to seed pod abortion. However, an ER protocol for butterfly weed has not been documented, so media type, days of stratification, seed/explant cleaning methods, embryo culture environmental conditions, and the effect of embryo maturity on success are unknown. The primary objectives of this project were to: (1) determine if it is possible to rescue embryos in the autotrophic stage through the development of an ER methodology; (2) assess the optimum embryo maturity to harvest seed and excise embryos for maximum growth of germinated embryos; and (3) determine if ER methods offer an improvement over seed germination rates using traditional seed germination in soilless substrates determine. We hypothesized, based on previous research conducted on another milkweed species (indian ipecac), that media with less nutrition would be superior in successful germination of autotrophic embryos. We also predicted that germination rates of rescued embryos would exceed those observed under traditional commercial settings, and embryos harvested in the heterotrophic phase of development would fail to produce viable plantlets.

Materials and Methods

Parent and Seed production. Stock plants of butterfly weed were produced from three wild-collected seed pods from a single plant (Oglethorpe County, GA) in Aug. 2017, dry stored until Jan. 2018, and cool moist stratified at 2 °C for 6 weeks. Seeds were germinated in Mar. 2018 at the University of Georgia Athens campus, College Station Greenhouse Complex (lat. 33.9480°N, long. 83.3773°W; USDA Zone 8a) in 804 inserts (T.O. Plastics, Minneapolis, MN) containing 100% perlite (Carolina Perlite Co. Inc., Gold Hill NC). Supplemental light was provided by

light-emitting diode (LED) arrays (Fluence Spyder with PhysioSpec, Fluence Technologies Inc., Austin, TX); a *PPFD* (photosynthetic photon flux density) of 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a 14-h daylength was maintained until ambient daylength reached 14-h (18 May in Athens, GA). Seedlings were transplanted into trade 1-gal containers (Classic 400, Nursery Supply, Agwam, MA) with a 75% pine bark mulch, 20% compost, and 5% perlite media mix (Foothills Compost, Grayson, GA) and grown in a glasshouse with temperatures of 24 °C (day) and 20 °C (night). Plants were topdressed with 21 g of 16N-3.5P-7.5K slow release fertilizer (Pursell, Sylacauga, AL) and hand watered daily to container capacity with municipal water. Once flowering occurred, controlled intraspecific pollinations of butterfly weed were made in the same greenhouse environment to generate seed pods. Harvested pods were taken into the lab and seeds were separated from the pod to be surfaced sterilized.

Experimental Design of Study one: Effects of Media Type on ER Success.

The first study was performed to determine optimal culture media type for the germination and regeneration of autotrophic embryos harvested at 90 DAP. Media type served as the treatment effect and included MS Basal Salt mixture (Sigma-Aldrich, St. Louis, MO), MS Basal Salt Mixture at half strength (½ MS) (Sigma-Aldrich), and WPM (Sigma-Aldrich). Protocols for preparation were followed as published in PhytoTechnology Laboratories™ technical information guide (2008) with pH adjusted to 5.7-5.9. After preparation, media were poured into either 100x15-mm petri dishes (Thermo Fisher Scientific, Waltham, MA), or sterilized cups (12FCB 12oz PET clear cup with DLKC16/24NH clear dome lid, Southeastern Paper Group INC., Atlanta, GA). Once plated and cooled, plates were stored in a dark location held at 25 ± 2 °C day/night temperatures until use. A single seed pod was harvested at 90 DAP, containing 117

seeds. Seeds were randomly divided into three equal groups of 39, to be placed on the three media types. Seed designated for each media treatment were then randomly subdivided into three replications, with 13 seeds in each replication. After randomly dividing seed from each treatment into replications, seeds were taken into the lab, surface sterilized, excised, and plated on MS, ½ MS, or WPM media filled petri dishes.

Experimental Design of Study two: Effects of Embryo Maturity on ER Success.

The second study utilized a media type from the first study (½ MS), to examine its ability to successfully germinate embryos at various DAP, whereby embryo maturity was the treatment effect. In this study, two additional embryo maturities of 60 and 30 DAP were examined, and data from 90 DAP embryos in study one were used to discern differences among embryo maturities. A single seed pod was harvested at each point of embryo maturity, containing between 72-117 seed. The pod harvested at 90 DAP contained 117 seeds, of which 39 randomly selected seeds were used for statistical analysis in this study, subdivided into three replications, with 13 seeds in each replication. The seed pod harvested at 60 DAP contained 72 seeds, divided into four replications of 18 individually plated embryos. The pod harvested at 30 DAP contained 100 seeds. To maintain some homogeneity of replication numbers, only 72 randomly selected seeds were chosen from this seed pod and divided into four replications of 18. After randomly dividing seeds from each treatment into replications, seeds were taken into the lab, surface sterilized, excised, and plated on ½ MS filled petri dishes.

Preparation of the Germination Control. To compare ER germination rates in both studies to industry standard germination rates, a single pod was harvested at maturity (natural splitting of

the pod). The mature seeds were immediately cold moist stratified in damp sand (All-purpose Builder's Sand; Lowe's, Charlotte, NC) at 2 °C for 30 d. A randomized complete block of 39 seeds randomly selected from the pod (split into three replications of 13) were germinated in in 804 inserts (T.O. Plastics) with 100% perlite (Carolina Perlite Co.) inside a greenhouse at the University of Georgia Athens campus, College Station Greenhouse Complex. Supplemental light was provided by LED arrays delivering a *PPFD* of 250 μmol·m^{-2}·s^{-1} and a 14-h daylength that was maintained until ambient daylength reached 14-h (18 May in Athens, GA). Seed trays were misted with municipal water for 10 s every 10 m with temperatures at 24 °C (day) and 20 °C (night). Germination percent was recorded 30 d after sowing.

Seed Surface Sterilization. Surface sterilization occurred in a laboratory environment. Seeds were initially rinsed under tap water for 5 min before being placed into a sterile 500-mL bottle filled with room temperature sterile water and 1 mL of dish soap (Dawn; Procter & Gamble, Cincinnati, OH). The solution of soap and water was gently shaken for 4 min, then poured out, catching the seeds in a sterilized strainer. Seeds were rinsed with reverse osmosis water for 2 to 3 min to ensure the removal of residual soap. The next steps occurred under a laminar flow hood and all equipment was sterilized. Seeds were placed into a 100-mL beaker filled with a 75% ethanol solution and agitated for 30 s. Seeds were then transferred to another 100-mL beaker filled with a 10% Roccal (Pfizer, New York City, NY) solution and stirred for 1 min. Seeds were transferred to a 500-mL beaker filled with a 1.5% bleach solution (Clorox, Oakland, CA) and agitated for 3 min. Immediately after the bleach wash, seeds were transferred to another 500-mL beaker filled with double deionized (DI) water. Seeds were agitated for 3 min and the DI washing process was repeated three times in different 500-mL beakers. At this point the seeds

were considered disinfected and could be stored in petri dishes sealed with laboratory film (Parafilm, Sigma-Aldrich) until excision.

Embryo Rescue. After surface sterilization, embryos were excised under a laminar flow hood using a sterile scalpel on sterile filter paper; a dissecting microscope was used to aid in visibility of embryos. Embryos of butterfly weed are small (0.6 cm long), so an initial lateral cut was made around the outside edge of seeds to avoid cutting into the embryo. After the initial cut the thin testa (seed coat) was peeled away from the embryo using forceps. Using either the scalpel or forceps, embryos were gently removed and placed on the pre-determined media type in 100x15-mm petri dishes (Thermo Fisher Scientific, Waltham, MA). It should be noted that even though the dividing hilum scar appears to be an easier place to make the incision, a vertical excising cut down the middle of the seed will often puncture the embryo. Immediately after plating embryos on media treatments in 100x15-mm petri dishes (Thermo Fisher Scientific), dishes were placed in a dark room for 3 d at 22 ± 2 °C day/night temperatures to break chemical dormancy. Embryos were transferred to a climate-controlled environment held at 22 ± 2 °C day/night temperatures, and 16/8-h light/ dark photoperiod with a *PPFD* of 50 μmol·m^{-2}·s^{-1} provided by cool white fluorescent tubes. Once an embryo had an actively growing radicle, embryos were re-plated to a sterilized cup (12FCB 12oz PET clear cup with DLKC16/24NH clear dome lid, Southeastern Paper Group INC., Atlanta, GA) and grown out for 100 d in the first study and 70 d in the second study. It should be noted that embryos were initially germinated in petri dishes due to laboratory space limitations but could also be germinated directly in clear PET cups to minimize labor.

Data Collection and statistical analysis. Data collection for both studies included: average longest root length (centimeters), average shoot length (centimeters), germination percentage, and number of explants successfully generated (defined as reaching a height of 7.62 cm). To prevent contamination, embryos were not removed from their sterile containers to take measurements, with the exception of the final measurement date. Media on which embryos were placed was clear, and measurements were as accurate as possible; on petri dishes root lengths were measured with a ruler from below, and in sterile cups from the side to below depending on root development. Shoot lengths were measured from the top of petri dishes or the side of sterile cups. Measurements were collected this way to reduce distortion of root and shoot lengths, obtaining the most accurate data possible without exposing developing embryos to unnecessary contamination. Root length was measured from root tip to where it joins either callus or meristem tissue. Data collection began 10 d after plating embryos and continued at 10-d intervals for 50 d in both studies. Shoot length was measured from the base of callus or meristem tissue to the uppermost meristematic tissue. Shoot length data collection in the first study began at 60 d and continued at 10-d intervals for 100 d. Shoot length data collection in the second study began at 30 d and continued at 10-d intervals for 70 d. Germination was defined as the number of seeds that successfully began radicle elongation and was reported as percentage data. Successful explant number was measured as plants alive in vitro at 100 d in the first study and 70 d in the second study. Statistical analysis for both studies was performed using JMP (version 13.0; SAS Institute, Cary, NC). Data was analyzed to determine normality and homogeneity, with one-way analysis of variance (ANOVA) and separation of treatment means using Tukey's HSD (honestly significant difference) used in both studies to analyze root length, shoot length, and successful explant number variances. Germination percentage data was log transformed prior to the one-

way ANOVA analysis to determine significance, and then back transformed for reporting. Only data showing significant differences ($P \leq 0.05$) among treatments are reported.

Results and Discussion

Study one: Effects of Media Type on ER Success.

Average root length (± SE) on ½ MS media (3.50 ± 0.51 cm), was greater when compared to MS media (1.43 ± 0.33 cm) (Fig. 4.1). WPM was similar to MS and ½ MS media, with an average root length of 1.98 ± 0.36 cm (Fig. 4.1). Differences between root lengths became significant as early as 20 d after excision and maintained the separation through day 50 (Fig. 4.1). At 100 d, MS and ½ MS had similar shoot growth, and both had greater shoot lengths (9.28 ± 1.91 cm and 14.76 ± 1.74 cm, respectively) compared to WPM media (2.75 ± 1.05 cm) (Fig. 4.2). When assessing combined root and shoot growth results, ½ MS media had both the greatest root growth (3.50 ± 0.51 cm) and shoot growth (14.76 ± 1.74 cm). Root lengths of indian ipecac reached 14.46 cm when plated on ½ MS media at a similar maturity (Sahai et al., 2010). Pendulous wax flower (*Hoya wightii*), achieved similar shoot growth (9.1 cm) in MS media at similar maturity (Lakshmi et al., 2010). Our results indicate that MS media is ideal for the promotion of root and shoot growth, with ½ MS being superior for butterfly weed.

While comparisons of growth parameters among media types are useful, to be a commercially viable ER technique, ER must be more effective when compared to the industry standard of germination in soilless substrate under greenhouse conditions. This study determined that by comparing germination percentages on MS, ½ MS, and WPM to the control (seeds germinated in perlite), ½ MS (97.43% ± 2.00%) and MS (94.88% ± 4.29%) had higher germination rates

compared to WPM (82.05% ± 3.1%) or perlite (79.5% ± 3.09%) (Table 4.1). Commercially, high germination rates are important, but survivability of plantlets is equally critical. When comparing the percentage of plants that survived 100 d (harvest date), ½ MS was superior (89.75% ± 5.50%) to MS (51.00% ± 9.74%) and WPM (56.41% ± 4.26%) (Table 4.1). Similar results have been documented in mountain laurel (*Kalmia latifolia*) (Li and Zhang, 2018), rhododentron (*Rhododendron* sp.) (Eeckhaut et al., 2007), rose (*Rosa* cvs.) (Marchant et al., 1993), and Japanese holly (*Ilex crenata*) (Yang et al., 2015), whereby ER significantly increased germination rates compared to seed germination in soilless substrates. When assessing results including germination rate, root growth, and shoot growth, ½ MS was the superior media for germination and growth of 90 DAP butterfly weed embryos.

Study two: Effects of Embryo Maturity on ER Success

Literature frequently describes ER as occurring a few days to a few weeks after pollination (Sharma et al., 1996; George et al., 2008). However, in study two the objective was to determine the age whereby embryos were incapable of either germination or growth (heterotrophic developmental stage). The methodology employed in this study was developed previously for ER of orchids. Orchids have similarly complex reproductive systems to milkweeds and share extremely low seed germination rates (Tsuchiya-Itaru, 1954; Wyatt and Broyles, 1994). Orchid pods require 112-224 d to mature, but prematurely harvesting pods at 100-150 d after fertilization for ER drastically improves germination success rates (Knudson, 1922). The average amount of time a butterfly weed pod takes to reach maturity after pollination is 118.0 ± 4.2 d (unpublished data). Therefore, when testing efficacy of the ER protocol developed in study one, the determination was made to perform ER on seeds at 90, 60, and 30 DAP. The goal was to

determine if 30 DAP and/or 60 DAP embryos could be germinated and grown out without the aid of PGRs (in autotrophic growth phase). One significant change made to data collection in the second study was to begin collecting shoot measurements at day 30 (rather than day 60) to better capture shoot growth curves that are more in-line with commercial needs. Typically, tissue cultured plant material is removed from sterile conditions and grown to a maximum of 7.6-12.7 cm before being sold and/or transplanted (Mulhern, 2010). In study one, it was not anticipated that shoot growth would occur as quickly as it did. Root length measurements remained the same. Overlap in measurement dates from study one allowed embryo growth comparisons from embryos of different ages (90, 60, and 30 DAP), affording a more complete picture of growth and development.

Across the three embryo maturities, root length of embryos rescued 60 DAP was longer (6.20 ± 0.36 cm) than those harvested at 90 DAP (3.50 ± 0.51 cm) or 30 DAP (0.16 ± 0.02 cm) (Fig. 4.3). Embryos harvested at 90 DAP had longer root lengths (3.50 ± 0.51 cm) than the 30 DAP (0.16 ± 0.02 cm) treatment (Fig. 4.3). Embryos harvested 60 DAP had greater shoot length (8.16 ± 0.44 cm) than those harvested at 90 DAP (5.38 ± 0.69 cm) or 30 DAP (0.33 ± 0.02 cm) (Fig. 4.4). Root length differences became significant as early as day 10 after excision and maintained the separation through duration of the study (Fig. 4.3). As observed in study one, germination rates for all ER DAP treatments on ½ MS media were greater (30 DAP – 100% ± 0.00%, 60 DAP – 100% ± 0.00%, and 90 DAP – 97.43% ± 4.47%) than germination of mature seed on perlite (control – 72.38% ± 6.91%) (Table 4.2). Survival rates were similar across all treatments (30 DAP – 100% ± 0.00%, 60 DAP – 95.83% ± 1.73%, and 90 DAP – 89.74% ± 5.50%), but survivability does not equate to successful embryo development (Table 4.2, Fig. 4.4). For example, the 30 DAP treatment had the highest survival rate (100%), but the smallest root (0.16

± 0.02 cm) and shoot length (0.33 ± 0.02 cm) (Figs. 4.3 and 4.4, Table 4.2); making it the least successful treatment. Overall, embryos harvested 60 DAP yielded the greatest root (6.20 ± 0.36 cm) and shoot growth (8.16 ± 0.44 cm) compared to other treatments, with comparable germination and embryo survival rates (Fig. 4.3 and 4.4, Table 4.2).

These results indicate that butterfly weed embryos reach an autotrophic stage by 60 DAP, 58 d earlier than when pods are typically harvested (118.0±4.2 d). From a commercial standpoint, this could significantly reduce the time needed to produce a salable liner. Using traditional seed-based propagation, a commercial grower would harvest seeds 118 DAP, stratify for 30 d, germinate under greenhouse conditions (requiring 30 d), and grow out for 30 d to reach a salable size; totaling 208 d (29.7 w) invested in propagation. Conversely, seeds required for ER could be harvested 60 DAP and require 60 additional days in culture to reach a salable size of 7.62 cm (Fig. 4), for a total of 120 d (17.1 weeks) invested in propagation. In this scenario, ER results in a reduction in butterfly weed propagation time of 88 d (12.6 weeks).

This study documented a successful protocol for ER in butterfly weed; including surface sterilization techniques, excision methods, stratification times, media type/strength, and embryo harvesting time. Previous studies either investigated embryogenesis of species in the milkweed family (but not milkweed species), or organogenesis of milkweed from leaf or meristem tissue. To our knowledge this is the first study to investigate ER of embryonic tissue from seeds, and the first to document the effect of embryo maturity on the successful development of explants in culture. As methods for ER vary from species to species, this study aimed at providing a commercial ER protocol for butterfly weed, with the hope that this protocol may be applicable to

other milkweed species. Results indicated that ½ MS media yielded ER seedlings with greater root and shoot lengths with comparable survival rates compared to MS and WPM. This was likely due to milkweed species natural affinity for soils with low nutritional content, which most closely aligned with ½ MS media (Stevens, 1945). These results indicate that ER could be a commercially viable alternative to germination of milkweed species in soilless substrates, reducing propagation time by 88 d (12.6 w) and as a result possibly increasing profitability. Another goal of this study was to determine at what point embryo maturity affected embryo germination and growth. Embryos rescued at 30 DAP resulted in the lowest root and shoot growth, while 60 DAP yielded the highest root and shoot growth. While there was no difference in germination percentages among embryos of differing maturities or among differing media types, ER germination rates were uniformly higher than germination rates of fully mature seed sowed in soilless substrate. These findings provide a starting point for improved commercial propagation of butterfly weed, as well as potentially offering a viable propagation protocol for threatened and/or endangered milkweed species to enhance restoration efforts.

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Figure 4.1. Effect of ½ strength Murashige and Skoog media (½ MS), full strength Murashige and Skoog media (MS), and Woody Plant Media (WPM) on root length (cm) over time when culturing butterfly weed embryos harvested 90 d after pollination (DAP).[z] Bars represent means with standard error at $P < 0.05$. Letters indicate significance at day 50 based upon Tukey's HSD at $P \leq 0.05$.[y] 1 cm = 0.3937 inch.

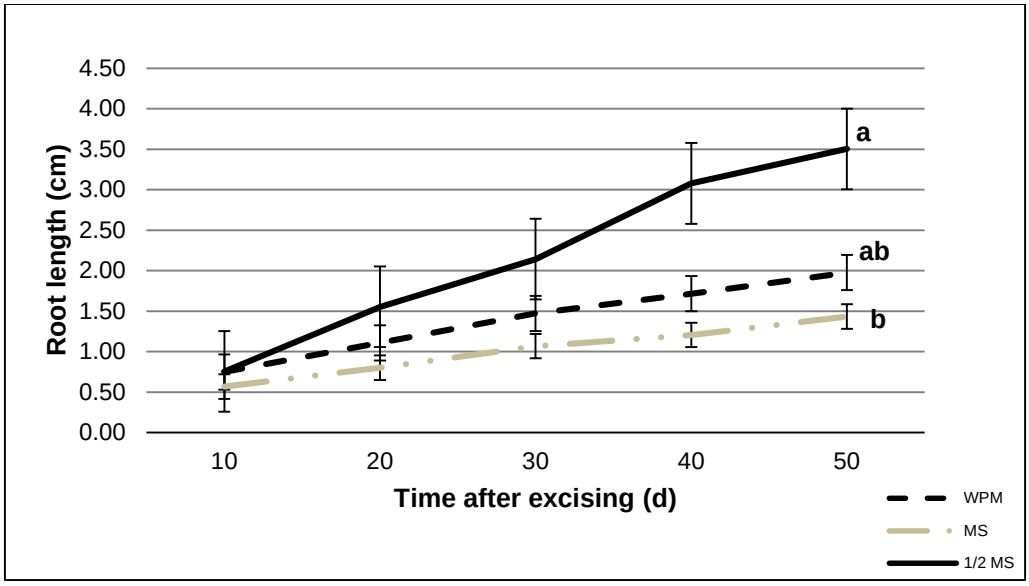

Figure 4.2. Effect of ½ strength Murashige and Skoog media (½ MS), full strength Murashige and Skoog media (MS), and Woody Plant Media (WPM) on shoot length (cm) over time when culturing butterfly weed embryos harvested 90 d after pollination (DAP). [z] Bars represent means with standard error at $P < 0.05$. Letters indicate significance at day 100 based upon Tukey's HSD at $P \leq 0.05$. [y] 1 cm = 0.3937 inch.

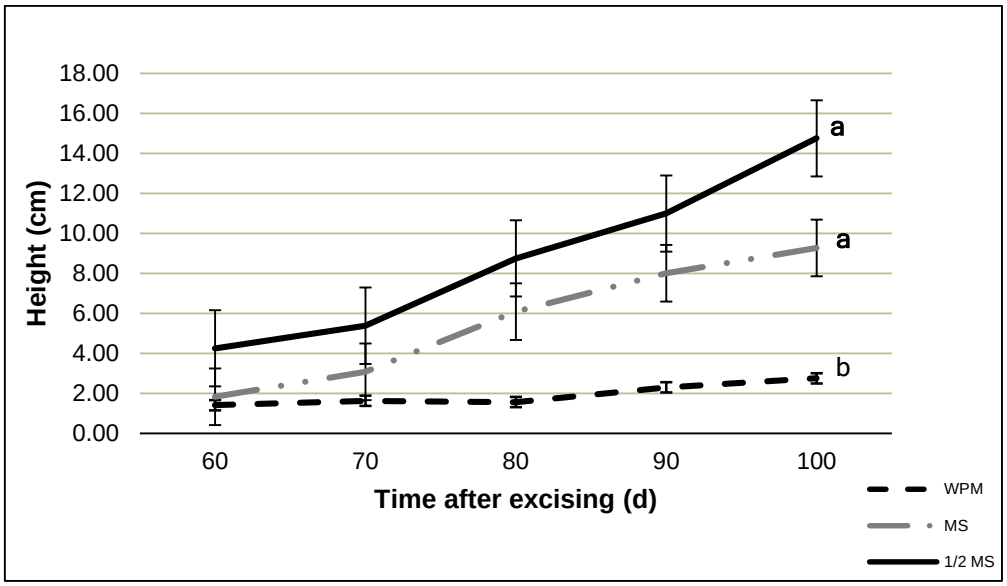

Figure 4.3. Effect of butterfly weed embryo maturity on root length using ½ MS media. Embryo maturities of 90 d after pollination (DAP), 60 DAP, and 30 DAP are displayed. Days after excising refers to the number days accrued after the successful removal of the embryo from the seed coat. [z] Bars represent means with standard error at $P < 0.05$. Letters indicate significance at day 50 based upon Tukey's HSD at $P \leq 0.05$. [y] 1 cm = 0.3937 inch.

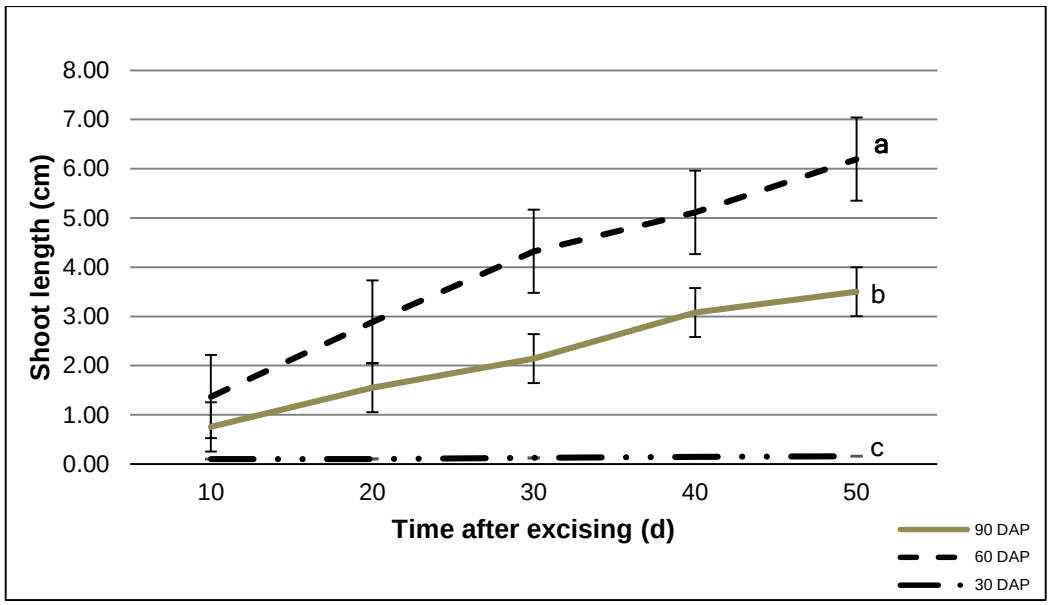

Figure 4.4. Effect of butterfly weed embryo maturity on shoot length at 70 days after excising using ½ MS media. Embryo maturities of 90 d after pollination (DAP), 60 DAP, and 30 DAP are displayed. Days after excising refers to how many days accrued after the successful removal of the embryo from the seed. [z] Bars represent means with standard error, and letters above bars represent groupings based upon Tukey's HSD at $P \leq 0.05$. [y] 1 cm = 0.3937 inch

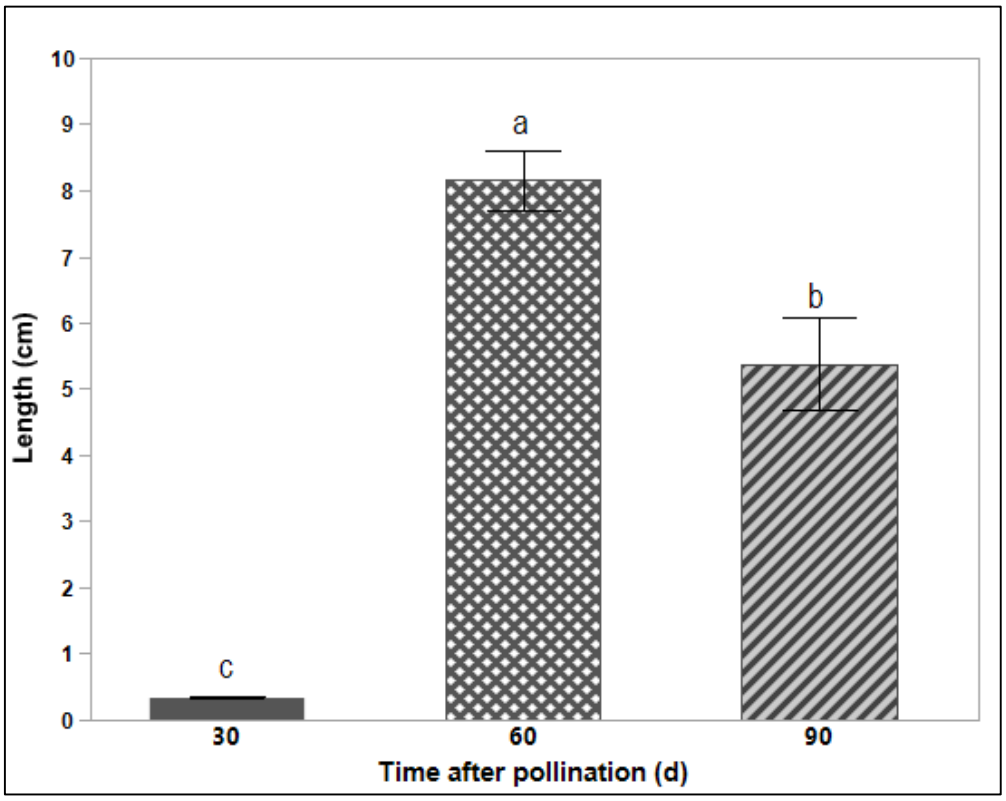

Table 4.1. Effect of ½ strength Murashige and Skoog media (½ MS), full strength Murashige and Skoog media (MS), Woody Plant Media (WPM), and germination of fully mature butterfly weed seed on perlite under greenhouse conditions (control) on germination rates and survival rates of plantlets.

Media	df	Germination (no.)	Germination (%)	Final Survival (no.)	Survival Success (%)
WPM	39	32	82.05 bc [z]	22 b	56.41 b
MS	39	37	94.88 ab	20 b	51.00 b
1/2 MS	39	38	97.43 **a**	35 **a**	89.74 **a**
Perlite	39	31	79.5 c	--	--

[z] Letters by means represent groupings based upon Tukey's HSD at $P \leq 0.05$.

Table 4.2. The effect of butterfly weed embryo maturity (90, 60, and 30 d after pollination; DAP) on germination and survival rates are reported; in addition to germination rates of seed sown in perlite under greenhouse conditions (control).

DAP	N	GERMINATION (NO.)	GERMINATION (%)	FINAL SURVIVAL (NO.)	SURVIVAL SUCCESS (%)
90	39	38	97.43 **a**[z]	35 **a**	89.74 **a**
60	72	72	100 **a**	69 **a**	95.83 **a**
30	72	72	100 **a**	72 **a**	100 **a**
PERLITE	39	31	72.38 b	--	--

[z] Letters by means represent groupings based upon Tukey's HSD at $P \leq 0.05$.

CHAPTER 5

Use of Flow Cytometry to Assess Total Genomic Content for *Asclepias spp.*

Introduction

Asclepias is a member of the Apocynaceae family, the dicot counterpart of the Orchidaceae. *Asclepias* has 130 species native to North America, six to South America, and up to 250 species in Africa. (Blackwell, 1964; Fishbein, 1996; Fishbein, 2008; Goyder 2001; Stevens, 1983; Woodson, 1954; Wyatt and Broyles, 1994). Known for its ecological importance to a number of migratory North American butterfly species (Hutchings, 1923; Brower, 1969; Brower et al., 1972; Malcolm, 1991), the family possesses one of the most complex reproductive structures aside from orchids in the plant kingdom (Wyatt, 1976). The family's unique reproductive strategy has evolved to promote nearly exclusive outcrossing in nature, yet interspecific hybridization has been rarely reported (Bookman, 1984; Weitemier et al., 2015). Phylogenetic studies in *Asclepias* have been made both on physiological and genetic differences between species. Currently, species in the genus have been divided into 17 different clades (Agrawal and Fishbein, 2006, 2008; Agrawal et al., 2008; Fishbein, 1996; Fishbein et al., 2011). These previously published studies indicate a division across the family into broadly geographic clades that could imply a high degree of relatedness among species within these clades. However, not all species within clades share the same affinity towards interspecific hybridization, raising questions as to what other factors may be at play in limiting interspecific compatibility (Lewis et al., 2021). While phylogenic relationships are tentative indicators of how genetically similar Asclepias spp. are to one another, flow cytometry (FCM) may offer important additional insights into genetic similarities and fertility barriers among species used in conjunction with other species genomic comparison methods.

Flow cytometry can estimate genome level, general ploidy, nuclear replication, and endopolyploidy (Doležel et al., 2007). FCM research has been used in the Asclepiadoideae family (a subfamily of Apocynaceae) and *Asclepias spp.* to determine ploidy and total genomic content for several species. These species include *A. curassavica* L., *A. fascicularis* Decne., *A. incarnata* L., *A. latifolia* Torr., *A. salicifolia* Lodd., *A. speciosa* Torr., *A. syriaca* L., *A. verticillata* L., and *A. tuberosa* L.; all being diploid (Darling and Wylie, 1956 and Moyer, 1936). In addition, it appears that 22 *Asclepias* species have a base chromosome count of 11 (2n=2x=22) (Darling and Wylie, 1956; Gadella et al., 1969; Heiser and Whitaker, 1948; CCDB database, 2021). A 2001 study by Albers and Maeve determined that in an analysis of over 650 species within the Apocynaceae family, only 3% deviated from the x=11 chromosome count, and polyploidy was not observed. With only a small number of species in the family documented, there is still work to be done to ensure that ploidy levels are consistent across the among species. Hybridization and natural ploidy variations could be detected and utilized for improved commercial production by further exploring the ploidy of additional species.

Total genomic content, measured ordinarily using propidium iodide (PI), is usually characterized by C-value. C-value is the number of base pairs (picograms) of DNA present in a whole chromosome complement (Swift, 1950; Greilhuber et al., 2005). Two stains can be utilized to estimate genomic content via FCM. DAPI offers an efficient approximation of AT base pairs and subsequently genome size, in addition to ploidy. Conversely, PI will bind to AT and GC regions, providing estimations of total DNA content and genome size with greater accuracy (Doležel et al., 2007).

Published reports documenting total genomic content for *Asclepias* have been minimal. Those species having total genomic content documented include *A. curassavica* L., *A. incarnata*,

A. syriaca, A. tuberosa, and A. verticillata (Bai et al., 2012). Based on the available literature, the relatively small genome size *Asclepias* is intriguing. In angiosperms, 1.4 pg – 3.5 pg (2C value) is considered to be a very small to small genome (Soltis et al., 2003). *Asclepias* genome size (2C values) ranges from 0.7 – 1.1 pg, with *A. verticillata* recorded as the smallest and *A. tuberosa* the largest (Bai et al., 2012). Given that total DNA content among species with published genome sizes can vary from 40 – 70 % (Verloove et al., 2017), it is important to understand the range of sizes utilizing a larger complement of species. However, the known differences in DNA content may explain why there are have been few documented naturally occurring interspecific hybrids (Kephart et al., 1988; Zonneveld, 2019).

The objective of this study was to determine the total genomic content of 15 *Asclepias* species native to North America and four synthetic hybrids between the maternal parent *A. tuberosa* and pollen parents, including *A. hirtella, A. purpurascens, A. speciosa,* and *A. syriaca.* Results will increase the number of reported and documented species and could potentially be used to aid and identify hybridization opportunities between species in the future.

Materials and Methods

Plant Material: The species utilized and measured for this study were: *A. amplexicaulis* Sm., *A. angustifolia* Schweigg, *A. asperula* Decne., *A. curassavica, A. fascicularis, A. hirtella* Woodson, *A. incarnata, A. latifolia, A. linaria* Cav., *A. purpurascens* L., *A. speciosa, A. subverticillata* Gray, *A. syriaca, A. tubero*sa, *A. variegata* L., and *A. viridis* Walter. Four hybrids were also analyzed: *A. tuberosa* x *A. hirtella, A. tuberosa* x *A. purpurascens, A. tuberosa* x *A. speciosa,* and *A. tuberosa* x *A. syriaca* (Table 5.1). Seeds were obtained from various sources (Table 5.1) and subsequently cold-moist stratified for 30 days at 3-4 °C in a washed builder's sand substrate. Upon removal from stratification, seeds were germinated in Jan. 2020 at the

University of Georgia Athens campus, College Station Greenhouse Complex (lat. 33.9480°N, long. 83.3773°W). Seeds were placed at surface level in 804 inserts (T.O. Plastics, Minneapolis, MN) containing 100% perlite (Carolina Perlite Co. Inc., Gold Hill, NC) to a depth of 5.2 cm, with a topdressing of vermiculite (TX401, BWI, Greer, SC) of 0.635 cm depth. Supplemental light was provided by light-emitting diode (LED) arrays (Fluence Spyder with PhysioSpec, Fluence Technologies Inc., Austin, TX), providing a *PPFD* (photosynthetic photon flux density) of 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the substrate surface and 14-h daylength (Albrecht and Lehmann, 1991). After planting, seed trays were placed on a mist bench with a misting cycle applying municipal water (pH 6.2, alkalinity of 11 ppm) set at 6 s every 10 min. Greenhouse temperatures were maintained at 25 °C days and 18 °C at night. Upon germination and expansion of the first set of true leaves, seedlings were transplanted into 804 inserts (T.O. Plastics) held by 1020 greenhouse trays (T.O. Plastics) filled with 80% milled peat (Sungro Peat Moss Grower Grade Orange, Agwam, MA) and 20% perlite (Carolina Perlite Co. Inc., Gold Hill, NC). Once established, seedlings were transplanted into 3.97 L (#1) containers (Classic 400, Nursery Supply, Agwam, MA) containing an 80% bark (3/8" particle size) and 20% milled peat (Foothills Compost, Gainesville, GA). Seedlings were irrigated with municipal water and fertilized twice a week with Peter's 20N- 4.4P-16.6K liquid soluble fertilizer (Scotts Co., Marysville, OH) at 100 ppm.

DNA C-values and Internal Standard

Immature leaf tissue was employed in the determination of DNA content and ploidy in this study. Samples were harvested from actively growing stems with newly expanded leaves 4 – 6 w after germination. Collection time varied slightly due to differences in leaf size among species. For consistent results, only one plant was used per species from which all samples were taken. Fresh leaf material was harvested and processed for each species immediately after collection.

The internal standard used was *Pisum sativum* L. 'Ctirad,'. This genotype has a published C-value closest to the estimated C-value of *Asclepias* spp. (0.1- 3.0 pg) (Bai et al., 2012). Pisum *sativum* 'Ctirad' was germinated and grown alongside *Asclepias* plants in Jan. 2020, and newly expanded leaf tissue was harvested for use employing identical techniques.

CyStain PI Absolute P kit (Sysmex America Inc., Lincolnshire, IL) was used to prepare all samples. Approximately 1 cm^2 of the species of interest and the internal standard *Pisum sativum* 'Ctirad' were coarsely chopped with a new razor blade in 500 µl of iced extraction buffer in 100x15-mm petri dishes (Thermo Fisher Scientific, Waltham, MA) for 90 s. The solution was then filtered through a 30 µm filter (CellTrics, Sysmex America Inc., Lincolnshire, IL) and into a 3.5 ml sample tube (Sysmex America Inc., Lincolnshire, IL) to isolate somatic nuclei from other cellular debris. Without centrifuging, 2 ml of staining buffer was added, followed by 12 µl of PI and 6 µl of RNase stock solutions. PI-stained samples were incubated in darkness for at least 30 min at 4 °C (refrigerator temperature). Samples estimating C-values were then run on a CytoFLEX S flow cytometer (Beckman Coulter, Hialeah, FL) operating at 488 nm at the University of Georgia Athens Center for Tropical and Emerging Global Diseases Cytometry Shared Resource Laboratory Coverdell Center. Five replicates of each accession were prepared and analyzed. A minimum of 5000 particles were analyzed for each sample. Sample runs were rejected if the coefficient of variation (cv) was greater than 3 % (Marie and Brown, 1993). Further analysis to determine differences between genome sizes across all species was performed with JMP statistical software (version 13.0; SAS Institute, Cary, NC). Data was analyzed to determine normality and homogeneity, with one-way analysis of variance (ANOVA) and separation of treatment means using Tukey's HSD (honestly significant difference). Only data showing significant differences ($P \leq 0.05$) among treatments are reported.

The 2C-value was calculated using the formula:

(mean of sample peak/mean of standard peak) x 2C DNA amount (pg) of the standard.

Reported 2C DNA for *Pisum sativum* 'Ctirad' was 9.09 pg (Dirihan et al., 2013)

Results and Discussion

The 2C genome size ranged from 0.645 – 1.239 pg among species examined in this study (Table 5.2), with *A. tuberosa x A. hirtella* hybrid having the largest genome size and *A. subverticillata* having the smallest. Those species classified as having small genome sizes (between 0.645 and 0.659 pg) included *A. subverticillata* (0.645 pg), *A. curassavica* (0.652 pg), *A. angustifolia* (0.66 pg), and *A. fascicularis* (0.69 pg) (Table 5.2). *A. linaria, A. purpurascens*, and *A. speciosa* shared slightly larger genome sizes, ranging from 0.781-0.819 pg in size (Table 5.2). *A. syriaca, A. variegata*, and *A. viridis* had similar and slightly larger genome sizes, ranging from 0.91-0.954 pg (Table 5.2). Species with 2C levels ranging from 1.038-1.070 pg (Table 5.2) included *A. latifolia, A. tuberosa x A. syriaca* hybrid, *A. tuberosa x A. speciosa hybrid, A. hirtella*, and *A. tuberosa x A. purpurascens* hybrids. *A. asperula* and *A. tuberosa* had the largest DNA content of any species observed in this study, being 1.12 and 1.15 pg in size (Table 5.2). The largest genome measured in this study was the hybrid between *A. tuberosa* and *A. hirtella*, being 1.239 pg in size (Table 5.2). We did not find differences between *A. linaria* and *A. subverticillata* found naturally growing at high elevations versus low elevations (Table 5.2).

Despite slightly varying methods among this study and the published work of Bai et al. (2012) and Kubešová et al. (2010), results were similar to species analyzed in their studies. Using the same internal standard, Bai et al. (2012) estimated the DNA content of *A. incarnata* to have a 2C value of 0.7 pg, similar to our finding of 0.734 pg. In the Bai et al. (2012) study, *Asclepias tuberosa* was 1.1 pg in size, similar to the findings of this research at 1.15 pg. Similarly, for

Asclepias syriaca, the genome size estimated by Bai et al. (2012) was 0.9 pg, similar to this study's estimated value of 0.91 pg. Kubešová et al. (2010) used a differing standard, *Solanum lycopersicum* L. (tomato), but still had a similar 2C value in *A. syriaca* (0.84 pg) to that measured in this study (0.91 pg). Bainard et al., 2012, used *Glycine max* (soybean) as an internal standard when estimating DNA content of *A. syriaca,* yet still reported similar values (0.86 pg) to those in this study (0.91 pg). This consistency among results from multiple published reports utilizing varying FCM protocols is encouraging. Furthermore, this consistency infers that results obtained on other taxa measured in this study can be presumed (at a minimum) precise. Our expansion to include more species than are currently found may also help breeders in future work on the genus.

Regarding ploidy, Kubešová et al. (2010) offer the only report on ploidy in *Asclepias.* In that study, *A. syriaca* was determined to be diploid with a genome size of 0.91 pg. Their calculated DNA content mirrored that of *A. syriaca* in this study and was in the range of all observed species (Table 5.2). Therefore, it can be assumed that the estimated ploidy of all other species observed in this study are diploid, as there appear to be no significantly larger genome sizes observed. Changes in ploidy (e.g. tetraploidy) can be observed through significantly greater DNA content estimations compared to their diploid counterparts in ornamental species with autopolyploid genotypes (Lattier et al., 2019; Rothleutner et al., 2016). Based on this data, the assumption of diploid was made, and the equation from Dirihan et al. (2013) was used to confirm that no polyploidy was observed from this study (Table 5.2). Further research attempts were made to visually karyotype via root and pollen squashes to confirm and validate chromosome counts and estimated ploidy. However, clear images to make definitive chromosome counts were unsuccessful due to the species' extremely small chromosomes. It did appear from this preliminary work that both *A. syriaca* and *A. tuberosa x A. syriaca* hybrids had the same

chromosome counts (2n=22). However, due to imperfect imaging, we do not base any results on those findings (Lewis, 2021).

In a study conducted by Lewis et al. (2021), interspecific hybrids were attempted using *A. tuberosa* as the maternal parent and seven other species as pollen parents. Of seven pollen-donor species, only four successfully hybridized with *A. tuberosa*; *A hirtella, A. purpurascens, A. speciosa,* and *A. syriaca.* A potential reason for the failure of *A. currisavica, A. fascicularis,* and *A. incarnata* (as paternal parents) to hybridize with *A. tuberosa* could be a difference in genome size. Referring to Table 5.2, with *A. tuberosa* as the maternal parent, species that failed to make successful hybrids with *A. tuberosa* (1.15 pg) did have smaller genome sizes (0.65-0.73 pg). In addition, based on the AFLP based phylogeny study by Weitemier et al. in *Asclepias* (2015), species unable to hybridize with *A. tuberosa* were in a more distantly related clade. Of the four species that did hybridize, all were located within approximately the same clade (K and L), while the three that failed to hybridize were significantly removed (clade F). Our results for genome size add an additional layer to predict future successful hybridizations. Knowing both genetic and genome size similarities may be a valuable resource when planning hybridization work. While correlation does not equal causation, genome size may play a role in the success of controlled and natural interspecific hybridization events via a lack of chromosomal pairing.

Typically, successful interspecific hybrids between species have intermediate genome sizes, as seen in *Cirsium, Cornus,* and *Magnolia* (Bures et al., 2004; Parris et al., 2010; Shearer and Ranney, 2013). Interestingly, the hybrid *A. tuberosa x A. hirtella* measured in this study deviated from this trend, having a genome size greater than either parent (Table 5.2). Similar phenomena have been documented in *Helianthus* (sunflower) hybrids and *Elaesis* (Oil palm) hybrids, where the interspecific hybrids also had up to 50% more DNA than the parent species (Baack et al.,

2005; Camillo et al., 2014). Baack et al. (2005) made one hypothesis that could apply here: recombination of two genomes adapted for vastly different environments leads to the sometimes temporary increase in genome size. *Asclepias hirtella* possesses a different morphology from *A. tuberosa*, having pinnate leaves, smaller floral size, strong apical dominance, and greater average height. *A. tuberosa* has more obovate leaves, larger floral structures, denser branching habits, and relies on tuberous roots for reproduction. Ecologically, *A. hirtella* is found primarily in soils with higher available moisture and pH levels in the Midwest. Conversely, *A. tuberosa* is typically found throughout its range in microenvironments with low soil moisture availability and low pH. The hybridization of these two vastly different species could have resulted in the increased genome size compared to the other paternal species that successfully hybridized with *A. tuberosa*. From a molecular standpoint, genomic content increase in diploid plants has also been linked to differences in intron size and transposon copy number (Baack et al., 2005). Thus, while an increase in genome size compared to the parents is uncommon in hybrids, it is not unprecedented. As the two species are dissimilar, a combination of both genetically based and abiotically influenced factors likely affected the change in genome size. Differences in genome sizes have also been attributed to differences in frost resistance (MacGillivary and Grime, 1995), calyx size (MeaGher and Costich, 1996), and elevation (Bottini et al., 2000 and Reeves et al., 1998). However, we do not believe these abiotic factors significantly influence *Asclepias,* as we did not find differences in genome size between *A. linaria* and *A. subverticillata* grown at high elevations versus low elevations (Table 5.2).

Genome sizes calculated during this project should be helpful for plant breeders in predicting and those species of Asclepias with enhanced potential for success when attempting interspecific hybridization. Those species with similarities in chromosome size contribute to functional meiosis and bivalent pairing between genomes, thus making higher hybridization success

possible (Rothleutner et al., 2016). Our research serves as a reference point and reveals that *Asclepias* species have a small reference genome size that varies among species.

This study documents the total genomic content of 15 *Asclepias* species and four hybrids. In general, the range of *Asclepias* species tends to be between 0.645 pg and 1.24 pg. Based on previous research and results discovered in this study, it is also concluded that the estimated ploidy of all observed *Asclepias* species are diploids. Thus, there appear to be barriers to hybridization, whereby genome size is a good indicator of the potential success or failure of a natural or controlled hybridization event. In the future, breeders may be able to use genome size as guides to identify potential parents for successful crosses. These findings provide a starting point for future breeding efforts and expand the general knowledge surrounding *Asclepias* species. In doing so, impacts will benefit not only the commercial market but also pollinator relationships with Asclepias. Improving the variability of morphological features, the range at which the species can be found, and vigor would positively impact the monarch butterfly and other pollinators.

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Table 5.1. *Asclepias* species used and seed source locations. More than one seed source was utilized for this experiment to ensure the germination of at least one plant per species. For *Asclepias linaria* and *subverticillata*, seeds were acquired that had been collected from high elevation (HE) and low elevation (LE) populations.

Species	Seed source [w]	Location [w]
A. angustifolia	Terroir Seeds	Chino Valley, AZ
A. asperula	Terroir Seeds	Chino Valley, AZ
A. curassavica	Ball Horticulture Co.	Chicago, IL
A. fascicularis	USDA	Columbus, OH and Parlier, CA
A. hirtella	Everwilde Farms	Fallbrook, CA, WC
	Prairie Moon Nursery	Winona, MN
A. incarnata	Everwilde Farms	Fallbrook, CA,
	Prairie Moon Nursery	Winona, MN
A. latifolia	Terroir Seeds	Chino Valley, AZ
A. linaria (HE and LE)	Terroir Seeds	Chino Valley, AZ
A. purpurascens	Everwilde Farms	Fallbrook, CA,
	Wild collected	Indiantown/Lebanon Co., PA
A. speciosa	Prairie Moon Nursery	Winona, MN
	USDA	Columbus, OH and Parlier, Ca
A. subverticillata (HE and LE)	Terroir Seeds	Chino Valley, AZ
A. syriaca	Prairie Moon Nursery	Winona, MN
	USDA	Columbus, OH and Parlier, CA
A. tuberosa x A. hirtella	University of Georgia	Athens, GA
A. tuberosa x A. purpurascens	University of Georgia	Athens, GA
A. tuberosa x A. speciosa	University of Georgia	Athens, GA
A. tuberosa x A. syriaca	University of Georgia	Athens, GA
A. tuberosa	Wild collected	Oglethorpe County, GA
A. variegata	Wild collected	Rabun County, GA
A. viridis	Everwilde Farms	Fallbrook, CA

Table 5.2. The calculated 2C (pg) and (Mbp) with standard deviation values for all 19 *Asclepias* species and hybrids. High elevation (HE) and low elevation (LE) are reported, even though there is no difference between the two elevations in either species.

Species	2C-value (pg DNA)	Sig. Level[z]	S.D	(Mbp) [y]	Ploidy[x]
A. angustifolia	0.659	H	0.029	645.82	2
A. asperula	1.126	B	0.021	1103.58	2
A. curassavica	0.652	H	0.012	638.57	2
A. fascicularis	0.690	GH	0.011	676.49	2
A. hirtella	1.055	C	0.017	1033.80	2
A. incarnata	0.734	FG	0.011	718.93	2
A. latifolia	1.038	C	0.020	1017.34	2
A. linaria (HE)	0.781	EF	0.015	765.38	2
A. linaria (LE)	0.775	EF	0.008	759.50	2
A. purpurascens	0.798	E	0.032	781.55	2
A. speciosa	0.819	E	0.019	802.52	2
A. subverticillata (HE)	0.652	H	0.017	638.76	2
A. subverticillata (LE)	0.645	H	0.008	632.10	2
A. syriaca	0.910	D	0.013	891.97	2
A. tuberosa x A. hirtella	1.239	A	0.029	1214.42	2
A. tuberosa x A. purpurascens	1.073	C	0.016	1051.25	2
A. tuberosa x A. speciosa	1.049	C	0.018	1027.53	2
A. tuberosa x A. syriaca	1.040	C	0.024	1018.91	2
A. tuberosa [x]	1.150	B	0.018	1127.00	2
A. variegata	0.926	D	0.017	907.09	2
A. viridis	0.954	D	0.027	934.82	2

[z] Mean separation by Tukey's HSD (α=0.05, and $p < 0.05$). Means with the same letter are not significantly different from each other.

[y] Conversion rate of pg to million base pairs (Mbp) was 1 pg of DNA = 980 Mbp (Dolezel et al., 2003)

[x] The estimated ploidy level based on previous records and flow cytometry samples

CHAPTER 6

Inheritance of Flower Color, Pubescence, and Sap Color in *Asclepias* Interspecific Hybrids

Introduction

Asclepias sp. L (milkweed) is utilized by several migratory butterfly species (and other *Lepidoptera* sp.) as both an oviposition site (Hutchings 1923; Brower 1969) and food source (Fishbein and Venable, 1996). A steady decline of overwintering sites, breeding habitats, and food sources such as *Asclepias* sp. has led conservationists to advocate for *Asclepias* sp. to be planted in public and private areas to increase threatened or endangered Lepidoptera sp. population numbers (Luna and Dumroese, 2013). In addition to being the oviposition and critical forage source for larval Lepidoptera, *Asclepias* sp. also serves as a host to over 80 pollinator species. A member of the Apocynaceae family, over 100 species of *Asclepias* are native to nearly all physiographic regions in North America (Woodson, 1954; Wyatt and Broyles, 1994). This broad native range has led to a divergence in phenotypic and physiological traits within the genus and (in many cases) isolation of species. This isolation is common in *Asclepias* plants and results in very few naturally occurring hybrids that, when successfully produced, are most likely the result of habitat overlap (Wyatt and Broyles, 1992). The lack of naturally occurring interspecific hybrids in *Asclepias* is also due to complex floral biology within the genus. Asclepias boasts one of the most reproductively complex structures known in the plant kingdom (Wyatt, 1976). Pollen is transmitted via pollinia, or sac-like structures containing pollen. Pollinia are part of a “lock and key” pollination method (Stebbins, 1970), whereby successful pollination requires several critical steps. These include removal of pollinia from the pollen parent, desiccation of the pollinia to facilitate pollen release (typically during the flight of the pollinator), and precise placement of pollinia in the female parent’s stigmatic slit (requiring a

rotation of 90 degrees). Pollinia and stigmatic slit size vary significantly depending on species, and these differences are also credited to the limit of successful naturally occurring interspecific hybridizations (Wyatt and Broyles, 1994). However, some species have been successfully hybridized under controlled conditions (Kephart and Heiser, 1980), including *A. speciosa* Torr. × *A. syriaca* L. (Stevens, 1945), *A. exaltata* L. × *A. syriaca* (Wyatt and Hunt, 1991), *A. exaltata* x *A. quadrifolia* Jacq., and *A. purpurascens* L. × *A. syriaca* (Kephart et al., 1988). In this study, parent species were selected that possess traits suitable for commercial production. When selecting parental species, traits such as interesting floral morphology, ecological abundance, soil moisture tolerance, hybridization ability based on previous research, genetic relatedness, and branching capability were considered.

Asclepias tuberosa L. was used as the maternal parent, and one-way crosses were performed in this study. Several circumstances dictated this choice. *A. tuberosa* is the most commonly grown *Asclepias* spp. commercially and does not produce a large amount of latex in its sap that some consumers dislike (Agrawal et al., 2008; Cullina, 2000). *A. tuberosa* also has naturally occurring branching, is a repeat fall bloomer, and is one of the shortest species in the genus (Cullina, 2000). Finally, the range in flower color for *A. tuberosa* ranges from golden yellow to dark orange and is very attractive. Of seven species that served as pollen parents, four resulted in a viable seed set and an F_1 population. These included *A. hirtella* Woodson, *A. purpurascens*., *A. speciosa*, and *A. syriaca* (Lewis et al., 2021). *Asclepias hirtella* was selected for its tolerance to wet and dry soil conditions (common in commercial production environments), green floral coloration, and lanceolate foliage (Cullina, 2000). *Asclepias purpurascens* was selected for its uniquely dark pink/purple floral coloration, extended bloom period, proven hybridization capability, and noticeable fragrance (Cullina, 2000; Kephart et al., 1988). *Asclepias speciosa* is the most widely used monarch host plant in the northern U.S., having the largest flowers in the

genus, with light pink coloration. It has been reported to hybridize with other *Asclepias* species when geographically overlapped and produces large amounts of nectar with strong fragrance (Cullina, 2000; Luna and Dumroese, 2013; Kephart et al., 1988; and Stevens, 1945). *A. syriaca* possesses strong fragrance, has naturally hybridized with other *Asclepias* species, possesses dark pink/mauve flowers, and has a broad native range (most of U.S. and USDA Hardiness Zones 4-9) (Cullina, 2000; Kephart et al., 1988; Stevens, 1945; and Wyatt and Hunt, 1991).

As all species of *Asclepias* are outcrossing, it would be illogical to assume that any parents used in these hybridization efforts would be entirely homozygous for any trait (Lipow and Wyatt, 2000). However, a standard hypothesis based on Mendel's first law of the inheritance of simply inherited traits indicates that for different phenotypes, the intermediate or dominantly inherited trait should be expressed and visible in all F_1 progeny (Lipow and Wyatt, 2000).

Pubescence, or having "hair" on leaf surfaces, refers to specialized cells extending from the epidermis of the leaf (or other plant organs). These hairs are called trichomes, and in *Asclepias,* these structures serve two functions (Agrawal et al., 2009). One function is the reflection of light away from the leaf surface to reduce heat load and solar radiation penetrating the leaf surface. Trichomes are vital given the arid, sunny, and dry regions *Asclepias* typically inhabit (Ehleringer et al., 1976). The second function is herbivore resistance, although many predators of *Asclepias*, such as aphids (*Aphidoidea* spp. Geoffroy) or monarchs (*Danaus plexippus*), have co-evolved to overcome this defense mechanism (Agrawal et al., 2009). Of the species selected to be hybridized, *A. purpurascens* and *A. hirtella* are glabrous (lacking trichome "hairs" on leaf surfaces) (Agrawal et al., 2009). *A. tuberosa, A. syriaca*, and *A. speciosa* are all pubescent, with *A. tuberosa* having the densest foliar pubescence (Agrawal et al., 2009). Pubescence can vary in quality and quantity depending on the tissue type, plant age, location on the plant, and plant

growing conditions (Johnson, 1975). These variances make it difficult to accurately define a plant as “pubescent” or “non-pubescent”, as its absence or presence can change over time, based on environmental conditions or based on the plant's location. No research has documented the inheritance of pubescence in *Asclepias*, but there has been work in other species. Pubescence is a dominant trait controlled by a single gene in rice (*Oryza sativa* L.) (Zheng et al., 2013), wheat (*Triticum aestivum* L.) (Love and Craig, 1924), pepper (*Capsicum annuum* L.) (Shuh and Fontenot, 1990), *Lotus corniculatus* L. (Hinckley and Keim, 1960), and lentil (*Lens culinaris* Medik.) (Khosravi et al., 2012). Comparing previous observations from other taxa with those of Asclepias, a study by Agrawal et al. in 2009 looked at the phylogeny of pubescence in 43 *Asclepias* species, and approximately half (20) were non-pubescent. While a small sample of the *Asclepias* genus, results indicate that pubescence in Asclepias F1 interspecific hybrids may not be a dominant phenotype, as it is rarely expressed in the larger sample of *Asclepias* species. *Asclepias* is unique in that the genus falls within the 10% of flowering plant species that produce latex sap (Agrawal and Konno, 2009). Produced in specialized cells called laticifers, the latex coagulates when exposed to air and contain varying levels of cardiac glycosides (Agrawal and Konno, 2009). These cardenolides, or cardiac glycosides, are astringent and toxic at high consumption levels when consumed by a wide array of insects and mammals. As a result of consuming foliage containing cardenolides, insects such as butterflies confer protection from predation (Malcom, 1991). While it is a protective mechanism, studies have shown that excess latex consumption can increase monarch butterfly caterpillar mortality by up to 33% when feeding upon *Asclepias* spp. (Zalucki et al. 2001; Zalucki & Malcolm 1999). It has also been observed that this latex production is phenotypically plastic. Specifically, increased latex production was documented after adding fertilizer (under greenhouse growing conditions) or due to herbivory damage (Agrawal and Konno, 2009). White latex sap is almost ubiquitously present

among all *Asclepias* sp., except for *A. tuberosa*, which has the same chemical components but clear sap. From both a commercial and ecological standpoint, researchers have noted the importance of understanding the inheritance of latex sap (Züst et al., 2019). Latex sap inheritance is important from the consumer perspective as latex sap is both sticky and can stain surfaces, two factors that could reduce consumer demand.

Within Asclepiadaceae, an Apocynaceae subfamily that contains the milkweed genera, floral color variability includes every color except black. Even blue can be found in the South American blue milkweed (*Oxypetalum coeruleum*). Despite the variation in floral color observed among species within the family, color variation within individual species is minimal. In a study reported by Woodson in 1964, color variation in *A. tuberosa* was documented in populations from New Mexico, the U.S. to the northern border of New York, U.S. Woodson determined that floral color ranged from light yellow to reddish-yellow. These floral colors are determined by several pigments produced by unique biochemical synthesis pathways within the plant. Anthocyanins, responsible for expressing pinks, reds, and purples, are found in species such as *A. speciosa, A. syriaca*, and *A. purpurascens* (Woodson, 1964). Carotenoids responsible for expressing orange and yellow hues are dominant in *A. tuberosa*. Inheritance of flower color is typically a qualitative trait that usually exhibits simple inheritance influenced by a few genes (Bernardo, 2014). However, utilizing parents with differing color pigment pathways makes predicting inheritance patterns and color variations complicated.

The objective of this study was to determine the inheritance of several phenotypic traits. These traits include pubescence, sap color, and flower color. In doing so, we hope to expand the body of knowledge about *Asclepias* and provide a baseline of inheritance patterns to assist future breeding efforts within Asclepias.

Materials and Methods

This work is a continuation of the study documented in Lewis et al. (2021), which details parent production (seed sources and growing conditions) and methodology used to conduct successful interspecific hybridization among seven species of *Asclepias*. Lewis et al., 2021 also documents optimal pollination times, time to pod maturity, number of seeds per pod, stratification requirements, and germination rates of hybrid seeds. Of the seven pollen parent species used in this study, four successfully developed hybrid seeds when *A. tuberosa* was the maternal parent. Those successful pollen parents included *A. hirtella, A. purpurascens, A. speciosa*, and *A. syriaca.* Hybrid seed germination occurred between Nov. 2018 and late Feb. 2019 at the University of Georgia Athens campus, College Station Greenhouse Complex (lat. 33.9480°N, long. 83.3773°W) in 804 inserts (T.O. Plastics, Minneapolis, MN) containing 100% perlite (Carolina Perlite Co. Inc., Gold Hill, NC) with a topdressing of vermiculite (TX401, BWI, Greer, SC) to a 0.635 cm depth. Supplemental light was provided by light-emitting diode (LED) arrays (Fluence Spyder with PhysioSpec, Fluence Technologies Inc., Austin, TX), providing a *PPFD* (photosynthetic photon flux density) of 250μmol·m^{-2}·s^{-1} and 15-h daylength. Seed trays were placed on a mist bench with a misting cycle applying municipal water (pH 6.2 and alkalinity of 11 ppm) at 6 s every 10 m. Greenhouse temperatures were maintained at 25 °C day and 18 °C night. Upon germination and expansion of the first set of true leaves, hybrid seedlings were transplanted into 804 inserts (T.O. Plastics, Minneapolis, MN) held by 1020 greenhouse trays (T.O. Plastics) filled with 80% milled peat (Sungro Peat Moss Grower Grade Orange, Agwam, MA) and 20% perlite (Carolina Perlite Co. Inc., Gold Hill, NC). Once established, hybrid seedlings were transplanted into 3.97 L (#1) containers (Classic 400, Nursery Supply, Agwam, MA) containing an 80% bark (1 cm particle size), 20% milled peat mix (Foothills Compost, Gainesville, GA). Throughout hybrid seedling germination, establishment, and transplanting

stages, seedlings were irrigated with municipal water and fertilized twice weekly with Peter's 20N- 4.4P-16.6K liquid soluble fertilizer (Scotts Co., Marysville, OH) at 100 ppm. Additional chemical inputs in 2019 (Rowland Chemical Co., Athens, GA) included sprays for aphids (Bifenazate and Abamectin "Sirroco") and spider mites (Spinosad "Conserve SC", Chlorfenapyr "Pylon", Acequinocyl "Shuttle O", Spinetoram "Xxpire", Bifenthrin "Upstar", Abamectin "Lucid", Etoxazole "Tetrasan", Bifenazate "Floramite SC", and Chlorpyrifos "Duraguard ME") (Rowland Chemical Co., Athens, GA). Chemical control sprays for thrips not overlapping with ones already mentioned under spider mites: Acetamiprid "TriStar", Flonicamid "Aria", and Nouvaluron "Pedestal" (Rowland Chemical Co., Athens, GA). Active ingredients were rotated to avoid tolerance buildup in the pest population. In 2020 beneficial insects were used in conjunction with pesticides to maintain better control over pest population numbers. Beneficials for aphid control included *Aphidius colemani* Viereck, *Chrysoperla rufilabris* Burmeister, and *Hippodamia convergens* Meneville (IPM Laboratories Inc, Locke, NY). Spider mite beneficial controls included *Phytoseiulus persimilis* Evans and *Galendromus occidentalis* Nesbit (IPM Laboratories Inc, Locke, NY). Thrip biological controls used were *Amblyseius cucumeris* Oudemans and *Stratiolaelaps scimitus* Womersley (IPM Laboratories Inc, Locke, NY). Used in conjunction with biological control agents were Bifenazate "Floramite SC" and Acequinocyl "Shuttle O" for spider mites as well as Flonicamid "Aria" with Cyclaniliprole and Flonicamid "Pradia" for thrips and aphids. Scouting occurred daily, and releases occurred approximately twice a month, varying in beneficial type and quantity on temperature and season.

Data Collection and Analysis. Approximately 4,000 hybrid genotypes germinated and grew to maturity in 3.97 L (#1) containers (Classic 400, Nursery Supply, Agwam, MA). Measurements were taken twice during each genotype's life cycle to capture phenotypic traits with accuracy and precision. The first was at three months after germination (juvenile), and the second was at six

months (at maturity – flowering initiated). Flower color data was recorded one day after the flower opening before noon using the Royal Horticultural Society 2007 Colour Chart (V^{th} Edition, RHS.org, Surrey) as a reference. Based on the online RHS colorimeter tool, colors were transformed into red, blue, and green values (RHS, 2021). In addition, pubescence and sap color (white or clear) data were collected and recorded as binomial data, with 1 representing presence and 0 representing an absence of the trait.

Measurements were taken on F_1 hybrids, including 1,244 genotypes from *A. tuberosa* x A. *hirtella* (×*hirtella*), 624 genotypes from *A. tuberosa* × *A. syriaca* (×*syriaca*), 1256 genotypes from *A. tuberosa* × *A. speciosa* (×*speciosa*.), and 792 genotypes from *A. tuberosa* × *A. purpurascens* (×*purpurascens*). When collecting data from F_1 populations, the numbering of accessions allowed for tracking genotypes back to individual seed pods from which the individual originated, along with dates of hybridization, seed collection, and germination. When analyzing flower color, inheritance patterns were investigated at seed pod (full-sib genotypes) and parental species levels. When assessing phenotypes originating from individual seed pods, only those with at least 15 successful successions were utilized to ensure the significance of results. Given differences in germination rates, flowering times, pest pressure, and other environmental factors, flower color succession numbers varied from pollen parent hybrid groups. Therefore, flower color inheritance was only analyzed within individual pollen parent hybrid groups and not between groups to reduce extraneous error. After data collection, 1072 ×*hirtella*, 716 ×*speciosa*, 412 ×*syriaca*, and 542 ×*purpurascens* genotypes had complete data records and were analyzed based upon these parameters.

All data collection occurred in a controlled environment greenhouse structure with uniform environmental conditions. Regulating temperature, light, and production inputs (e.g., soil and fertility) across all F_1 genotypes was performed to minimize environmental impacts on observed

phenotypes. F_1 genotypes were arranged in a completely randomized design within the greenhouse. Statistical analysis was performed using JMP (version 16.0; SAS Institute, Cary, NC). Data analyzed to determine normality and homogeneity, with one-way analysis of variance (ANOVA) and separation of treatment means using Tukey's HSD to analyze differences among and within populations. Afterward, a Chi-Squared test was performed if ANOVA results were significant and potential inheritance patterns could be estimated. Pubescence and sap color were analyzed using Goodness of fit, or Chi-square (χ^2) with a critical value of $\chi^2_{0.05,1}$=3.84 for the two observed phenotypes (Dowdy et al., 2004)

Results and Discussion

Pubescence

Each of the hybrid F_1 groups was found to have varying inheritance ratios. Pollen parent ×*hirtella* F_1 genotypes fit the phenotypic ratio of 1:15 for pubescence/glaucous, having Chi-square values less than the critical value at 0.24 and 3.84, respectively (Table 6.1). This ratio is known as duplicate gene action and indicates that if pubescence is expressed, it results from a double homozygous recessive genotype inheritance (Bernardo, 2014). Despite fitting a 1:15 ratio for duplicate gene action, the actual inheritance ratio is 0:1, due to one pod appearing to be a self instead of a hybrid (Figure 6.1). Once the outlier pod genotypes were removed, the subsequent Chi-square test did confirm that the ×*hirtella* hybrid population fit a 0:1 inheritance ratio (Aa × AA = 0:1) (χ^2 = 0.46 and p-value = 0.49), indicating glaucousness is dominantly inherited, and the hybrid population did recombine as an F_1 population should.

Pollen parent ×*speciosa* F_1 genotypes fit the phenotypic ratio of 1:3 for pubescence to glaucous inheritance (χ^2= 1.65 and p= 0.20) (Table 6.1). For ×*speciosa* hybrids, glaucous is a dominant trait inherited from heterozygous parents controlled by a single gene (Aa × Aa = 1:3) (Bernardo,

2014). Pollen parent ×*syrica* F_1 genotypes fit the phenotypic ratio of 1:1 of pubescence to glaucous inheritance (χ^2= 0.75 and p= 0.39) (Table 6.1). Assuming pubescence is recessively inherited in the heterozygous form from *A. tuberosa* (Aa), one would see this inheritance ratio if *A. syriaca* was in a homozygous recessive state (Aa × aa = 1:1). Pollen parent ×*purpurascens* F_1 genotypes best fit the phenotypic ratio of 9:7 (χ^2= 2.27 and p= 0.13) (Table 6.1). Duplicate recessive epistasis ratio (9:7), also referred to as complementary epistasis, is a form of interallelic interaction. Epistasis is the expression of one allele being affected by the expression of one or more independently inherited alleles. In a duplicate recessive epistasis inheritance instance, pubescence is still a recessive allele. However, if the allele is present at either of two loci, recessive alleles mask the expression of the dominant glaucous allele (Bernardo, 2014 and Fehr, 1991). Overall, pubescence is a recessively inherited trait for all hybrids (Table 6.1).

Sap Color

Inheritance ratios differed among hybrid pollen parent groups. Pollen parent ×*hirtella* F_1 genotypes initially fit the phenotypic ratio of 1:15 (duplicate gene action) with white sap being dominant, having Chi-square values less than the critical value at 0.17 and 3.84 respectively (p= 0.68) (Table 6.2). However, on further inspection of the 63 genotypes that had clear sap, over a third of those genotypes originated from a singular pod, appearing to be a self (Figure 6.1). Once removed, ×*hirtella* F_1 genotypes fit a 1:0 ratio, with white sap dominantly inherited over clear sap (χ^2 = 0.88, p= 0.35). Pollen parent ×*speciosa* and ×p*urpurascens* F_1 genotypes fit a 1:0 inheritance ratio, having χ^2 and p-value cutoffs within the limits (χ^2= 1.66, p= 0.20; χ^2= 0.96, p=0 .54) (Table 6.2). Similar to the ×*hirtella* F_1 genotypes, the ×*syriaca* F_1 genotypes appeared to initially fit a 1:15 inheritance ratio (χ^2= 0.96, p= 0.541) (Table 6.2). However, of the 40 genotypes that inherited clear sap, 26 came from two pods. Once the two outlier pods were removed, ×*syriaca* fell into a 1:0 white sap to clear sap inheritance ratio (χ^2= 0.29, p= 0.59).

While two hybrid groups did not initially fit the expected inheritance ratios, given how few hybrids exuded clear sap when wounded, white sap is a dominantly inherited trait. Across all hybrids, clear sap is recessively inherited, with white sap being dominant and expressed in almost all F_1 genotypes.

Flower Color

Looking at the distribution of flower color across the entire ×*hirtella* F_1 population, there appear to be three primary RHS Colour Chart ranges expressed in this F_1 population (Figure 6.2a); yellow (23-26 range), dark reddish-pink (35-42 range), and light pink (48-56 range). Looking at the distribution of flower color within and among ×*hirtella* hybrid pods, only pod 13 has a significantly different average floral color from the rest ($\mu = 24.38$ and $p < 0.0001$) (Figure 6.1). Pod 13 had an average flower color of 24.4, appearing to be a case where every surviving genotype inherited a yellow flower color from *A. tuberosa*, with no distribution of other colors present. There was no difference between pods and their average flower colors (Figure 6.1). Across the hybrid group, only 90 of the 1072 ×*hirtella* hybrids (8.3%) inherited a yellow coloration from *A. tuberosa* (Figure 6.3a).

Distribution of flower color across the ×*purpurascens* F_1 population indicated pink hues appear to be inherited with a higher frequency than yellow hues (Figure 6.4 and Figure 6.2b). There are a few genotypes that inherited a yellow/orange color (20-30 range), and some genotypes inherited coloration in the orange/pink to red/pink range (35-45) (Figure 6.2b and Figure 6.3b). However, most genotypes inherited pink coloration (47-56) with varying degrees of intensity (Figure 6.3b). When analyzing the distribution of flower color among pods compared to the parents, ×*purpurascens* F_1 genotypes had less color variation, similar to ×*hirtella* hybrids. Pods 9,8 and 13 were found not to be different from *A. purpurascen*s (flower color 74) ($p = 0.054$) (Figure 6.4), having average flower color of the pod of 53.1, 52.6, and 52.4. Pods 2, 6, 7, 10, 11,

and 12 differed from both parents and were similar; ranging in color from 51.9 to 50.24 (Figure 6.4) ($p = 0.027$). Pods 1, 3, 4, and 5 were not different from *A. tuberosa* flower color (28) and ranged from 49.9 to 46.2 ($p = 0.23$). Of the 544 hybrid genotypes observed, 29 genotypes were yellow/orange (5.33%), 36 genotypes orange/pink to red/pink (6.62%), and 479 were pink (88.05%) (Figure 6.4 and Figure 6.3b). While pink appears to be a dominantly inherited color in F_1 progeny, given the wide range of pinks of various intensities inherited, we surmise inheritance of pink intensity is a result of the gene dosage effect (Figure 6.5b).

The general distribution of flower color across the entire ×*speciosa* hybrid population appears to be four colors inherited with higher frequency than others (Figure 6.2c and Figure 6.3c). The four groups roughly correspond to yellow/orange (20-32 range), a light reddish pink (35-40 range), a bubblegum pink (47-52 range), and light pink (54-55 range) (Figure 6.2c and Figure 6.3c). The average color value across pods within the ×speciosa hybrids showed differences among the hybrids and the parents and among the hybrid pods. Pods 5,6 and 15 (51.4 to 51.7 average) were different compared to pods 3,10, 11,12, 13, and 14 (35.6 to 43.1 average) ($p < 0.001$) (Figure 6.6). Pod 7 (50.9) differed from pods 11,12,13, and 14 ($p = 0.002$), and pods 1 and 2 (49.4 and 50.4) from 11,12, and 13 ($p =0.002$) (Figure 6.6). However, while the inheritance of flower color slightly differed from pod to pod, in laymen's terms, pink coloring was the dominant color inherited throughout the ×*speciosa* hybrid population (Figure 6.5c). Only 85 of 893 ×*speciosa* genotypes (9.5%) were morphologically identical to *A. tuberosa* and had recessively colored yellow flowers.

Looking at the general distribution of flower color across the entire ×*syriaca* F_1 population, the range of observed colors expanded past the range of the two parents (Figure 6.7 and Figure 6.2d). The mean color value of *A. syriaca* was 36, a light mauve color, and *A. tuberosa* a vibrant orange at 28. Progeny resulting from the hybrid loosely fell into three groups: yellow/orange (13-

32), orange/pink (34-45), and pink (47-56) (Figures 6.6, 6.2d, and 6.3d). Based on flower colors within each pod, there were color differences among pods (Figure 6.7) ($p = 0.007$). Pods 1, 2, 4, and six had an average flower color range of 48.0 – 51.4 and differed from all the other pods ($p = 0.0021$, Figure 6.7). Pods 3, 5, 6, and *A. syriaca* were found to be in a similar range between 33.1 – 37.4 ($p < 0.001$) (Figure 6.7). Pods 7, 8, and *A. tuberosa* were in the last group, averaging between 27.6 – 28.8 ($p < 0.003$) (Figure 6.7). A yellow/orange flower color inheritance occurred in 201 out of 412 genotypes, or 48.7% of the hybrids (Figure 6.3d). Reddish pink flower color appeared in 19.6% of the progeny, and pink in 31.5% of the progeny. If you were to combine the reddish-pink and pink phenotype groups, you would get an approximately 1:1 ratio of inheritance of pink to orange in the ×*syriaca* hybrids ($\chi^2 = 0.024$) (Figure 6.3d and Figure 6.5d).

The consequences of selfing being present in the F_1 hybrids were not deleterious yet unexpected. Given the morphology of *Asclepias*, unintentional selfing was unexpected. The pollinia sit in the floral structure and are physically removed from the pistils. Thus, all plants grown under greenhouse conditions were not exposed to pollinators to make crosses. While self-fertility has been previously documented in *Asclepias speciosa* (Bookman, 1984) and *Asclepias syriaca* (Kahn and Morse, 1991), *Asclepias purpurascens* and *Asclepias tuberosa* have low levels of self-fertility (Wyatt and Broyles, 1994; Wyatt 1976). Curiously, we did not observe greater levels of selfing in some hybrids. To date, research regarding self-pollinations in *Asclepias* species indicates that a pollination event occurred before fertilization and subsequent fruit production initiated. For self-pollination to occur, pollinia must still be removed from the anther chamber, have at least one pollinia inserted inside the stigmatic slit, and then have enough pollen cells germinate to fertilize the ovary to avoid pod abortion entirely. To our knowledge, the research team did not accidentally make selves during the hybridization process. Therefore, we can only assume that an errant pollinator performed the few self-pollinations noted.

An additional phenomenon noted during data analysis was the interaction of the two pigment pathways expressed in hybrids. Most clearly seen in the ×*hirtella* hybrids, initial floral pigments were varying shades of pink. Under higher heat stress and as florets aged, the floral color would shift to a yellow golden color for several days before completely senescing (Figure 6.1). *Asclepias hirtella* is green in color, but when the flowers are new, there is anthocyanin accumulation at the tips of the petals that degrade over time. This phenomenon has also been observed in *Brunfelsia calycina, Cotinus coggygria,* and *Arabidopsis thaliniana* (Oren-Shamir and Levi-Nissim, 1997; Rowan et al., 2009; and Vaknin et al., 2005). A combination of natural downregulation and reduced synthesis of anthocyanin due to heat, high UV levels for extended periods, and flower age result in loss of pigmentation in the floral structures. We believe we see a similar scenario take place in the ×*hirtella* and ×*purpurascens* hybrids. As the high temperature and light levels within the greenhouse compound with the increasing age of the floral structure, the anthocyanin pigments begin to degrade, lightening the floral color. However, the carotenoid pathway inherited from *A. tuberosa* is still expressed throughout the floral cycle. Carotenoids have been reported as being extremely resilient under high temperatures and can even be upregulated over time (Kim et al., 2021; Nisar et al., 2015; and Wahid, 2007). These facts point to a theory that in ×*hirtella* and ×*purpurascens* F_1 genotypes, there is a codominant pigment expression pathway. Both the anthocyanin and carotenoid pathways from hybrid parents are expressed in the progeny, but depending on environmental conditions, to what degree either color range is represented can change over time or based on the environment. We do not see this same phenomenon expressed in ×*syriaca* or ×*speciosa* F_1 genotypes. The ×syriaca hybrids appear to inherit one pigment pathway or the other, and ×*speciosa* hybrids are all dominantly expressing the phenotype of the pollen parent. Overall, while one would have expected each

hybrid group to inherit phenotypic traits similarly across the groups, this study did reveal significant phenotypic diversity among the parental species and their hybrids.

This study documents research on the inheritance of essential phenotypic traits across hybrid populations resulting from controlled crosses made between *Asclepias tuberosa* and *A. hirtella, A. purpurascens, A. speciosa,* and *A. syriaca*. When assessing presence versus absence of pubescence, glaucous was determined to be a dominantly controlled trait and inherited across all hybrid groups. *Asclepias hirtella* hybrids inherited pubescence at a 0:1 ratio, *A. speciosa* at a 1:3 ratio, *A. syriaca* at 1:1, and *A. purpurascens* at a 9:7 ratio. White sap was also a dominantly controlled trait and was expressed at a 1:0 ratio across all hybrid groups. Flower color inheritance varied depending on the pollen parent combination used to create the segregating populations. Both ×*hirtella* and ×*purpurascens* had intermediate floral color but codominantly inherited both parents' anthocyanin and carotenoid pathways. Expression of the anthocyanin pathway began at bud break, but as environmental factors degraded the pigmentation, the carotenoid pigments remained. Degradation led to flowers changing color as they aged from pink to yellow in ×*hirtella* and bicolored progeny in ×*purpurascens.* The ×*speciosa* hybrids dominantly inherited flower coloration from the pollen parent *A. speciosa,* with little to no variation in the progeny. Finally, ×*syriaca* inherited flower colors from both parents in a 1:1 ratio. Approximately half the genotypes were yellow or orange, and the other half was pink in various hues. These findings provide a starting point for future breeding efforts in *Asclepias,* elucidating the probable inheritance of specific phenotypic traits if other interspecific hybridization attempts are made.

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Table 6.1: *Asclepias* F_1 hybrids pubescence inheritance (Pubescent/Glaucous) ratios tested using Chi-square test with P values. $X^2_{0.05,1}$= 3.84 and α = 0.05. Bolded values indicate inheritance ratios that have acceptable χ^2 values.

Pollen Parent	N	Pubescent	Glaucous	Expected Ratio	Pubescent (Exp.)	Glaucous (Exp.)	X^2	P-value
***A. hirtella*[z]**	972	64	908	0:1	0.0	972.0	5.74	0.02
				1:15	61.4	919.6	0.24	***0.63***
A. speciosa	878	203	675	**1:3**	219.5	658.5	1.65	***0.20***
A. syriaca	479	249	230	**1:1**	239.5	239.5	0.75	***0.39***
A. purpurascens	583	346	237	1:1	293.0	293.0	20.38	0.0001
				9:7	327.9	255.0	2.27	***0.13***

[z] After removal of the outlier pod, a Chi-square test confirmed that *A. hirtella* does fit the 0:1 pubescence to glaucous inheritance ratio (X^2 = 0.46 and p-value = 0.49).

Table 6.2: *Asclepias* F_1 hybrids sap color inheritance (White or Clear) ratios tested using Chi-square test with P values. $X^2_{0.05,1}$= 3.84 and α = 0.05. Bolded values indicate inheritance ratios that have acceptable χ^2 values.

Pollen Parent	N	White Sap	Clear Sap	Expected Ratio	White Sap (Exp.)	Clear Sap (Exp.)	X^2	P-value
***A. hirtella*[z]**	972	909	63	1:0	972.0	0.0	5.06	0.03
				15:1	911.3	60.7	0.17	*0.68*
A. speciosa	880	875	5	**1:0**	880.0	0.0	1.66	***0.20***
***A. syriaca*[y]**	478	438	40	1:0	478.0	0.0	3271.03	0.001
				15:1	448.2	29.8	3.66	*0.04*
A. purpurascens	587	569	15	**1:0**	587.0	0.0	0.96	***0.54***

[z] After removing the outlier pod, a Chi-square test confirmed that *A. hirtella* does fit the 1:0 white sap to clear sap inheritance ratio (X^2 = 0.88, p= 0.35).

[y] After removing the two outlier pods, a Chi-square test confirmed that *A. syriaca* does fit the 1:0 white sap to clear sap inheritance ratio (X^2= 0.29, p= 0.59).

Figure 6.1. Average flower color (±Standard Deviation) of *Asclepias tuberosa* x *A. hirtella* hybrids based on pods with over 15 surviving genotypes analyzed compared to the pollen parent *A. hirtella* and the maternal parent *A. tuberosa*. Color values were determined using the Royal Horticultural Society Colour Chart metrics.

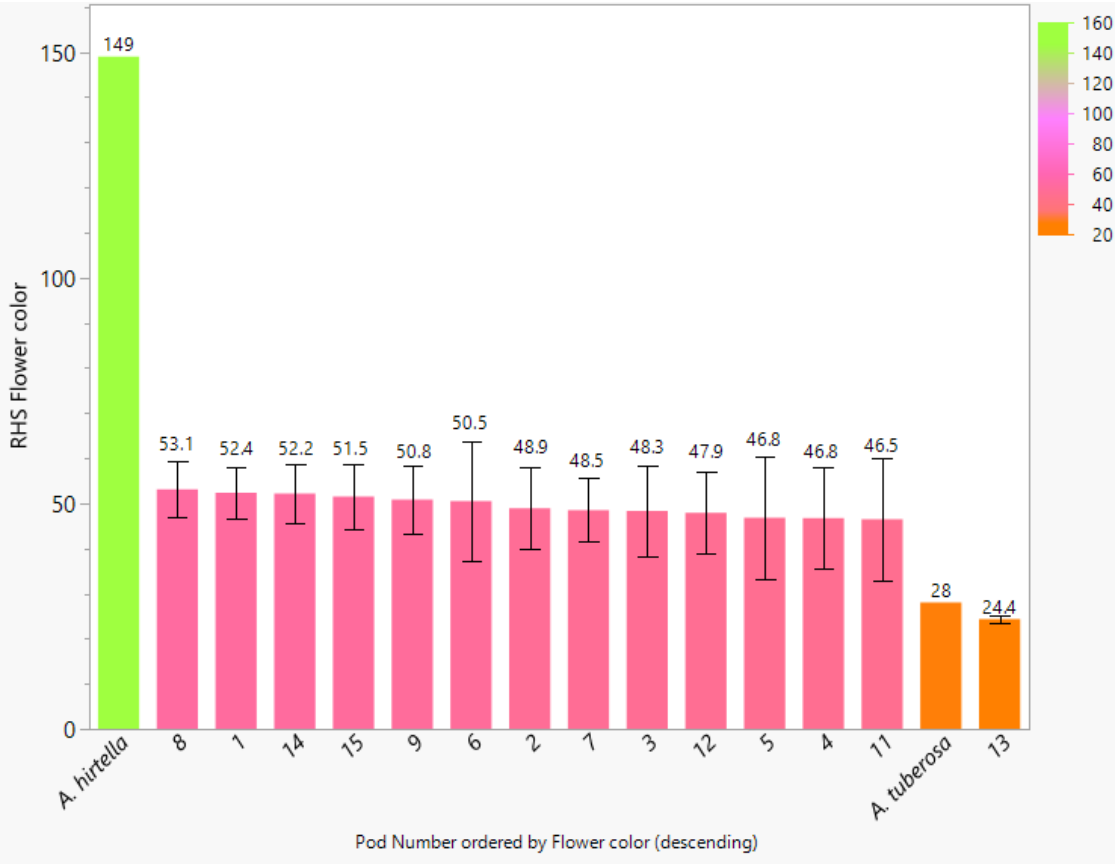


Figure 6.2. The overall distribution of flower colors as based on the Royal Horticultural Society Colour metrics. A. *A. tuberosa* × *A. hirtella*, B. *A. tuberosa* × *A. purpurascens*, C. *A. tuberosa* × *A. speciosa*, and D. *A. tuberosa* × *A. syriaca*.

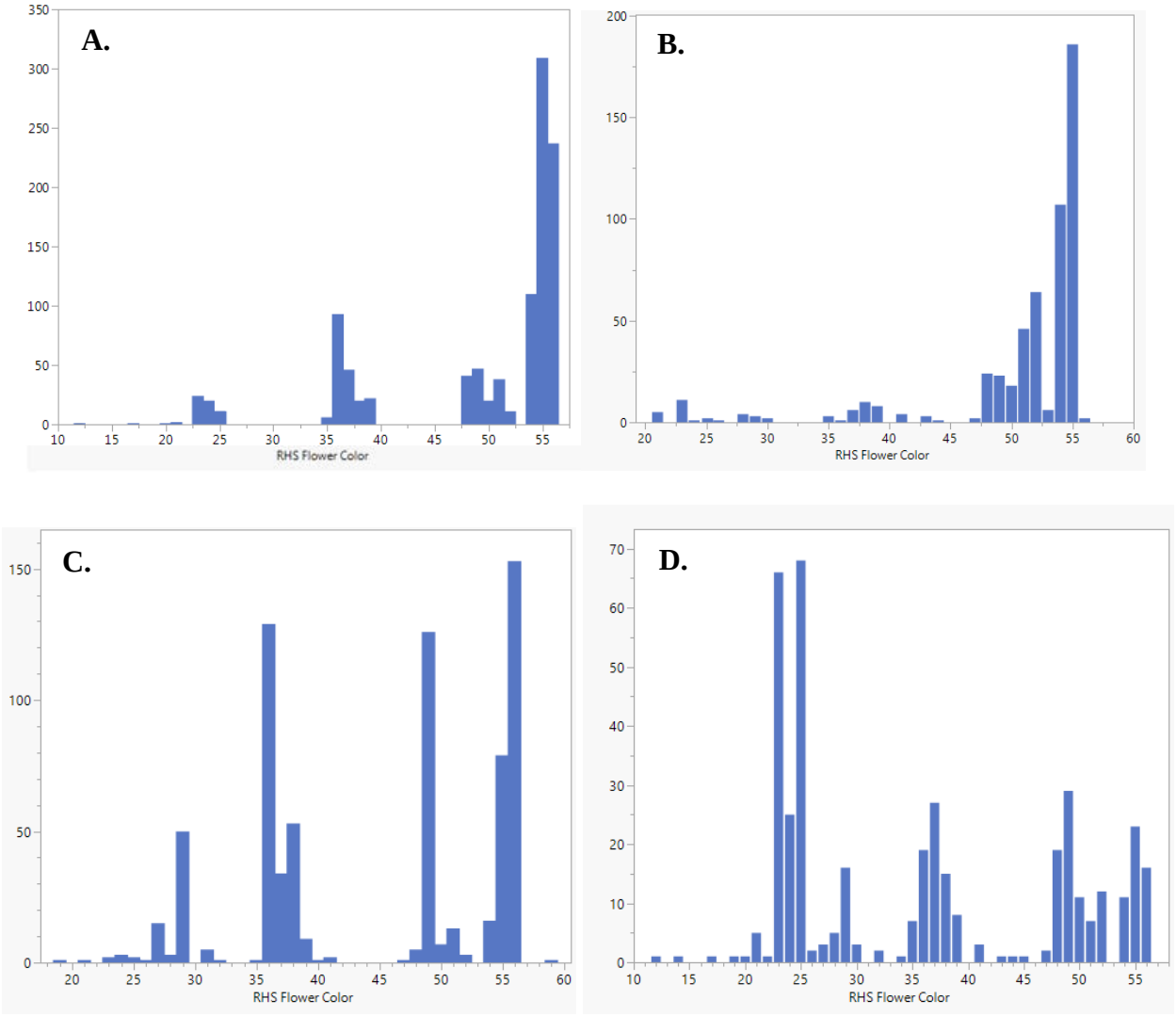


Figure 6.3. Distribution of the relative flower color based on their RHS color metric number for all genotypes across the four *Asclepias* hybrid groups. A. *A. tuberosa* × *A. hirtella*, B. *A. tuberosa* × *A. purpurascens*, C. *A. tuberosa* × *A. speciosa*, and D. *A. tuberosa* × *A. syriaca*.

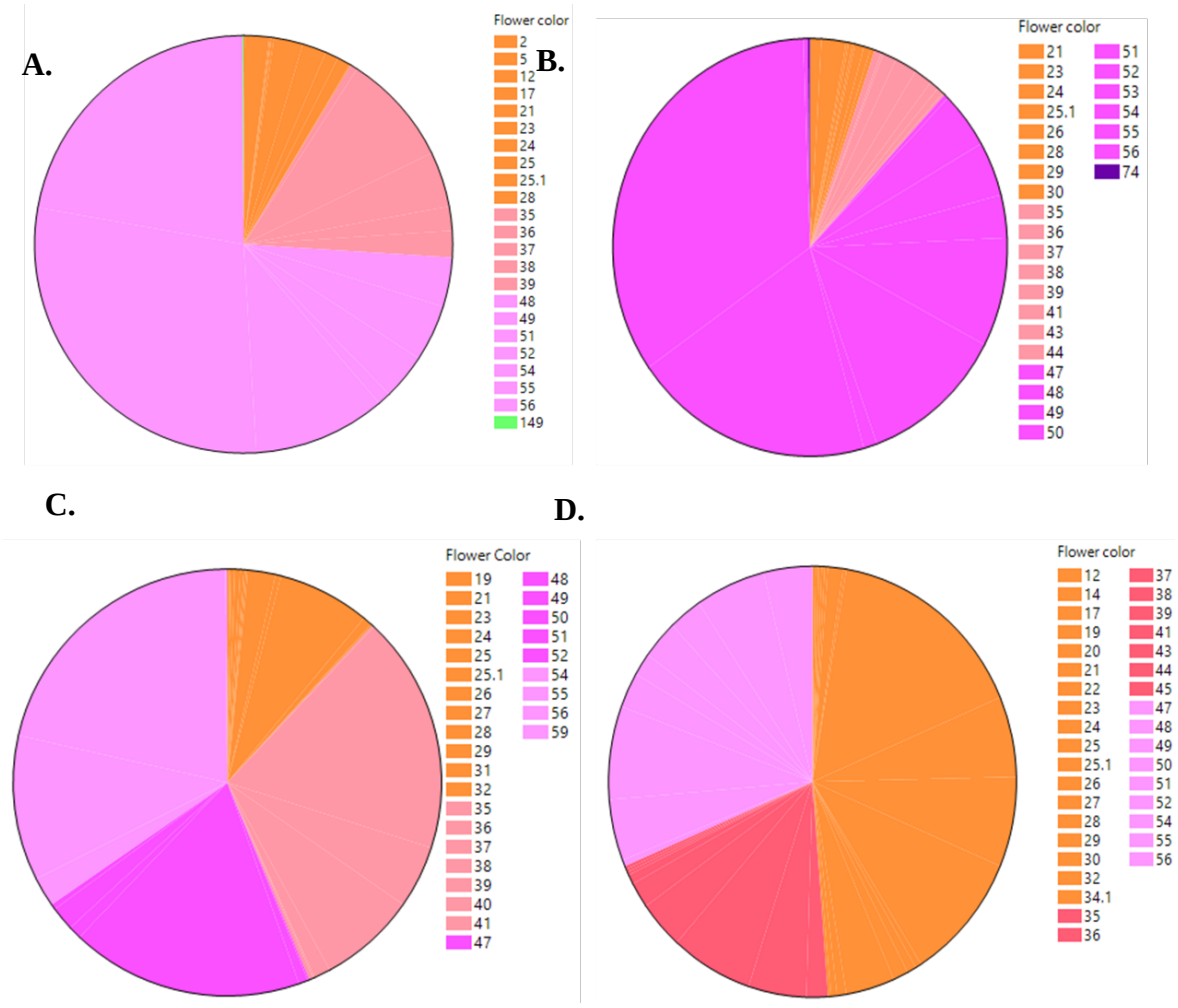


Figure 6.4. Average flower color (±Standard Deviation) of *Asclepias tuberosa* x *A. purpurascens* hybrids based on pods with over 15 surviving genotypes analyzed compared to the pollen parent *A. purpurascens* and the maternal parent *A. tuberosa*. Color values were determined using the Royal Horticultural Society Colour Chart metrics.

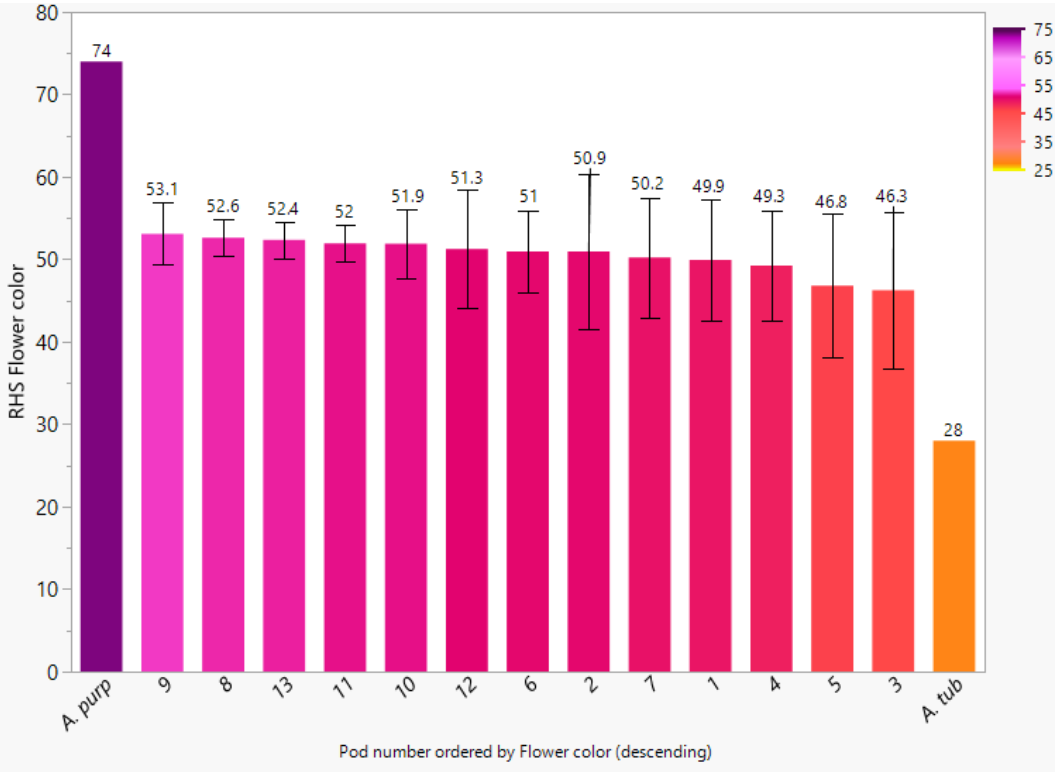


Figure 6.5. Sample of the observed flower color diversity within the four hybrid groups. *Asclepias tuberosa*, the maternal parent, is always pictured to the right. Measurement increments are in cm. A. *A. tuberosa* × *A. hirtella*, B. *A. tuberosa* × *A. purpurascens*, C. *A. tuberosa* × *A. speciosa*, and D. *A. tuberosa* × *A. syriaca*. For A. we also included the pollen parent *A. hirtella* for size and color reference.

Figure 6.6. Average flower color (±Standard Deviation) of *Asclepias tuberosa* x *A. speciosa* hybrids based on pods with over 15 surviving genotypes analyzed compared to the pollen parent *A. speciosa* and the maternal parent *A. tuberosa*. Color values were determined using the Royal Horticultural Society Colour Chart metrics.

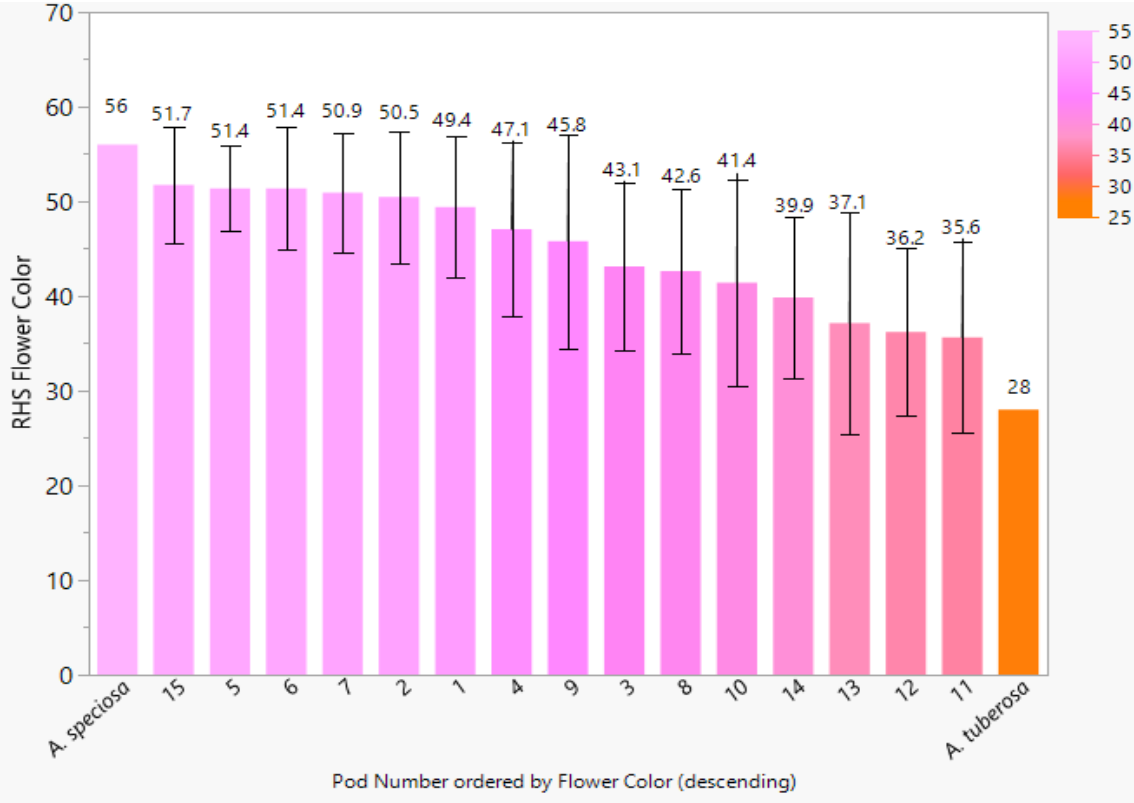
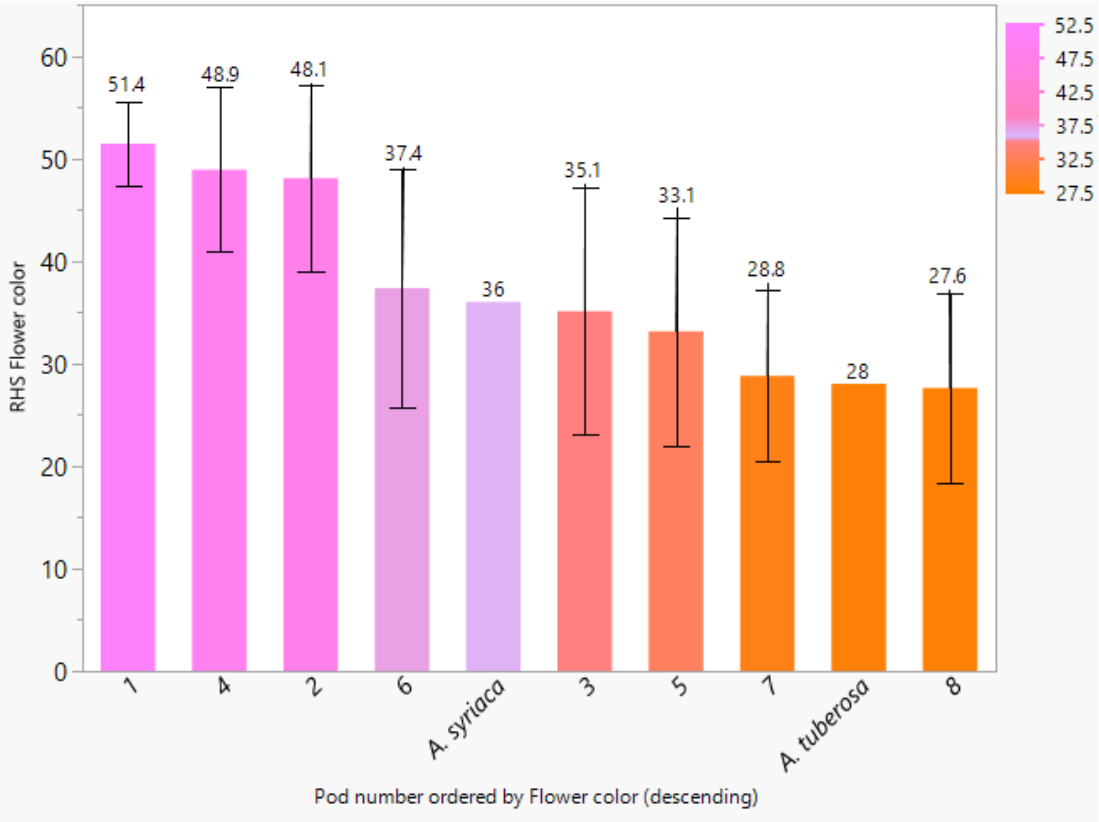


Figure 6.7. Average flower color (±Standard Deviation) of *Asclepias tuberosa* x *A. syriaca* hybrids based on pods with over 15 surviving genotypes analyzed compared to the pollen parent *A. syriaca* and the maternal parent *A. tuberosa*. Color values were determined using the Royal Horticultural Society Colour Chart metrics.



CHAPTER 7

Inheritance of Foliage Traits and Flower Height in Interspecific Hybrids of Asclepias

Introduction

Asclepias spp. L. is a diverse herbaceous perennial genus in the Apocynaceae family, with 130 species native to North America and the Caribbean (Woodson, 1954; Fishbein et al., 2011). Having such an expansive geographical range has resulted in enormous phenotypic diversity within the family. Species can range in height from 30-180 cm and have extremely lanceolate to obovate foliage. Flower size among species spans from 0.3-1.5 cm in size, and floral colors can range the entire color spectrum from red to purple. Additionally, there is a wide range among species in the genus to various abiotic tolerances (Lewis et al., 2021). Therefore, ornamental plant breeders could utilize this diversity in morphology and physiologic responses to abiotic stressors among species to create genotypes with unique ornamental phenotypes and improved abiotic tolerances. However, until a commercially viable protocol for interspecific hybridization was developed as part of this project (Lewis et al., 2021), the development of elite cultivars of Asclepias had yet to be explored.

The genus Asclepias has an extensive native range spanning most of North America, yet species are often isolated due to evolutionary (physiographic) boundaries. Habitat degradation and low seed yield rates have also contributed to a reduction in species range and subsequent habitat overlap (Pleasants and Oberhauser, 2012). When species overlap, interspecific hybridization rates remain low, with fruit set often less than five percent (Wyatt and Broyles, 1994). For such a heavily outcrossing species, the low observed fertilization rate is unusual and generally can be attributed to low soil fertility levels that cause pods to abort before maturity due to abiotic stress or incomplete fertilization (Robertson, 1887; Woodson, 1941; and Wyatt, 1976).

To compound this problem, morphologically, *Asclepias* is one of the most reproductively complex species in the plant kingdom (Wyatt, 1976). Much like Orchidaceae, *Asclepias* does not produce loose pollen grains. Instead, pollen is encapsulated in two pollen packets called pollinium housed inside the main structure of the flower called the gynostegium (Stebbins, 1970). There is an opening in the gynostegium called the stigmatic slit that leads to two independent ovaries, of which one must be penetrated for successful fertilization to occur. In natural habitats, pollinia are removed via a pollinator's rake and comb (on legs), dried for a short period (during flight), rotated 90 degrees, and then inserted into the stigmatic slit of the maternal parent to facilitate fertilization. To further complicate this process, pollinia and stigmatic slit size vary depending upon species, and these differences are purported to limit successful naturally occurring interspecific hybridization via pollinator activity (Wyatt and Broyles, 1994).

It is understandable that with so many barriers to interspecific hybridization, few studies have reported upon naturally observed interspecific hybridizations (Wyatt and Broyles, 1992). However, some species have been successfully hybridized under controlled conditions (Kephart and Heiser, 1980). Examples of successful interspecific hybridization under controlled conditions include *A. speciosa Torr.* × *A. syriaca L.* (Stevens, 1945*), A. exaltata L.* × *A. syriaca* (Wyatt and Hunt, 1991*), A. exaltata x A. quadrifolia* Jacq., and *A. purpurascens L.* × *A. syriaca* (Kephart et al., 1988). As all of these successful crosses occurred from species in the same phylogenetic clade (Weitemier et al., 2015), a study by Lewis et al., 2021 attempted to develop interspecific hybrids among parents within and among across the clades documented by Weitemier et al. (2015).

Building upon previous research by Lewis et al. (2021), this study utilized *A. tuberosa* L. as a maternal parent, with seven other species serving as pollen parents. Pollen parents included

A. curassavica L., *A. fascicularis* Decne., *A. hirtella* Woodson, *A. incarnata* L., *A. purpurascens*., *A. speciosa,* and *A. syriaca*. Of those six pollen parents, four successfully developed hybrid seed. These successful paternal species included *A. hirtella*, *A. purpurascens*., *A. speciosa,* and *A. syriaca*. Upon further investigation, the four successful paternal species were classified as being in the same clade as *A. tuberosa*, based upon Weitemier et al. (2015).

Upon the successful interspecific hybridization of *A. tuberosa* (maternal parent) and four paternal species, an inherent next step was to investigate the phenotypic impact of independent assortment among hybrid populations. Studies documenting segregation among hybrid genotypes resulting from controlled crosses have been documented for select traits (Kephart et al., 1988; Lewis et al., 2021, and Wyatt and Broyles, 1994). However, this study questioned if F_1 genotypes would exhibit similar intermediate tendencies as previously noted in pubescence, production of latex sap, and floral pigment production. Furthermore, given the diversity and range in other morphological traits measurable in *Asclepias* species, would intermediate inheritance of traits such as leaf morphology, flower size, and plant height be visible. Especially given there were much more visibly distinct differences observed among parents and progeny.

In other species, inheritance of flower size can vary among species. For example, in broccoli (*Brassica oleracea* L. var. *italica*) and cabbage (*Brassica oleracea* L. var. *capitata*) hybrids, it was determined that corolla, petal width, stamen, and anther length were controlled by additive-dominance epistasis (Shu et al., 2015). Similarly, in *Plumbago auriculata* Lam. × *Plumbago auriculata* ‘Alba’ hybrids, flower size was controlled by a pair of additive-dominant-epistatic major genes (Shen et al., 2020). In three interspecific rose hybrids, flower size, petal size, black spot resistance, and powdery mildew resistance were controlled by additive gene action (no dominance) (Shupert, 2006). Looking at research done on *Asclepias*, a study

done by Kephart et al., 1988, hybrids were made between two distinct *Asclepias* species: *A. exaltata* and *A. syriaca*. Kephart's study found that the F_1 population was intermediate of the two-parent species regarding flower size, which would make sense, given that both species were comparative in size.

In contrast, the species used in our hybrid crosses have floral sizes ranging from 0.3 cm to 0.7 cm in length, from reflexed petal to hood tip. In interspecific *Asclepias* hybrids, we wanted to see if there would be a similar intermediate inheritance pattern observed by Kephart et al. (1988), or if other inheritance patterns emerge from the varied hybrid crosses. Inheritance of height is unique because it can behave as either a quantitative or qualitative trait. For example, in peas, the difference in height is tied to a single major gene that affects internode length (Bernardo, 2014). Similar instances of a single major gene affecting height can be found in maize, rice, wheat, and sorghum (Bernardo, 2014). However, plant height is generally a quantitative trait if heights are normally segregating across the population, controlled by multiple genes, with progeny heights segregating with little pattern (Bernardo, 2014). Inheritance of leaf length and leaf width are also quantitative traits, and thus inheritance should follow a normal distribution in the F_1 progeny (Allard, 1960). If correct, leaf length and width should have a bell-like distribution, with a small percentage having small narrow leaves, a small percentage with large, broad leaves, and the majority falling somewhere in the middle of the range (Bernardo, 2014). Additionally, environmental effects or the (GxE) effect will likely be higher for leaf length, width, and plant height, as heat and water stress can be controlled under greenhouse growing conditions but not eliminated.

One of defining characteristics of *Asclepias* is the critical lack of branching. When seen in the wild or commercial production, most species send up only one stem at a time, and regardless of

pruning measures taken, they fail to send out auxiliary branches to increase branching. A result of this habit is the colloquial classification of *Asclepias* as a weed. For example, in *A. syriaca*, each node of the main stem has two auxiliary buds. Of the two buds, the weakest is aborted, leaving only one viable auxiliary bud if the apical meristem is damaged or removed. Thus, even if pruning was applied to the apical meristem to induce increased branching, there remains only one bud for the plant to branch from (Nolan, 1969). Officially termed bifurcation, this phenomenon has been observed in other species such as *A. speciosa, A. hirtella, A. purpurascens,* and *A. curassavica* L. A species with an exception to this phenomenon is *A. tuberosa*. It is one of the few rare *Asclepias* species that does respond well to pruning and is capable of multiple auxiliary branching events without pruning. In trying to determine the potential inheritance of branching from other ornamentals, *Helianthus annus* L., another species with limited branching, is one where extensive work has been done. That body of work determined that branching can be determined by a singular dominant gene (Putt, 1940; Shull, 1908), duplicate dominant genes (Hockett and Knowles, 1970), three dominant genes (Gavrilova and Anisimova, 2003), and even a singular recessive homozygous (Vedmedeva, 2018). Thus, inheritance of branching can be complicated and complex and highly varied depending on the species used. As is the common theme with much of this paper, there is no literature on branching inheritance regarding *Asclepias*. Therefore, this will probably be the first time inheritance of branching will be observed and measured in *Asclepias.*

This study aims to determine the mode of inheritance for morphological traits in *Asclepias* hybrid's height, leaf length, width, branching, and flower height. It is expected that inheritance will be determined by multiple genes and be intermediates of the parents.

Materials and Methods

Production of the parents and the methods used in the hybridization process are elucidated in Lewis et al., 2021. Of the six *Asclepias* species used as pollen parents, four species successfully hybridized with *Asclepias tuberosa*: *A. hirtella, A. purpurascens, A. speciosa,* and *A. syriaca.* Seeds collected from the hybridizations were germinated either directly after harvest or 30 d post-stratification. Between Nov. 2018 to late Feb. 2019, hybrid seedlings were grown at the University of Georgia Athens campus, College Station Greenhouse Complex (lat. 33.9480°N, long. 83.3773°W) in 804 inserts (T.O. Plastics, Minneapolis, MN) containing 100% perlite (Carolina Perlite Co. Inc., Gold Hill, NC) with a topdressing of vermiculite (TX401, BWI, Greer, SC) to a 0.635 cm depth. Supplemental light was provided by light-emitting diode (LED) arrays (Fluence Spyder with PhysioSpec, Fluence Technologies Inc., Austin, TX), providing a *PPFD* (photosynthetic photon flux density) of 250μmol·m^{-2}·s^{-1} and 15-h daylength. Hybrid seedlings were placed on a mist bench with a misting cycle applying municipal water (pH 6.2 and alkalinity of 11 ppm) at 6 s every 10 m. Greenhouse temperatures were maintained at 25 °C day and 18 °C night. Upon germination and expansion of the first set of true leaves, hybrid seedlings were transplanted into 804 inserts (T.O. Plastics) held by 1020 greenhouse trays (T.O. Plastics) filled with 80% milled peat (Sungro Peat Moss Grower Grade Orange, Agwam, MA) and 20% perlite (Carolina Perlite Co. Inc.). Once established, hybrid seedlings were transplanted into 3.97 L (#1) containers (Classic 400, Nursery Supply, Agwam, MA) containing an 80% bark (1 cm particle size), 20% milled peat mix (Foothills Compost, Gainesville, GA). Hybrid plants were irrigated with municipal water and fertilized twice a week with Peter's 20N- 4.4P-16.6K liquid soluble fertilizer (Scotts Co., Marysville, OH) at 100 ppm. Additional chemical inputs in 2019 (Rowland Chemical Co., Athens, GA) included sprays for aphids: Bifenazate, Abamectin

"Sirroco", spider mites: Spinosad "Conserve SC", Chlorfenapyr "Pylon", Acequinocyl "Shuttle O", Spinetoram "Xxpire", Bifenthrin "Upstar", Abamectin "Lucid", Etoxazole "Tetrasan", Bifenazate "Floramite SC", and Chlorpyrifos "Duraguard ME"(Rowland Chemical Co., Athens, GA). Thrips sprays not overlapping with ones already mentioned under spider mites: Acetamiprid "TriStar", Flonicamid "Aria", and Nouvaluron "Pedestal" (Rowland Chemical Co., Athens, GA). Active ingredients were rotated to avoid tolerance buildup in the pest population, and in 2020 beneficials were used in conjunction with pesticides to maintain better control over pest population numbers. Beneficials were obtained through IPM labs (IPM Laboratories Inc, Locke, NY), with aphid controls being *Aphidius colemani* Viereck, *Chrysoperla rufilabris* Burmeister, and *Hippodamia convergens* Meneville. Spider mite beneficial controls were *Phytoseiulus persimilis* Evans and *Galendromus occidentalis* Nesbit. Thrip biological controls used were *Amblyseius cucumeris* Oudemans and *Stratiolaelaps scimitus* Womersley. Used in conjunction with Bifenazate "Floramite SC" and Acequinocyl "Shuttle O" for spider mites, and Flonicamid "Aria" with Cyclaniliprole and Flonicamid "Pradia" for thrips and aphids, better success with pest control was observed in the 2020 season. Scouting occurred daily, and releases occurred approximately twice a month, varying in beneficial type and quantity on the temperature and season.

Data Collection and Analysis. After the germination of all the hybridized seeds, approximately 4,000 genotypes survived to be potted up into 3.97 L (#1) containers. Measurements were taken at two periods, three months after emergence from germination (juvenile), and six months (at maturity) to capture morphological features accurately and uniformly. Due to a significant spider mite and thrip infestation in mid-July and early September of 2019, a third of the hybrid population had to be cut back to limit the severity of spread. Those genotypes missed the six-

month measurements and were kept and placed into cold storage mid-November with environmental conditions consisting of 96% relative humidity and 3-4 °C. Over three years, we have determined that adequate chilling hour requirements to induce flowering of *Asclepias* to be eight weeks. Once removed, these remaining hybrids were moved into the greenhouse and grown out until they reached maturity at 6 m. Measurements were then taken and completed in July 2020. Parameters measured included: flower color, pubescence, sap color, height, leaf length, leaf width, branching, and flower height. This paper will not cover the inheritance patterns found for flower color, pubescence, and sap color. Instead, those results can be found in Lewis et al., 2022 (submitted for review). Total height, leaf length, and leaf width parameters were measured in cm, from soil base to apex of the primary apical meristem, petiole to tip, and margin to margin. Flower height was measured in cm, from the base of the gynostegium to the horn tip, ignoring the reflexed petal segment. Branching was measured empirically on a scale of zero to five, 0= no branching, 1= one lateral branching event, 2= two lateral branching events observed, 3= multiple branching observed in the lower half of the plant, 4= multiple branching events present on the lower two-thirds of the plant, and 5= multiple branching events observed in all locations on meristems. All noted parameters were recorded for both the hybrid genotypes and the parents used in the crosses (Table 7.1).

Of the nearly 4,000 hybrid genotypes, after measurements were finished; 1244 genotypes from *A. tuberosa* × A. *hirtella* (×*hirtella*), 624 genotypes from *A. tuberosa* × *A. syriaca* (×*syriaca*), 1256 genotypes from *A. tuberosa* × *A. speciosa* (×*speciosa.*), and 792geotypes from *A. tuberosa* × *A. purpurascens* (×*purpurascens*). Data on the hybrids recorded from which pod each genotype originated, which seed from a said pod, and the date the cross was made. Inheritance patterns were investigated at a pod level and between pollen parents to investigate significant

differences. When analyzing the pollen parents, 500 randomly selected genotypes were selected from each pollen parent to reduce extraneous error from vastly different population sizes. Between pods, differences were analyzed by taking pods with at least 15 successions to ensure results had enough statistical power to be significant while capturing the full extent of variation. The number of pods analyzed per pollen parent varied due to germination and survivability differences; thus, conclusions between pollen parents were not analyzed using the same data set. All data collection occurred in a controlled environment greenhouse structure with uniform environmental conditions. Regulating temperature, light, and production inputs (e.g., soil and fertility) was performed to minimize environmental effects on the production of F_1 phenotypes. F_1 genotypes were arranged in a completely randomized design within the greenhouses. Statistical analysis was performed using JMP (version 13.0; SAS Institute, Cary, NC). Data were analyzed to determine normality and homogeneity, with one-way analysis of variance (ANOVA) and separation of treatment means using Tukey's HSD with alpha at $\alpha = 0.05$ to analyze differences. In addition, Anderson-Darling Goodness of Fit tests were performed to test for normal distributions of data, and transformations of data to fit normality using Logarithmic and Exponential fits were trialed but not used in the final analysis. As there was no significant difference in results, only p-values of the Anderson-Darling Goodness of fit test using normal distributions were reported.

Results and Discussion

Comparisons across the four hybrid groups to *A. tuberosa*, the maternal parent, were conducted for the commercially important traits of height and branching. *Asclepias tuberosa* had an average overall height of 37.8 ± 5.6 cm, only significantly differing from the ×*speciosa* hybrid's average

height of 62.9 ± 0.7 cm (p = 0.001) (Figure 7.1). While the other hybrid groups, excluding ×*speciosa,* did not have taller heights than *A. tuberosa,* all four groups were significantly different. The ×*speciosa* hybrids with an average height of 62.9 ± 0.7 cm were taller than ×*syriaca* hybrids at 52.7 ± 0.7 cm (p = 0.0001) (Figure 7.1). In turn, ×*syriaca* hybrids were taller than ×*hirtella* hybrids, which averaged 48.3 ± 0.7 cm in height (p = 0.0001) (Figure 7.1). Lastly, ×*purpurascens* hybrids were shorter than the other hybrid groups, with an average height of 42.8 ± 0.7 cm (Figure 7.1). Therefore, ×speciosa hybrids were the tallest on average across the hybrid groups, followed by ×*syriaca*, ×*hirtella,* and then ×*purpurascens*. However, only ×*speciosa* significantly differed in average heights compared to *A. tuberosa* (Figure 7.1). Branching has somewhat similar results. *Asclepias tuberosa* has an average branching score of 3.00, and while none of the hybrid groups differed in scoring to *A. tuberosa,* they did differ amongst each other. Closest in average branching to *A. tuberosa* was ×*hirtella* hybrids, with an average branching score of 2.7 ± 0.07 (Figure 7.2). Differing from ×*hirtella* were the ×*purpurascens* hybrids, with an average branching score of 2.12 ± 0.07 (Figure 7.2). Reduced branching score then went to ×*speciosa* hybrids, having a 1.8 ± 0.07 score, and lastly, the ×*syriaca* hybrids with a 1.5 ± 0.07 score (Figure 7.2). With none of the hybrids groups differing significantly from *A. tuberosa* regarding their branching scores, while there were branching differences between the hybrid groups, no significant increase or decrease in branching was detected.

With morphological differences between the parents utilized in these hybridizations, further analysis of inheritance was evaluated based on the individual hybrid group. For the ×*hirtella* hybrids, none of the parameters fit a normal distribution. For height, the average ×*hirtella* value is 47.67 ± 12.84 cm (standard deviation), closer to the maternal parent's average height value of 37.8 ± 5.6 cm than the paternal parent *A. hirtella* at 99.44 ± 14.42 (Table 7.1). Based on

skewness values, ×*hirtella* hybrids are relatively symmetrical, only slightly skewed towards *A. hirtella*, having a skewness value of +0.43. While height does not fit a normal distribution curve, it is still relatively uniform and slightly favors heights closer to *A. hirtella* than *A. tuberosa* (Table 7.2 and Figure 7.3a). Leaf length of ×*hirtella* hybrids had an average length of 7.40 ± 2.33 cm (Table 7.1), compared to 8.11 ± 1.80 cm of *A. tuberosa* and 9.62 ± 1.15 cm in *A. hirtella*. Based on the calculated skewness values, ×*hirtella* hybrids values have a +0.71 skewness to the right (Table 7.2 and Figure 7.4a). Given that both parent values are slightly higher than the average ×*hirtella* value, this was not surprising. Skewness values indicate that the hybrids performed approximately intermediate of the parents and had more outliers to attribute for the reduced average value (Table 7.2). Leaf width increased in the hybrids compared to the parents. *Asclepias tuberosa* had an average leaf width of 1.40 ± 0.48 cm, *A. hirtella* at 0.78 ± 0.24 cm, and ×*hirtella* hybrids at 0.91 ± 0.61 cm (Table 7.1). The distribution of all ×*hirtella* hybrids was skewed to the right (+4.09), with most genotypes inheriting a leaf width more similar to *A. tuberosa* than an intermediate or *A. hirtella* size (Table 7.2, Figure 7.4a). Flower height in the parents averaged 0.5 cm for *A. tuberosa* and 0.2 cm for *A. hirtella* (Table 7.1). The average flower height of the ×*hirtella* hybrids was 0.34 ± 0.12 cm, over half of the population (363/546) having either 0.30 or 0.35 cm flower heights (Table 7.1 and Figure 7.5a). A skewness value of +1.84 reflects the earlier observation that values skewed to the right favor larger flower heights while still being intermediate between the two parents (Table 7.2 and Figure 7.5a). Lastly, branching score values for *A. tuberosa* averaged 3.00 ± 1.15, *A. hirtella* at 0.00, and ×*hirtella* hybrids at 2.48 ± 1.78 (Table 7.1 and Figure 7.6a). Opposite of other parameters analyzed in this group, the skewness value was -0.12, indicating that the data was roughly symmetrical, and branching was less likely to be inherited in ×*hirtella* hybrids in an intermediate

fashion compared to other phenotypic traits (Table 7.2, Figure 7.6a). Overall, height, leaf length, and branching trended towards normal distributions, as one would see in an F_1 hybrid population. Leaf width and flower height did show indications of inheriting major genes that influenced an increase of width and flower size, being more similar to *A. tuberosa* than *A. hirtella*.

For the ×*purpurascens* hybrids, many of the same trends were present as with the ×*hirtella* hybrids. Overall, none of the parameters measured fell into a normal distribution based on a goodness of fit test, but many parameters were close. Average height of *A. tuberosa* was 37.80 ± 5.60 cm, *A. purpurascens* 45.53 ± 9.51 cm, and ×*purpurascens* was 42.68 ± 13.14 cm (Table 7.1). While distribution did not pass the test or normality, the skewness of the data was only +0.93. The skewness value indicated a slight increase of size but was only moderately skewed towards *A. purpurascens* (Table 7.2, Figure 7.3b). Leaf length averages were 8.11 ± 1.80 cm for *A. tuberosa*, 14.93 ± 0.29 cm for *A. purpurascens*, and 9.22 ± 2.53 cm for ×*purpurascens* (Table 7.1). Skewness was only reported at +0.14 or reasonably symmetrical, with a larger variance in values of 6.25 cm (Table 7.2, Figure 7.4b). Leaf width was similar in that while there appeared to be significant differences in average widths, 1.40 ± 0.48 cm for *A. tuberosa*, 7.36 ± 1.21 cm for *A. purpurascens*, and 2.71 ± 1.08 cm for ×*purpurascens*, distribution across the population looked close to normal. The skewness value was only +0.60, with a variance of 1.22 cm (Table 7.2). Flower height averages were 0.50 cm for both *A. tuberosa* and *A. purpurascens*, and ×*purpurascens* was 0.51 ± 0.07 cm (Table 7.1, Figure 7.5b). Skewness was higher, coming in at +1.66, indicating a skewness towards larger floral sizes or being of equal or slightly larger size than either parent. Lastly, branching values were 3.00 ± 1.15 for *A. tuberosa*, 0.33 ± 0.58 for *A. purpurascens*, and 2.12 ± 1.61 for ×*purpurascens* (Table 7.1 and Figure 7.6b). Data was fairly symmetrical, having a skewness value of +0.22, although looking at the distribution, it appears to

be randomly distributed (Table 7.2, Figure 7.6b). In general, for ×*purpurascens* hybrids, all parameters except for flower height were normally distributed, intermediates of both parents. Flower height was the one exception whereby flower size inheritance was equal or greater size than the parents.

As with the previous hybrid groups, none of the parameters we took data on fit a normal distribution based on a goodness of fit test, even if the distributions looked to be normally distributed. Looking at average heights across the parents and hybrids, *A. tuberosa* had an average height of 37.80 ± 5.60 cm, *A. speciosa* at 103.5 ± 25.47 cm, and ×*speciosa* hybrids at 64.09 ± 17.12 cm (Table 7.1). Here the skewness value was +0.53, indicating a slight skewness to the right, but overall heights were intermediate of the parents (Table 7.2, Figure 7.3c). Leaf length averages were 8.11 ± 1.80 cm for *A. tuberosa*, 12.04 ± 1.05 cm for *A. syriaca*, and 10.59 ± 2.70 cm for ×*syriaca* hybrids (Table 7.1). The overall distribution of values was reasonably symmetrical, as the skewness value was +0.27, hybrids only slightly inheriting leaf lengths more similar to *A. speciosa* than *A. tuberosa* (Table 7.2, Figure 7.4c). Leaf width averages were 1.40 ± 0.48 cm for *A. tuberosa*, 5.07 ± 0.91 cm for *A. speciosa*, and 2.67 ± 1.11 cm for ×*speciosa* hybrids (Table 7.1). The overall distribution of values was slightly skewed to the right at a value of +0.91, indicating a slight bias towards inheritance of widths more similar to *A. speciosa* than *A. tuberosa* (Table 7.2, Figure 7.4c). Flower height averaged at 0.50 cm for *A. tuberosa*, 0.91 ± 0.1 cm for *A. speciosa*, and 0.68 ± 0.17 cm for ×*speciosa* hybrids (Table 7.1). Differing from previous hybrid groups, ×*speciosa* is slightly skewed to the left, at -0.21, with an overall variance in the height of 0.03 cm (Table 7.2, Figure 7.5c). Lastly, average branching for *A. tuberosa* was 3.00 ± 1.15, 0.00 for *A. speciosa*, and 1.64 ± 1.50 for ×*speciosa* hybrids (Table 7.1). The distribution of all hybrid progeny indicates a slight skewness to the right of +0.84,

favoring *A. tuberosa*'s branching values ever so slightly (Table 7.2, Figure 7.6c). Overall, the ×*speciosa* hybrids were intermediate of the parents for all parameters with the potential exception of branching, where higher levels of branching were inherited.

With the ×*syriaca* hybrids, none of the parameters fit a normal distribution based on a goodness of fit test. However, in this hybrid cross, the leaf length and leaf width parameters came close to achieving normality, with p-values of 0.0014 and 0.002. The average height for *A. tuberosa* was 37.80 ± 5.60 cm, *A. syriaca* was 113 ± 17.20 cm, and ×*syriaca* was 52.62 ± 17.03cm (Table 7.1). The skewness of data for height was calculated to be +0.47, being relatively symmetrical, and if there is any skewness, it being towards *A. syriaca* heights instead of *A. tuberosa* (Table 7.2, Figure 7.3d). Leaf length averages were 8.11 ± 1.80 cm for *A. tuberosa*, 13.4 ± 1.69 cm for *A. syriaca*, and 11.20 ± 3.43 cm for ×*syriaca* hybrids (Table 7.1). While not fitting normality, the skewness was -0.04, with most hybrids having intermediate lengths compared to the parents (Table 7.2, Figure 7.4d). Leaf width averages for *A. tuberosa* were 1.40 ± 0.48 cm, *A. syriaca* at 3.77 ± 0.84 cm, and ×*syriaca* hybrids at 2.82 ± 1.18 cm (Table 7.1, Figure 7.4d). Almost fitting a normal distribution, the skewness to the data was +0.43 with a variance of 1.22 cm, which means that leaf width inheritance in the hybrid progeny was approximately intermediate of the parents (Table 7.2). Flower heights were also intermediate in the hybrid progeny, with parents having average heights of 0.50 cm for *A. tuberosa*, 0.43 ± 0.05 cm in *A. syriaca*, and 0.48 ± 0.06 cm in ×*syriaca* (Table 7.1, Figure 7.5d). Skewness was 0.01 with a variance of 0.003 cm, with three groupings of flower sizes seen in the progeny (Table 7.2, Figure 7.5d). Branching averages were 3.00 ± 1.15 cm in *A. tuberosa*, 0.00 in *A. syriaca*, and 1.46 ± 1.61 in ×*syriaca* (Table 7.1). The distribution was slightly skewed to the right, to a degree of +0.84, indicating a slight increase of branching compared to *A. syriaca* but not outperforming *A. tuberosa* (Table 7.2, Figure 7.6d).

Overall, ×*syriaca* hybrids followed an approximately normal distribution for all parameters, being intermediates of the parents.

By and large, all hybrid F_1 populations performed as we expected. Allard (1960) and Bernardo (2014) noted that inheritance of quantitative traits should follow a normal distribution in the F_1 population. While statistically none of our parameters across any hybrid group fit into a normal distribution, hybrid genotypes averaged intermediate values of the parents. If major genes had controlled some traits, we would have expected to see higher levels of skewness than any observed in this study. One point of interest was the performance of ×*purpurascens* hybrids for flower height. Unlike other traits, inheritance of flower height was the same or exceeded the size of either parent used in the cross. By definition, a hybrid that outperforms the parents would be the expression of heterosis (East, 1936). Typically, heterosis is seen as increases in yield, flower number, stem thickness, leaf number, or branching in hybrid populations (East, 1936). Therefore, we hypothesized that heterosis could be observed in the F1 population of ×purpurascens hybrids in this particular case. A study by Krieger et al. (2010) on tomatoes partially supported this hypothesis, as they observed that flower number and number of inflorescences were determined by heterosis. They determined that the promotion of flowering was due to heterosis. While we did not think to record inflorescence numbers on our hybrids, we did observe a significant increase in floral production in the hybrids compared to the parents. Knowing that flower number increase is caused by heterosis, and flower height can be influenced, heterosis may have caused the increased flower height in ×*purpurascens* (East, 1936 and Krieger et al., 2010).

This study reported the inheritance patterns of the foliar traits height, leaf length, leaf width, flower height, and branching between four hybrid groups of *Asclepias*. Compared to their maternal parent, A. tuberosa, only ×*speciosa* differed significantly in height between the hybrid

groups. For branching across the four groups, none of the hybrids inherited higher or significantly lower branching amounts than *A. tuberosa*. Within the hybrid groups, approximately normal distributions for height, leaf length, and flower height were seen in the ×*hirtella* hybrid group. Leaf width and Flower height did show indications of inheriting major genes that influenced an increase of width and flower size, being more similar to *A. tuberosa* than *A. hirtella*. For ×*purpurascens,* all parameters except flower height were normally distributed, being intermediates of both parents. Flower height was the one exception whereby flower size inheritance was equal or greater size than the parents. In both ×*speciosa* and ×*syriaca* hybrids, all parameters measured had intermediate values between the two parents and were not normally distributed but trended in a normal distributional fashion. These findings indicated that in regards to quantitative traits observed in these four hybrid groups of *Asclepias*, typical quantitative inheritance occurred. Most quantitative traits are controlled by two or more genes, leading to the wide range of values seen in the progeny. Knowing that an F_1 population yields progeny intermediate of the parents is still helpful. It provides a reference for commercial growers and breeders to know what to expect when working on this species.

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Table 7.1. Comparisons of the straight species measurements on different traits in comparison to their hybrids. *Asclepias tuberosa* is reported first as it was the maternal parent for all crosses, and standard deviations are reported.

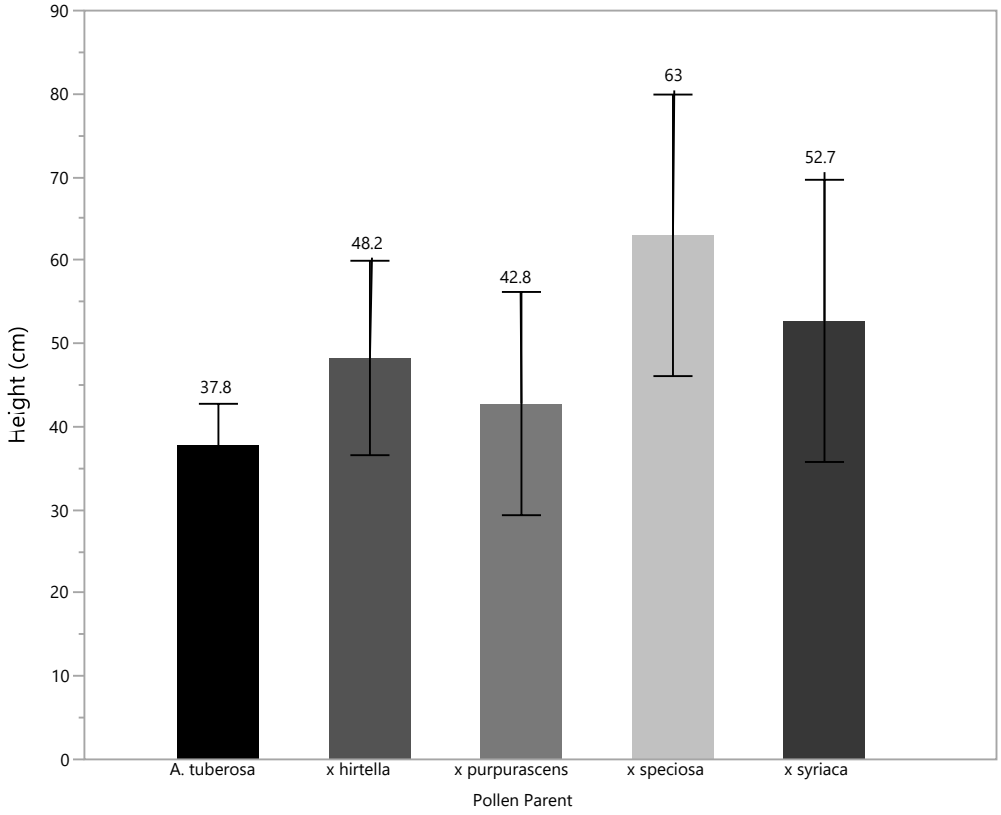
Asclepias types	Height (cm)	Leaf length (cm)	Leaf width (cm)	Flower height[z] (cm)	Branching (0-5)
A. tuberosa	**37.80 ± 4.93**	**8.11 ± 1.80**	**1.40 ± 0.48**	**0.50**	**3.00 ± 1.15**
A. hirtella	**99.44 ± 14.42**	**9.62 ± 1.15**	**0.78 ± 0.24**	**0.20**	**0**
A. purpurascens	**45.53 ± 9.51**	**14.93 ± 0.29**	**7.36 ± 1.21**	**0.50**	**0.33 ± 0.58**
A. speciosa	**103.50 ± 25.47**	**12.04 ± 1.05**	**5.07 ± 0.91**	**0.91 ± 0.10**	**0**
A. syriaca	**113.00 ± 17.20**	**13.40 ± 1.69**	**3.77 ± 0.84**	**0.43 ± 0.05**	**0**
A. tuberosa x *A. hirtella*	47.67 ± 12.84	7.40 ± 2.33	0.91 ± 0.61	0.34 ± 0.12	2.48 ± 1.78
A. tuberosa x *A. purpurascens*	42.68 ± 13.14	9.22 ± 2.53	2.71 ± 1.08	0.51 ± 0.07	2.12 ± 1.61
A. tuberosa x *A. speciosa*	64.09 ± 17.12	10.59 ± 2.70	2.67 ± 1.11	0.68 ± 0.17	1.64 ± 1.50
A. tuberosa x *A. syriaca*	52.62 ± 17.03	11.20 ± 3.43	2.82 ± 1.18	0.48 ± 0.06	1.46 ± 1.61

[z] Flower height refers to a singular floret measured from the base of the hood to the horn tip. Reflexed petals were not included in this measurement as most observers would not assume them to be part of the "flower". Additionally, the petals were easily damaged, which inhibited precise measurement.

Table 7.2. Skewness and Variance values for the distributions of *Asclepias* hybrid groups on different quantitative traits.

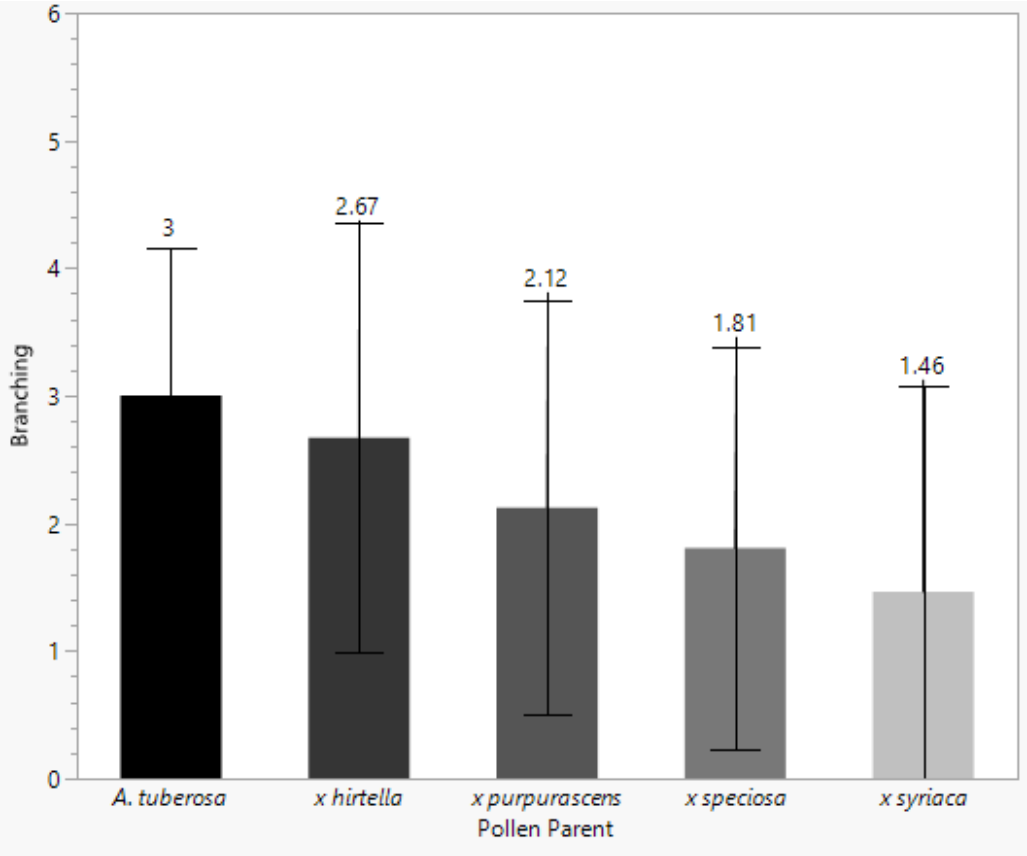
Asclepias types	Height (cm)		Leaf length (cm)		Leaf width (cm)		Flower height (cm)		Branching (0-5)	
A. tuberosa x *A. hirtella*	0.43	165.03	0.71	3.80	4.09	0.19	1.84	0.004	-0.12	3.05
A. tuberosa x *A. purpurascens*	0.93	172.58	014	6.25	0.60	1.22	1.66	0.004	0.22	2.59
A. tuberosa x *A. speciosa*	0.53	295.64	0.27	7.04	0.91	1.25	-0.21	0.03	0.51	2.25
A. tuberosa x *A. syriaca*	0.47	294.43	-0.04	10.58	0.43	1.4	0.01	0.003	0.84	2.62

Figure 7.1. Comparison of the hybrids mean heights to the maternal parent, *Asclepias tuberosa.* Hybrids are reported based on the pollen parent . Standard deviations are shown .



Error bar represents one standard deviation from the mean.

Figure 7.2. Comparison of the hybrids mean branching value to the maternal parent, *Asclepias tuberosa.* Hybrids are reported based on the pollen parent used in the cross. Standard deviation bars are shown.



Error bar represents one standard deviation from the mean.

Figure 7.3. Distribution of height (cm) across *Asclepias* hybrid groups: A. *A. tuberosa* x *A. hirtella*, B. *A. tuberosa* x *A. purpurascens*, C. *A. tuberosa* x *A. speciosa*, and D. *A. tuberosa* x *A. syriaca*. The superimposed line indicates the goodness of fit for the data set.

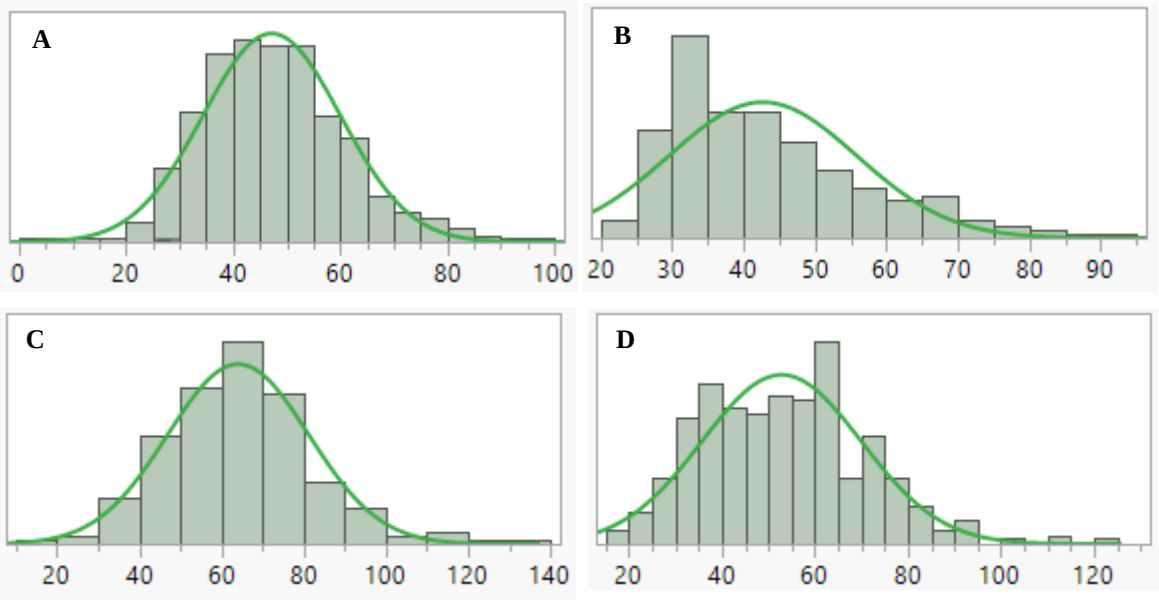


Figure 7.4. Samples of leaf length and widths of the parents and their hybrids (cm). A. *A. tuberosa*, x*hirtella* hybrids, *A. hirtella*. B. *A. tuberosa*, x*purpurascens*, *A. purpurascens*. C. *A. tuberosa*, x*speciosa*, *A. speciosa*. D. *A. tuberosa*, x*syriaca*, *A. syriaca*.

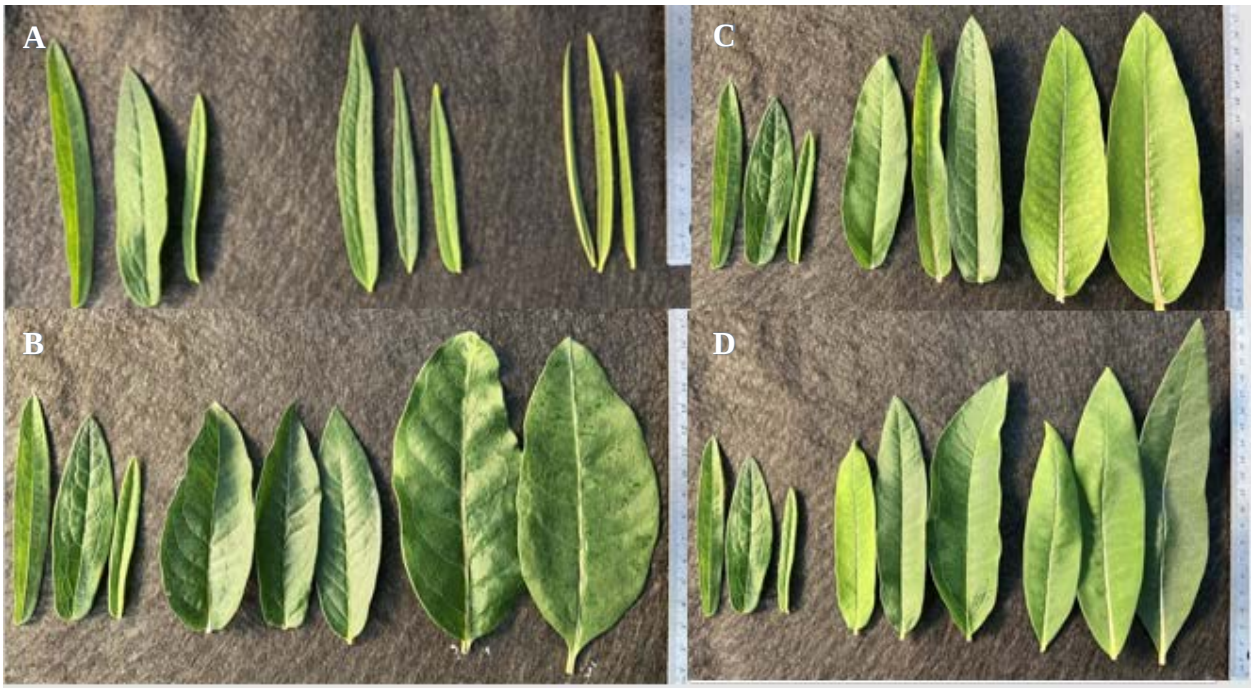


Figure 7.5. Distribution of flower height (cm) across *Asclepias* hybrid groups: A. *A. tuberosa* x *A. hirtella*, B. *A. tuberosa* x *A. purpurascens*, C. *A. tuberosa* x *A. speciosa*, and D. *A. tuberosa* x *A. syriaca*. The superimposed line indicates the goodness of fit for the data set.

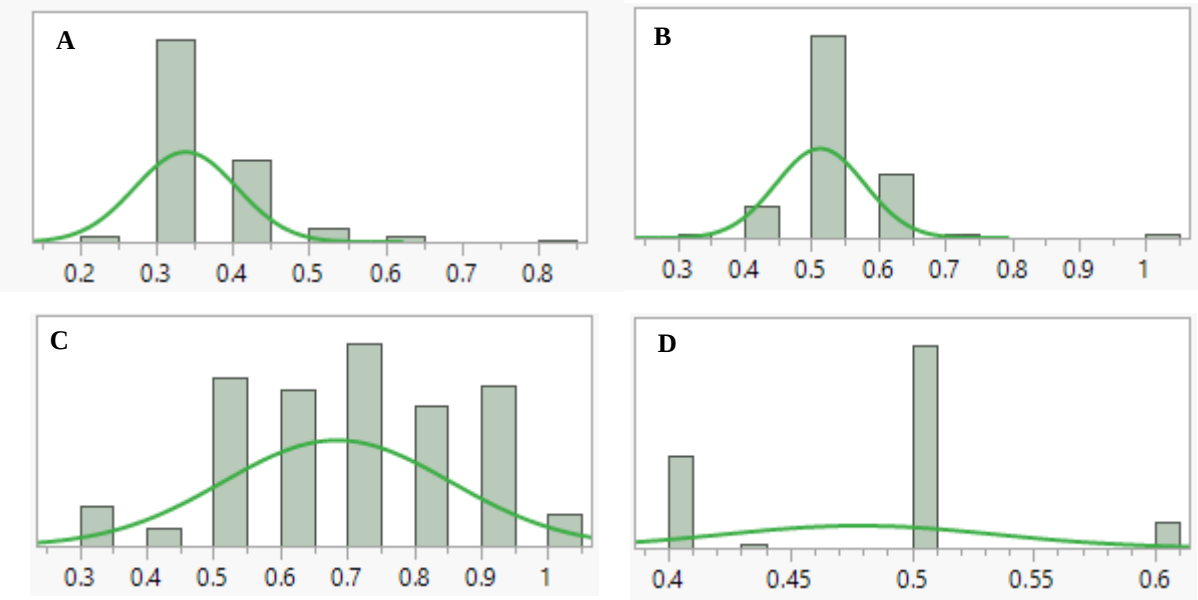
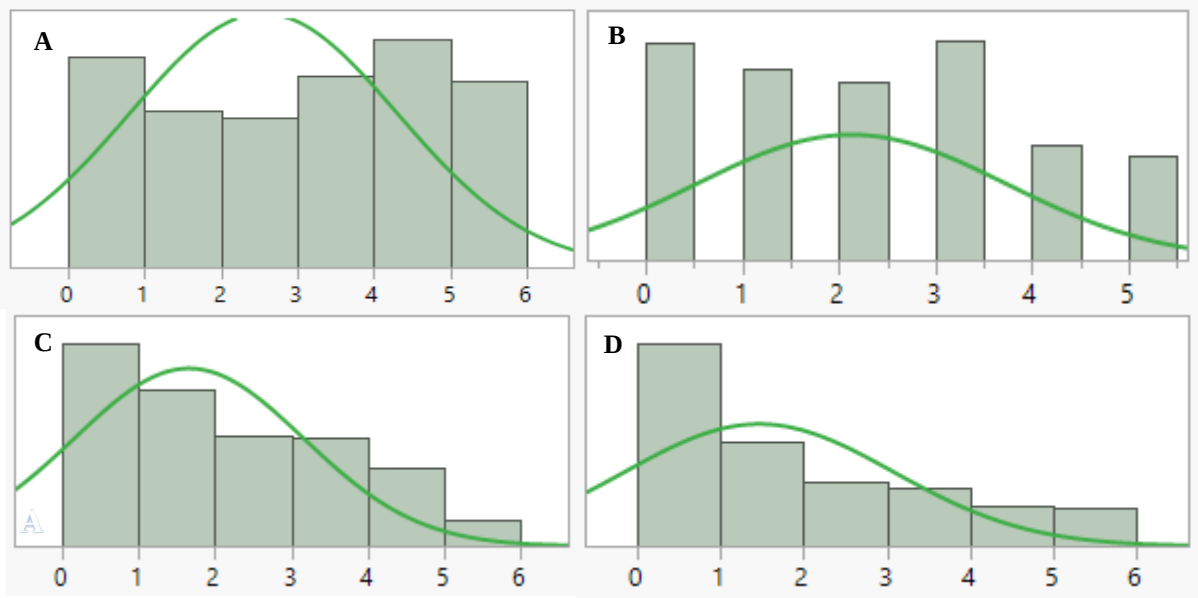


Figure 7.6. Distribution of branching values across *Asclepias* hybrid groups: A. *A. tuberosa* x *A. hirtella*, B. *A. tuberosa* x *A. purpurascens*, C. *A. tuberosa* x *A. speciosa*, and D. *A. tuberosa* x *A. syriaca*. The superimposed line indicates the goodness of fit for the data set.



CONCLUSIONS

Milkweed (*Asclepias* sp.) is a critical pollinator genus across North America and is a host plant for many butterfly species, notably the monarch butterfly (*Danaus plexippus*). However, commercial production of *Asclepias* is currently limited to a few species. Most species lack commercial traits and have minimal branching habits, excessive height, and reduced color variation. In the interspecific hybridization study, a commercially viable *Asclepias* species, butterfly weed (*Asclepias tuberosa* L.), was used as a maternal parent. Three different pollination methods were trialed in an attempt to create interspecific hybrids. Pollination methods included a traditional method, a pollen-solution-based method, and a novel inverted pollinia method. The inverted pollinia method increased pollination success rates fourfold among intraspecific crosses of *A. tuberosa*. Once pollination methods were optimized, *A. tuberosa* was used as the maternal parent, and one-way crosses were made to seven other *Asclepias* species using the inverted pollinia method. Of the seven species that were used as pollen donors, four successfully developed hybrid seeds; green milkweed (*Asclepias hirtella* Woodson), purple milkweed (*Asclepias purpurascens* L.), showy milkweed (*Asclepias speciosa* Torr.), and common milkweed (*Asclepias syriaca* L.). As germination methods vary significantly among *Asclepias* species, three methods of germination were trialed on seed developed via interspecific hybridizations; direct-seeded, cold-moist stratification, and embryo rescue. Of the three methods, cold-moist stratification was superior to direct seeding and embryo rescue. This research was the first documented case of a controlled interspecific hybridization event among these species.

In the vegetative propagation study, cuttings of *Asclepias tuberosa* were taken at two maturities and treated with potassium salt of indole-3-butyric acid (K-IBA) or 1-naphthaleneacetic acid (NAA) at varying concentrations to determine if a commercially viable protocol could be developed. Comparing cuttings taken from container-grown mature stock plants and juvenile seedlings, propagules originating from mature plants resulted in higher survival, likely because of enhanced carbohydrate reserves and photosynthetic capacity. Exogenous auxin (K-IBA or NAA) treatments did not affect cutting survival or root number and length across both maturities of cuttings. The only growth parameter influenced by a specific hormone and concentration was cutting height, which was greater when K-IBA at 1000 or 3000 ppm concentrations were employed, compared to the control with no hormone applied. Ultimately, vegetative propagation can be achieved with commercially acceptable success using vegetatively mature cuttings without the application of exogenous hormones. These findings should afford commercial and conservation-based producers of *A. tuberosa* a more rigid, science-based, simplified, and cost-effective propagation protocol. In addition, as *Asclepias* contains 108 recognized species, this study should afford propagators and researchers of *Asclepias* spp. a platform on which to base future studies. With a successful vegetative propagation protocol established, future propagation studies of *Asclepias* species could further enhance commercial production and enhance conservation efforts.

Complementing the hybrid study and parallel to the propagation study in terms of commercial integration importance, the embryo rescue project aimed to increase *Asclepias* production. Through embryo rescue, genotypes previously lost due to lack of nutrients, incomplete fertilization, late-term abortion, or inability to break dormancy and successfully germinate could be saved and grown in a commercial setting. Our embryo rescue project

determined the optimal growing media (study one) and embryo maturity (study two) to recover mature seedlings from excised embryos of *Asclepias tuberosa* and compared the results to those of traditional methods of seed germination (in a soilless substrate). Study one investigated three different media: Murashige and Skoog (MS) medium at full strength, half strength, and woody plant medium. MS medium at half strength was optimal for *A. tuberosa* germination and maturation, with greater root and shoot lengths at the time of harvest compared to MS and WPM. The higher success of half-strength MS over the other treatments was likely due to the natural affinity of milkweed species for soils with low nutritional content, most closely aligned with ½ MS medium supplements. These results indicated that ER could be a commercially viable alternative to germination of milkweed species in soilless substrates, reducing propagation time by 88 d (12.6 weeks) and, as a result, possibly increasing profitability. Another goal of this study was to determine at what point embryo maturity affected *A. tuberosa* embryo germination and growth. In study two, the effects of MS medium at half strength on embryo maturation 90, 60, and 30 days after pollination (DAP) were investigated. Embryos rescued at 30 DAP resulted in the lowest root and shoot growth, whereas 60 DAP yielded the highest growth. The optimal time to harvest embryos was 60 DAP; embryos at 30 DAP were capable of germination but not maturation. A similar increase in germination rates was observed for all embryo maturities than seed germinated using a soilless substrate. A mean germination rate of 97.4% was observed using embryo rescue, but it was 72.3% with mature seed germinated in a soilless substrate typical of commercial production. Although there was no statistically significant difference between germination percentages among embryos of differing maturities or medium types, ER germination rates were uniformly higher than fully mature seed sowed in a soilless substrate.

The protocol developed for this study should help standardize production, reduce propagation time, and improve milkweed's commercial acceptance and profitability. This study documented a successful protocol for ER in *A. tuberosa*, including surface sterilization techniques, excision methods, stratification times, medium type/strength, and embryo harvesting time. Previous studies investigated either embryogenesis of species in the *Asclepias* family (but not specific *Asclepias* species) or the organogenesis of *Asclepias* spp. from leaf or meristem tissue. To our knowledge, this is the first study to investigate ER of embryonic tissue from seeds, and it is the first to document the effect of embryo maturity on the successful development of explants in culture. Because methods for ER vary from species to species, this study aimed at providing a commercial ER protocol for *Asclepias tuberosa*, with the hope that this protocol may apply to other milkweed species. In doing so, further research could use this information to create a viable propagation protocol for threatened or endangered milkweed species to enhance restoration efforts.

Interest in plant species of importance to pollinator health has been increasing over the last five years. As a result, species that would typically be understudied have come to the forefront to be further investigated. For example, milkweed, or *Asclepias*, is a plant native to North America with great importance to various pollinators, especially the monarch butterfly (*Danaus plexippus* L.). While exhaustive research has been conducted on *Asclepias* flower morphology, seed production, and pollinator impact, little cytological work has been done. Knowing the genome size of species can predict their ability to hybridize and the genetic variability within a genus. During the interspecific hybridization study, one question was why certain species successfully hybridized with A. tuberosa and why others did not. One of the possible reasons could be significant differences in genome size, leading to research being

conducted to investigate this hypothesis. Our study used 15 different *Asclepias* species and four interspecific *Asclepias* hybrids, and using propidium iodide, the total genomic content was calculated. We found that the 2C genome size ranged from 0.65 to 1.24 picograms. Of that range, species that had previously been incapable of hybridizing with *A. tuberosa* were found to have significantly smaller genome sizes. In addition, all species used in this study were predicted to be diploids, removing any possibility of interploidy incompatibility being another barrier against hybridization. Thus, there appear to be barriers to hybridization, whereby genome size is a good indicator of the success or failure of a potential hybrid. In the future, breeders may be able to use genome size as guides to identify potential parents for successful crosses. These findings provide a starting point for future breeding efforts and expand the general knowledge surrounding *Asclepias* species. To our knowledge, our research doubles the number of species with their genomic content reported in the literature and is the first to report 2C values for interspecific hybrids.

After doing all previous work and studies on *Asclepias*, we finally got to look at the inheritance of different traits in our F_1 hybrids from the crossing of *Asclepias tuberosa* to *Asclepias hirtella, Asclepias purpurascens, Asclepias speciosa,* and *Asclepias syriaca*. We first began the analysis of the hybrids to determine the inheritance of qualitative traits. Morphological traits analyzed were the inheritance of pubescence, sap color, and flower color. Pubescence was recessively inherited across all hybrid groups, with a cross between *A. tuberosa* and *A. purpurascens* having a duplicate recessive epistasis inheritance ratio. *Asclepias hirtella* hybrids inherited pubescence at a 0:1 ratio, *A. speciosa* at a 1:3 ratio, *A. syriaca* at 1:1, and *A. purpurascens* at a 9:7 ratio. White sap color was dominantly inherited across all hybrid groups, with genotypes exhibiting clear sap looking identical to the maternal parent. Sap color was

expressed at a 1:0 ratio across all hybrid groups regardless of pollen parent used in the cross. Flower color was found to be inherited differently depending on the pollen parent used in the hybrid cross. *Asclepias hirtella* hybrids inherited an intermediate color of the two parents, while *Asclepias purpurascens* hybrid coloration favored *A. purpurascens* with additive gene action effects. Both ×*hirtella* and ×*purpurascens* had intermediate floral color but codominantly inherited both parents' anthocyanin and carotenoid pathways. Expression of the anthocyanin pathway began at bud break, but as environmental factors degraded the pigmentation, the carotenoid pigments remained. Degradation led to flowers changing color as they aged from pink to yellow in ×*hirtella* and bicolored progeny in ×*purpurascens.* The ×*speciosa* hybrids dominantly inherited flower coloration from the pollen parent *A. speciosa*, with little to no variation in the progeny. Finally, ×*syriaca* inherited flower colors from both parents in a 1:1 ratio. Approximately half the genotypes were yellow or orange, and the other half was pink in various hues. These findings provide a starting point for future breeding efforts in *Asclepias*, elucidating the probable inheritance of specific phenotypic traits if other interspecific hybridization attempts are made.

Lastly, we began looking at possible inheritance patterns of some quantitative traits of the *Asclepias* hybrids. Hybrids from the crossing of *Asclepias tuberosa* to *Asclepias hirtella, Asclepias purpurascens, Asclepias speciosa,* and *Asclepias syriaca* were analyzed to determine the inheritance of quantitative traits. Traits investigated were; height, leaf length, leaf width, flower height, and branching values of the hybrids and parents used in the crosses. It was discovered that in comparison to their maternal parent, *A. tuberosa*, only *A. tuberosa* x *A. speciosa* differed in height between the hybrid groups. While the hybrids of *A. hirtella, A. purpurascens,* and *A. syriaca* were similar in size to *A. tuberosa* (37.9 – 52.7cm), *A. speciosa*

were significantly taller than *A. tuberosa* (63.0 cm). For branching across the four groups, none of the hybrids differed from *A. tuberosa* (3.00 ± 1.15). Approximately normal distributions for height, leaf length, and flower height were seen in the hybrids of *A. hirtella*. Leaf width and flower height did show indications of inheriting significant genes that influenced an increase of leaf width and flower size, being more similar to *A. tuberosa* than *A. hirtella*. For hybrids of *A. purpurascens*, all parameters except for flower height were normally distributed, being intermediates of both parents. Flower height inheritance was of equal or greater size than both of the parents. Hybrids of both *A. speciosa* and A. *syriaca* had approximately normal distributions for all parameters measured in the hybrid populations. These findings indicated that typical quantitative inheritance occurred. Most traits are controlled by two or more genes, leading to the wide range of values seen in the progeny. Knowing that an F_1 population yields progeny intermediate of the parents provides a frame of reference for commercial growers and breeders to know what to expect when working with these species.

In conclusion, interspecific hybridizations between *Asclepias* species are possible. Depending on the parents used, their genome size, how readily they propagate, and how they inherit ornamentally desirable traits will determine their value to the commercial market. Our study was fortunate to happen upon species hybrid crosses that would work, with beneficial inheritance of commercially desirable traits, and genotypes well suited to most growing environments. This work provides a base for future work with a species that previously was not thought to hybridize. Future challenges will arise around breeding, propagation, and fertility because that seems to be the hallmark of the *Asclepias* genus. Given the importance of the genus to pollinator health, challenges in reproduction and proliferation impact both insects and humans alike. However, the exciting outcomes of this project provide future breeders incentives and

ideas of what is possible and what could be achieved in later generations. Future results will benefit humans and the yards they decorate with Asclepias plus the pollinators who depend on them to live and prosper.