BREEDING AND PROPAGATION OF *LAGERSTROEMIA* CULTIVARS

by

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(Under the Direction of Donglin Zhang)

ABSTRACT

Crape myrtle (*Lagerstroemia* spp.) is widely utilized in landscape owing to its diverse flower colors, extended blooming period, attractive bark, and ease of maintenance. Targeted breeding has produced elite semi-dwarf, disease-tolerant cultivars such as ‘Pristine Crystal’, ‘Pristine Lilac’, and ‘Pristine Ruby’. Notably, these cultivars exhibit exceptional clean foliage during the summer season, enhanced disease resistance, and semi-dwarf characteristics that further facilitate their use in landscape settings. Despite decades of breeding work on crape myrtle, fundamental cytogenetic parameters, including genome size and chromosome number, have remained inadequately defined. Using a refined fluorescent-staining protocol we confirmed that *Lagerstroemia* species possess $2n = 48$ chromosomes, and flow-cytometric analyses showed genome sizes of 0.70–0.79 pg across the genus. Interspecific hybridization proved critical for trait enhancement. From 3,126 controlled crosses among elite cultivars and *L. speciosa* we obtained 731 fruits; ‘Pristine Lilac’ was the most effective maternal parent, whereas ‘Pristine Crystal’ and ‘Pristine Ruby’ excelled as pollen donors. For hybrids 21H026, 21H040, and 21H043, Woody Plant Medium plus moderate 6-benzylaminopurine (BA), indole-3-butyric acid (IBA), and gibberellic acid (GA_3) produced the longest shoots and strongest roots, while MS-based media maximized shoot multiplication. A leaf-derived explant flowered in vitro within 60 d, indicating

potential for annual, first-year-blooming cultivars. To expand genetic diversity, we optimized ethyl methanesulfonate (EMS) mutagenesis. EMS concentration was the chief determinant of germination and survival; exposure time showed genotype-specific effects, with LD_{50} values differing significantly between 6- and 12-h treatments, underscoring the need for cultivar-tailored protocols. Finally, we generated a high-quality, haplotype-resolved genome of *L. speciosa* using PacBio Revio long reads, Hi-C scaffolding, and RNA-seq support. Although *k*-mer analysis estimated a 348 Mb genome, flow cytometry converged on ~367 Mb per haploid genome. The two haplotypes span 343.4 Mb and 317.0 Mb with scaffold N50s of 13.95 Mb and 12.75 Mb, respectively, and BUSCO completeness >93%. Repeat annotation showed ~40% repetitive content. This reference genome, together with the cytogenetic, tissue-culture, and EMS datasets, provides a robust platform for identifying genes controlling ornamental traits, disease resistance, and stress tolerance, thereby accelerating future crape myrtle breeding.

INDEX WORDS: *Lagerstroemia*, crape myrtle, new cultivar, interspecific hybridization, mutation breeding, traditional breeding, EMS, whole genome sequencing

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Literature review

Introduction

Lagerstroemia is one of the most popular landscape plants in mild-climate regions because of its easiness of propagation and cultivation, long blooming period, and diversity of plant habits from miniature potting plants to tall trees (Pooler, 2007). *Lagerstroemia* is native to southern China, southeast Asia, and Japan for timber and ornamental use for more than 1500 years (Huxley, 1992; Pooler, 2006; Pounders et al., 2013; Wang et al.,2014). *Lagerstroemia* was introduced to Europe in mid 1600s and thereafter to the southeastern United States in late 1700s (Egolf and Andrick, 1978).

Lagerstroemia is in the loosestrife family (Lythraceae), which comprises at least 53 species (Furtado and Montien, 1969). Lythraceae is a family of flowering plants including herbaceous plants, shrubs, and trees. The larger genera are *Cuphea* (containing 275 spp.), *Lagerstroemia* (56 spp.), *Nesaea* (50 spp.), *Rotala* (45 spp.), and *Lythrum* (35 spp.) (Judd et al., 2008). Lythraceae is widely distributed around the world in the tropics. Among all the species, the most popular ones are *Lagerstroemia indica* L. and *L. indica* × *L. fauriei* Koehne hybrids (Pounders and Sakhanokho, 2015). Initiated in the 1960s, the U.S. National Arboretum's *Lagerstroemia* breeding program has released 29 cultivars, of which 22 are *L. indica* × *L. fauriei* hybrids that exhibit high levels of powdery-mildew tolerance (Pooler 2006).

Lagerstroemia indica is native to China, Indochina, Himalayas, and Japan, and commonly known as crape myrtle. The height of the crape myrtle is ranging from 4.6 to 7.6 m. Leaf blade is

in elliptic, oblong, obovate, or suborbicular shape with 2.5 to 7 cm long and 1.5 to 4 cm blade. In nature, the flower color is usually rose to red. Breeders put a lot of effort to expand the flower color to include white, pink, mauve, lavender, and purple. Crape Myrtle is easy to grow in medium moisture under full sun condition from USDA zone 6 to 9 (Dirr, 1998). Normally, the growth of new stems ranges from 61 to 122 cm in one growing season (Pooler, 2006).

Lagerstroemia fauriei is native to Japan and commonly called Japanese crape myrtle. It is an upright, multi-stemmed, deciduous shrub or tree with height from 6 to 11 m and can grow up to 20-30 m tall. The white flowers are very showy during the blooming period in summer. The cinnamon-colored bark is attractive during the winter. The Japanese crape myrtle is much colder hardy and can survive from USDA zone 6 to 10 (Dirr, 1998). It prefers the full sun with good drainage and grows fast. The Japanese crape myrtle is resistant to powdery mildew and most of the *L. indica* × *L. fauriei* cultivars inherit the resistance. However, cultivars with *L. fauriei* parentage are more susceptible to crape myrtle aphid in northern Florida (Mizell and Knox, 1992). Recent phylogenetic study on chloroplasts of different *Lagerstroemia* species showed that *L. fauriei* and *L. subcostata* are close relatives, which implies that we could further breed disease resistant cultivars with *L. subcostata*. (Dong et al., 2021; Gu et al., 2019; Xu et al., 2017).

Lagerstroemia speciosa, commonly known as queens crape myrtle, was first categorized with *L. indica* and *L. grandiflora* Roxb. and excluded by Blume in 1856 (Furtado and Srisuko, 1969). It is an up-right, deciduous tree that grow in tropical and subtropical areas including India, Japan, North Burma, Eastern Himalayan regions, and China (Furtado and Srisuko, 1969; Gilman and Watson, 2014). Leaf is oblong or elliptic oblong in shape with 5 to 19.5 cm long and 4 to 8.5 cm broad. Flowers are bright pink to lavender and often very showy in a large, axillary, or terminal panicle (Gilman and Watson, 1993; Randall, 2012).

There is a demand for the dwarf cultivars since most *Lagerstroemia* species are originated in tropical and subtropical regions, therefore cannot tolerant the cold temperature in the northern United States. Because crape myrtle is a woody ornamental that rarely flowers in its first year, developing dwarf, container-friendly cultivars capable of blooming within a single growing season would enable its use as an annual in colder regions. One important trait for pot crape myrtle is the ability to grow and flower under artificial light conditions. Flower buds' development of dwarf cultivars can take one to three months depending on temperature and day length (Goi and Tanaka, 1976; Motoki et al., 1972). Some of the dwarf cultivars can revert to normal growth habits due to sports or reversions (Byers, 1997). The sports are usually branches that have larger leaves and flowers, and longer internodes (Pooler, 2006). The sports can be pruned off and may show up in the following years. However, it is still not clear whether it is caused by environmental, chromosomal, physiological, or epigenetic factors (Pooler, 2006).

Lagerstroemia was only used as shrubs or small trees in landscape and recently have been associated with potting or dwarf plants (Pooler, 2007). Byers (1997) classified cultivars into four categories (>20 feet, 10-20 feet, 5-10 feet, and <5 feet) based on the mature plant height. According to 'Manual of Woody Landscape Plants', the *Lagerstroemia* cultivars were categorized to three categories based on plant height. The semi-dwarf cultivars are 5- to 12-foot-tall including 'Acoma', 'Caddo', 'Hopi', 'Pecos', 'Tonto', and 'Zuni'. The intermediate cultivars are 13- to 20-foot-tall including 'Apalachee', 'Comanche', 'Lipan', 'Osage', 'Sioux', and 'Yuma'. The tree type cultivars are 23- to 33-foot-tall including 'Biloxi', 'Choctaw', 'Miami', 'Muskogee', 'Natchez', 'Tuscarora', 'Tuskegee', and 'Wichita'. In contemporary ornamental horticulture, consumer demand increasingly favors compact, semi-dwarf, and dwarf cultivars. As a result, breeding for smaller plant stature has become a top priority in our laboratory's crape myrtle (*Lagerstroemia*)

program. By selecting and crossing lines that naturally exhibit reduced internode length, limited height, and controlled branching, we aim to develop attractive, space-efficient cultivars that meet the evolving preferences of both home gardeners and commercial landscapers. This approach not only aligns with market needs for container-friendly and low-maintenance plants but also leverages the genetic diversity within *Lagerstroemia* to introduce novel colors, disease resistance, and other desirable traits into more compact phenotypes.

Germplasm

Until now, there are 217 valid named crape myrtle cultivars and about half of the number are widely used commercially in the U.S., Europe, Asia, or Australia (Pooler, 2007). The majority of the cultivars are selections of *L. indica* and hybrids of *L. indica*. The other parent of most *Lagerstroemia* hybrids is *L. fauriei*, which provides the source of powdery mildew resistance. In 1954, John Creech introduced seeds of *L. fauriei* to the U.S. through USDA (Creech, 1985). The USDA GRIN-Global system now preserves 71 accessions, including 9 direct species and 44 cultivars. According to Flora of China, there are totally 15 species in China and the majority of the species located in the southern part of China. By partnering with the USDA and other international institutions, we can access *Lagerstroemia* species of interest for targeted breeding. Such collaborations facilitate the sharing of diverse germplasm—an essential factor in improving disease and pest resistance, which currently ranks as our top breeding priority. Beyond resistance traits, we are also intensifying efforts to refine ornamental characteristics such as flower color, flower size, blooming period, and bark color, all of which influence market appeal.

Existing cultivars provide a valuable genetic baseline for these improvements. For instance, the “Ebony” series—renowned for its striking dark foliage—offers novel leaf color genes that can be integrated into new breeding lines, thereby expanding the palette of ornamental traits available

to breeders. Ultimately, leveraging both global germplasm and established cultivars ensures a broader genetic base, accelerates innovation in cultivar development, and addresses evolving consumer demands for improved aesthetics and durability under diverse environmental conditions.

Flower Traits

There are very few studies on the flower color of *Lagerstroemia*. The previous research indicated that the anthocyanin pigments in *L. indica*, *L. fauriei*, and *L. × amabilis* are 3-glucosides of delphinidin, petunidin, and malvidin, which is known to contribute to purple and blue flower color (Egolf and Santamour, 1975; Harborne, 1965). Earlier, some researchers thought it was not possible to achieve the true red flower due to the absence of red pigments including pelargonidin and cyanidin. However, interspecific hybridization has broadened the flower color in *Lagerstroemia* breeding. The hybrids of three species including *L. indica*, *L. fauriei*, and *L. limii* by Dr. Egolf resulting two red cultivars ‘Arapaho’ and ‘Cheyenne’ (Pooler, 2006). Although interspecific crosses among *Lagerstroemia* species have successfully broadened the available color palette (e.g., expanding reds, purples, and pinks), achieving truly novel hues such as vibrant yellows or blues remains elusive through conventional breeding. The genus and its close relatives lack the necessary biochemical pathways to synthesize pigments (e.g., carotenoids for bright yellows and certain anthocyanin modifications for blues) that would confer these specific flower colors. Consequently, transgenic, or gene-editing approaches may be required to introduce the requisite biosynthetic enzymes or regulatory elements. By incorporating genes from other plant families capable of producing these pigments, breeders could potentially overcome the inherent pigment limitations of *Lagerstroemia*, opening the door to a more diverse range of flower colors for ornamental use.

Other important flower traits are the blooming period and the ability to rebloom. In USDA hardiness Zone 7, the blooming period starts from middle June to late September, with peak bloom in early August (Pooler, 2006). However, different blooming periods have been observed from different cultivars and open-pollinated species (Jamaludheen et al., 1995; Knox and Norcini, 1992). Thus, selection for blooming periods is possible due to the variability in *Lagerstroemia* germplasm. Besides the variability in bloom periods, it is also possible to create reblooming cultivars with mutation (Whitcomb et al., 1984) or sterile triploids. By focusing on both color innovation and extended bloom traits, we can develop *Lagerstroemia* cultivars with strong, unique appeal in an increasingly competitive ornamental market.

Flow Cytometry

Flow cytometry (FCM) has become a powerful and versatile tool in plant science, particularly for ornamental breeding. This technique provides rapid and accurate information on genome size, ploidy levels, cell cycle progression, and the presence of hybrids—all of which are vital for breeding and genetic improvement programs at a low cost. FCM is a high-throughput method that analyzes cells or particles as they pass through laser-based detectors. In plant breeding, the primary application involves measuring the nuclear DNA content of cells from young leaf tissue or other plant organs. By staining nuclei with fluorescent dyes (e.g., propidium iodide, DAPI), researchers can estimate genome size and determine ploidy levels. FCM was first used for plant study in 1973 (Heller, 1973) on *Vicia faba*. However, due to the hard replication, it was not widely recognized until the 1980s. Galbraith (1983) designed a new buffer (Galbraith's buffer) that worked well with most plants and made it easy to use for plant studies.

In ornamental breeding, manipulating ploidy is common to develop new cultivars with improved traits such as larger flowers, altered color, increased disease resistance, and other novel

traits because of the gene dosage effect. Ma et al. (2015) used FCM to determine the ploidy level of *Chrysanthemum* species. Flow cytometric analyses have established genome sizes for several ornamentals—including *Coreopsis*, *Malus*, *Pelargonium*, *Phlox*, and *Rudbeckia*—thereby providing valuable benchmarks for comparative genomics (Jourdan et al. 2014; Plaschil et al. 2022; Zhang et al. 2024).The genome size data can further help ornamental breeding programs by identifying suitable parents for crosses, monitoring stability in newly generated hybrids, and assisting in phylogenetic and taxonomic studies. However, there are challenges to conduct FCM such as the part of plants, staining dye selection, internal standard, and secondary metabolites. The biggest challenge is the secondary metabolites like phenolics, tannins, and mucilage in ornamental species that can interfere with nuclear isolation and DNA staining. Thus, pre-treatments or buffer modifications may be needed (Loureiro et al., 2007). Flow cytometry is a powerful tool for precisely determining nuclear DNA content, which in turn enables accurate ploidy analysis—an essential step in many breeding and genomic studies. While flow cytometry has been widely adopted for ploidy assessment in various horticultural crops, there is a notable absence of English-language protocols tailored for *Lagerstroemia*. Consequently, designing and optimizing a specialized buffer for nuclear isolation in this genus is a critical first step. The right buffer must account for tissue characteristics, secondary metabolites, and any species-specific challenges that could interfere with accurate fluorescence measurements. Establishing a reliable protocol will facilitate more robust breeding strategies, helping researchers track hybrid status, identify polyploids, and expedite the selection of superior ornamental traits in crape myrtle.

Cytogenetics and Phylogenetics

Cytogenetics is the study of chromosomes, their structure, function, and behavior, which plays a crucial role in ornamental plant breeding. By understanding chromosome numbers, karyotype organization, and the presence of structural variations or polyploidy, breeders can more effectively manipulate genetic traits to improve flower color, plant architecture, disease resistance, and other ornamental qualities. In ornamental horticulture, cytogenetic techniques—including fluorescence in situ hybridization (FISH) and genomic in situ hybridization (GISH)—are possible to determine chromosome numbers, generate detailed karyotypes, and uncover additional genomic features. FISH uses fluorescent probes to locate specific DNA sequences (e.g., 5S and 45S ribosomal DNA regions) on chromosomes. In ornamentals, FISH reveals the location of rDNA or other marker genes, aiding in the identification of homologous chromosomes and interspecific hybrid verification (Amann and Fuchs, 2008). GISH, on the other hand, has been extensively applied to clarify the genomic composition of hybrids by allowing the direct visualization of parental genomes within a single nucleus. This powerful molecular cytogenetic tool is also instrumental in elucidating the origin and evolution of natural allopolyploids, as it can distinguish between subgenomes that have arisen from distinct species (Silva and Souza, 2013). From cytogenetics information, breeders can gain valuable information on genetic stability and diversity, enabling the development of improved cultivars with novel and desirable traits.

Many *Lagerstroemia* species within the genus offer a diverse range of traits, creating opportunities for breeders to enhance characteristics like flower color, flower size, bark color, and disease resistance. However, species within the genus may not always be compatible due to differences in their gene pools. Therefore, understanding chromosome numbers, chromosome structure, and phylogenetic relationships among distinct species and cultivars is essential for

successful hybridization and breeding efforts. There are only a few species of *Lagerstroemia* that have known chromosome numbers, including *L. indica* (2n = 48), *L. parviflora* (2n = 48), *L. speciosa* (2n = 48 or 2n = 50), and *L. duperreana* (2n = 48) (Ali, 1977; Bowden, 1945; Guha, 1972; Mehra, 1976; Nanda, 1972; Singhal and Gill, 1984). Most recent studies indicated that the chromosome numbers for *L. caudata*, *L. fauriei*, and *L. limii* are all 2n = 48. Furtado and Srisuko (1969) classified the taxonomy of *Lagerstroemia* spp. based on morphological characteristics of overall growth habit, leaf characteristics, and reproductive structures. There are three sections, i.e., 1) *L.* sect. *Sibia*, 2) *L.* sect. *Adambea*, and 3) *L.* sect. *Trichocarpidium*. A more recent study using chloroplast genome to analyze the phylogenetic relationship among *Lagerstroemia* species (Dong et al., 2021). The study concluded that there were four Clades in the genus of *Lagerstroemia*, while Clade I was sister to Clade II, and Clade III was sister to Clade IV. There were four taxa in Clade I, including *L. intermedia*, *L. siamica*, *L. speciosa*, and *L. venusta*. Clade II included six taxa, namely, *L. calyculata*, *L. floribunda*, *L.* sp. 01, *L.* sp. 02, *L. tomentosa*, and *L. villosa*. Clade III consisted of three taxa: *L. fauriei*, *L. limii* and *L. subcostata*. There were seven taxa in Clade IV, including *L. anhuiensis*, *L. caudata*, *L. excelsa*, *L. glabra*, *L. guilinensis*, *L. indica*, and *L.* sp. 03.

Knowledge of *Lagerstroemia* taxonomy and genetics is crucial for selecting parents that are both compatible and capable of transmitting target traits—such as enhanced flower color, bloom size, and disease resistance—to their offspring. In our own work, we leverage the most recent cytogenetic and phylogenetic data to refine cross combinations and anticipate where potential barriers to hybridization may arise. Species with differing chromosome numbers or structures may have reduced fertility or produce non-viable seeds, so knowing the karyotypes in advance helps us avoid or mitigate these incompatibilities. Traditional morphology-based taxonomy might group species differently from chloroplast genome analyses. By consulting both methods, we can identify

distant relatives more accurately, which may yield novel traits (e.g., new flower hues, bark textures) through interspecific hybridization.

Pest and Disease

Even though crape myrtle performs well in the southern US, it has several pests and disease problems. Granulate ambrosia beetles usually fly in late winter and early spring depending on locations (Chappell et al., 2012). Flea beetles can be a big problem in production but not on landscaping plants (Pettis et al., 2004). The adult beetles can cause rapid defoliation, but the larvae can only feed on herbaceous plants from Onagraceae and Lythraceae families (Pettis and Braman, 2007).

Lagerstroemia cultivars are only susceptible to several fungi and insect pest diseases. Powdery mildew (*Erysiphe australiana*) is the worst and most widespread disease on *Lagerstroemia spp.* and cultivars (Chappell et al., 2012). The disease usually occurs on warm days and cool nights with high humidity. Powdery mildew can decrease the aesthetic value of crape myrtle by showing white spots on leaves, shoots, and flowers. Cercospora leaf spot (*Pseudocercospora lythracearum*) has been observed in cultivars of *L. indica*, *L. fauriei*, and *L. indica* x *fauriei* hybrid in the southern United States (Hagan, 2001). The cercospora leaf spot disease like rainy or heavy dew with warm and cloudy weather. The symptoms start from the bottom leaves with small, brown spots. The spots keep getting larger and spread to the whole canopy, leaves turn yellow to red and eventually drop. There are some cultivars selected by breeders for good disease resistance on these fungus diseases (Chappell et al., 2012; Thurn et al., 2019).

Recently, crape myrtle bark scale (CMBS) is spreading in the southeastern United States and reduced the aesthetic value of crape myrtle (Gu et al., 2014). CMBS is a phloem-feeding insect

that can feed on *Callicarpa*, *Hypericum*, *Lagerstroemia*, and *Spiraea* (Xie et al., 2021). Black sooty mold is the other problem that CMBS can cause to crape myrtle reduce the leaf and flower growth. Some of the chemical treatments can reduce the population of CMBS (Vafaie, 2019). However, the long-term use of chemicals might cause potential damage to humans and beneficial insects. Until now, there has not been an efficient way to control CMBS. Different species and cultivars have been screened for CMBS resistance and *L. speciosa* showed high resistance to CMBS (Wu et al., 2021).

Conventional Breeding

Hybridization

Interspecific hybridization usually leads to heterosis—also known as hybrid vigor—to potentially improve yield, growth rate, biomass, and fertility relative to either parent species. This approach is particularly valuable in ornamental breeding, where novel phenotypic variation is highly prized for expanding cultivar diversity. However, interspecific crosses sometimes encounter fertilization barriers that hinder embryo development. In such cases, embryo rescue can be employed to bypass post-fertilization obstacles and nurture hybrid embryos that would otherwise fail to mature under normal conditions.

Embryo resuce has proven successful in numerous woody ornamentals, including *Buddleja*, *Hibiscus*, *Hydrangea*, and *Rhododendron* (Eeckhaut et al., 2007; van Laere, 2008). By isolating embryos at a suitable developmental stage and culturing them in vitro, breeders can circumvent incompatible endosperm formation or other physiological mismatches. In the context of *Lagerstroemia*, integrating embryo rescue into a hybridization program could be invaluable when crossing distantly related or chromosome-number-mismatched species, thereby broadening the

gene pool and accelerating the creation of cultivars with improved growth, ornamental traits, and disease resistance.

Many of the cultivars are derived from the crosses between *L. indica* and *L. fauriei* because of the powdery mildew resistance. Starting from 1978, the National Arboretum released 22 hybrid cultivars named after Indian tribes. Losing fertility is often associated with crosses between different species (Poehlman, 1987). Sterility might be beneficial if superior cultivars can be selected from F1 hybrids thus no further invasiveness concerns. Fertility analysis was done between hybrids of *L. indica* and *L. speciosa* (Ju et al., 2019). Sterile hybrids had no pollen germinated whereas fertile hybrids had 25.90% pollen germination rate. The similarity of pollen germination and pollen tube elongation between the sterile and fertile progenies indicated that backcrosses were not restricted by pre-zygotic barriers since pollen tubes grew through the style to penetrate the ovaries (Kuligowska et al. 2015). However, all the fertilized ovaries from the sterile plants aborted seven days after pollination which indicated that the barriers occurred during the post-fertilization phases (Ju et al., 2019). The first dark leaf crape myrtle (Crape myrtle 'Chocolate Mocha') was carried out by breeders from the Mississippi State University with *L. indica* Whit IV ('Red Rocket') being the maternal parent and *L. indica x fauriei* 'Sarah's Favorite' being the male parent (USOOPP21540P2). The cultivar has bubble gum pink flower color and unique dark brown/red-purple leaf color. The traits of interest were stable with asexual propagation.

The crape myrtle cultivars 'Arapaho' and 'Cheyenne' were selected in 1996 from seedlings that originated from controlled crossing made in 1989 ('Arapaho') and 1990 ('Cheyenne') (Pooler, 2006). *L. limii* were used as a parent of the hybridization process in 1986 to broaden the genetic base. Crosses resulting in 'Arapaho' and 'Cheyenne' were made in 1960, 1968, 1979, 1986, 1989, and 1990. The two released cultivars have been evaluated in various locations including Alabama,

Arkansas, California, Florida, Georgia, Mississippi, New Jersey, North Carolina, Oregon, Pennsylvania, South Carolina, Tennessee, Texas, and Virginia. Both cultivars were registered in 2003 by the U.S. The released cultivars had purple-black leaves and a range of flower colors with intermediate growth habits.

My research seeks to further expand the ornamental palette of crape myrtle while addressing known limitations like sterility barriers and restricted color pathways. By leveraging the lessons learned from past hybrid introductions—particularly the importance of strategic parent selection, genetic diversity, and robust evaluation across multiple climates, I aim to introduce novel traits by combining diverse species (including *L. indica*, *L. fauriei*, *L. limii*, and *L. speciosa*) to develop lines with improved aesthetics (flower color, foliage color), disease resistance, and compact growth habits. Employ advanced techniques (e.g., embryo rescue, cytogenetic analysis) to facilitate successful hybridization, even among distantly related or chromosome-mismatched species.

Open Pollination

Open pollination refers to the natural transfer of pollen by wind or biotic agents (e.g., insects, birds) under uncontrolled conditions (Kearns and Inouye, 1993). In ornamental breeding, open pollination can be both a deliberate and an incidental strategy to introduce genetic variability. While controlled pollination methods are often used to ensure specific parental crosses, open pollination can enrich breeding material by capturing diverse genetic recombination present in the environment.

Unlike controlled hybridization which requires manual emasculation, controlled pollination, and isolation, open pollination requires less labor and fewer resources, making it accessible for initial screening of a large breeding population. Historically, many popular ornamentals were

derived from open pollination varieties. According to Google Patent (USPP11836P2), the two parents of the most popular rose cultivar (Knock Out® Roses) in the United States were seedlings of open pollination varieties of 'Carefree Beauty' and 'Razzle Dazzle'. Most ornamental plants collected from the wild undergo open pollination before being selected by plant hunters and breeders for further breeding and commercial development. Robert Fortune, a prominent Scottish botanist and plant collector, played a crucial role in introducing numerous ornamental species from China to England. His contributions significantly shaped Western horticulture by providing novel genetic material for breeding programs. Notable examples of plants introduced by Fortune include *Pleioblastus fortunei*, *Rhododendron fortunei*, and *Rosa fortuniana* (Watt, 2017). These species were first propagated and selected for desirable traits before being incorporated into breeding efforts, ultimately leading to improved ornamental cultivars.

Mutagenesis

Mutagenesis is another way to breed for new cultivars. Mutation can generate random variations, resulting in mutant plants with novel morphological traits (Loewe and Hill, 2010). Two major mutagens are being used to induce mutation: physical and chemical mutagens. Until now, there are over 3275 mutant varieties in more than 220 plant species that have been officially recognized worldwide (http://mvd.iaea.org/). Physical mutagens include X-rays, gamma rays, ultraviolet light, alpha particles, beta particles, particles from accelerators, neutrons, ion beam irradiation, and ion beam implantation, cosmic irradiation, and laser beam irradiation. Chemical mutagens include ethyl methane sulfonate (EMS), diethyl sulfate (DES), ethylene imine (EI), propane sultone, N-methyl-N-nitroso urethane (MUN), and sodium azide (Gottschalk and Wolff, 1983). According to the FAO/IAEA database, there have been 465 mutants released that are propagated vegetatively. Most of the mutants are ornamental plants, and few are fruit trees. Among

the ornamental plants, *Chrysanthemum* (187), *Alstroemeria* (35), *Dahlia* (34), *Streptocarpus* (30), *Begonia* (25), *Dianthus* (18), and *Rhododendron* (15) have been studied the most in mutation breeding (Maluszynski et al., 1992).

EMS was used to induce mutation on crape myrtle. Whitcomb (1984, 1985) has used EMS to induce two cultivars, 'Centennial Spirit' and 'Prairie Lace'. These two mutant varieties were both developed by treating the seeds with 4% of EMS for 1 hour and selecting from the second generation of open pollinated seeds. 'Centennial Spirit' tends to have thicker leaves, strong stems, resistance to powdery mildew, tolerance to low temperature and drought. The main improvement of 'Prairie Lace' is sterility. A natural yellow leaf mutant of *L. indica* was found at the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, China in 2003 (Wang et al., 2016). The unique characteristic of the cultivar is that the leaves are yellow all the time. The leaf color of all clones was observed for 8 years and remains stable.

Conventional hybridization can significantly expand genetic diversity in *Lagerstroemia*, yet certain novel traits—particularly unique leaf variegation or dramatic flower color shifts—may be elusive through crossing alone. In such cases, chemical mutagenesis using ethyl methanesulfonate (EMS) offers a complementary approach. EMS induces random point mutations in the plant's genome, potentially altering key genes responsible for pigment biosynthesis, leaf morphology, or flower architecture. When carefully optimized, EMS treatments can yield new phenotypes that would otherwise be difficult or impossible to achieve via interspecific hybridization.

From a commercial perspective, leaf and flower mutations are particularly valuable in ornamental markets, where visually striking foliage or unusual blooms can command premium prices. An EMS protocol designed for *Lagerstroemia* would typically involve determining the appropriate seed or tissue dose, exposure time, and post-treatment recovery conditions to balance

mutation frequency with survivorship. Mutagenized populations are then screened for desirable traits—such as altered leaf pigmentation or novel flower forms—before undergoing further propagation and evaluation for horticultural performance. By integrating EMS mutagenesis with traditional breeding, researchers can broaden the phenotypic range of crape myrtle cultivars, driving innovation and diversity in ornamental horticulture.

Polyploidy

Polyploidy is the condition that cells contain more than two sets of chromosomes per cell, which is a unique characteristic in plant evolution. Polyploidy spontaneously happens in nature, with an incidence range from 30% to 35% (Stebbins, 1971) up to 70% (Masterson, 1994) for angiosperm. Polyploid plants tend to have lower growth rate, late flowering, and longer flowering period compared to their diploid counterparts (Levin, 1983). There are two types of polyploids: autopolyploid and allopolyploid. Autopolyploids contain multiple copies of basic set of chromosomes of the same genome while allopolyploids have combined genomes from distinct species (Acquaah, 2007). Because autopolyploids contain more than two copies of chromosome, meiosis may result in univalents and multivalents whereas the diploid counterparts normally form bivalents (Acquaah, 2007). Univalents and multivalents can also lead to sterile since chromosomes cannot pair.

In ornamental plant breeding, induction of polyploids has been widely used to enlarge plant organs (Caporali et al., 2014; Kehr, 1996; Ranney, 2006), increase plant vigor (Ranney, 2006), and breeding for sterility (Contreras et al., 2009; Ranney, 2006). There are mainly three different mitotic polyploidization methods: meristem treatment with mitotic inhibitor solution at the apex of seedlings for 2-5 days, pulse treatment with explants soaked in relatively high concentration of mitotic inhibitor for 6 h-12 days, and chronic treatment that explants are put on a medium with

relatively low concentration of mitotic inhibitor for 15-70 days (Eeckhaut et al., 2018). Colchicine was the first mitotic inhibitor used on plants to double the number of chromosomes (Blakeslee and Avery, 1937). Later, some herbicides like oryzalin, trifluralin, pronamide, and propham were discovered to be mitotic inhibitors (Bartels and Hilton, 1973). Polyploidy has been widely used in ornamental breeding for quite a few species including *Escallonia rosea* (Denaeghel et al., 2015), *Cercis glabra* (Nadler et al., 2012), *Lavandula ×intermedia* (Urwin, 2014), *Vitex agnus-castus* (Ari et al., 2015), *Ligustrum japonicum* (Fetouh et al., 2016), *Hebe* 'Oratia Beauty' (Gallone et al., 2014), *Prunus lusitanica* (Schulze and Contreras, 2017), *Populus spp.* (Xu et al., 2016), *Echinacea purpurea* (Li et al., 2016), *Gerbera jamesonii* (Gantait et al., 2011), *Rudbeckia subtomentosa* (Oates et al., 2012), *Lychnis senno* (Nonaka et al., 2011), *Gentiana decumbens* (Tomiczak et al., 2015), *Trollius chinensis* (Zhang et al., 2016), *Crocosmia aurea* (Hannweg et al., 2013), *Tulipa gesneriana* (Podwyszynska, 2012), etc.

Several studies have been conducted to develop polyploid crape myrtle. Zhang et al. (2010) designed three treatments: 1) explants were immersed in liquid medium supplemented with 15 and 20 mM colchicine for 24 hours on shaker at 100 rpm; 2) explants were placed on solid medium supplemented with 125 and 250 μM for 30 days; and 3) explants were incubated in liquid medium supplemented with 250, 500, and 750 μM colchicine for days with 100 rpm shaking. They found out that the third treatment worked best on inducing polyploidy of *L. indica*. Song (2012) determined tetraploid and hexaploidy *L. indica* and plants with higher ploidy level tend to have wrinkled and thicker leaves, less stoma frequency, and larger guard cells. Ye et al. (2010) induced *L. indica* cultivars with various concentrations of colchicine with different durations. They found out that the morphological variations of 0.5% and 0.8% colchicine treatments were significantly higher than 0.2% treatment, but there was no significant difference between 0.5% and 0.8%

treatments. The leaf size, pollen size, and flower size of the tetraploids were significantly larger than the diploid counterparts. However, the pollen vigorous of the tetraploids was lower than the diploids.

Marker assisted breeding

Markers have been used on crape myrtle studies focusing on limited species including *L. caudata*, *L. fauriei*, *L. indica*, *L. limii*, *L. speciosa*, and *L. subcostata* (Liu et al., 2013;). SSR markers have been used to study genetic diversity in *Lagerstroemia*. Cai et al. (2011) studied genetic distance in 26 *Lagerstroemia* cultivars from 151 allele and categorized the cultivars into two groups. Later studies on 96 genotypes showed genetic diversity and categorized the 96 genotypes to 3 clusters (He et al., 2012). *L. indica* transcriptome data was also used to identify EST-SSR markers (Ye et al., 2019). Among the 1200 synthesized primer pairs, 761 EST-SSRs were successfully amplified and only 245 EST-SSRs showed polymorphism. The first genetic map of *Lagerstroemia* derived from the cross between *L. caudata* and *L. indica* 'Xiang Xue Yun' generated from 383 AFLP primers and 150 SSR primers (He et al., 2013). The transcriptome analysis of two crape myrtle cultivars 'Carolina Beauty' and 'Natchez' generated new hypotheses of powdery mildew resistance (Wang et al., 2015). Zhou et al. (2021) developed the linkage map on BC2 population of *L. fauriei* × *L. indica* "Pocomoke" cross. Three QTLs related to plant height and two QTLs related to internode length were found in this study which could help fast screening dwarf hybrids. Ye et al. (2016) was trying to identify and validate the SNP markers to dwarf traits. Two markers are highly correlated with the internode length trait and one marker highly correlated with the primary lateral branch height.

Whole Genome Sequencing

While traditional breeding has produced numerous cultivars, whole-genome sequencing (WGS) offers new prospects for accelerated genetic improvement. By providing genome-wide insights into gene content, structure, and function, WGS paves the way for:

1. Marker-assisted selection for traits such as flower color, extended bloom time, and disease resistance (e.g., against powdery mildew).

2. Comparative genomics to clarify evolutionary relationships within *Lagerstroemia* and with other members of the Lythraceae family.

3. Identification of candidate genes underlying ornamental traits, enabling more targeted breeding strategies.

Although whole-genome sequencing (WGS) is a powerful tool, its application in ornamental breeding has only recently gained traction. As sequencing costs have decreased, WGS has become more accessible for smaller projects, including ornamental breeding. WGS enables a better understanding of chromosome numbers in species, particularly since many woody ornamental plants possess many chromosomes but small genome sizes. Traditional cytogenetic methods, such as chromosome squashes, can be challenging for accurately determining chromosome numbers and structures. Additionally, woody ornamental plants often produce higher levels of secondary metabolites compared to annuals and perennials, which can interfere with flow cytometry studies. By leveraging WGS, ornamental breeders can more precisely estimate genome sizes and better analyze genetic complexity. Due to their natural reproductive habits, many woody ornamentals exhibit high levels of heterozygosity and polyploidy. Sequencing heterozygous genomes is crucial for capturing the full range of genetic variation, particularly for traits like disease resistance. Furthermore, polyploidization and diploidization are significant evolutionary processes in plants.

Sequencing polyploid genomes provides insights into genome duplication events and speciation mechanisms, contributing to both breeding advancements and evolutionary studies (Li et al., 2021).

Until 2025, there are 21 species in the Lythraceae family that have been sequenced from NCBI database, including three *Lagerstroemia* species (*L. speciosa*, *L. excelsa*, and *L. fauriei*). The WGS of *L. indica* and *L. speciosa* indicated that they are both diploid with 48 chromosomes (Wan et al., 2024; Zhou et al., 2023). The WGS can help find putative resistance (R) genes and defense-related pathways, offering clues for breeding crape myrtle varieties with improved resistance to powdery mildew and other pathogens and pests. Molecular markers derived from WGS (e.g., single nucleotide polymorphisms, SNPs) allow breeders to rapidly screen seedling populations for desirable genetic variants associated with flower color, bloom duration, growth habit, and disease resistance. Although CRISPR-Cas technology is new to ornamental crops, it offers significant potential for developing new cultivars with precise genetic modifications. By targeting genes associated with ornamental traits (such as flower color, plant architecture, and fragrance) and disease resistance, CRISPR-Cas allows breeders to introduce desirable traits without the need for hybridization (Liu et al., 2024). This precision breeding approach can significantly reduce the time required for cultivar development compared to traditional breeding methods, making it a valuable tool for accelerating genetic improvements in ornamental plants.

Crape myrtles (*Lagerstroemia* spp.) face increasing pest and disease pressures in regions where natural predators are scarce or absent, complicating conventional control measures. To address these challenges, researchers are looking beyond traditional breeding approaches toward genome-informed strategies. By mining whole-genome datasets, it becomes possible to identify candidate resistance genes linked to insect or pathogen defense. Once located, these genes can be rapidly integrated into breeding pipelines via marker-assisted selection (MAS), enabling breeders

to track and incorporate resistance alleles with greater precision. This approach not only expedites the development of resistant cultivars but also reduces reliance on chemical pesticides, contributing to more sustainable landscape management and preserving the ornamental quality of crape myrtles.

Propagation

Propagation is a particularly important way to increase the number of plants. There are four ways to propagate woody plants: seed, cuttings, grafting, and tissue culture (Dirr and Heuser, 2006). Seed is very important for both breeding and propagation. A seed carries the genetic information that is from the parents. For woody plants, since most of the plants are heterozygous, seedlings will show variation which will benefit the breeding work. Once the desired traits are obtained, vegetative propagation (cuttings, grafting, or tissue culture) will be applied to maintain the characteristics of the trees. For most species, seed moisture should be around 5 to 12% and the best temperature to store seeds should be 41 °F (Dirr and Heuser, 2006).

Cuttings, on the other hand, are used to maintain the cultivars for woody plants. Cutting propagation can be overly complicated since there are a lot of factors that can affect cutting success including time of the year, temperature, humidity, climate, cutting quality, etc. There are several types of cuttings including leaf, stem, and root. Leaf and root cutting are barely used for woody propagation (English, 1981; Fordham, 1969; Orton, 1978). Most woody plants are propagated by stem cuttings. Stem cuttings can be divided into softwood cuttings, semi-hardwood cuttings, and hardwood cuttings. For *Lagerstroemia* cultivars, soft wood cuttings are usually done during the growing season and hardwood cuttings are done during the winter season.

Grafting is one of the oldest plant propagation methods that can date back to more than 2000 years ago (Young, 1983). It is the process of the two pieces of living plant parts to unite and

continuously grow to one plant. There are several factors that can affect the success of the grafting: 1) the scion and rootstock are compatible; 2) the vascular cambium of the scion is contact directly with that of the rootstock; 3) physiological stage for both scion and rootstock are ideal; 4) cut surfaces is covered to protect from desiccation right after grafting; and 5) grafted plants are proper cared for a period of time after grafting. There are three types of grafting: detached scion graftage, approach graftage, and repair graftage. Recently, grafting has been used on crape myrtle cultivars in China. One cultivar called 'Xiangyun' were found seedless when buds grafted to other cultivars (Wang et al., 2014).

Tissue culture

Tissue culture is defined as the aseptic culture of cells, tissues, organs or seeds under controlled nutritional and environmental conditions (Thorpe, 2007). The first attempt in tissue culture happened in early 1900 to maintain mesophyll cells in culture based on postulates (Haberlandt, 1902).

The first stage is to prepare the donor plant. To increase the success of tissue culture, donor plants should be cultivated under ideal conditions with proper irrigation, nutrition, temperature, and humidity (Cassels and Doyle, 2005). Pretreatment with plant growth regulators may improve the morphogenic response during the *in vitro* establishment (García et al., 1999).

The second stage is introduction and establishment. This stage is the most difficult stage since the next following steps depend on the phytosanitary and quality of the established explants (George and Debergh, 2008). The superficial disinfection methods of the tissues depend on the types of explants. The most popular disinfectants are sodium hypochlorite (Tilkat et al., 2009), calcium hypochlorite (García et al., 1999) and ethanol (Singh and Gurung, 2009). However, woody plants usually need more drastic disinfection treatment such as mercuric chloride (Hussain and

Anis, 2009). After transferring the explants to the media, it is important to control the emission of phenolic compounds by the explants. The disinfection procedure can damage the plant tissue and phenolic compounds are used to protect the tissue. There are many ways to approach the goal by adding additional antioxidants such as activated charcoal, citric acid, ascorbic acid, nicotinic acid, and L-cysteine to the media (Shekhawat et al., 1993). Explants that grow in the tubes are generally showing reduced growth, increased branching, and reduced elongation which is also known as hyperhydricity (Bairu et al., 2009). Plant growth regulators can cause hyperhydricity in tissue culture in several species (Ziv, 1991; Fraguas et al., 2004; Toth et al., 2004; Fraguas et al., 2009).

The third stage is multiplication. The purpose of this stage is to increase the growth of the branches to increase the number of units until the desired number is achieved (Saini and Jaiwal, 2002). It is very important to study the combination of auxin and cytokinin for better results (García et al., 1999; Tilkat et al., 2009). The way to manage explants and the position that explants on the media can play an important role to improve the morphogenetic response (García et al., 1999; Papafotiou and Martini, 2009).

The fourth stage is to induce root for the explants. Explants may simultaneously form roots in the media when the condition is good. However, it is important to carry out the protocol for root induction and development of strong roots with different hormone combinations. There are several factors that can affect rooting: auxin: cytokinin rates (Leonardi et al., 2001); the type of explant (Hiregoudar et al., 2005); the number of subcultures (Ríos et al., 2005); the pH of the culture media; and the concentration of sucrose (Newell et al., 2005).

The last stage is *ex vitro* adaptation or plant acclimatization. At this stage, plants must adapt to the environment outside laboratory conditions. It is important to manage the light intensity, substrate moisture, and temperature of the plants. The humidity of the plant should be high since

the *in vitro* media humidity is very high. The humidity can keep reducing as the plants adapt to new environments.

Tissue culture is very important to agricultural and horticultural industries as it can mass propagate genotypes of interest in a relatively brief time. Tissue culture also allows people to propagate disease free plants. For woody plants, meristem tissue culture has been proven to be an efficient way to eliminate viruses (Popesku et al., 2010; Tan et al., 2010). Tissue culture is a good way to manipulate ploidy level. *In vitro* polyploidy can double the chromosome number as well as maintaining the genetic background of the plant of interest. Lastly, tissue culture is a good tool for genetic transformation. Without tissue culture, it is almost impossible to have an efficient regeneration protocol for genetic transformation.

A lot of research has been done on tissue culture with different *Lagerstroemia* species. ZoBAYED (2000) has done tissue culture research on nodal explants of *L. speciosa* and *L. thorellii*. Duan et al. (2013) has done research on stem explants for *L. indica* tissue culture. Quraishi et al. (1997) worked on micropropagation of *Lagerstroemia parviflora* through axillary bud. There are also many more papers on tissue culture of different *L. indica* cultivars with different plant parts. However, most scientists use different hormone combinations to achieve the best results.

Propagation techniques are critical in ornamental breeding, as every newly developed cultivar must be reproducible at scale for commercial release. Whether through traditional methods (e.g., cuttings, grafting) or modern tissue culture and micropropagation, reliable propagation ensures that the desired traits—such as novel colors, compact growth habits, or resistance to pests—remain stable in each plant generation. In transgenic or gene-editing programs, efficient callus induction is essential for introducing target genes and regenerating transformed plants. Without robust

propagation protocols, even the most promising new genotypes cannot be deployed or evaluated widely, hindering both research progress and commercial success.

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CHAPTER 2

Cytogenetics study of *Lagerstroemia* taxa

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Abstract.

Crapemyrtle (*Lagerstroemia spp.*) is a popular ornamental genus known for its vibrant floral displays, exfoliating bark, and extended blooming period. However, most commercial cultivars are derived from a narrow genetic base and remain susceptible to various pests and diseases, such as powdery mildew and crape myrtle bark scale. In this study, we investigated the chromosome number and genome size of eight *Lagerstroemia* species—*L. indica*, *L. fauriei*, *L. limii*, *L. loudonii*, *L. microcarpa*, *L. speciosa*, *L. tomentosa*, and *L. villosa*—to improve breeding strategies aimed at introducing broader genetic diversity. A modified fluorescent staining protocol was developed and optimized to identify the optimal enzyme digestion time for root tip preparations (60 minutes), resulting in clear chromosome spreads. Flow cytometry was employed to determine genome sizes, which ranged from 0.71 to 0.79 pg. Testing four different DNA extraction buffers (LB01, Woody Plant buffer, MB01, and a commercial kit) showed that MB01 provided the most reliable results, with low coefficients of variation and minimal interference from secondary metabolites. These findings confirmed the close genomic relationships among *Lagerstroemia* species, highlighting their potential for interspecific hybridization and further breeding. The protocols developed herein can facilitate further cytogenetic and genomic studies, contributing to the development of disease-resistant and ornamental *Lagerstroemia* cultivars with enhanced horticultural value.

Introduction

Lagerstroemia is a genus widely cultivated for its long blooming period, exfoliating bark, and diverse flower colors (Furtado and Srisuko, 1969). Most popular cultivars are derived from *L. indica* and *L. indica* × *L. fauriei* hybrids. However, the genetic background of these cultivars is highly limited to a few species, which poses challenges for future cultivar development. This

limitation underscores the importance of understanding genetic diversity within the genus for effective breeding programs. Presently, existing cultivars suffer from persistent disease and pest issues—such as powdery mildew and crape myrtle bark scale—highlighting the necessity of introducing genetic material from other species to enhance resistance.

Studying plant chromosomes is crucial for understanding plant evolution, taxonomy, and breeding potential. The basic chromosome number for several species in the Lythraceae family is reported as x = 8 (Tobe et al., 1986; Raven, 1974). According to the Kew Garden Plants of the World Online database, there are 63 accepted species and subspecies of *Lagerstroemia*. However, chromosome numbers have been reported for only six species: *L. reginae* Roxb. (2n = 48), *L. speciosa* (2n = 48; 2n = 50), *L. duperreana* (2n = 48), *L. indica* (2n = 48; 2n = 50), *L. parviflora* (2n = 48), and *L. floribunda* (2n = 48) (Ali, 1977). Understanding chromosome structure and number is essential for advancing breeding efforts. Given that x = 8 is a common basic chromosome number, it is likely that most *Lagerstroemia* species are polyploids with 2n = 6x = 48. Interestingly, the closely related species *Duabanga grandiflora* also possesses 2n = 48 chromosomes. However, previous studies have suggested that the basic chromosome number for the genera *Sonneratia* and *Duabanga* is n = 12, which might be the ancestral number for the order (Graham et al., 1993).

Chromosome number and ploidy levels in woody plants can be determined through traditional cytological methods such as root squashes and modern techniques like flow cytometry. However, the small chromosome size and genome complexity of many woody plants make traditional methods challenging. Fluorescent staining using ribosomal DNA (rDNA) probes can effectively determine both chromosome number and ploidy level. Tandemly repeated 45S rDNA and 5S rDNA sequences, which are highly conserved across plant species, have been widely used to study

genome evolution and polyploid origins (Volkov et al., 2017; Gomes et al., 2014). In *Lagerstroemia*, there are limited reports on the use of fluorescent staining, with some studies using stem tips instead of root tips for analysis.

Previously, high levels of secondary metabolites in these species interfered with DNA staining, causing flow-cytometric analysis to be ineffective. Flow cytometry for *Lagerstroemia* species was first successfully performed in 2020. The study revealed that the genome size of *Lagerstroemia* species ranges from 341 to 370 megabase pairs (Mb) per haploid genome (1C) (Liu et al., 2020), consistent with earlier whole genome sequencing results that estimated the 1C genome size of *L. indica* at 329.14 Mb (Zhou et al., 2023). The whole genome sequencing also confirmed the diploid nature of *L. indica*.

A key challenge in plant flow cytometry is selecting an appropriate buffer. Unlike animal cells, plant cells possess rigid cell walls that complicate nuclei extraction and passage through the flow cytometer nozzle (Fomicheva and Domblides, 2023). Additionally, secondary metabolites such as polyphenols, tannins, and terpenes can interfere with DNA staining, compromising results. Studies have shown that the concentration of secondary metabolites varies across plant tissues. For example, *L. loudonii* leaves contain around 4% secondary metabolites, stems more than 3%, stem bark less than 2%, while fruit tissues may contain over 10% (Faramayuda et al., 2021). Due to the high secondary metabolite content in leaves, the choice of plant tissue and buffer solution is critical for obtaining accurate flow cytometry results in *Lagerstroemia*.

In this study, we aim to identify the most effective buffer for performing flow cytometry in *Lagerstroemia* species, addressing the challenges posed by the secondary metabolites and optimizing nuclear isolation techniques for future genomic research and breeding applications.

Materials and Methods

Plant Materials

Eight *Lagerstroemia* species (*L. indica*, *L. fauriei*, *L. limii*, *L. loudonii*, *L. microcarpa*, *L. speciosa*, *L. tomentosa*, and *L. villosa*) were selected for cytogenetic analysis using root tips and for flow cytometry using young leaves.

Root tips were collected on a sunny morning between 9:00 and 10:00 am. Approximately 1 cm was cut from the distal end of the primary root and placed into 1.5 mL tubes containing ~100 µL of deionized water water. The root tips were treated at 4 °C for 4 hours with 8-hydroxyquinoline supplemented with cycloheximide to arrest mitosis. Afterward, they were rinsed three times with tap water and transferred to Farmer's solution (ethanol: acetic acid = 3:1) at room temperature overnight. Once the treatment was complete, root tips were rinsed again, then stored in 70% ethanol at 4 °C until further use.

In a fluorescent staining study, enzyme digestion times (30, 60, and 90 minutes) were tested using five root tips per species to determine optimal conditions. Before digestion, root tips were immersed in 1× citric buffer for at least 10 minutes. Each tip was then lightly dried, and about 1 mm was sliced from the distal end. The slices were transferred to tubes containing FISH enzyme on ice. The fluorescent in situ hybridization (FISH) enzyme mixture was prepared by combining 0.025 g of pectolyase Y-23, 0.05 g of cellulase Onozuka R-10, and 2.43 g of 1× citric buffer, then adjusting the final volume to 2.5 mL. The resulting solution was subsequently aliquoted into 10 µL portions for future use. Digestion was carried out at 37 °C for the specified duration (30, 60, or 90 minutes), after which tubes were immediately placed on ice. To wash and remove cell walls, 100% EtOH was added and carefully pipetted out without discarding the root tip slices. After two rinses, about 4 µL of EtOH remained, to which 32 µL of 100% acetic acid was added. A metal

pick was used to crush the slices for 1–2 minutes until the suspension appeared cloudy and no solid material was visible.

Flow cytometry was then performed using three leaves per accession, analyzing at least 3,000 nuclei per sample, with a coefficient of variation (CV) below 10 considered acceptable. Newly emerged leaf tissue (~1 cm^2) was chopped into 1–2 mm^2 pieces, then placed into 1,000 µL of nuclei extraction buffer (LB01, Woody Plant buffer, or MB01) (Doležel *et al.*, 1989; Loureiro et al., 2007; Sadhu et al., 2016). The detailed composition of the nuclei isolation buffers were listed in Table 1.1. When using CyStain PI Absolute P (Sysmex Partec, Görlitz, Germany), only 500 µL of the reagent was required. Samples were gently mixed for about 30 seconds, filtered through a 30 µm mesh, and supplemented with 6 µL of RNase and 12 µL of propidium iodide (PI) staining solution unless CyStain reagents were already present, in which case no additional staining solution was needed. The homogenate was analyzed at the University of Georgia Center for Tropical and Emerging Global Diseases (CTEGD) Cytometry Shared Resource Laboratory using a CytoFLEX Flow Cytometer (Beckman Coulter Life Sciences, Indianapolis, IN). Genome sizes were calculated based on the formula Sample 2C genome size = (mean of the sample peak ÷ mean of the standard peak) × 2C genome size of the standard. Tomato 'Stupické polní rané' (2C = 1.96 pg) served as the external standard for all analyses (Doležel et al., 2007).

Results and Discussion

Cytogenetic Analysis

Adapting a maize FISH protocol for use in *Lagerstroemia* root tips yielded high-quality chromosome spreads, but only after determining the optimal enzyme digestion time. Initially, three treatment durations—30, 60, and 90 minutes—were compared to identify the interval that would

reliably break down cell walls without damaging chromosome structure. Observations under fluorescence microscopy showed that with the fixed concentration of FISH enzyme, 30 minutes of digestion was insufficient, leaving residual cell walls and causing partial clumping of chromosomes, while 90 minutes produced noticeable fragmentation, with individual chromosomes appearing fuzzy or even lost. Consequently, a 60-minute digestion was chosen as the balance point, enabling clear visualization of discrete chromosomes and minimal background interference (Figs. 1.1-1.3).

These optimized conditions allowed precise chromosome counts for several *Lagerstroemia* species, with particular focus on *L. loudonii* and *L. microcarpa*, both of which were documented for the first time to have 2n = 48 chromosomes. Additional observations on *L. excelsa*, *L. limii*, *L. tomentosa*, and *L. villosa* corroborated previous studies by confirming the same diploid number (2n = 48). These findings reinforce the prevailing hypothesis that x = 8 is a fundamental base chromosome number throughout *Lagerstroemia*. Despite the consistent 2n = 48 count, it proved challenging to construct detailed karyotypes or to definitively assess ploidy levels, due to the small and morphologically similar chromosomes characteristic of the genus. Nonetheless, defining these chromosome numbers in multiple species provides an important baseline for future interspecific crosses, potential polyploid breeding strategies and genome sequencing projects.

Flow Cytometry Analysis

Parallel to the cytogenetic work, flow cytometry was conducted to quantify genome size in the same *Lagerstroemia* species using young leaf tissue. The analysis demonstrated that most of the species tested shared very similar 2C genome sizes, ranging from 0.71 to 0.79 pg (Table 1.2). While these values were closely clustered, a notable contrast emerged when examining *Hemia salicifolia*, a closely related species with fewer chromosomes (2n = 16) but a larger genome (2C =

1.10 pg). This discrepancy highlights that chromosome count does not invariably correlate with total DNA content, underscoring the importance of integrating both cytogenetic data (e.g., chromosome numbers) and genomic data (e.g., flow cytometry) for a more holistic understanding of *Lagerstroemia* diversity.

In optimizing the flow cytometry protocol, four different nuclei isolation buffers—MB01, LB01, Woody Plant buffer, and CyStain—were evaluated for their capacity to release intact nuclei free of secondary metabolite interference. Although LB01 and Woody Plant buffer have proven suitable in other plant species, they generated large extraneous "junk" peaks for *Lagerstroemia*, making the critical 2C peaks difficult to discern and inflating the coefficient of variation (CV). CyStain, a commercial reagent designed to work across a wide range of taxa, offered some improvement but still yielded suboptimal histograms, likely due to the high concentrations of phenolic compounds typically found in woody tissues. In contrast, MB01 consistently produced well-defined peaks with low CV values (Figs. 1.4-1.5). The superior performance of MB01 may result from its higher polyvinylpyrrolidone (PVP) content, which binds phenolics and other secondary metabolites, and from the inclusion of β-mercaptoethanol, which mitigates oxidative damage. The superior performance of MB01 in nuclei isolation likely reflects its formulation: it retains the core components of LB01 but at higher concentrations, thereby enhancing membrane disruption and phenolic binding. In contrast, the elevated Triton X-100 level in Woody Plant Buffer (WPB) appears to generate excessive cellular debris, producing "junk" peaks during flowcytometric analysis. Moreover, the antioxidant capacity- provided by sodium metabisulfite in WPB is either sub-optimal at the concentration used or intrinsically less effective than the βmercaptoethanol- present in MB01, resulting in poorer preservation of nuclear DNA integrity and diminished signal quality. Through these optimizations, flow cytometry provided robust estimates

of genome size for multiple *Lagerstroemia* species, laying the groundwork for more precise breeding decisions and molecular analyses in the future.

Breeding Significance and Future Applications

This study's cytogenetic findings (2n = 48 for all species tested) hold vital importance for breeding programs. In general, species sharing the same chromosome number are more likely to produce viable offspring due to compatible chromosome pairing during meiosis. For instance, *L. indica* and *L. speciosa* crosses have successfully generated viable hybrids (Pounders et al., 2007). This compatibility is particularly relevant for introducing disease and pest resistance genes into commercial cultivars. Powdery mildew and crape myrtle bark scale are widespread problems in the genus, affecting both ornamental value and overall plant health.

Certain wild species (e.g., *L. speciosa*) have desirable traits like large flowers (6-7 cm), extended panicles (17-42 cm), and moderate bark scale resistance (Wu et al., 2020; Meerow et al., 2015). By confirming that *L. speciosa* shares the same chromosome number with commonly cultivated species, breeders can systematically incorporate its traits into new hybrid cultivars. Furthermore, our results are consistent with previous reports demonstrating that genome sizes of different species and cultivars are quite similar, further supporting the potential for wide hybridization within the genus (Liu et al., 2020).

Flow cytometry also provides a rapid way to screen progenies for ploidy level, allowing breeders to identify and select triploid or tetraploid individuals that may exhibit hybrid vigor or other desirable traits (e.g., larger flowers, improved disease resistance). By employing the optimized MB01 buffer, researchers and breeders can more reliably assess genome sizes and ploidy levels in *Lagerstroemia*.

Conclusion and Future Perspectives

In this study, we developed and optimized a fluorescent staining protocol for cytogenetic analysis in *Lagerstroemia*, demonstrating that a 60-minute enzyme digestion yields clear chromosome spreads. We also identified MB01 as an effective buffer for flow cytometry, enabling accurate genome size estimations in the presence of secondary metabolites. Our findings confirm that multiple *Lagerstroemia* species, including *L. loudonii*, *L. microcarpa*, *L. limii*, *L. tomentosa*, and *L. villosa*, all share 2n = 48 chromosomes. This confirmation underscores the potential for broad hybridization within the genus, vital for breeding ornamental cultivars with improved disease resistance and desirable floral characteristics.

Looking ahead, incorporating advanced molecular cytogenetic techniques, such as FISH (fluorescence in situ hybridization) using rDNA or oligonucleotide probes, will provide finer resolution of chromosomal structures. Likewise, whole-genome sequencing combined with cytogenetic data can reveal the evolutionary relationships and speciation patterns within the Lythraceae family. Finally, as the crape myrtle bark scale and powdery mildew continue to challenge growers, interspecific hybridization and embryo rescue techniques will become increasingly important. By leveraging new genomic tools and cytogenetic insights, breeders can develop next-generation *Lagerstroemia* cultivars that combine ornamental beauty with robust pest and disease resistance.

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Table 1.1 Nuclei isolation buffers and their compositions.

Buffer	Composition
LB01	15 mm Tris; 2 mm Na_2EDTA; 0·5 mm spermine.4HCl; 80 mm KCl; 20 mm NaCl; 15 mm β-mercaptoethanol; 0·1 % (v/v) Triton X-100; pH 7·5
MB01	20 mm MOPS; 2·5 mm Na_2EDTA; 0·7 mm spermine.4HCl; 80 mm KCl; 20 mm NaCl; 1 % (w/v) PVP; 0·5 % (v/v) β-mercaptoethanol; 0·2 % (v/v) Triton X-100; pH 7·4
WPB	200 mM Tris, 4 mM MgCl2, 2 mM Na2EDTA, 86 mM NaCl, 10 mM sodium metabisulphite, 1 % PVP-10, 1 % (v/v) Triton X-100.

Table 1.2 Estimated genome size of different *Lagerstroemia* taxa and *Heimia salicifolia*. The data are the means ± SDs (n=5). The different lowercase letters indicate significant differences among the various species based on Duncan's test at $P < 0.05$.

Specie	Genome size (pg)	SD
Hemia salicifolia	1.10 a	± 0.04
L. excelsa	0.73 cd	± 0.00
L. fauriei	0.71 cd	± 0.01
L. limii	0.75 c	± 0.01
L. loudonii	0.75 c	± 0.02
L. macrocarpa	0.79 b	± 0.01
L. speciosa	0.75 c	± 0.02
L. tomemtosa	0.72 cd	± 0.01
L. villosa	0.70 d	± 0.01

Figure 1.1. Fluorescent staining of different *Lagerstroemia* species under 30 min enzyme digestion. A. *L. excelsa*, B. *L. fauriei*, C. *L. limii*, D. *L. loudonii*, E. *L. macrocarpa*, F. *L. speciosa*, G. *L. tomentosa*, H. *L. villosa.* Scale bar = 10 µm.

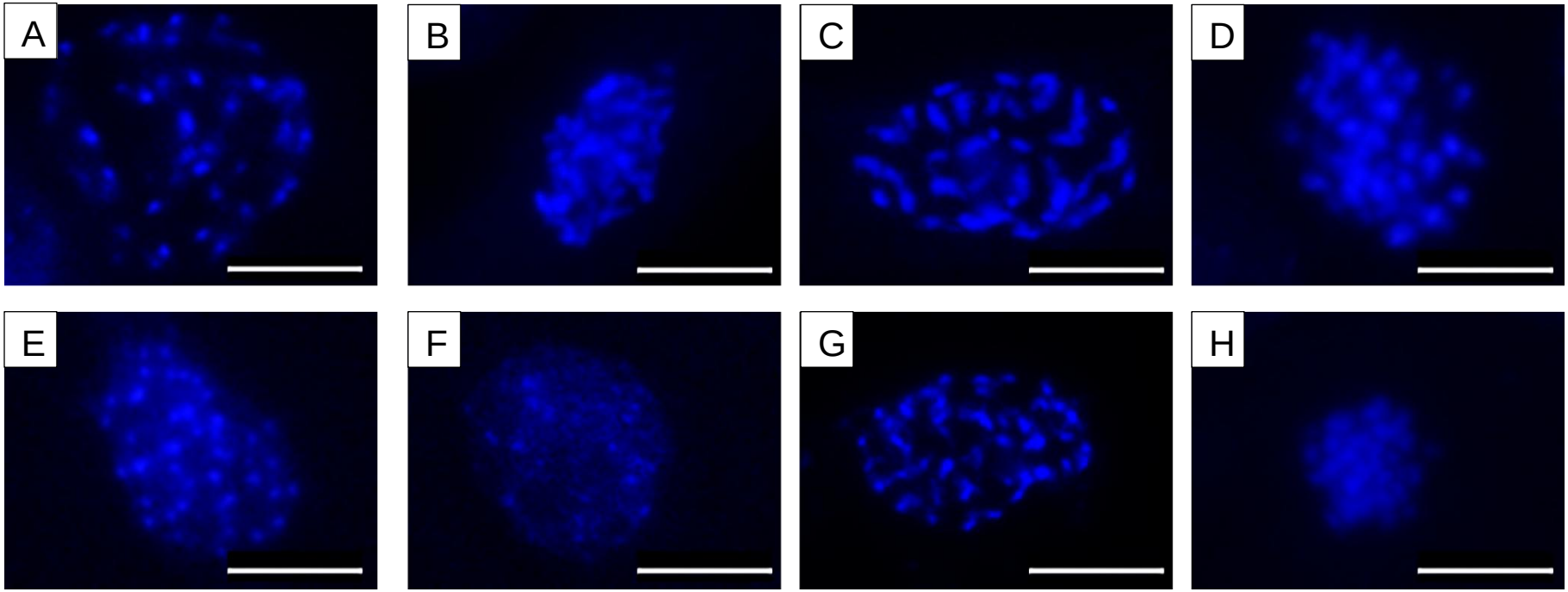


Figure 1.2. Fluorescent staining of different *Lagerstroemia* species under 60 min enzyme digestion. A. *L. excelsa*, B. *L. fauriei*, C. *L. limii*, D. *L. loudonii*, E. *L. macrocarpa*, F. *L. speciosa*, G. *L. tomentosa*, H. *L. villosa*. Scale bar = 10 µm.

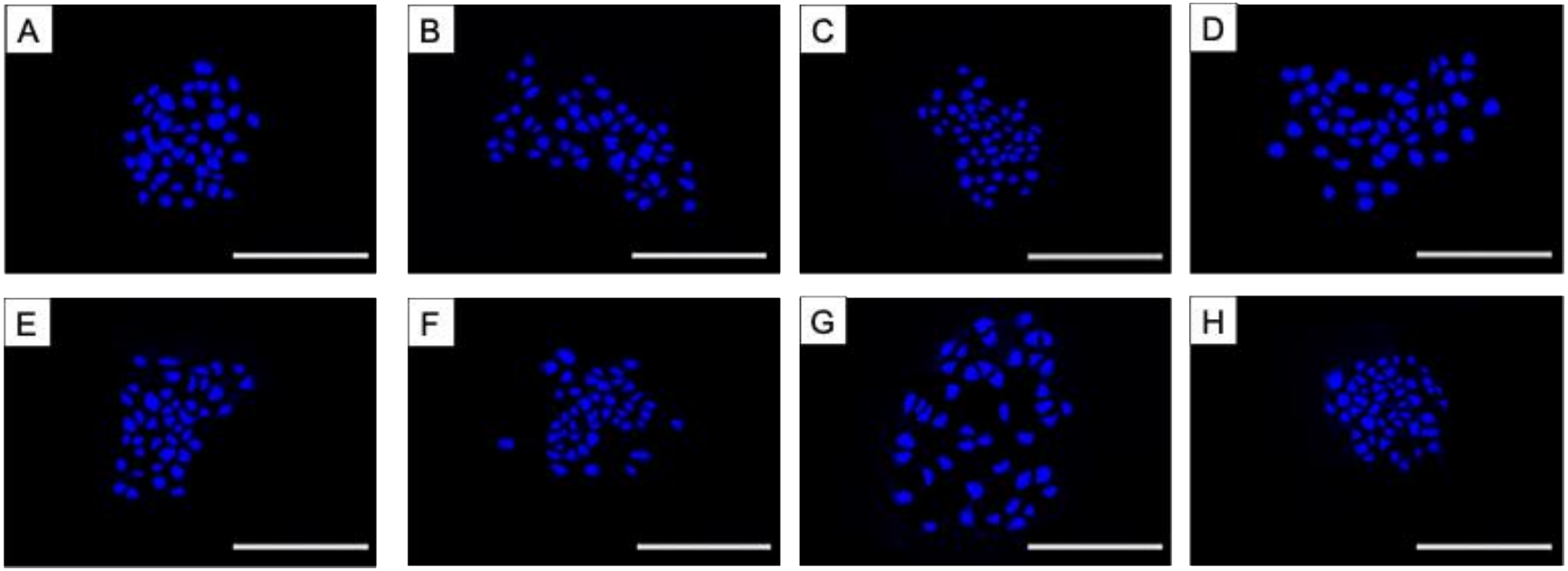


Figure 1.3. Fluorescent staining of different *Lagerstroemia* species under 60 min enzyme digestion. A. *L. excelsa*, B. *L. fauriei*, C. *L. limii*, D. *L. loudonii*, E. *L. macrocarpa*, F. *L. speciosa*, G. *L. tomentosa*, H. *L. villosa*. Scale bar = 10 µm.

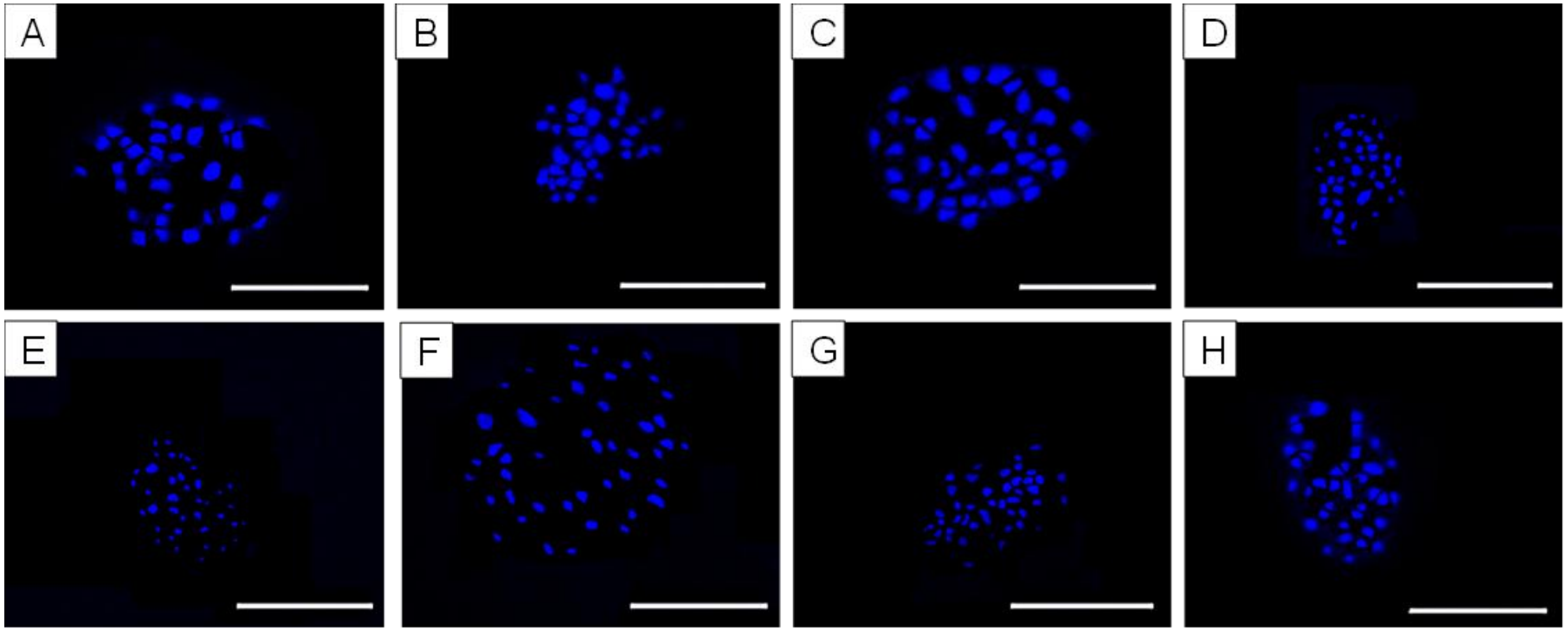
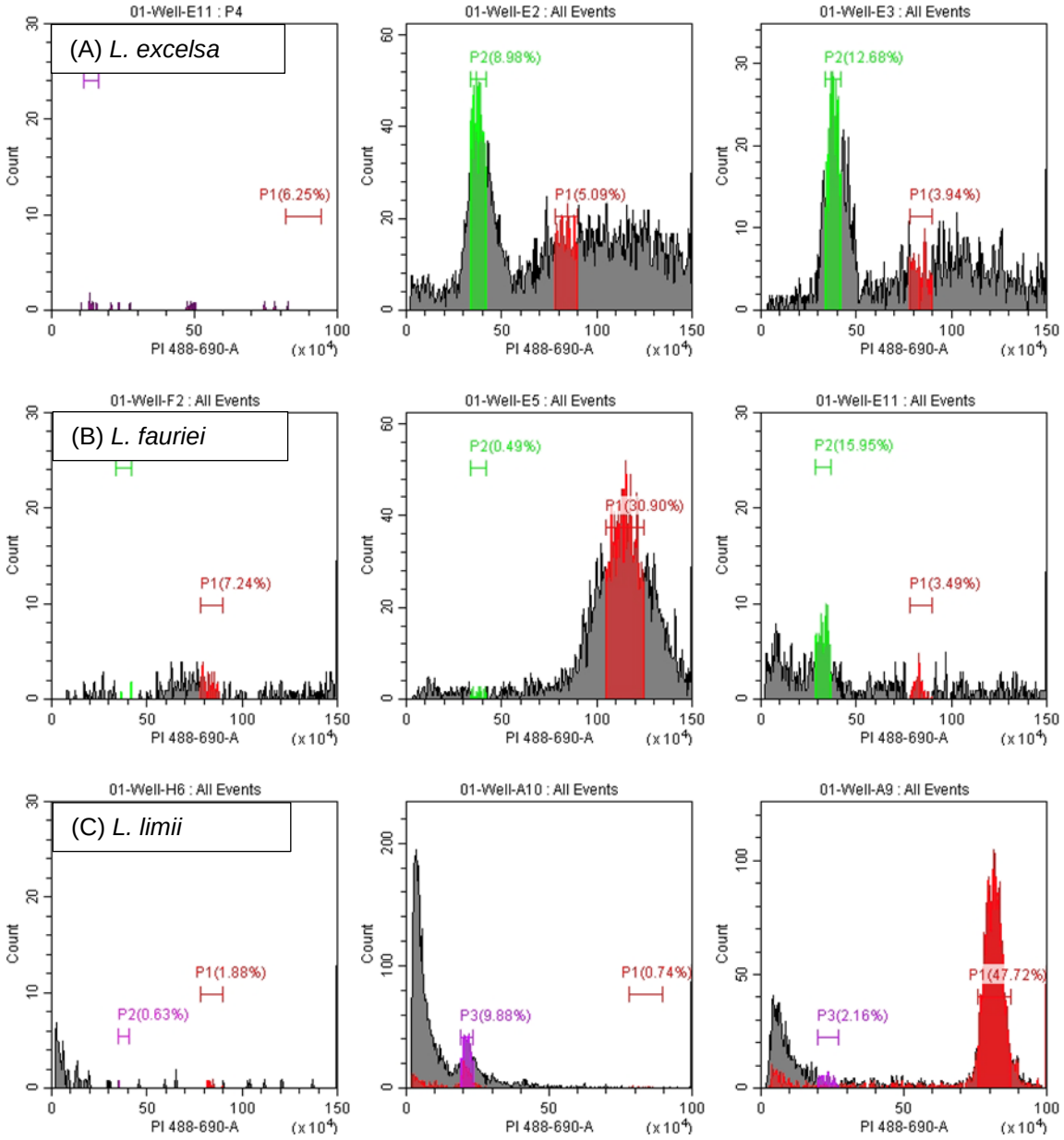
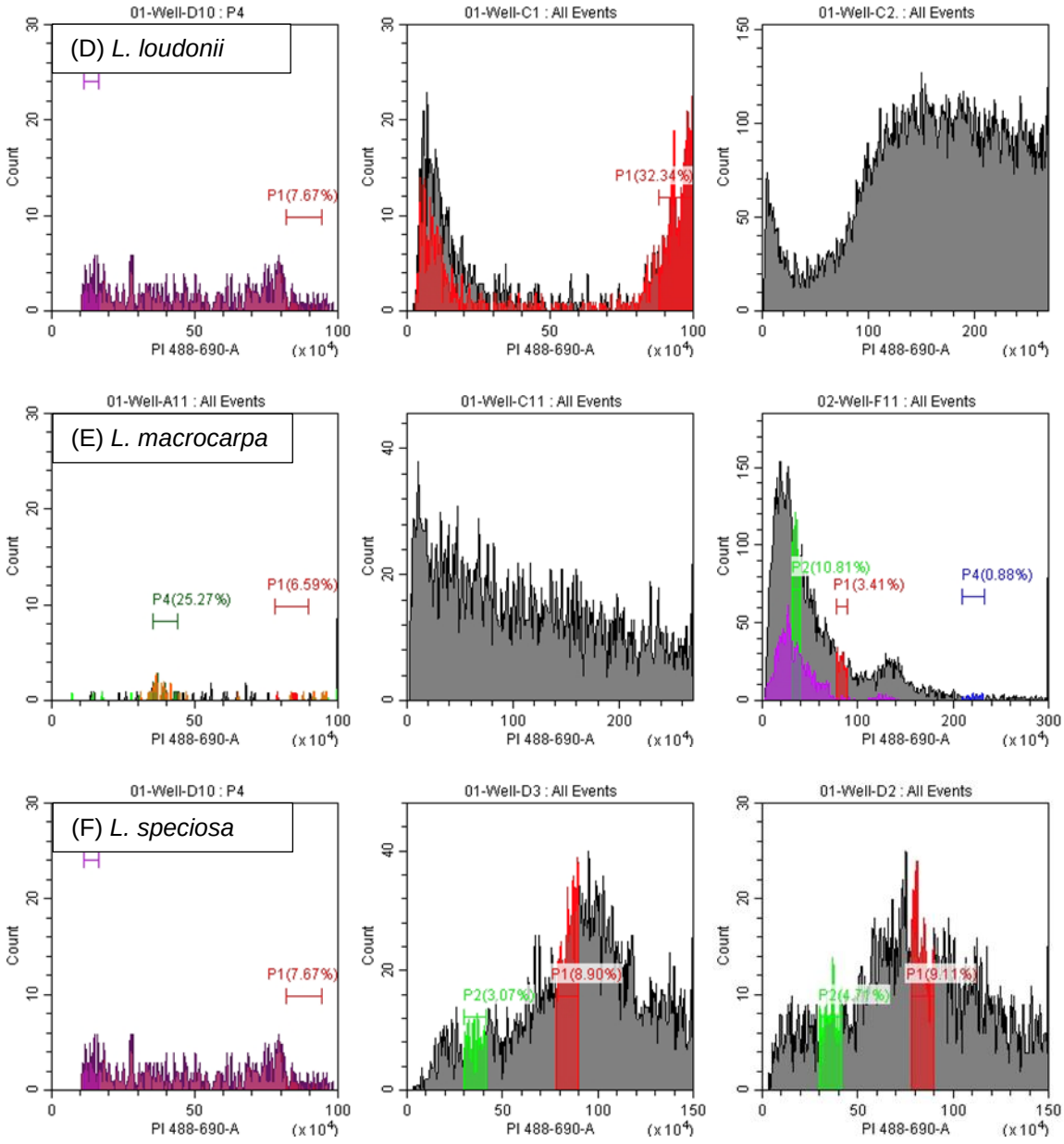


Figure 1.4. Flow cytometry results using CyStain, LB01 and Woody Plant buffers. A. *L. excelsa*, B. *L. fauriei*, C. *L. limii*, D. *L. loudonii*, E. *L. macrocarpa*, F. *L. speciosa*, G. *L. tomentosa*, H. *L. villosa*.




(D) L. loudonii
01-Well-D10 : P4
P1(7.67%)
01-Well-C1 : All Events
P1(32.34%)
01-Well-C2. : All Events
(E) L. macrocarpa
01-Well-A11 : All Events
P4(25.27%)
P1(6.59%)
01-Well-C11 : All Events
02-Well-F11 : All Events
P2(10.81%)
P1(3.41%)
P4(0.88%)
(F) L. speciosa
01-Well-D10 : P4
P1(7.67%)
01-Well-D3 : All Events
P2(3.07%)
P1(8.90%)
01-Well-D2 : All Events
P1(9.11%)
Count
PI 488-690-A
(x10^4)

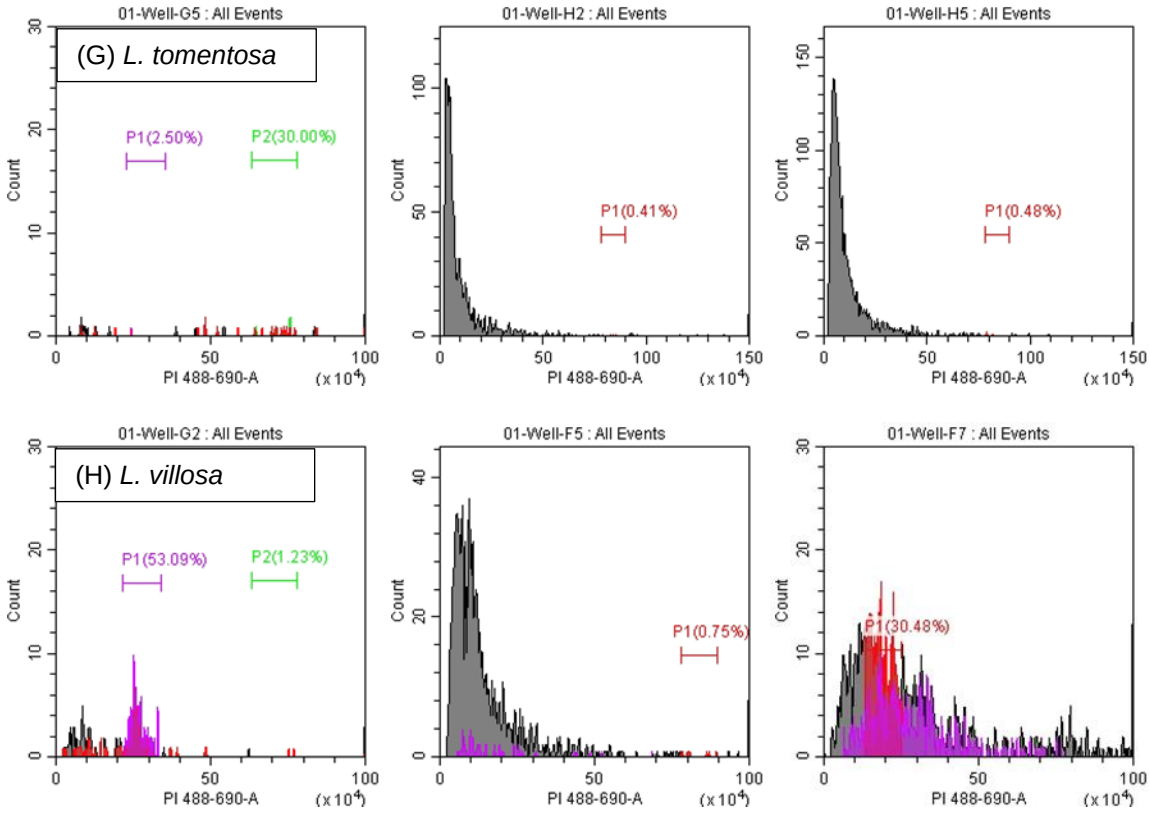
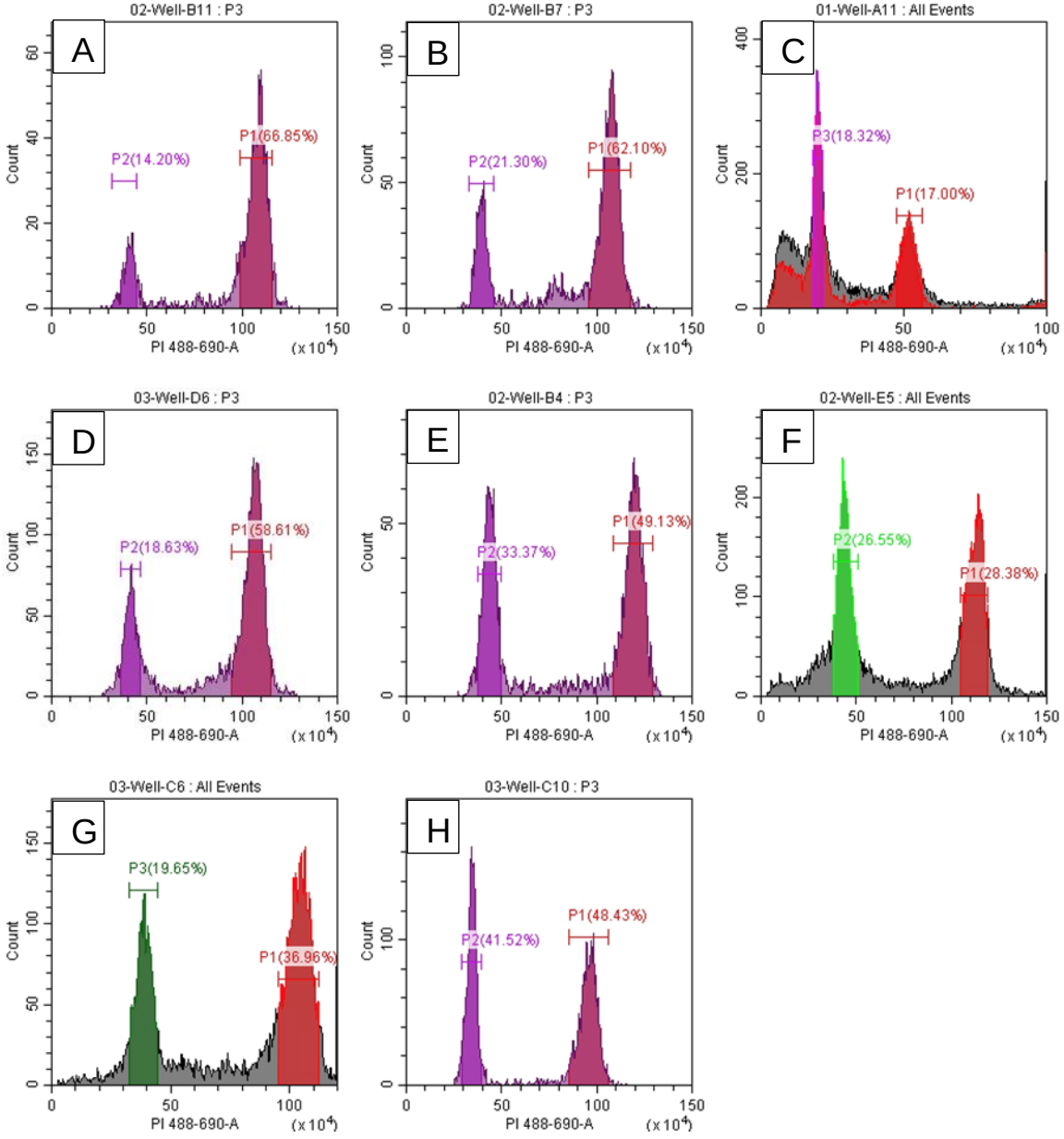

01-Well-G5 : All Events
(G) L. tomentosa
P1(2.50%)
P2(30.00%)
Count
PI 488-690-A
(x10^4)
01-Well-H2 : All Events
P1(0.41%)
Count
PI 488-690-A
(x10^4)
01-Well-H5 : All Events
P1(0.48%)
Count
PI 488-690-A
(x10^4)
01-Well-G2 : All Events
(H) L. villosa
P1(53.09%)
P2(1.23%)
Count
PI 488-690-A
(x10^4)
01-Well-F5 : All Events
P1(0.75%)
Count
PI 488-690-A
(x10^4)
01-Well-F7 : All Events
P1(30.48%)
Count
PI 488-690-A
(x10^4)

Figure 1.5. Flow cytometry results using MB01 buffer. A. *L. excelsa*, B. *L. fauriei*, C. *L. limii*, D. *L. loudonii*, E. *L. macrocarpa*, F. *L. speciosa*, G. *L. tomentosa*, H. *L. villosa*



CHAPTER 3

Compatibility of Cross-Hybridizing *Lagerstroemia* Taxa

Yue Y, Zhang D. To be submitted to *Scientia Horticulturae*.

Additional index words. *Lagerstroemia*, crape myrtle, cross-hybridization, compatibility

Abstract.

Interspecific hybridization is a pivotal strategy for developing novel *Lagerstroemia* cultivars with enhanced ornamental traits and disease resistance. In this study, 3126 controlled crosses were performed among elite crape myrtle cultivars (e.g., 'Pristine Crystal', 'Pristine Lilac', 'Pristine Ruby', D03-34) and *L. speciosa*, resulting in 731 harvested fruits. From a total of 20862 seeds, 5470 were deemed viable. Pollen viability assessments revealed that 'Pristine Crystal', 'Pristine Ruby', and *L. speciosa* produced abundant and highly viable pollen, whereas D03-34 generated limited amounts, and 'Pristine Lilac' exhibited no pollen dehiscence. These findings align with subsequent fruit-set data, which showed that crosses using 'Pristine Lilac' or D03-34 as pollen donors had significantly reduced fruit production. Logistic regression confirmed strong genotype effects on fruit set ($p<0.0001$), and chi-square analysis similarly demonstrated significant differences in viable seed production and germination across crosses ($p<0.0001$). Key crosses such as 'Pristine Ruby' × D02-52 and 'Pristine Lilac' × *L. speciosa* achieved high germination percentages (72% and 68%, respectively), highlighting their potential for efficient seedling establishment. In contrast, multiple cross combinations exhibited ≤3% germination. Introducing the germination value (GV) provided additional insight into both the rate and completeness of germination, and it was highly correlated ($R^2=0.92$) with the final germination percentage. Overall, 'Pristine Lilac' emerged as a superior maternal parent, while 'Pristine Crystal' and 'Pristine Ruby' were more effective as paternal parents. Future directions include testing seed treatments, refining tissue culture protocols to enhance germination, and evaluating cold hardiness and pest resistance to expand the adaptability of Lagerstroemia hybrids to diverse growing regions.

Introduction

Cross-hybridization plays a pivotal role in ornamental breeding by facilitating the incorporation of desirable traits—such as disease and pest resistance, improved floral display, and extended blooming periods—across related species (Hancock, 2012). In general, successful hybridization events depend on genetic compatibility, floral biology (e.g., synchrony in flowering time and compatibility of floral structures), and post-pollination mechanisms that control embryo development (Rosas-Guerrero et al., 2011). Despite the inherent challenges, breeding programs using cross-hybridization have led to numerous breakthroughs in the development of superior ornamental cultivars.

Crape myrtle (*Lagerstroemia* spp.) is a prime example of an ornamental genus that has benefited from cross-breeding initiatives. Native to Asia and introduced into the United States in the 18th century, crape myrtles are valued for their vibrant summer blooms, attractive bark, and adaptability to varied climatic regions (Dirr, 2009). Early work by Egolf at the U.S. National Arboretum laid the groundwork for systematic crape myrtle breeding, focusing on enhancing disease resistance, especially against powdery mildew (*Erysiphe lagerstroemiae*), and improving floral traits (e.g., flower color, blooming period) (Egolf and Andrick, 1978). Subsequent research further demonstrated the feasibility of interspecific hybridization among *Lagerstroemia indica*, *L. fauriei*, and other species, leading to the creation of widely grown hybrid cultivars (Pooler, 2006; Pooler and Dix, 1999; Pounders et al., 2007).

There are many factors that can affect cross-compatibility in crape myrtle. At the floral level, overlapping bloom periods and pollinator attraction are crucial for effective pollen transfer (Dirr, 2009). For example, *L. fauriei* typically begins blooming in late May to early June. However, unlike some other crape myrtle species, it does not have the ability to rebloom later in the season.

On the other hand, most *L. indica* cultivars can bloom from June all the way to August and September. Several studies have been carried out to investigate cross-compatibility in *Lagerstroemia*. These studies aim to understand the breeding potential and genetic compatibility between distinct species or cultivars, which is crucial for developing new hybrids with desirable traits such as improved disease resistance, unique flower colors, and adaptability to various climates. Pounders et al. (2006) conducted research on self- and cross-hybridization study on different *Lagerstroemia* cultivars. Their results indicated that the seed pot set was lower in self-pollination on 'Catawba', 'Whit IV', 'Tonto' and 'Tuscarora' cultivars. As a result, hybrids between *L. indica* and *L. fauriei* exhibit inbreeding depression. Compatibility studies have also been conducted on crosses between *L. indica* and *L. speciosa*. Earlier study indicated that high frequency of univalents and bivalents with abnormal meiotic behavior prohibits further breeding from F1 hybrids (Ali, 1977; Datta and Jena, 1977). Later study showed that due to the high sterility, breeding with F1 can only be executed with backcrossing or sib mating (Pounders et al., 2007).

The tangible outcomes of these breeding programs are seen in modern crape myrtle cultivars that combine the cold hardiness of *L. fauriei* with the diverse color palette of *L. indica* (Dirr, 2009). Breeders now also target improved tolerance to drought, enhanced ornamental traits (e.g., growth habit, bark coloration), and pest and disease resistance—factors increasingly important in urban horticulture (Pooler, 2006). As breeders broaden the gene pool through continued interspecific and intergeneric hybridization, the potential for novel improvements in *Lagerstroemia* and other ornamentals grows.

In the past decades, there has been a rising concern of crape myrtle bark scale (Marwah et al., 2021). The crape myrtle bark scale was first found in Texas in 2004 and soon spread to over 14 states (Gu, 2018). The pest could be a big threat to crape myrtle and other ornamental plants

because it does not have natural enemies. Therefore, breeding for pest resistance is crucial for ensuring the future success and sustainability of crape myrtle cultivation. By developing cultivars with enhanced resistance to pests, growers can reduce reliance on chemical controls, improve plant health, and maintain the ornamental value of crape myrtles in diverse landscapes. Very few studies have done research on crape myrtle bark scale resistance. Wu et al. (2020) conducted research on host suitability for crape myrtle bark scale among different crape myrtle species. They concluded that *L. speciosa* has the least numbers of male and female crape myrtle bark scales which made it possible for breeding resistance.

In this study, we aim to investigate the compatibility among various elite cultivars at the UGA Horticultural Farm, as well as the compatibility between these elite cultivars and *L. speciosa*. This research seeks to enhance our understanding of the breeding process and facilitate the development of improved crape myrtle hybrids with desirable traits.

Materials and Methods

The cultivars selected for the compatibility study were a subset of those currently utilized as parent plants in the crape myrtle breeding program at the UGA Horticultural Farm. 'Pristine Crystal' and 'Pristine Lilac' originated from crosses between Delta Jazz® and Dazzle Me Pink® seedlings, while 'Pristine Ruby' and D03-34 resulted from a hybridization between 'Crescent Moon' and 'Ebony Fire' (Yue et al., 2025). Seedlings of *L. speciosa* were obtained from USDA-GRIN. All plants used for hybridization were field grown at the UGA Horticultural Farm in Watkinsville, GA. In the summer of 2023, reciprocal crosses were performed among the three elite cultivars, as well as crosses using the four cultivars as female parents (♀) and *L. speciosa* as the male parent (♂).

The controlled pollination process was carefully managed to prevent unwanted pollination and ensure accurate hybridization. One day before the flower opening, panicles were covered with mesh bags to exclude pollinators. Early in the morning, before 6:00 AM, flower buds were manually opened, and anthers were removed from both the cultivars and *L. speciosa* before dehiscence. Flower petals were also removed to facilitate pollination. At around 7:00 AM, when pollen began to dehisce, a fine-tip brush was used to apply pollen to the stigmas. The crossed panicles were then re-covered with mesh bags to prevent pollinator interference. To further investigate compatibility mechanisms, a pollen staining and pollen tube germination study was conducted. Pollen was collected early in the morning and stored in a Petri dish with silica gel desiccant (Dry & Dry, Brea, CA) for four hours. Pollen viability was assessed using acetocarmine staining (TCI America, Portland, OR) for two hours, followed by using Olympus DP74 at 4x for microscopic examination. Viable pollen percentages were determined by counting 100 pollen grains per sample across three replicates. For pollen tube germination, a pollen germination medium that design for *Lagerstroemia* was freshly prepared containing 200 g/L sucrose, 150 mg/L boric acid, and 20 mg/L $CaCl_2$. Pollen was applied to the germination medium for two hours, and the percentage of pollen tube formation was recorded under Olympus DP74 at 10x. To ensure accuracy, three replicates were conducted, with 100 pollen grains counted per replicate.

Five flowers from each cross combination were collected at 1, 4, 8, and 24 h after pollination and placed into Carnoy's solution (acetic acid:ethanol; 1:3, v:v). After 24 hours of fixation flowers were transferred to 70% ethanol at 4 °C before use. Before staining, flowers were rinsed in distilled water for 30 minutes and then softened using 8 N NaOH for 1 hour to facilitate better visualization of pollen tubes. Flowers were rinsed in distilled water for 30 minutes and then transferred to a 0.1% (w/v) aniline blue solution in 0.1 N K_3PO_4 for 1 hour, following the protocol of Martin (1959).

After staining, the stigma and style were placed on a microscope slide with a drop of aniline blue stain, gently squashed under a cover slip, and observed under a microscope at 10x (DP74; Olympus America, Inc., Melville, N.Y.) for pollen tube analysis. A completely randomized design was employed, with the flower panicle serving as the experimental unit. Within each panicle, flowers were crossed with a designated male parent, and the number of pollinations per panicle was recorded daily. A minimum of 100 pollinations per parental combination was conducted for the cultivar crosses, while at least 80 pollinations were performed for crosses between cultivars and *L. speciosa*, using a minimum of 10 panicles per combination. In fall 2023, pods were harvested once they turned dark brown. In spring 2024, seeds were extracted, and viable seeds were counted for seed germination study. During summer 2024, a completely randomized design was implemented for the germination study, with four replicates, each containing 25 seeds. Germination data were collected over a 30-day period, and germination values were calculated at the conclusion of the study.

Results

Pollen viability varied significantly among different cultivars and Lagerstroemia speciosa. Abundant pollen production was observed in ‘Pristine Crystal’, ‘Pristine Ruby’, and *L. speciosa*, whereas only a small amount of pollen was produced by D03-34, and no pollen dehiscence was observed in ‘Pristine Lilac’ (Table 2.1, Fig. 2.1). The pollen staining of ‘Pristine Crystal’, ‘Pristine Ruby’, and *L. speciosa* were very high and showed no significant difference (Fig. 2.3A). Additionally, pollen tube germination was also successfully observed in ‘Pristine Crystal’, ‘Pristine Ruby’, and *L. speciosa*, indicating their potential for successful fertilization (Table 2.1, Fig. 2.2). However, pollen tube germination levels varied with ‘Pristine Ruby’ and *L. speciosa* showing significantly higher germination than ‘Pristine Crystal’ (Fig. 2.3B).

During the summer of 2023, 3126 artificial crosses were performed. By the end of the season, 731 fruits were collected to evaluate seed set, and viable seeds were counted in winter 2023. From these efforts, 20862 seeds were harvested in total, with 5470 confirmed as viable. Of all the crosses, 2779 came from crosses among 'Pristine Crystal', 'Pristine Lilac', 'Pristine Ruby', D03-34, and *L. speciosa* which yielded a total of 10314 seeds and 2484 viable seeds (Table 2.2). As shown in Table 2.2, the fruit set was significantly lower from those cross combinations that use 'Pristine Lilac' and D03-34 as pollen donors which coordinated with the results from pollen staining and pollen tube germination. A Chi-square test of fruit vs. no fruit across the 16 crosses was highly significant (χ^{15}_{2} = 387.2, $p < 0.0001$), indicating strong genotype effects on fruit set. Likewise, viable vs. nonviable seeds across the 12 seed-producing crosses also differed significantly ($\chi^{11}_{2} \approx$ 530, $p < 0.0001$).

A summary of fruit-set percentage (±SE), average seeds per fruit (±SE), and viable seeds per fruit (±SE) for all 16 cross combinations is presented in Table 3. Each metric is accompanied by group letters derived from post-hoc multiple-comparison tests (Tukey's HSD at α=0.05), indicating which crosses differ significantly within each metric. Logistic regression of fruit set (n = total pollinations across all crosses) revealed a highly significant effect of cross combination ($p < 0.0001$). Pairwise contrasts identified three major groupings (Table 2.3, Fig. 2.4). The highest fruit-set rates were observed in crosses 'Pristine Ruby' × 'Pristine Crystal', 'Pristine Crystal' × 'Pristine Ruby', and 'Pristine Crystal' × *L. speciosa*, with mean percentages of 54.5 ± 4.1%, 41.5 ± 4.5%, and 40.0 ± 4.9%, respectively (Fig 4A). A mid-range cluster ('Pristine Lilac' × *L. speciosa*, D03-34 × 'Pristine Crystal', etc.) showed intermediate fruit-set frequencies (20–41%) with no significant differences among them. In contrast, nine crosses displayed consistently low or zero

fruit set (≤20.7%), including D03-34 × ‘Pristine Lilac’, D03-34 × *L. speciosa*, ‘Pristine Ruby’ × D03-34, and ‘Pristine Lilac’ × D03-34, which failed to produce any fruit.

Among the successful fruits (i.e., excluding crosses with 0% fruit set), a one-way ANOVA on total seed counts (log-transformed to meet normality assumptions) indicated a strong cross effect ($p < 0.001$). Post-hoc Tukey tests revealed significant differences among cross combinations, with D03-34 × ‘Pristine Ruby’ yielding the most seeds per fruit, whereas ‘Pristine Ruby’ × ‘Pristine Lilac’ yielded the fewest seeds (Table 2.3, Fig 2.4B).

When performing viable seed counts, the same post-hoc procedure yielded similar patterns. D03-34 × ‘Pristine Crystal’ and D03-34 × ‘Pristine Ruby’ produced the highest mean viable seeds (~10–11 viable seeds per fruit), whereas ‘Pristine Ruby’ × ‘Pristine Lilac’ produced the fewest mean viable seeds (Table 2.3, Fig 2.4C).

Overall, the final integrated table (Table 2.3) underscores three critical findings: (1) fruit-set probabilities vary drastically among cross types, with certain combinations consistently achieving higher success rates; (2) seed output per fruit is not necessarily proportional to fruit set, as some crosses with moderate fruit-set percentages still yield comparatively high seed counts; and (3) viable seed production shows patterns similar to total seed counts, further highlighting which crosses ultimately produce more high-quality seeds. This approach offers a holistic view of cross performance and enables detailed, evidence-based recommendations for breeding or conservation strategies.

A chi-square analysis was applied to the seed germination study, and it revealed very high significance ($p < 0.0001$) among different crosses (Table 2.4, Fig 2.6A). ‘Pristine Ruby’ × D02-52 (72% ± 2.3%) and ‘Pristine Lilac’ × *L. speciosa* (68% ± 17.7%) emerged as the most promising crosses for high germination success. Several crosses in the 20–34% range clustered together,

indicating moderate germination potential. Due to the lower germination percentage of these crosses, more seeds are needed to generate enough seedlings for future selection and breeding work. A substantial number of crosses exhibit ≤3% germination, indicating they might be incompatible for further breeding. While the germination value (GV) integrates multiple parameters beyond simple germination percentage, the two were highly correlated in this study (R^2=0.92, Fig. 2.5). The GV analysis revealed that the two cross combinations, 'Pristine Ruby' × D02-52 and 'Pristine Lilac' × *L. speciosa*, exhibited significantly higher GV compared to other combinations, indicating superior germination speed and uniformity (Table 2.4, Fig. 2.6B). The cumulative germination patterns across different cross combinations showed that hybrid seeds began germinating as early as day 7 and reached completion by day 30 (Fig. 2.7). The peak germination period for most crosses occurred between days 7 and 25, highlighting a critical window for seedling emergence and establishment.

Pollen tube staining analysis revealed that 'Pristine Lilac' and D03-34 did not produce any detectable pollen when used as paternal parents (Fig. 2.8A, Fig. 2.8B). This observation aligns with previous findings on pollen grain staining, suggesting that these genotypes may not be viable pollen donors. In contrast, when 'Pristine Crystal' and 'Pristine Ruby' were used as paternal parents, pollen grains were clearly observed on the stigma, indicating the potential for successful hybridization (Fig. 2.8C, Fig. 2.8D). Pollen tubes from 'Pristine Crystal' and 'Pristine Ruby' were clearly observed 24 hours after crossing with 'Pristine Lilac' and 'Pristine Crystal', indicating successful fertilization (Fig. 2.8E, Fig. 2.8F).

Discussion

Interspecific hybridization is a key breeding strategy for developing new cultivars by introducing desirable traits such as disease resistance, novel flower colors, and extended blooming

periods from wild species into cultivated varieties. This approach has been widely utilized in various horticultural crops, especially ornamental species such as *Lycoris* (Yang et al., 2023), *Iris* (Li et al., 2024), *Hydrangea* (Chen and Chen, 2022), *Lantana* (Deng et al., 2020), *Myrica* (Lubell-Brand et al., 2021), and *Michelia* (Han et al., 2014). Hybridization is also a primary method for developing new crape myrtle (*Lagerstroemia*) cultivars, with the most commercially available cultivars resulting from controlled cross-hybridization. The U.S. National Arboretum has introduced more than 20 interspecific hybrid crape myrtle cultivars (Pooler, 2006), and this number continues to rise as breeding efforts advance. These hybrid cultivars have played a significant role in breeding programs aimed at improving powdery mildew resistance, serving as valuable genetic resources for developing disease-resistant *Lagerstroemia* varieties.

Therefore, it is important to understand the mechanisms behind hybridization. The initial step involves selecting appropriate parental genotypes. In theory, choosing superior parents should result in enhanced F_1 hybrids. However, since the selected parents are already hybrids, they may exhibit reduced fecundity, potentially limiting further breeding efforts (Kéry et al., 2000). We observed no pollen produced by ‘Pristine Lilac’ and D03-34, which means they are not suitable as paternal parents. ‘Pristine Lilac’ originated as a seedling derived from a cross between Delta Jazz® and gamma-ray-irradiated Dazzle Me Pink®. Gamma-ray irradiation can cause mutations, often recessive (Ahloowalia and Maluszynski, 2001), that remain hidden until segregation. D03-34 was a hybrid seedling resulting from a cross between ‘Crescent Moon’ and ‘Ebony Fire’. ‘Ebony Fire’ itself was developed from a cross where a hybrid seedling of ‘Whit VII’ and ‘Arapaho’ served as the female parent, while ‘Chocolate Mocha’ was the male parent (Pounders et al., 2013). ‘Arapaho’ carries genetic contributions from L. limii, which may contribute to reduced fertility. Additionally,

‘Chocolate Mocha’ was derived through gamma-ray irradiation, potentially introducing recessive mutations linked to low fertility that can affect its progeny during segregation.

Pre-zygotic and post-zygotic barriers must be overcome during hybridization to achieve successful crosses. A previous study on self- and cross-pollination compatibility in crape myrtle (Pounders et al., 2006) found no evidence of self-incompatibility among different cultivars, a finding consistent with our results. Additionally, they reported that pollen tubes reached the ovules within 24 hours following cross-pollination, a pattern also observed in our study and corroborated by other interspecific hybridization research (Ju et al., 2019). The subsequent study (Ju et al., 2019) further demonstrated differential cross-compatibility preferences when using *L. speciosa* as a parent. A molecular study on F_1 interspecific hybridization between *L. indica* and *L. speciosa* identified potential genes associated with sterility (Yang et al., 2024). Using virus-induced gene silencing (VIGS), researchers selectively silenced *LiDMC1* and *LiASY1* in the paternal parent ‘Ebony Ember’, leading to a significant reduction in pollen viability. These findings suggest that such genes play key roles in pollen development, and their suppression may contribute to reduced fertility in F_1 hybrids.

Our results on cross combinations and fruit set clearly demonstrated a pre-zygotic barrier when *L. speciosa* was used as the paternal parent in crosses with D03-34 and ‘Pristine Ruby’. This implies that reproductive incompatibility at the pollen–pistil interaction stage may hinder successful fertilization in these specific hybrid combinations. A previous study on interspecific hybridization between *L. indica* and *L. speciosa* also reported considerable variation in pod set across different cross combinations when *L. speciosa* was used as the paternal parent (Pounders et al., 2007), indicating that reproductive compatibility may differ among parental pairings due to genetic or physiological barriers.

A related study on tomato hybridization described different levels of hybrid seed failure, categorized as negligible, intermediate ("weak"), and near-complete ("strong") (Roth et al., 2018). They found near-complete seed inviability in all cross combinations except one. This specific intrinsic post-zygotic isolation was potentially attributed to a thin endosperm layer, leading to embryonic developmental arrest and preventing successful seed maturation. To test whether a similar mechanism applies here, an in vitro seed germination study should be conducted to supply supplemental resources and evaluate whether endosperm limitations are preventing seed germination. This approach could clarify whether thin endosperm directly causes embryonic developmental arrest and subsequent hybrid seed failure.

The goal of this study is to develop new cultivars with novel traits, which require hybrid seeds to germinate. Previously, limited research has focused on seed germination in *Lagerstroemia* species. Therefore, understanding the germination traits of different cultivars is crucial for optimizing propagation strategies and supporting breeding programs aimed at producing improved varieties. Detailed knowledge of germination behavior can offer insights into factors affecting seed viability, dormancy, and overall seedling vigor.

A study on *L. speciosa* and *L. floribunda* revealed that gibberellic acid and potassium nitrate were not key factors in promoting seed germination. Instead, higher temperatures (35/20 °C) under a 16/8-hour light/dark photoperiod significantly enhanced germination (Hung et al., 2004). This suggests that optimal environmental conditions, rather than chemical treatments, play a more critical role in promoting germination in these tropical species (Furtado and Srisuko, 1969). Another study on *L. indica* using ultrasound treatment reported low germination percentages (3% to 9%), which further decreased with increasing ultrasound exposure (Ivanova and Stoilov, 2022).

One probable reason for this low success rate is the high proportion of inviable seeds, as the study did not discard non-viable seeds prior to treatment.

Lagerstroemia species typically produce 30–60 seeds per pod; however, many lack a fully developed embryo and consist primarily of wing-like structures, which contributes to low germination rates. A related study on F_1 seed germination between American elite cultivars and Chinese native Lagerstroemia cultivars showed that cross combinations significantly influenced germination, with percentages ranging from 44% down to 0% (Chen et al., 2023). These results underscore the importance of parental genotype selection, suggesting that genetic compatibility plays a major role in seed viability and germination potential.

Our results demonstrated a wide range of germination percentages, from 0% to 72%. Notably, interspecific hybrids exhibited distinct germination patterns, implying that genetic background and cross compatibility strongly influence seed viability and germination success. To gain a deeper understanding of germination traits across different cross combinations, we introduced the germination value (GV) as a metric to evaluate both germination speed and completeness (Czabator, 1962). This approach provides a more comprehensive assessment of germination performance and facilitates better comparisons of hybrid viability and seedling establishment across diverse genetic backgrounds.

We found that two specific cross combinations had significantly higher GV than others, providing valuable insights for optimizing future hybridization strategies. Also, most seeds completed germination within 30 days, aligning with Hung et al. (2004). Our preliminary studies similarly suggest that extending germination tests beyond 30 days is unlikely to yield more viable seedlings.

Conclusion and Future Directions

In this study, we conducted controlled crosses among elite Lagerstroemia cultivars and identified promising maternal and paternal parents for future breeding efforts. Our findings suggest that ‘Pristine Lilac’ is best used as a maternal parent due to its limited pollen production. In contrast, ‘Pristine Crystal’ and ‘Pristine Ruby’ demonstrated high pollen production, making them suitable paternal parents.

For interspecific hybridization with *L. speciosa*, ‘Pristine Lilac’ emerged as the most viable maternal parent, as no fruit set was observed when using ‘Pristine Ruby’ or D03-34 as maternal parents, indicating strong incompatibility in these crosses. Although ‘Pristine Crystal’ yielded more seeds than ‘Pristine Lilac’ when used as a maternal parent, the germination rate remained extremely low. To address seed germination challenges, we plan to explore various treatments and tissue culture techniques to enhance seed viability and germination success.

We also observed that interspecific hybrids display distinct intermediate growth habits between their parental species, warranting further morphological evaluation. Because most *Lagerstroemia* species and cultivars thrive in tropical to subtropical climates, breeding for cold hardiness remains a significant challenge, particularly in regions such as Watkinsville, Georgia, where winter conditions may not be optimal for tropical species and their hybrids.

Finally, the increasing prevalence of crape myrtle bark scale (Acanthococcus lagerstroemiae) in the southeastern United States underscores the urgency of breeding for pest-resistant cultivars. Developing Lagerstroemia lines with enhanced resistance to this invasive pest is critical for maintaining their ornamental value and ensuring long-term sustainability.

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Table 2.1. Pollen staining and pollen tube germination of elite cultivars and *L. speciosa*. The data are the means ± SEs (n=3). The different lowercase letters indicate significant differences among the various taxa based on Duncan's test at $P < 0.05$.

Taxa	Pollen staining (%)	Pollen tube germination (%)
Pristine Crystal	92.3 ± 1.5 a	37.0 ± 4.4 b
Pristine Ruby	95.7 ± 1.9 a	66.0 ± 7.9 a
L. speciosa	89.3 ± 2.0 a	73.0 ± 5.0 a
Pristine Lilac	0.0 ± 0.0 b	0.0 ± 0.0 c
D03-34	0.0 ± 0.0 b	0.0 ± 0.0 c

Table 2.2. Cross number, fruit set, seed set, and viable seed of elite cultivars and *L. speciosa*.

Cross	Cross	Fruit set	Seed set	Viable seed
Pristine Ruby × D03-34	160	0	0	0
Pristine Ruby × Pristine Crystal	145	79	2144	365
Pristine Ruby × Pristine Lila	132	2	42	1
D03-34 × Pristine Crystal	125	39	1371	423
D03-34 × Pristine Lilac	110	0	0	0
D03-34 × Pristine Ruby	141	15	535	154
Pristine Crystal × Pristine Lilac	111	23	586	77
Pristine Crystal × Pristine Ruby	118	49	1430	304
Pristine Crystal × D03-34	108	11	298	71
Pristine Lilac × Pristine Crystal	122	35	989	466
Pristine Lilac × Pristine Ruby	110	35	910	103
Pristine Lilac × D03-34	125	0	0	0
Pristine Ruby × *L. speciosa*	80	5	144	32
D03-34 x *L. speciosa*	86	0	0	0
Pristine Lilac × *L. speciosa*	88	31	716	200
Pristine Crystal × *L. speciosa*	100	40	1149	288
total	1861	365	10314	2484

Table 2.3. Fruit set, seed set, and viable seed among cross combinations. The data are the means ± SEs. The different lowercase letters indicate significant differences among the various crosses based on Duncan's test at $P < 0.05$.

Cross	Fruit set (%)	Average seeds per fruit	Viable seeds
Pristine Ruby × Pristine Crystal	54.5 ± 4.1 a	33.0 ± 2.0 a	10.0 ± 1.5 ab
Pristine Crystal × Pristine Ruby	41.5 ± 4.5 ab	28.0 ± 1.5 abc	9.0 ± 1.2 abc
Pristine Crystal × *L. speciosa*	40.0 ± 4.9 ab	25.0 ± 2.2 bc	8.0 ± 1.1 bc
Pristine Lilac × *L. speciosa*	35.2 ± 5.1 b	20.0 ± 3.0 c	6.0 ± 1.5 c
Pristine Lilac × Pristine Ruby	31.8 ± 4.4 b	30.0 ± 2.1 ab	8.0 ± 1.3 bc
D03-34 × Pristine Crystal	31.2 ± 4.1 b	36.0±2.5 a	11.0 ± 1.2 a
Pristine Lilac × Pristine Crystal	28.7 ± 4.1 b	22.0 ± 2.2 bc	6.0 ± 1.4 c
Pristine Crystal × Pristine Lilac	20.7 ± 3.9 bc	24.0 ± 2.1 bc	7.0 ± 1.3 bc
D03-34 × Pristine Ruby	10.6 ± 2.6 c	35.0 ± 3.1 a	10.0 ± 1.5 ab
Pristine Crystal × D03-34	10.2 ± 2.9 c	29.0 ± 2.5 ab	8.0 ± 1.7 bc
Pristine Ruby × *L. speciosa*	6.3 ± 2.7 c	25.0 ± 2.1 bc	9.0 ± 1.3 abc
Pristine Ruby × Pristine Lilac	1.5 ± 1.1 c	18.0 ± 3.0 c	4.0 ± 2.0 d
D03-34 × Pristine Lilac	0.0 ± 0.0 c	—	—
D03-34 × *L. speciosa*	0.0 ± 0.0 c	—	—
Pristine Ruby × D03-34	0.0 ± 0.0 c	—	—
Pristine Lilac × D03-34	0.0 ± 0.0 c	—	—

Table 2.4. Germination percentage and germination value of *Lagerstroemia* spp. crosses. The data are the means ± SEs (n=100). The different lowercase letters indicate significant differences among the various crosses based on Duncan's test at $P < 0.05$.

Cross	Germination value	Germination percentage %
Pristine Lilac × *L. speciosa*	2.650 ± 0.866 a	68 ± 17.7 a
Pristine Ruby × D02-52	2.420 ± 0.299 a	72 ± 2.3 a
Pristine Lilac × D02-52	0.553 ± 0.116 b	34 ± 4.8 b
D03-34 × D02-45	0.528 ± 0.265 b	30 ± 11.4 b
Pristine Crystal × D02-52	0.422 ± 0.145b	33 ± 5.7 b
Pristine Ruby × D03-14	0.385 ± 0.113 b	30 ± 6.2 b
D03-34 × D02-52	0.297 ± 0.203 b	21 ± 9 bc
D03-34 × D03-14	0.224 ± 0.149 b	20 ± 7.1 bc
Pristine Ruby × Pristine Crystal	0.208 ± 0.144 b	20 ± 8.2 bc
Pristine Lilac × Pristine Crystal	0.012 ± 0.012 b	2 ± 2 c
Pristine Crystal × D03-14	0.012 ± 0.012b	3 ± 3 c
Pristine Crystal × *L. speciosa*	0.005 ± 0.002 b	3 ± 1 c
D03-34 × Pristine Crystal	0.003 ± 0.003 b	1 ± 1 c
D03-34 × Pristine Ruby	0.000 ±0.000 b	0 ± 0 c
Pristine Crystal × Pristine Ruby	0.000 ±0.000 b	0 ± 0 c
Pristine Lilac × Pristine Ruby	0.000 ±0.000 b	0 ± 0 c

Figure 2.1. Pollen staining of elite cultivars and *L. speciosa*. A. Pristine Crystal, B. Pristine Ruby, C. *L. speciosa*.

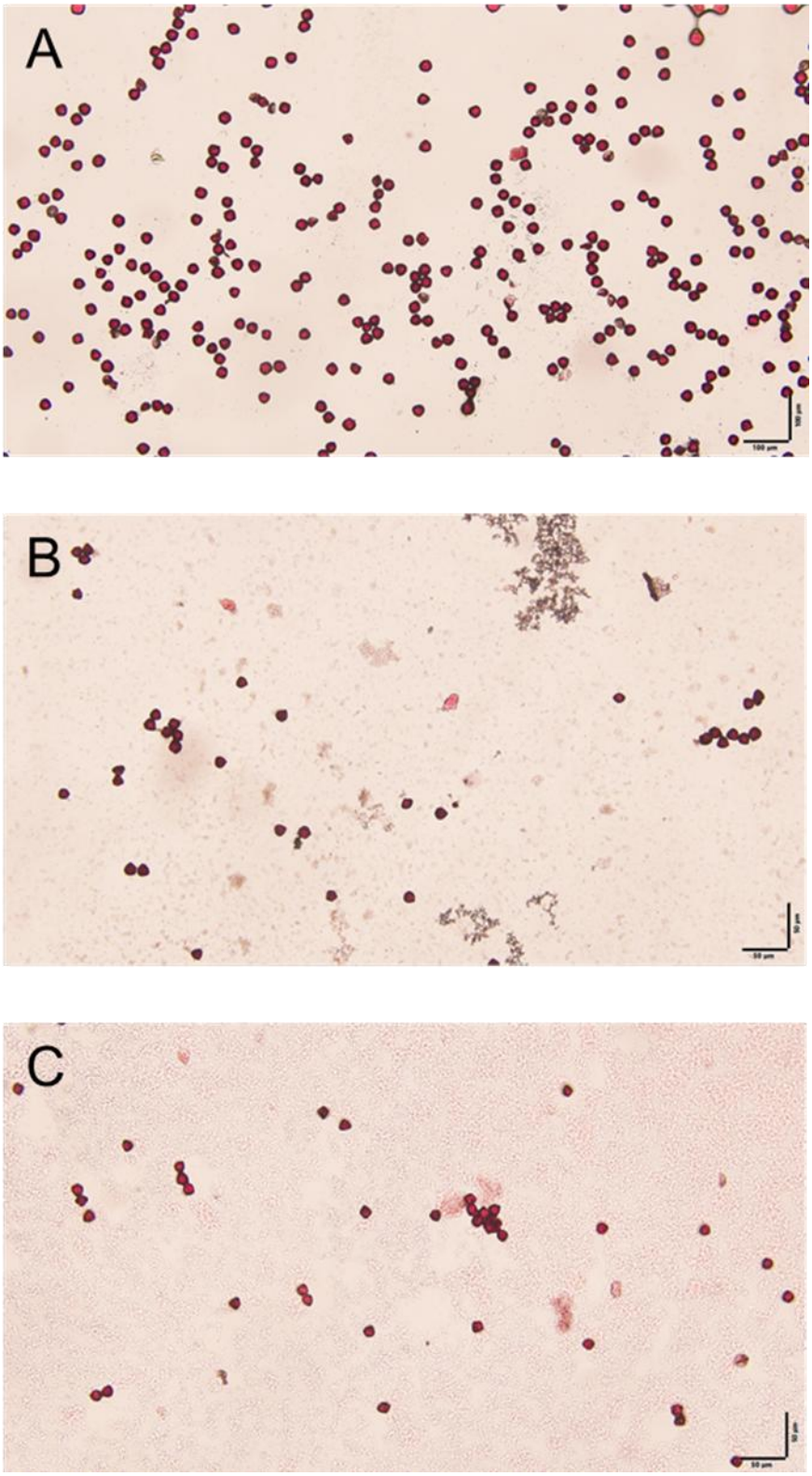


Figure 2.2. Pollen tube germination of elite cultivars and *L. speciosa*. A. Pristine Crystal, B. Pristine Ruby, C. *L. speciosa*.

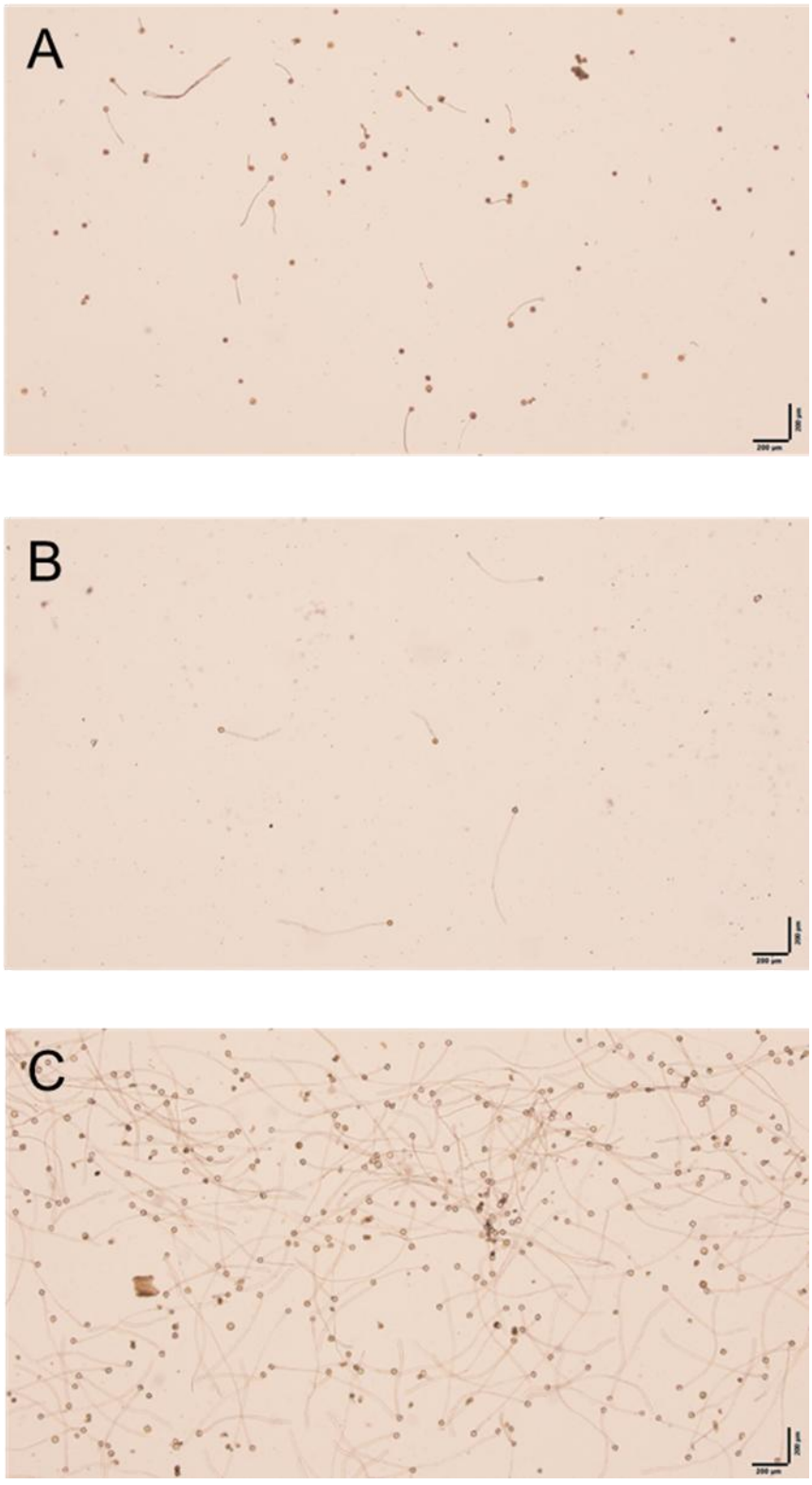


Figure 2.3. Pollen grain staining (A) and pollen tube germination (B) among cultivars and *L. speciosa.* The data are the means ± SEs (n=300). The different lowercase letters indicate significant differences among the various crosses based on Duncan's test at $P < 0.05$.

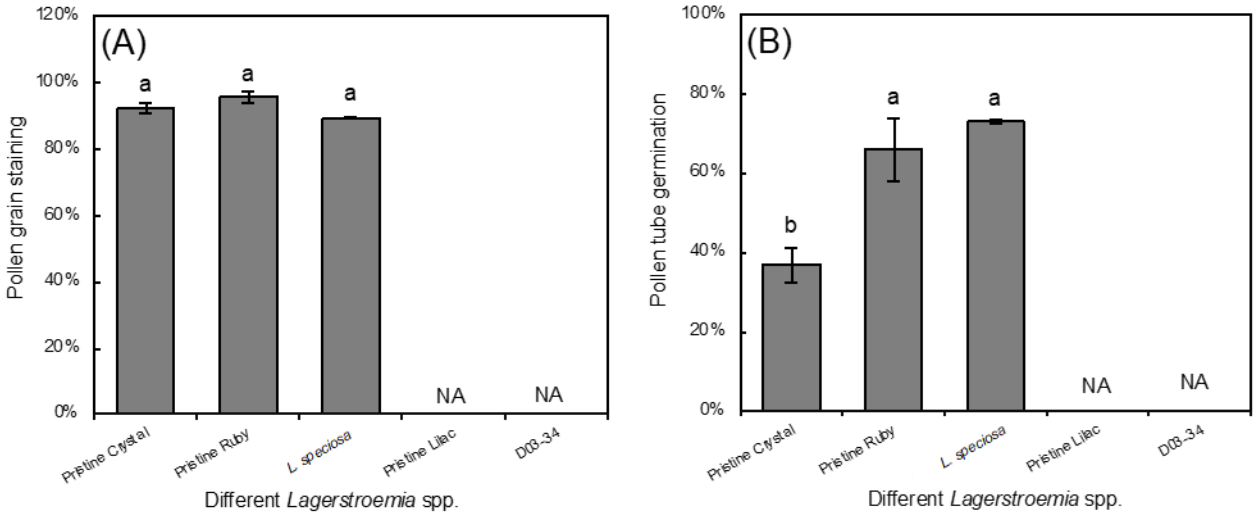


Figure 2.4. Fruit set % (A), average seed set (B), and viable seed (C) among cross combinations. The data are the means ± SEs. The different lowercase letters indicate significant differences among the various crosses based on Duncan's test at $P < 0.05$.

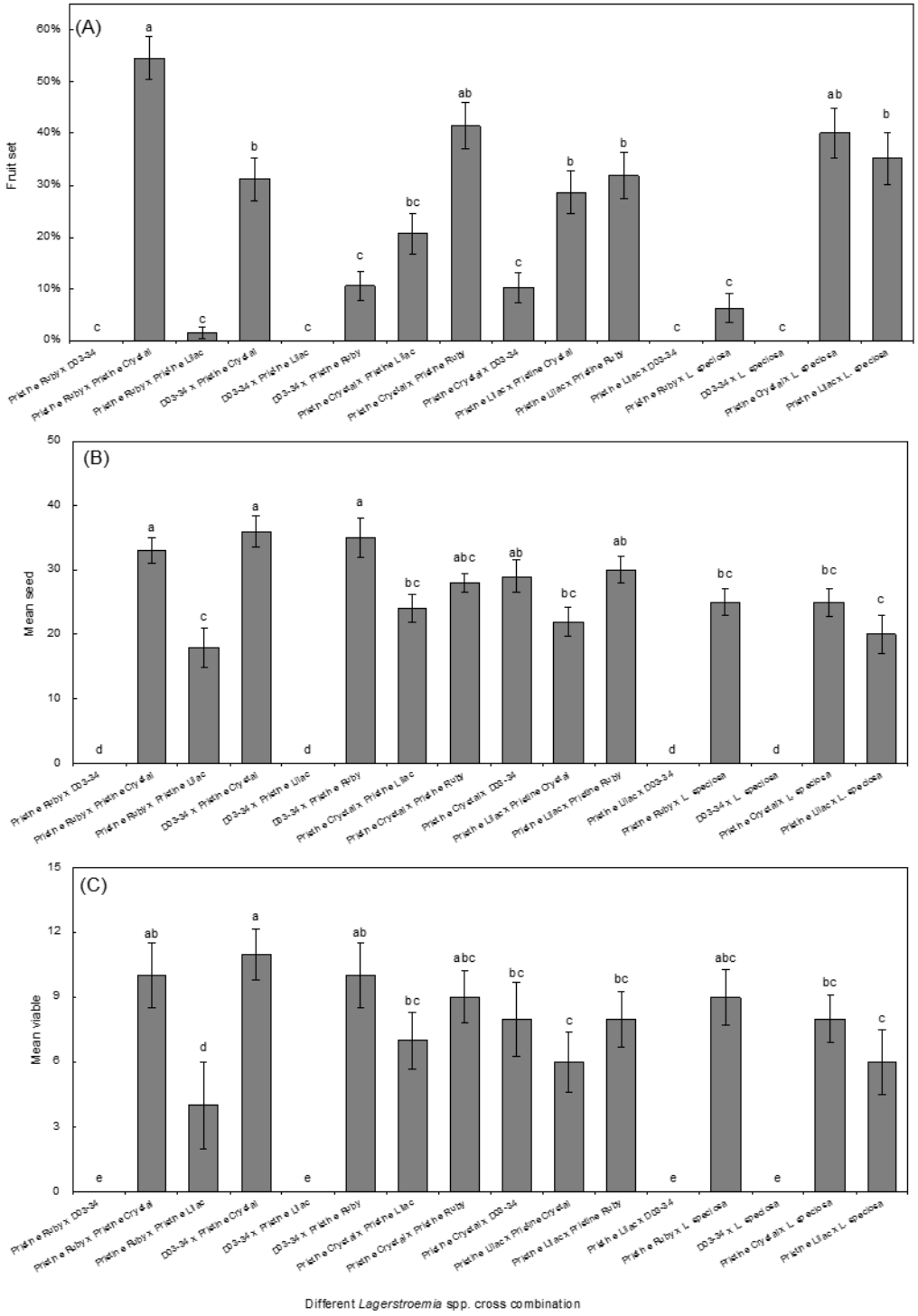


Figure 2.5. The correlation between germination value and germination percentage of *Lagerstroemia* spp. hybrids.

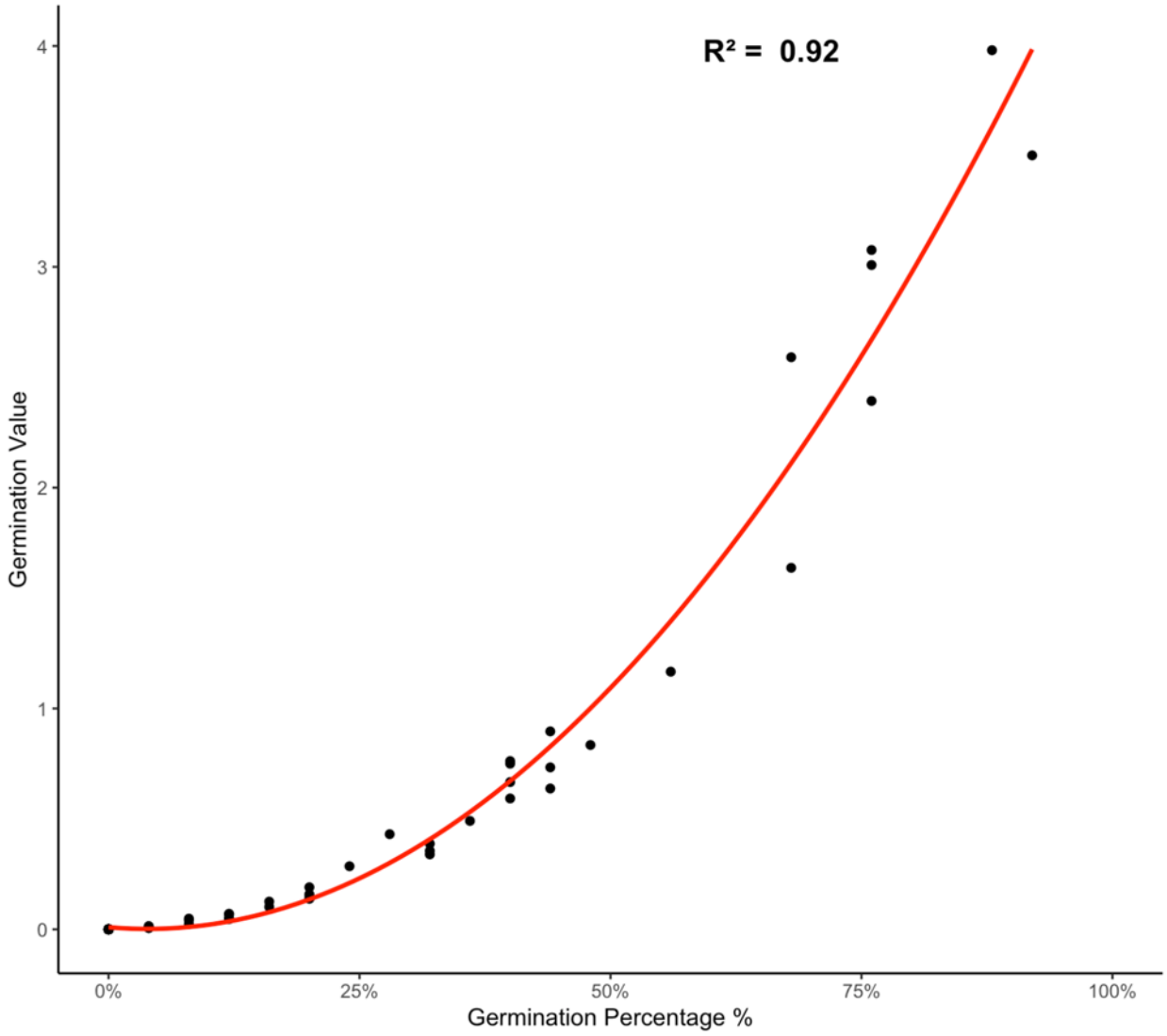


Figure 2.6. Germination value (A) and germination percentage (B) of *Lagerstroemia* spp. crosses. The data are the means ± SEs (n=100). The different lowercase letters indicate significant differences among the various crosses based on Duncan's test at $P < 0.05$.

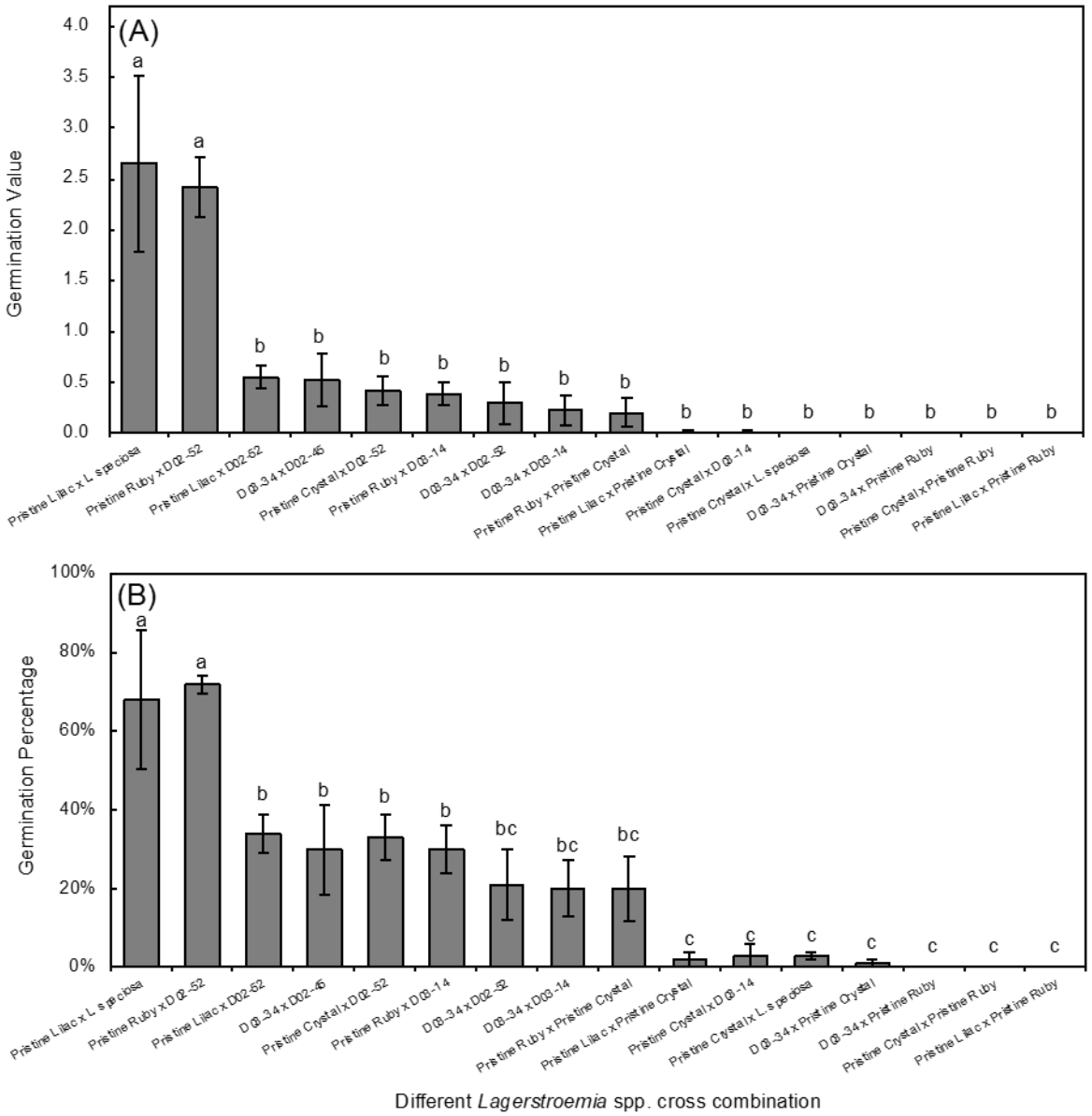


Figure 2.7. Cumulative germination percentage of the 30-day germination test on cross combinations.

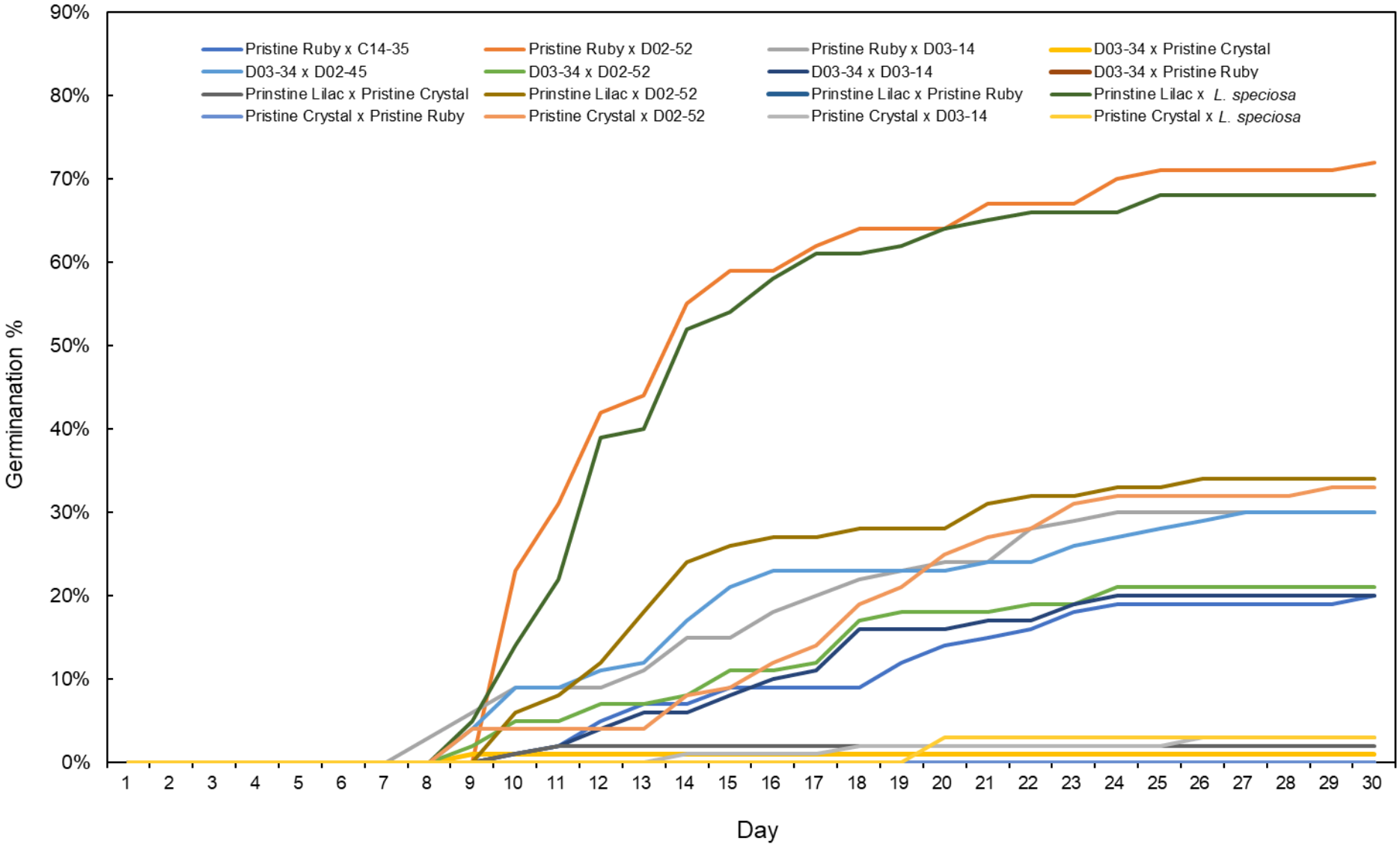
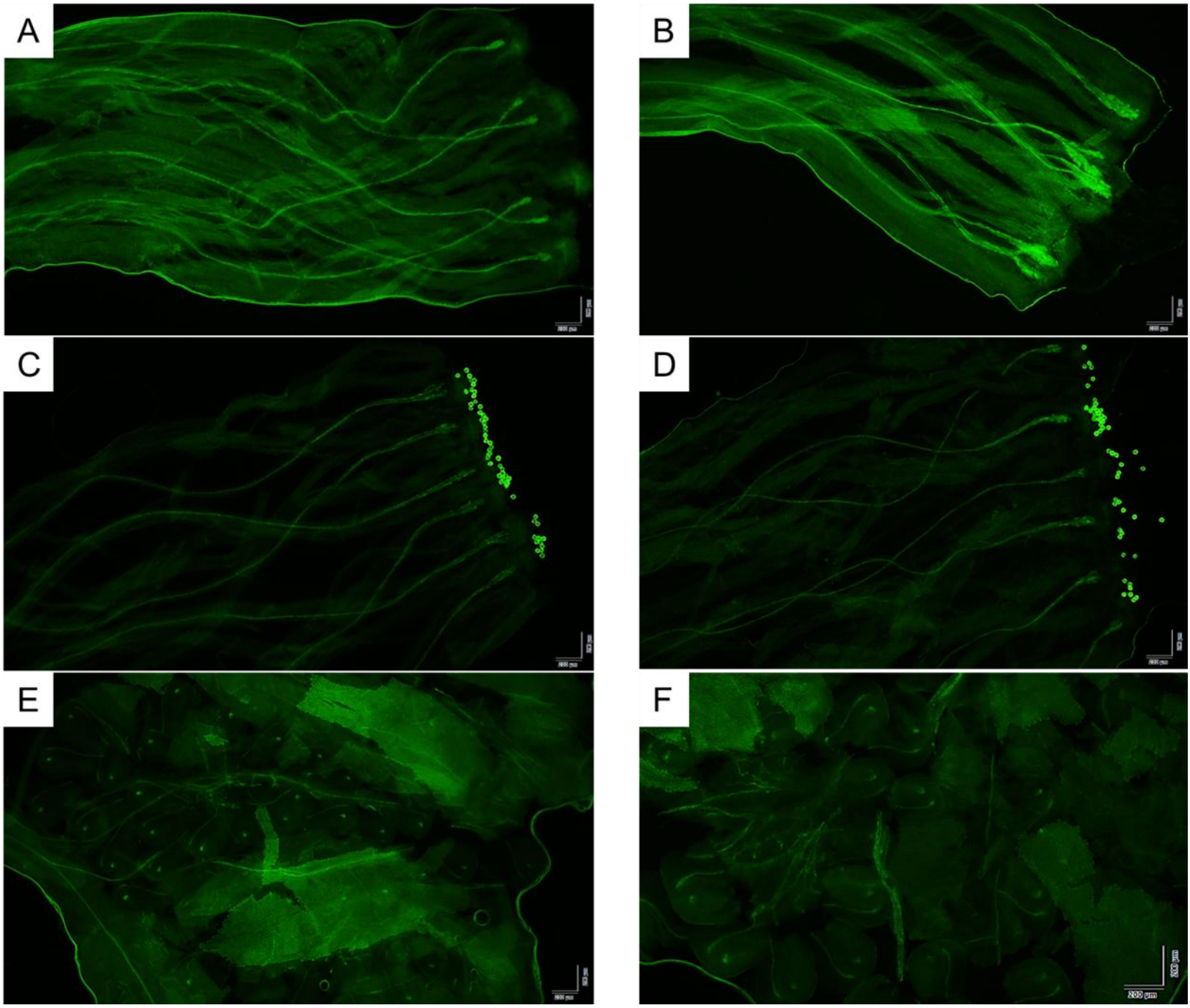


Figure 2.8. Pollen germination and pollen tube growth from cross combinations. A. ‘Pristine Crystal’ × ‘Pristine Lilac’, B. ‘Pristine Crystal’ × D03-34, C. ‘Pristine Ruby’ × ‘Pristine Crystal’, D. D03-34 × ‘Pristine Ruby’, E. ‘Pristine Lilac’ × ‘Pristine Crystal’ 24 h after pollination. F. ‘Pristine Crystal’ × ‘Pristine Ruby’.



CHAPTER 4

Micropropagation of One-Year Blooming *Lagerstroemia* Cultivars

Yue Y, Zhang D. To be submitted to *HortScience*.

Additional index words. new cultivars; *Lagerstroemia*; crape myrtle; tissue culture; propagation

Abstract.

Micropropagation presents a rapid, efficient strategy for propagating *Lagerstroemia* hybrids, particularly valuable for overcoming the limitations of conventional stem cutting methods and addressing disease pressures in the southeastern United States. In this study, we optimized seed-based sterilization protocols and evaluated various basal media and hormone regimens to enhance in vitro germination, shoot proliferation, and root induction. Hybrid seeds from three crosses (21H026, 21H040, and 21H043) were sterilized using different concentrations of sodium hypochlorite, with treatment concentration emerging as the critical factor influencing both germination and contamination rates. Furthermore, comparisons among full-strength MS, half-strength MS, and Woody Plant Medium (WPM) revealed that WPM supplemented with moderate levels of 6-benzylaminopurine (BA), indole-3-butyric acid (IBA), and gibberellic acid (GA_3) produced the longest shoots and most robust root systems, while MS-based media favored shoot multiplication. Notably, an unexpected early flowering response from leaf explants underscores the potential for rapid trait selection. These findings provide a versatile framework for efficient micropropagation in crape myrtle, paving the way for accelerated breeding cycles and the development of cultivars suitable for diverse environmental conditions.

Introduction

Micropropagation is an efficient way to fast propagate plants in a short time. It has been applied to many species including annuals, perennials, and woody plants. The micro-propagated plants are clones to the original plants thus they can inherit all the unique characters from the original plant. Micro-propagation can also save the propagation materials since each explant only needs a small piece of material from the original plant. Most of the crape myrtle cultivars are

propagated by stem cuttings. However, most crape myrtle cultivars can only survive in USDA zone 7 and above. Thus, there is a big gap for one-year blooming cultivars to be used as annual bedding plants and ground covers for zone 6 and lower states. Even though most crape myrtle cultivars form a lot of seed pods at the end of the blooming season, seed germination varies among different cultivars and the heterozygosity of F1 seeds make it hard for further experiments (chromosome doubling). The large amount of uniformity plantlets in a short period of time will be good for mutation experiments. The germination of F1 hybrids is also difficult due to the heterozygosity of woody plants. For example, the crosses between *Hibiscus syriacus* × *H. paramutabilis* and *H. syriacus* × *H. sinosyriacus* yielded no fruits (Van Laere et al., 2007). Embryo rescue was applied to, but seedlings were totally lost due to variegated and albinism. Therefore, embryo rescue might be an effective way to overcome the barrier.

Embryo rescue is to grow an immature or mature zygotic embryo under sterile conditions on a nutrient medium to obtain a viable plant (Bridgen, 1994). The major application of embryo rescue in plant breeding is the breeding of incompatible interspecific and intergeneric species. Outcrossing species are characterized by extensive abortion because of the failure of initial stages of tissue differentiation (Wiens et al., 1987). Seed production in inbreeding plants may exceed 80% of ovules compared to 20% in outcrossing plants (Wiens et al., 1987). The other reason for embryo abortion is the endosperm fails to develop properly (Hu and Wang, 1986). The endosperm usually develops poorly between interspecific crosses, intergeneric crosses, and different ploidy crosses (Bridgen, 1994). Small or young embryos that abort at the pilot stages are often difficult to isolate and the nutritional requirements of the young embryos vary a lot among species. Therefore, ovule culture methods might be an effective way to rescue the small or young embryos (Rangan, 1984). After pollination, ovaries will be kept and the calyx, corolla, and stamens will be removed. The

ovaries will be sterilized and cultured to the nutrient medium. Matured embryos perform well on media with 2% to 3% sucrose, whereas immature embryos grow better with 8% to 12% sucrose due to the mimicry of high osmotic potential within the young embryo sac (Bridgen, 1994). Plant growth regulators are not that important in embryo rescue. Exogenous auxins are not required for plant embryo growth in vitro (Norstog, 1979). Cytokinins are not effective when use alone but can promote growth and differentiation of embryos when combined with some auxins (Veen, 1963). Hormones should not be used for embryo rescue since they can cause structural abnormalities (Monnier, 1978).

The first application of embryo rescue in *Lagerstroemia* was reported by Wang et al. (2010) on *L. indica* and *L. speciosa* hybrids. Backcrosses between the hybrids and *L. indica* cultivars were attempted and no viable embryos were obtained from the crosses that the hybrids were female parents. However, some crosses that *L. indica* were used as female parents produced embryos. They then cultured the young embryos on a nutrient medium composed of half strength MS plus 30 g/L sucrose, 30 g/L glucose, MS vitamins, 3% coconut water, and 6 g/L Agargel and obtained two viable plants.

Our research focuses on two primary objectives: (1) germinating seeds produced by cross hybridization, and (2) rapidly propagating the most promising seedling lines that exhibit novel ornamental traits. Preliminary data collected in 2022 revealed distinct germination trends among various cross combinations, and subsequent potting trials demonstrated that certain hybrids not only flowered within three months of germination but also showed enhanced blooming performance. These early-flowering lines are especially valuable for breeding programs aimed at expediting selection cycles and introducing desirable traits more quickly.

However, in the southeastern United States, hot and humid conditions foster high levels of pathogen and pest pressure, making efficient disease control a key concern. Consequently, initiating micropropagation from seeds, rather than from potentially contaminated vegetative tissues, can mitigate microbial contamination and improve overall propagation success. Building on these findings, our strategy integrates systematic evaluation of seed-based germination protocols, selection of superior F_1 hybrids, and development of streamlined micropropagation methods. This approach not only accelerates the discovery and dissemination of improved crape myrtle lines but also addresses regional challenges associated with pest and disease management.

Materials and Methods

Hybrid seeds from 2021 were used as plant material to germinate in petri dishes to test germination percentage and contamination. We selected three different hybrid combinations including 21H026, 21H040, and 21H043 since they already showed the potential in the regular growing condition (Fig. 3.1). Completely randomized designs with 9 treatments and 5 reps (4 pseudo replicates per rep) were applied for sterilization. Seed explants were rinsed under running tap water in mesh bag for 30 min and then dipped to 70% ethanol for 30 seconds. The primary sterilant was commercial bleach (8.25% sodium hypochlorite), applied at three concentrations: 10%, 20%, and 30%. Each bleach concentration was tested at three intervals: 15, 30, or 45 minutes. After bleaching, seeds were rinsed three times in sterile distilled water to remove residual sterilant. The explants were then cultured on petri dishes (Falcon®, Tewksbury, MA) filled with 10 mL MS medium. The MS medium was made up by 4.4 g/L Murashige and Skoog Basal Salts with minimal organics (Sigma-Aldrich, St. Louis, MO), 30 g/L sucrose (Sigma-Aldrich, St. Louis, MO), 3 g/L Phytagel (Sigma-Aldrich, St. Louis, MO), and 2 ml/L Plant Preservative Mixture (PPM) (Plant Cell Technology, Washington, DC). The pH of the medium was adjusted to 5.8 before autoclaving

by using 0.1N NaOH. The medium was sterilized in autoclave at 121°C for 30 min before pouring to the petri dishes. Once germination was initiated, seedlings were sub-cultured monthly to ensure consistent growth and to facilitate further evaluation. Six months after initial germination, a subset of hybrids began flowering—a noteworthy observation that suggests certain seedlings may achieve blooming within their first year (Fig. 3.2). This early floral development underscores the potential for accelerated breeding cycles and may prove advantageous in selecting novel *Lagerstroemia* lines with rapid bloom characteristics.

To accelerate propagation of rapid-bloom seedlings, we focused on the top-performing hybrid seedling, designated 21H026. Three basal media—full-strength MS, half-strength MS (½ MS), and Woody Plant Medium (WPM)—were compared to identify optimal conditions for shoot proliferation. Each treatment included four culture jars (with five explants per jar) under different hormone regimes. As cytokinins, 6-benzylaminopurine (6-BAP) was tested at 0.5 mg/L and 1.0 mg/L. For auxin supplementation, indole-3-butyric acid (IBA) was applied at 0.05 mg/L and 0.1 mg/L. Gibberellic acid (GA_3) at 0 mg/L and 0.5 mg/L was incorporated to assess the impact on nodal segment elongation. By systematically comparing these media and hormonal concentrations, the study aims to identify the most effective protocol for rapidly multiplying elite *Lagerstroemia* lines exhibiting promising early-flowering traits. The whole experiment lasted 60 days and at the end of the experiment, shoot numbers, longest shoot length, root numbers, longest root length, and internode length were counted.

Results

Tissue culture responses were evaluated for three *Lagerstroemia* crosses—21H026, 21H043, and 21H040—under varying treatment durations and concentrations. Two primary parameters

were measured: germination rate (percentage of seeds that successfully germinated) and contamination rate (percentage of cultures exhibiting microbial contamination).

For Cross 21H026, concentration played a pivotal role in both germination ($F = 3.884$, $p = 0.033$) and contamination ($F = 3.692$, $p = 0.038$), whereas duration and the interaction between duration and concentration were not significant for either parameter (Table 3.1, Fig. 3.3). These results suggest that optimizing the treatment concentration is critical for improving germination outcomes and reducing contamination in this hybrid, while adjusting the length of exposure provided no additional benefit.

In Cross 21H043, no factors significantly affected germination rate (all $p > 0.10$). However, concentration had a highly significant influence on contamination ($F = 7.737$, $p = 0.002$), whereas duration and the duration × concentration interaction did not (all $p > 0.10$) (Table 3.1, Fig. 3.3). These data indicate that the principal avenue for lowering contamination in 21H043 is to optimize the concentration of the sanitizing or regulatory agents, without needing to alter treatment duration.

By contrast, Cross 21H040 exhibited a borderline effect of duration on germination rate ($F = 3.241$, $p = 0.055$), while concentration again showed a statistically significant impact ($F = 5.015$, $p = 0.014$) (Table 3.1, Fig. 3.3). In terms of contamination, concentration was significant ($F = 3.436$, $p = 0.047$), and duration had no measurable effect ($F = 0.655$, $p = 0.528$). No significant interaction was found in either parameter (all $p > 0.10$). Thus, although adjusting treatment time may slightly enhance germination, fine-tuning concentration remains the most robust strategy for improving tissue culture outcomes in 21H040.

Overall, the data underscores the dominant influence of treatment concentration on both germination and contamination across the three crosses, with treatment duration showing a limited or inconsistent role. These findings highlight the necessity of careful concentration optimization

in tissue culture protocols for newly developed *Lagerstroemia* hybrids, particularly when aiming to maximize germination success and minimize contamination.

For the best media for fast propagation, the hormones combination performed very differently. Shoot growth and root development varied markedly with medium composition and growth regulator combinations (Table 3.2., Fig. 3.4). The greatest shoot length (4.78 ± 0.16 cm) was obtained on WPM medium supplemented with 0.5 mg/L BA + 0.05 mg/L IBA + 0.5 mg/L GA, which was significantly higher ($p < 0.05$) than all other treatments. In contrast, the shortest shoots were observed on MS medium containing 1 mg/L BA + 0.1 mg/L IBA (1.61 ± 0.20 cm) and did not differ significantly from MS with 1 mg/L BA + 0.05 mg/L IBA + 0.5 mg/L GA (2.09 ± 0.12 cm). The lowest shoot elongation (1.6 cm) was observed in the MS + 1 mg/L BA + 0.1 mg/L IBA treatment, which was significantly different from all other treatments. Treatments with MS + 0.5 mg/L BA + 0.5 mg/L IBA, ½ MS + 0.5 mg/L BA + 0.1 mg/L IBA, and ½ MS + 1 mg/L BA + 0.05 mg/L IBA also resulted in short shoots, with lengths ranging between 2.3–2.7 cm.

Shoot multiplication was highest on MS medium with 1 mg/L BA + 0.1 mg/L IBA (10 ± 0.63 shoots) and on ½ MS containing 1 mg/L BA + 0.05 mg/L IBA (9.9 ± 0.60 shoots), both were significantly higher than the rest (Table 3.2, Fig. 3.5). By contrast, shoot numbers were minimal (1.7 ± 0.21) in WPM and MS media supplemented with GA and lower BA concentrations.

Root induction was strongly promoted in WPM-based treatments. The longest roots (up to 2.79 ± 0.78 cm) were recorded on WPM with 1 mg/L BA + 0.1 mg/L IBA, whereas no significant root formation (0 cm) occurred on ½ MS or MS media lacking adequate IBA levels (Table 3.2, Fig. 3.6). A similar trend was evident in root number, where WPM with BA and IBA produced 1.4–2.3 roots per explant, significantly higher than the 0–0.5 roots observed on most MS-based formulations (Table 3.2, Fig. 3.7).

Internode length was longest (0.75 ± 0.03 cm) in WPM with 0.5 mg/L BA + 0.05 mg/L IBA + 0.5 mg/L GA, suggesting that gibberellin supplementation contributed to shoot elongation. Treatments without GA exhibited shorter internodes (0.12–0.45 cm) (Table 3.2, Fig. 3.8).

Surprisingly, preliminary experiments using leaf explants yielded a noteworthy outcome. While only a small fraction of leaf tissues successfully initiated callus, one explant displayed remarkable development. Two weeks after being placed on WPM supplemented with 1.0 $mg \cdot L^{-1}$ BA and 0.1 mg/L^{-} IBA, callus formation became evident. By approximately seven weeks, shoot primordia had emerged from the callus; these rapidly progressed to form flower buds. Within 45 days of culture, a second bud emerged and opened, followed by a third bloom prior to day 60 and the initiation of a third flower bud (Fig. 3.10). Observing repeated flowering in vitro, especially in a woody ornamental such as *Lagerstroemia*, is extraordinarily rare and underscores the potential of leaf explants for rapid, high-impact trait selection or propagation.

Overall, these results indicate that WPM media, particularly those containing moderate levels of BA (0.5–1 mg/L), a small amount of IBA (0.05–0.1 mg/L), and, where desired, GA for elongation, are most effective for both shoot and root development. In contrast, MS-based media with higher BA concentrations favor shoot proliferation (increased shoot number) but often result in limited root initiation (Fig. 3.9).

Discussion

In this study, we established a protocol for rapid *in vitro* propagation under varied conditions, emphasizing the importance of starting with clean explants. Although nodal segments are commonly preferred for micropropagation, the first-year flowering plants we targeted were small and provided limited explant material. Moreover, the intense disease pressure in the southeastern United States posed an additional hurdle, since older tissues often harbor persistent fungal and

bacterial contaminants. Historically, mercury chloride has served as an effective surface disinfectant (Kumar et al., 2021; Zobayed, 2000), yet its high toxicity has curtailed its use in modern laboratories. Sodium hypochlorite, while safer, can be too weak at lower concentrations to eradicate microbial threats and can damage plant tissues at higher concentrations due to its corrosive, oxidizing properties.

Consequently, we selected seeds as the explant source. Seeds are typically stored under low-temperature, low-humidity conditions that impede disease survival, and the seed coat can mitigate potential tissue damage from sterilants like bleach. Indeed, sodium hypochlorite treatments have enhanced germination in other crops—such as cultivated tomatoes and *Solanum* relatives (Rick and Hunt, 1961; Rick and Borgnino, 1977)—and conifers, including Australian pine and Scotch pine (Wenny and Dumroese, 1987). Consistent with these findings, our results for crosses 21H026 and 21H040 revealed that higher bleach concentrations correlated with increased germination rates. This improvement likely stems from bleach eliminating or reducing pathogenic microorganisms and/or weakening the seed coat's physical or chemical barriers, thus fostering more uniform germination. In addition, bleach may remove inhibitory compounds that naturally curb germination until they degrade in the environment, thereby facilitating quicker and more synchronous seedling emergence.

Several studies have investigated the micropropagation of *Lagerstroemia* species, but most of this earlier work focused on genotypes other than *L. indica* and its related cultivars. A recent investigation by Zhang et al. (2023) tested four basal media—DWK, WPM, ½ MS, and MS—for an *L. indica* cultivar and reported that DWK produced the greatest plant height, followed by WPM, ½ MS, and MS. Although our findings aligned with theirs regarding superior performance of WPM

or ½ MS over full-strength MS, we did not evaluate DWK medium, leaving open the possibility that DWK might outperform the media tested here.

In the same study, combinations of NAA and BA were optimal for multiplication and plant height, with the best results achieved at 1.0 mg/L BA + 0.2 mg/L NAA (Zhang et al., 2023). By contrast, we used IBA as an auxin source, which contributed to differences in outcomes. Future protocols would benefit from systematically comparing NAA, IBA, and IAA to identify the most effective auxin for each *Lagerstroemia* genotype and desired propagation goal. Another noteworthy observation from Zhang et al. (2023) was the dynamic change in endogenous GA_3 content, peaking at around 250 ng/g at day 20, which points to a complex interplay between exogenous and endogenous hormones during in vitro culture.

Huang et al. (2022) similarly analyzed hormone fluctuations in an *L. indica* root induction study. They found that ½ MS medium was the most conducive for root formation, followed by ½ WPM and ½ DWK, and that IBA at 0.6 mg/L yielded 92.5% rooting with the longest roots (~2.29 cm). Their work also tracked GA_3 levels, peaking at day 10 with 450–500 µg/g—significantly higher than that reported by Zhang et al. (2023). Despite these varying endogenous hormone levels, our findings nonetheless confirm that exogenous GA_3 can improve plant height and internode elongation. These results echo an earlier study of 'Little Chief' crape myrtle, in which GA_3-enhanced media (1.0 mg/L BA + 0.02 mg/L NAA + 0.5 mg/L GA_3) improved branching and multiplication (Yamamoto et al., 1994). Although their experiment did not directly quantify shoot elongation, subsequent comparisons revealed that in vitro transplants significantly outgrew seedlings within four weeks, hinting at GA_3's contribution to accelerated plant height in tissue-cultured plants.

Previous studies in various plant species corroborate the finding that exogenous GA_3 can stimulate shoot elongation while hindering the outgrowth of lateral or tiller buds. In apple (*Malus domestica*), GA_3 did not promote axillary bud outgrowth or down-regulate branching repressor genes (MdTCP40, MdTCP33, and MdTCP16), as auxin depletion or cytokinins would, but instead elevated the expression of their transcriptional activators (MdSBP12, MdSBP18) (Tan et al., 2018). GA_3 application also decreased cytokinin-response regulators and auxin transport–related genes, thereby curbing cytokinin responsiveness and auxin export from the bud. Similarly, in herbaceous species such as barley (*Hordeum vulgare*), GA_3 inhibits tiller bud growth, whereas inhibiting GA synthesis with chlormequat chloride promotes tillering (Ma and Smith, 1992; Woodward and Marshall, 1988). Rice (*Oryza sativa*) trials demonstrated that GA_3 and NAA treatments repressed tiller bud development by modulating levels of indole-3-acetic acid and cytokinins (Liu et al., 2011). These observations help explain why, in our study, media supplemented with GA_3 consistently produced longer shoots yet reduced the total number of axillary buds or lateral shoots, whereas treatments lacking GA_3 favored more shoot initiations over elongation.

Taken together, these studies underscore the importance of considering both basal media composition (especially if DWK is available) and hormone regimens—particularly the choice of auxin and the addition of exogenous GA_3—in *Lagerstroemia* micropropagation protocols. Further research examining the endogenous hormone balance, especially GA_3 dynamics, would help refine these protocols, leading to improved shoot multiplication, elongation, and root induction in diverse crape myrtle genotypes.

In summary, our findings offer a versatile approach to in vitro propagation for a unique *Lagerstroemia* hybrid. Depending on the intended outcome, the choice of medium and hormone concentrations can be tailored to specific needs. For large-scale micropropagation where initial

stock material is scarce, ½ MS and MS media proved especially effective at stimulating multiple shoots, thereby maximizing plantlet production. Conversely, when ample stock plants are available, protocols geared toward generating larger, more robust explants may be preferable for subsequent potting and greenhouse acclimatization.

A particularly intriguing result was the successful regeneration of a leaf explant that not only formed callus and shoots in a brief period but also initiated flowering *in vitro*. This remarkable early flowering behavior warrants further investigation to determine whether repeated subculture maintains the 40-day flowering capacity or if the trait diminishes over successive propagation cycles. Should it remain stable, leaf-derived explants could offer a valuable system for rapid trait selection and commercialization of early-flowering crape myrtle lines.

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Table 3.1. Two-way ANOVA results of germination percentage and contamination of elite crosses.

Cross	Factor		Germination rate	Contamination rate
21H026	Duration	*F* value	1.171	0.033
		P value	0.325	0.968
	Concentration	*F* value	3.884	3.692
		P value	0.033*	0.038*
	Duration × Concentration	*F* value	0.339	0.775
		P value	0.849	0.551
21H043	Duration	*F* value	0.651	2.053
		P value	0.529	0.148
	Concentration	*F* value	1.698	7.737
		P value	0.202	0.002**
	Duration × Concentration	*F* value	1.628	1.026
		P value	0.196	0.412
21H040	Duration	*F* value	3.241	0.655
		P value	0.055	0.528
	Concentration	*F* value	5.015	3.436
		P value	0.014*	0.047*
	Duration × Concentration	*F* value	0.745	0.409
		P value	0.570	0.800

Table 3.2. Micropropagation growth index by different media and growth regulator combinations. The data are the means ± SEs (n=10).

The different lowercase letters indicate significant differences among the different treatments based on Duncan's test at $P < 0.05$.

Treatment	Longest shoot length (cm)	Shoot numbers	Longest root length (cm)	Root numbers	Internode length (cm)
WPM 0.5 mg/L BA+0.05 mg/L IBA	3.47 ± 0.26 c	3.40 ± 0.43 cd	1.86 ± 0.42 ab	2.30 ± 0.58 a	0.40 ± 0.04 b
WPM 0.5mg/L BA+0.1 mg/L IBA	3.29 ± 0.17 c	2.20 ± 0.13 de	2.21 ± 0.40 a	2.20 ± 0.44 a	0.37 ± 0.04 b
WPM 1mg/L BA+0.05mg/L IBA	4.13 ± 0.17 b	4.50 ± 0.27 c	2.23 ± 0.75 a	1.50 ± 0.52 ab	0.45 ± 0.05 b
WPM 1mg/L BA+0.1 mg/L IBA	4.08 ± 0.25 b	3.80 ± 0.55 c	2.79 ± 0.78 a	1.70 ± 0.50 a	0.42 ± 0.05 b
WPM 0.5 mg/L BA+0.05 mg/L IBA+0.5 mg/L GA	4.78 ± 0.16 a	1.70 ± 0.21 e	2.39 ± 0.80 a	1.40 ± 0.50 ab	0.75 ± 0.03 a
1/2 MS 0.5mg/L BA+0.05mg/L IBA	1.96 ± 0.11 de	7.90 ± 0.71 b	0.00 ± 0.00 c	0.00 ± 0.00 d	0.18 ± 0.03 cd
1/2 MS 1mg/L BA+0.05mg/L IBA	2.40 ± 0.16 d	9.90 ± 0.60 a	0.00 ± 0.00 c	0.00 ± 0.00 d	0.15 ± 0.03 d
1/2 MS 0.5mg/L BA+0.1mg/L IBA+0.5mg/L GA	3.00 ± 0.16 c	2.40 ± 0.16 de	1.87 ± 0.77 ab	0.50 ± 0.22 bcd	0.26 ± 0.02 cd
MS 1mg/L BA+0.1mg/L IBA	1.61 ± 0.20 e	10.00 ± 0.63 a	0.33 ± 0.25 bc	0.30 ± 0.21 cd	0.12 ± 0.02 d
MS 1mg/l BA+0.05mg/Ll IBA+0.5mg/L GA	2.09 ± 0.12 de	1.70 ± 0.21 e	0.00 ± 0.00 c	0.00 ± 0.00 d	0.13 ± 0.02 d

Figure 3.1. *Lagerstroemia* hybrid seedlings started to bloom three months after germination.

Figure 3.2. *Lagerstroemia* hybrid seedlings bloom in glass tube.

Figure 3.3. Germination percentage and contamination percentage of three different hybrids in MS medium. The data are the means ± SEs (n=20). The different lowercase letters indicate significant differences among hybrids based on Duncan's test at $P < 0.05$. The different upper letters indicate significant differences among hybrids based on Duncan's test at $P < 0.05$.

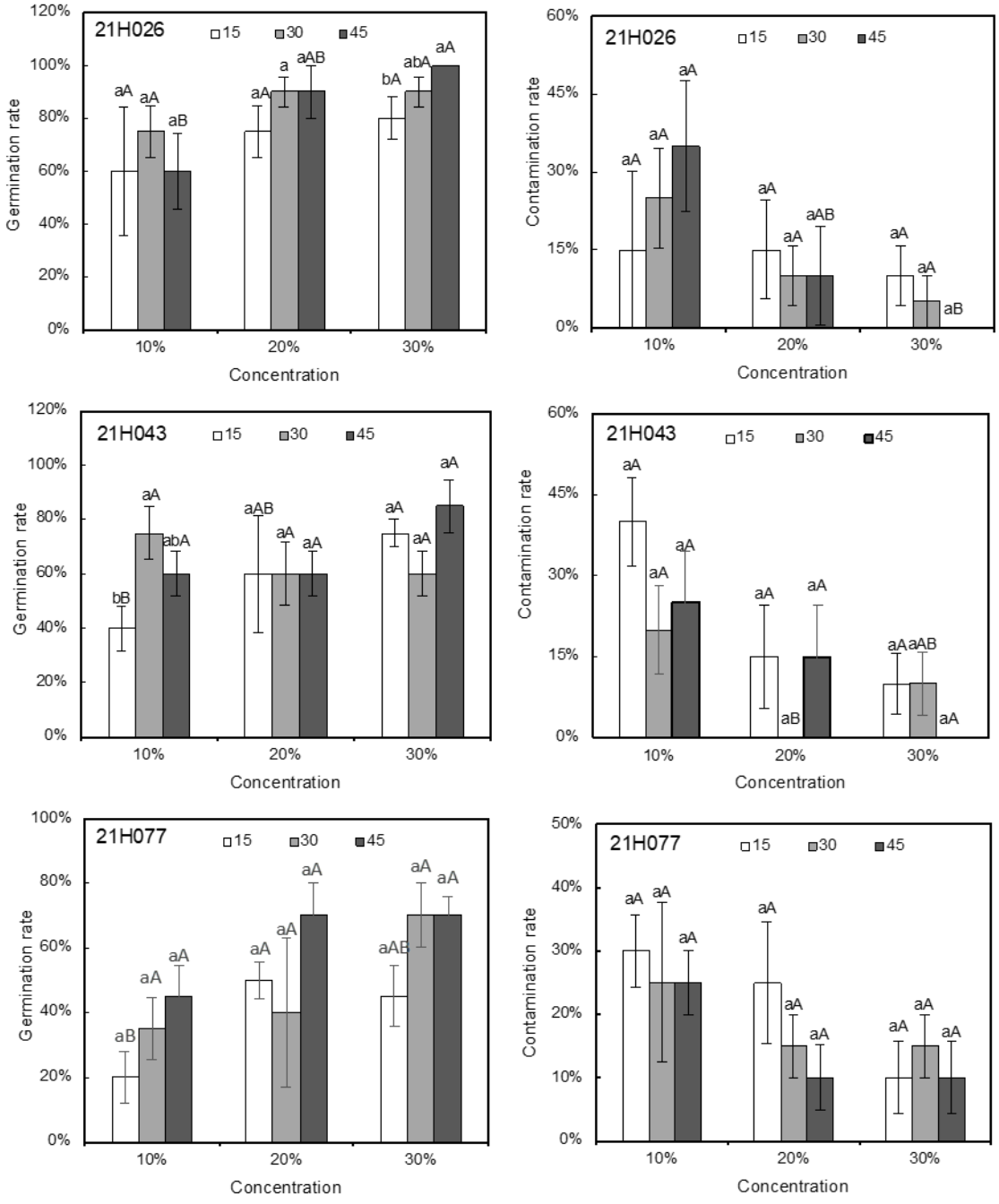

Figure 3.4. Longest shoot length by different media and growth regulator combinations. The data are the means ± SEs (n=10). The different lowercase letters indicate significant differences among treatments based on Duncan's test at $P < 0.05$.

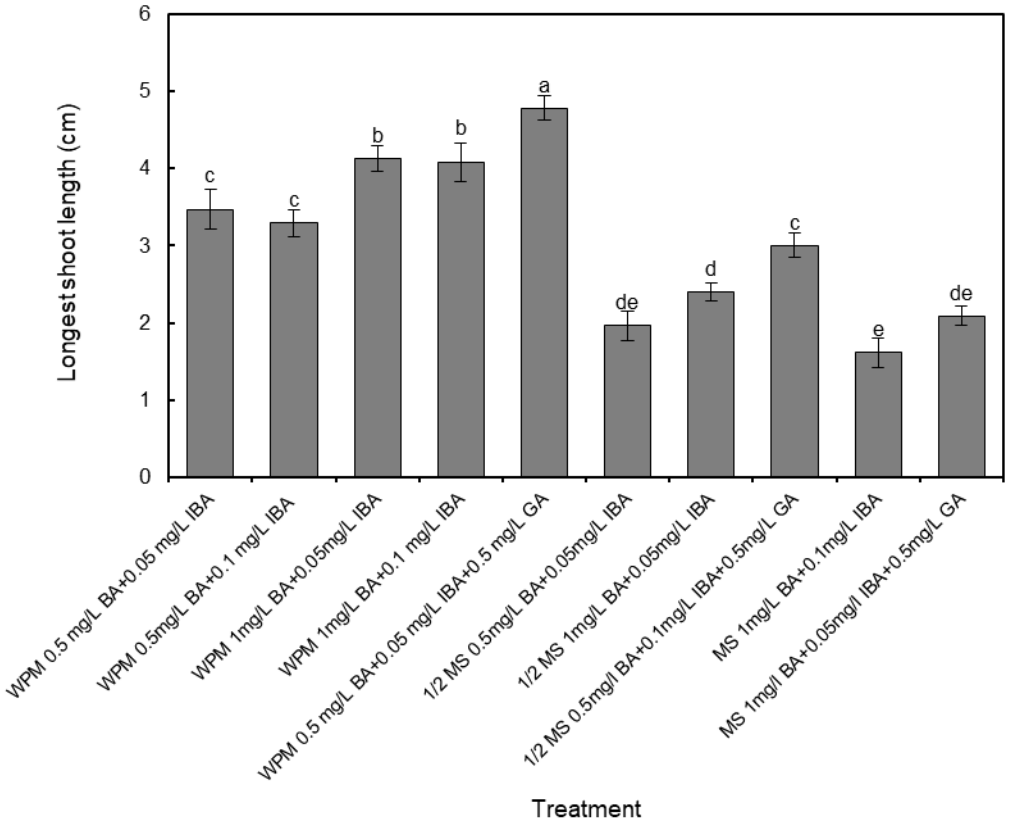

Figure 3.5. Shoot multiplication by different media and growth regulator combinations. The data are the means ± SEs (n=10). The different lowercase letters indicate significant differences among treatments based on Duncan's test at $P < 0.05$.

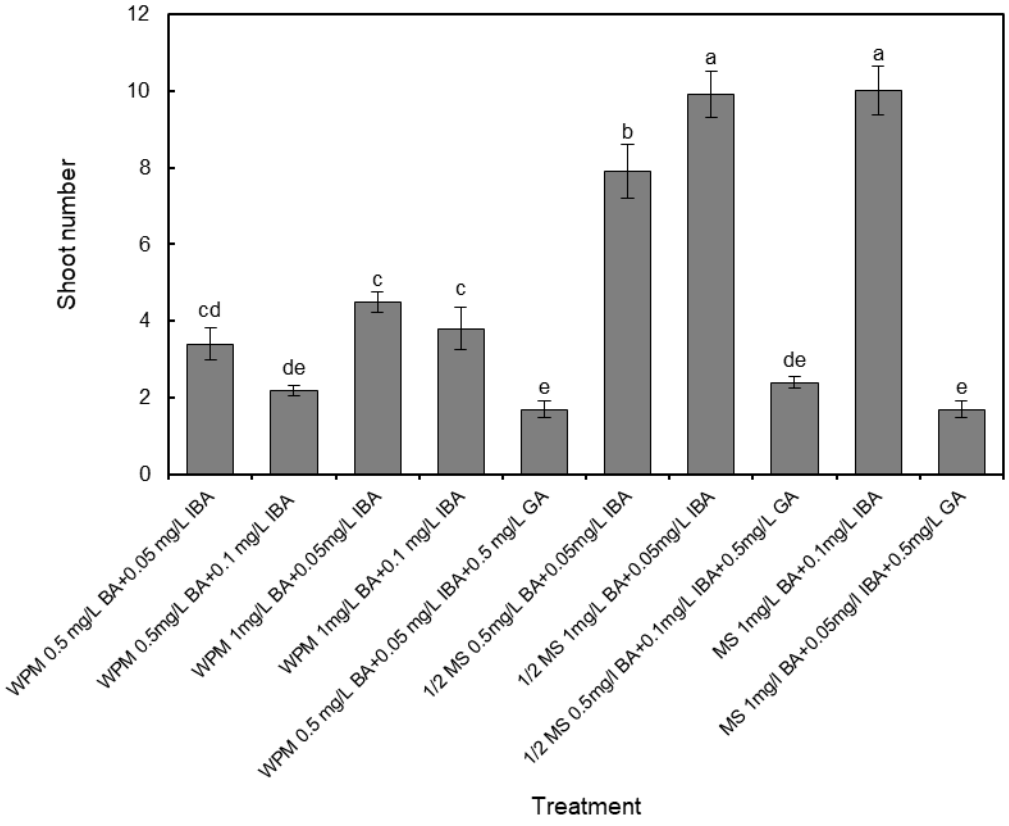

Figure 3.6. Longest root length by different media and growth regulator combinations. The data are the means ± SEs (n=10). The different lowercase letters indicate significant differences among treatments based on Duncan's test at $P < 0.05$.

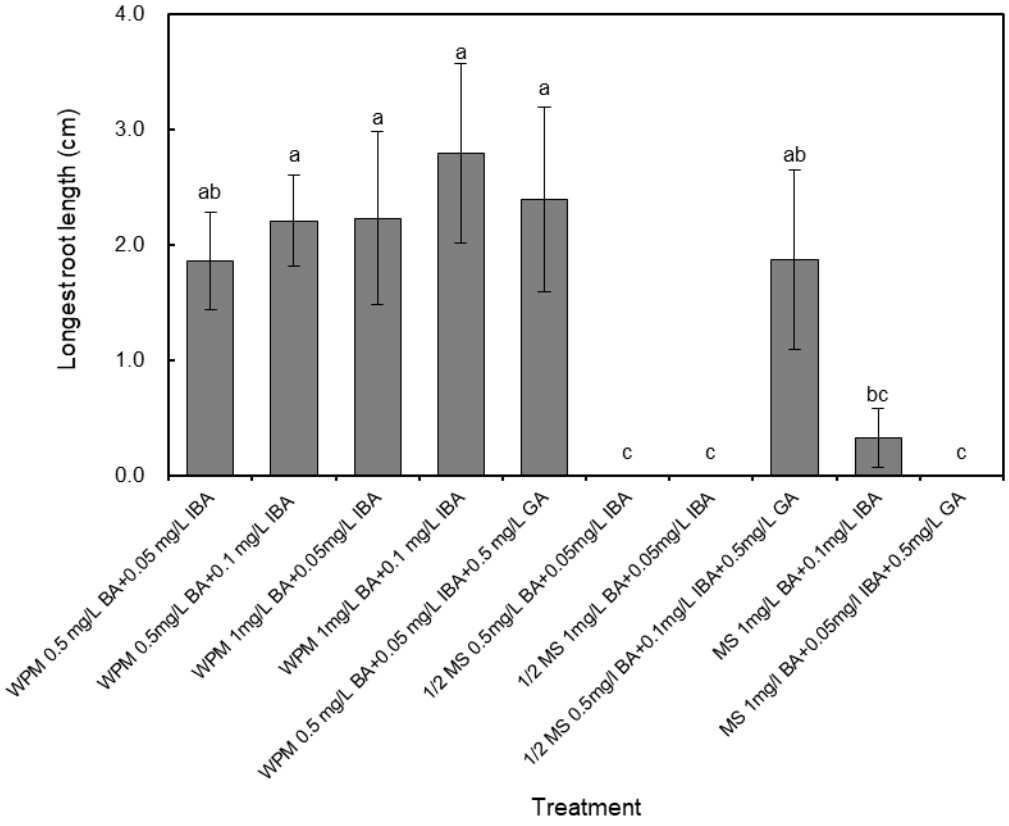

Figure 3.7. Root induction by different media and growth regulator combinations. The data are the means ± SEs (n=10). The different lowercase letters indicate significant differences among treatments based on Duncan's test at $P < 0.05$.

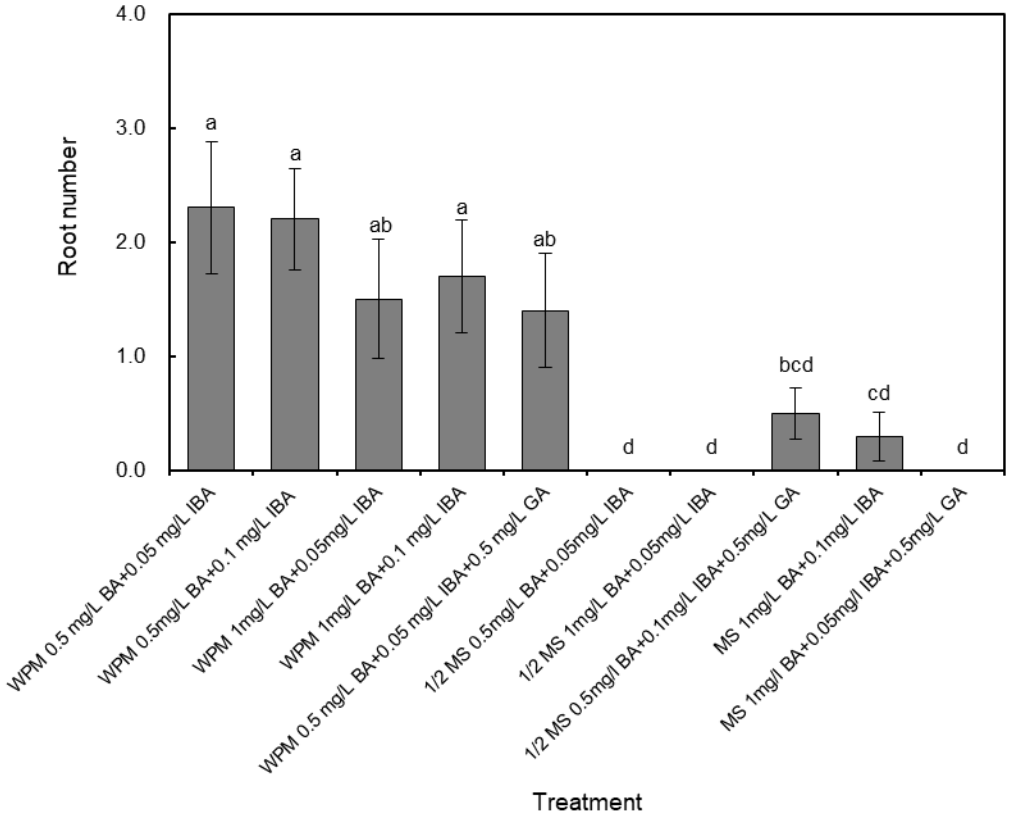

Figure 3.8. Internode length by different media and growth regulator combinations. The data are the means ± SEs (n=10). The different lowercase letters indicate significant differences among treatments based on Duncan's test at $P < 0.05$.

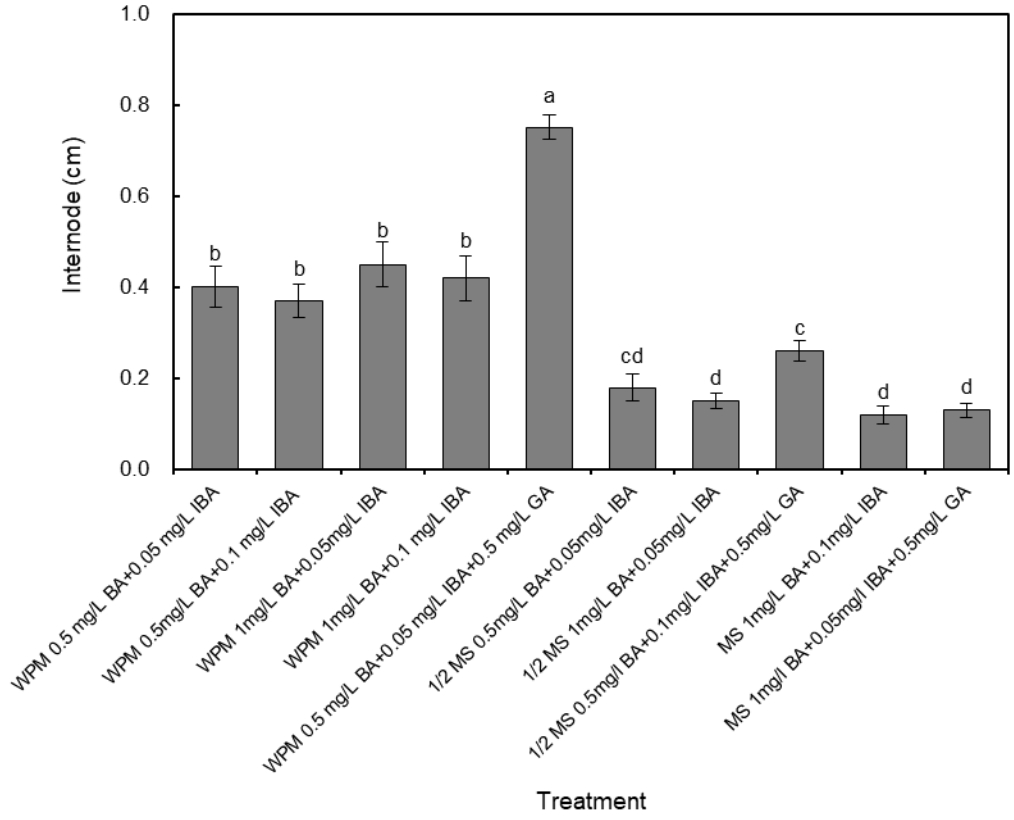

Figure 3.9. Growth habit of 21H026 from different growth media and growth regulator combinations. A. WPM 0.5 mg/L BA+0.05 mg/L IBA, B. WPM 0.5mg/L BA+0.1 mg/L IBA, C. WPM 1mg/L BA+0.05mg/L IBA, D. WPM 1mg/L BA+0.1 mg/L IBA, E. WPM 0.5 mg/L BA+0.05 mg/L IBA+0.5 mg/L GA, F, 1/2 MS 0.5mg/L BA+0.05mg/L IBA, G. 1/2 MS 1mg/L BA+0.05mg/L IBA, H. 1/2 MS 0.5mg/L BA+0.1mg/L IBA+0.5mg/L GA, I. MS 1mg/L BA+0.1mg/L IBA, J. MS 1mg/L BA+0.05mg/L IBA+0.5mg/L GA. Scale bar = 10 mm.

Figure 3.10. 21H026 induced from leaf explant showed two flowers and one flower bud 60 days after induction.

CHAPTER 5

Effects of EMS on the Germination and Seedling Growth of *Lagerstroemia* Taxa

Yue Y, Zhang D. To be submitted to *HortScience*.

Additional index words. new cultivars; *Lagerstroemia*; crape myrtle; EMS; mutation breeding

Abstract.

Crape myrtle (*Lagerstroemia* spp.) is prized in ornamental horticulture for its extended blooming season, vibrant floral displays, and unique bark. However, traditional breeding methods have struggled to incorporate novel traits such as unusual foliage coloration, compact growth habits, and enhanced flower pigmentation due to inherent genetic limitations. In this study, we developed a systematic EMS (ethyl methanesulfonate) treatment regimen to induce genetic variability in crape myrtle. Seeds from six elite hybrid crosses were pre-soaked and treated with EMS at concentrations of 0.25%, 0.5%, and 1.0% for either 6 or 12 hours. Germination and survival were monitored, and median lethal dose (LD_{50}) values were estimated using second-degree polynomial regression coupled with a bootstrap resampling approach. Results demonstrated that EMS concentration was the primary factor influencing germination and survival, although the impact of exposure time varied among genotypes. Notably, the hybrid 21H103 exhibited the highest EMS tolerance ($LD_{50} \approx 1.48\%$ at 6 hours and $\approx 0.99\%$ at 12 hours), while 21H043 was the most sensitive. The statistically significant differences in LD_{50} between exposure durations underscore the importance of tailoring EMS protocols to specific genetic backgrounds. This study establishes a foundational framework for EMS-induced mutagenesis in crape myrtle, paving the way for the development of cultivars with enhanced ornamental traits and potential disease resistance.

Introduction

Crape myrtle (*Lagerstroemia* spp.) stands out in ornamental horticulture for its extended blooming season, vivid floral displays, and visually distinctive bark, placing it among the most sought-after landscape trees and shrubs. Despite decades of progress through conventional

breeding and interspecific hybridization—yielding cultivars with improved disease resistance, novel color palettes, and enhanced cold tolerance—breeders continue to seek entirely new or rare traits such as novel foliage coloration, compact or dwarf growth habits, and more intense flower pigmentation. In many instances, these traits elude standard crossing methodologies, particularly when key pigment biosynthetic pathways or morphological genes are missing in the available germplasm or are tightly linked to unfavorable characteristics. Consequently, there is a need to explore alternative breeding approaches that circumvent these genetic constraints and further enrich the ornamental potential of crape myrtle.

Chemical mutagenesis using ethyl methanesulfonate (EMS) serves as a potent complement to traditional breeding strategies in a wide range of ornamental crops. By alkylating guanine residues and inducing G:C → A:T transitions (Melsen et al., 2021), EMS introduces numerous random single nucleotide polymorphisms (SNPs) (Greene et al., 2003). These SNPs can alter critical genes governing pigment biosynthesis, plant architecture, or stress tolerance, thereby creating phenotypic variants that may be difficult or impossible to obtain via conventional hybridization. Moreover, EMS typically induces fewer large-scale deletions than physical mutagens, which increases the likelihood of generating missense or nonsense mutations, causing change of function and loss of function mutant (Koornneef et al., 1982; Shikazono et al., 2005). Consequently, EMS has been widely adopted for ornamental breeding because its overall balance of beneficial to deleterious mutations tends to favor the rapid emergence of desirable traits (Gautam et al., 1992; Girija and Dhanavel, 2009).

Seeds represent the most common explant for EMS mutagenesis in ornamental breeding due to their ready availability and ease of handling. Nonetheless, reported EMS concentrations and exposure times differ markedly across plant species, reflecting variations in genome sensitivity

and metabolic detoxification pathways. At higher EMS concentrations, detrimental effects—such as lethality, sterility, or reduced regenerative capacity—can become prominent (Latado et al., 2004; Roychowdhury and Tah, 2011). Such outcomes undermine ornamental breeding objectives because vigorous plant survival and efficient propagation are essential for commercial success. Nevertheless, sterility can be an asset in specific cases involving potentially invasive ornamentals, where limiting reproductive capability is desirable for environmental stewardship. To strike an optimal balance between mutation efficiency and plant viability, many breeding programs determine the median lethal dose (LD_{50}), defined as the EMS treatment concentration and duration that yields 50% survival (Hohmann et al., 2005; Napoli and Ruehle, 1996). By targeting this threshold, researchers can maximize genetic variation while maintaining sufficient seedling numbers for downstream selection.

EMS-induced mutation has been applied to a variety of woody ornamental species with reported treatment concentrations ranging from 0.05% to 5% and durations spanning 1 to 48 hours (Melsen et al., 2021). For example, EMS treatment in *Ribes sanguineum* influenced leaf morphology, ultimately leading to the release of the cultivar 'Oregon Snowflake' (Contreras and Friddle, 2015). Additional phenotypic alterations—including dwarfism, thornlessness, leaf shape modifications, and variegation—have been observed in *Bougainvillea spectabilis* (Anitha et al., 2017). Despite these successes in other ornamentals, the use of EMS in crape myrtle (*Lagerstroemia* spp.) has received comparatively little attention. To date, only 'Centennial Spirit' and 'Prairie Lace' have been definitively associated with EMS mutagenesis, both arising from seeds soaked in 4% EMS for one hour (Whitcomb et al., 1984; Whitcomb, 1985). The surviving seedlings were then evaluated over several growing seasons, with breeders selecting lines based on criteria such as disease and pest resistance, flowering duration, and flower color intensity.

These prior examples illustrate EMS's potential to expand the crape myrtle gene pool but also highlight the need to refine protocols for optimal concentration and exposure time. In this study, we aimed to establish a systematic EMS treatment regimen for crape myrtle seeds. By investigating multiple EMS concentrations and treatment durations, we sought to identify conditions that maximize the emergence of desirable mutations—such as novel flower or foliage traits—while minimizing deleterious effects and maintaining robust seedling survival. Our results provide a framework to guide future EMS-based breeding efforts in *Lagerstroemia*.

Materials and Methods

In 2021, controlled hybridizations were conducted at the University of Georgia Horticultural Farm among several elite *Lagerstroemia* cultivars. Crosses such as 21H026, 21H040, 21H041, and 21H043, later shown to exhibit favorable attributes (e.g., early flowering, extended bloom duration, and resistance to common diseases), were selected for this study. Additionally, two interspecific hybrids involving *L. speciosa*, denoted 21H103 and 21H104, were included based on their larger foliage and inflorescences, which could prove advantageous for ornamental plantings in the southern United States. Seeds from these six crosses served as the plant material for EMS mutagenesis.

To allow a lower EMS concentration while maintaining mutation efficacy, seeds were subjected to longer soaking intervals. Prior to EMS treatment, seeds were imbibed in deionized water for 12 hours. EMS treatments were then administered at four concentrations—0% (control), 0.25%, 0.5%, and 1.0% for two durations (6 and 12 hours). Each treatment was replicated four times with 25 seeds per replicate. Immediately after EMS exposure, seeds were thoroughly rinsed under tap water for 30 minutes to remove residual chemicals.

Following treatment, seeds were sown in germination trays within a greenhouse maintained at 25/18°C (day/night). Germination was monitored for 30 days, after which seedlings were transplanted into 1.05 L containers filled with a 1:1 (v/v) mixture of PRO-MIX HP (Premier Tech Growers and Consumers, Quakertown, PA, USA) and perlite. Seedlings were grown under shade cloth in the greenhouse for 12 more weeks, when they were moved to 2.8 L pots for continued growth. Survival counts were then recorded, and survival proportions were converted to percentages for statistical analysis.

To estimate median lethal dose (LD_{50})—defined here as the EMS concentration corresponding to 50% survival—second-degree polynomial regression models were fitted to each cross at both 6-hour and 12-hour exposure intervals. A bootstrap resampling approach (n = 1000 iterations) was employed to assess significant differences in LD_{50} between the two exposure durations. For each iteration, Gaussian noise (σ = 5%) was added to survival percentages to simulate biological variation, after which LD_{50} was recalculated using the same polynomial regression model. This procedure provided empirical confidence intervals for the LD_{50} estimates and enabled a robust comparison across treatments and genotypes.

Results

Germination data were collected for six *Lagerstroemia* crosses—21H043, 21H040, 21H026, 21H104, 21H041, and 21H103—under varying treatments for time (duration) and concentration, as well as their interaction (time × concentration). Analyses of variance (ANOVA) revealed distinct patterns among the crosses (Table 4.1, Fig. 4.1). Significance was set at $p < 0.05$ (*), with $p < 0.01$ (**) indicating a highly significant effect. Germination in 21H043 was significantly influenced by concentration but showed no dependence on time or their interaction. Higher concentrations typically resulted in elevated germination percentages, suggesting that optimizing

solution strength is key for improving early seedling establishment in this cross. In 21H040, time clearly stood out as the major driver of germination ($p = 0.002$**), whereas concentration and the interaction were both near-significant but did not meet the 0.05 threshold. This outcome implies that extended or shortened exposure duration could be crucial to maximizing germination, whereas altering concentration alone had limited effects on seedling emergence. None of the factors or their interaction significantly affected 21H026. Germination rates remained relatively stable across all treatments, indicating that this cross may be inherently resilient to the range of tested durations and concentrations or that other unmeasured variables (e.g., seed vigor) play a more dominant role. In 21H104, both time and concentration influenced germination. Longer or more optimized durations, coupled with increased or precisely tuned solution strengths, produced significantly higher germination percentages. However, the lack of a significant interaction suggests that each factor operates independently, and no additional benefit arises from adjusting the two factors simultaneously in this specific cross. Germination in 21H041 was highly responsive to concentration, whereas time did not produce a notable effect. The strong concentration dependence likely underscores the importance of selecting an appropriate solution strength for promoting seedling emergence in this cross. Adjusting exposure duration offered no additional gains. For 21H103, concentration emerged as the sole significant factor. The marked rise in germination at specific concentration ranges indicates that fine-tuning solution strength is crucial for improving germination success. Neither the duration of treatment nor its interaction with concentration contributed meaningfully to germination rates.

From a practical breeding standpoint, these results highlight the importance of tailoring protocols for each cross. For crosses highly sensitive to time (e.g., 21H040, 21H104), modulating exposure duration may be a more critical strategy than adjusting EMS concentration. Conversely,

in crosses predominantly driven by concentration (e.g., 21H103, 21H041, 21H043), fine-tuning the EMS concentration is the key to enhancing germination success. Meanwhile, for crosses like 21H026—where neither time nor concentration induced significant changes—alternative factors such as seed maturity, storage conditions, or genetic variables may be more influential.

Survival percentages of six *Lagerstroemia* crosses—21H043, 21H040, 21H026, 21H104, 21H041, and 21H103—were analyzed to evaluate the effects of time, concentration, and their interaction (time × concentration). Among these six crosses, concentration emerged as the primary determinant of survival in four cases (21H026, 21H104, 21H041, 21H103), while time did not reach statistical significance in any cross (Table 4.1, Fig. 4.2). Notably, 21H043 and 21H040 showed no meaningful response to either factor, suggesting that their survival percentages may be more strongly governed by genetic background, initial seed quality, or other untested variables. This pattern underscores the importance of tailoring concentration protocols for specific genotypes, particularly those that demonstrate a clear dose-response in survival outcomes.

Polynomial regression models were applied to survival data for each Lagerstroemia cross under two exposure durations (6 h and 12 h), estimating the EMS concentration at which 50% of seeds survived (LD_{50}). The results (Table 4.2) reveal notable differences both among genotypes and between exposure times.

Across the 6 h treatments, 21H103 exhibited the highest EMS tolerance ($LD_{50} \approx 1.48\%$), followed by 21H040 (≈1.03%) and 21H104 (≈0.99%) (Fig. 4.3). In contrast, 21H043 showed the lowest LD_{50} (≈0.65%) at 6 h, suggesting it is more sensitive to EMS at shorter durations. For the remaining crosses—21H026 and 21H041—LD_{50} values fell within an intermediate range (≈0.75–0.78%).

Among the 12 h treatments, 21H103 again recorded the highest LD_{50} (≈0.99%), indicating its relatively robust response to extended EMS exposure (Fig. 4.4). Conversely, 21H043 continued to display the lowest estimated LD_{50} (≈0.51%), highlighting consistent EMS sensitivity across time points. The other crosses—21H040, 21H026, 21H104, and 21H041—clustered around LD_{50} values from 0.63% to 0.91%, reflecting moderate to high tolerance under 12 h treatments.

Overall, these estimates underscore a genotype-specific variation in EMS sensitivity, with 21H103 possessing notably higher EMS tolerance regardless of exposure duration, while 21H043 remains the most EMS-sensitive of the tested crosses. Such information is instrumental in selecting optimal EMS dosages for mutation breeding, as genotypes with higher LD_{50} may accommodate more aggressive mutagenic regimes without incurring excessive mortality.

A bootstrap approach was employed to evaluate the statistical difference in EMS LD_{50} estimates (i.e., the EMS concentration yielding 50% seed survival) between 6-hour and 12-hour treatments for each Lagerstroemia cross (Table 3). In this method, the survival data were resampled repeatedly (e.g., 1,000 iterations), adding random noise to simulate biological variation, and each resample was used to re-estimate the LD_{50} via polynomial regression. The resulting distribution of LD_{50} values allowed for constructing empirical confidence intervals and computing p-values for the difference in mean LD_{50} across the two exposure durations.

All six crosses showed highly significant differences ($p < 0.001$) between the 6-hour and 12-hour LD_{50} estimates, underscoring the influence of exposure time on EMS tolerance. For some cultivars (e.g., 21H043), the mean LD_{50} was notably higher at 6 hours (0.6665%) than at 12 hours (0.5121%), suggesting that prolonged EMS exposure led to increased seed mortality. Conversely, 21H026 had a slightly higher mean LD_{50} at 12 hours (0.8069%) compared to 6 hours (0.7593%),

indicating that, in this specific genetic background, additional soaking time did not dramatically escalate lethality within the tested concentration range.

Overall, the bootstrap analysis confirms that exposure duration can significantly shift the EMS LD_{50}, but the direction and magnitude of this shift vary by genotype. Such quantitative insights are pivotal for mutation breeding strategies, as they help breeders fine-tune EMS treatments to maximize useful genetic variation while minimizing excessive mortality.

Discussion

Recent findings underscore the efficacy of EMS as a mutagen in various ornamental species; however, research focusing on crape myrtle (*Lagerstroemia* spp.) remains comparatively sparse. Early studies acknowledged the potential of EMS for generating novel crape myrtle cultivars, but their optimal treatment parameters were not well-defined. More recently, Liang et al. (2025) investigated EMS mutagenesis in *L. speciosa,* a close relative of *L. indica*, by applying 1.2%–2.0% EMS for 8–12 hours. They reported a clear inverse relationship between both EMS concentration and exposure duration with seed germination and survival, identifying 1.8% for 10 hours or 1.6% for 12 hours as approximations of LD_{50}. In our study, the highest LD_{50} values appeared in 21H103 and 21H104, both hybrids bearing *L. speciosa* parentage. Their elevated tolerance likely reflects the inherited genomic background from *L. speciosa*, consistent with Liang et al.'s (2025) findings.

A similar trend was observed in *L. caudata,* where 0.78% EMS at 12 hours or 2.11% EMS at 4 hours constituted the approximate LD_{50} (Xu et al., 2022). Another EMS experiment in 'Bhagwa' pomegranate (*Punica granatum* L.), also within the Lythraceae family, concluded that 0.643 % EMS represented the LD_{50} for hardwood cuttings (Rawat et al., 2023). By contrast, *Gerbera* required only a 10-minute treatment at 0.65% EMS to reach LD_{50} (Ghani et al., 2014), underscoring the profound influence of species, genotype, and plant organ on EMS tolerance levels.

Collectively, these studies reinforce the genotype-specific nature of EMS sensitivity, even among closely related taxa. Variation in seed coat permeability, detoxification pathways, and genomic architecture can substantially modify the concentration–duration balance required to achieve LD_{50}. In *Lagerstroemia* breeding, this implies that protocols identified for one species or hybrid must be carefully validated—and potentially recalibrated—before broader application to other genetic backgrounds.

Although EMS-induced mutations can produce ornamental enhancements such as variegated foliage or dwarfing, many desirable variants do not manifest until the M_2 or later generations. In *Hydrangea*, for instance, several M_1 seedlings demonstrated dwarf and variegated phenotypes soon after germination (Greer and Rinehart, 2009), whereas *Sarcococca confusa* exhibited an irregular chlorophyll mutation only on a single branch of an M_1 plant (Hoskins and Contreras, 2019). Subsequent cuttings and seeds from that branch retained the chlorophyll mutation, underscoring how even a single mutated sector can provide heritable traits of horticultural interest. Conversely, other mutations may remain invisible in M_1 plants and only emerge in subsequent generations, as observed in *Salvia coccinea* M_2 populations that produced albino chlorophyll mutants (Maynard and Ruter, 2023). Because woody ornamentals, including crape myrtle, typically require multiple growing seasons before traits stabilize, a thorough evaluation of M_2 and M_3 generations is often necessary. Indeed, Dr. Whitcomb's EMS-derived crape myrtle cultivars took approximately seven to eight years to release (Whitcomb et al., 1984; Whitcomb, 1985).

Beyond ornamental traits, EMS mutagenesis also has potential to improve disease resistance in crape myrtle, which faces recurrent challenges such as powdery mildew, Cercospora leaf spot, and bark scale. In wheat, for example, large-scale EMS treatments allowed researchers to identify homozygous mutant lines conferring powdery mildew resistance (Ingvardsen et al., 2023).

Similarly, an EMS-mutated pea population yielded only seven resistant individuals by the M_3 generation—underscoring the low frequency and randomness of beneficial mutations (Sharma et al., 2025). Translating such strategies to crape myrtle could pave the way for more robust cultivars but would likely necessitate large seed populations and substantial labor resources. Unlike agronomic crops, ornamental breeding programs often operate under limited manpower and marker resources, further complicating screening efforts.

Overall, the absence of obvious morphological differences among M_1 seedlings in our current study does not exclude future variant discovery. As these plants mature, somaclonal or segmental mutations could manifest in traits like foliar coloration or architecture. Additionally, collecting seeds for M_2 and M_3 generations may be indispensable for capturing subtle or recessive mutations that only fully express once they are homozygous or have undergone additional segregation. Ultimately, combining EMS mutagenesis with thorough multi-generational evaluations offers a promising avenue for both aesthetic innovation and disease resilience in crape myrtle breeding.

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Table 4.1. Two-way ANOVA results of germination percentage and survival percentage among elite crosses.

Cross	Factor		Germination percentage	Survival percentage
21H043	Time	*F* value	0.651	0.098
		P value	0.430	0.758
	Concentration	*F* value	3.958	2.158
		P value	0.038*	0.144
	Time × Concentration	*F* value	0.705	0.284
		P value	0.507	0.756
21H040	Time	*F* value	13.714	0.002
		P value	0.002**	0.967
	Concentration	*F* value	3.310	2.158
		P value	0.060	0.144
	Time × Concentration	*F* value	3.074	1.339
		P value	0.071	0.287
21H026	Time	*F* value	1.135	0.051
		P value	0.301	0.824
	Concentration	*F* value	1.095	11.352
		P value	0.356	0.001**
	Time × Concentration	*F* value	0.631	1.755
		P value	0.543	0.201
21H104	Time	*F* value	12.985	0.045
		P value	0.002**	0.834
	Concentration	*F* value	4.358	10.702
		P value	0.029*	0.001***
	Time × Concentration	*F* value	1.435	0.374
		P value	0.260	0.693

(Continued table 4.1)

Cross	Factor		Germination percentage	Survival percentage
21H041	Time	*F* value	2.213	0.124
		P value	0.154	0.729
	Concentration	*F* value	8.616	5.905
		P value	0.002**	0.011*
	Time × Concentration	*F* value	0.118	0.605
		P value	0.889	0.557
21H103	Time	*F* value	0.000	1.826
		P value	1.000	0.193
	Concentration	*F* value	20.108	24.535
		P value	0.000***	0.000***
	Time × Concentration	*F* value	0.663	2.879
		P value	0.527	0.082

Table 4.2. Polynomial estimated LD_{50} EMS rates for elite crosses.

Cultivar	Exposure	Estimated LD_{50} EMS Rate (%)
21H043	6 h	0.6502
21H040	6 h	1.0314
21H026	6 h	0.7585
21H104	6 h	0.9913
21H041	6 h	0.7826
21H103	6 h	1.4815
21H043	12 h	0.5097
21H040	12 h	0.8388
21H026	12 h	0.7786
21H104	12 h	0.9110
21H041	12 h	0.6301
21H103	12 h	0.9873

Table 4.3. Bootstrapped polynomial estimates of LD50 EMS rates for elite crosses.

Cultivar	Mean LD_{50} (6 h)	Mean LD_{50} (12 h)	*p*-value
21H043	0.6665	0.5121	< 0.001
21H040	0.9424	0.8426	< 0.001
21H026	0.7593	0.8069	< 0.001
21H104	0.9909	0.9184	< 0.001
21H041	0.7786	0.6339	< 0.001
21H103	1.1613	0.9951	< 0.001

Figure 4.1. Germination percentage of elite crosses under different time and EMS concentration treatments. The data are the means ± SEs (n=4). The different lowercase letters indicate significant differences among elite crosses based on Duncan's test at $P < 0.05$. The different upper letters indicate significant differences among elite crosses based on Duncan's test at $P < 0.05$.

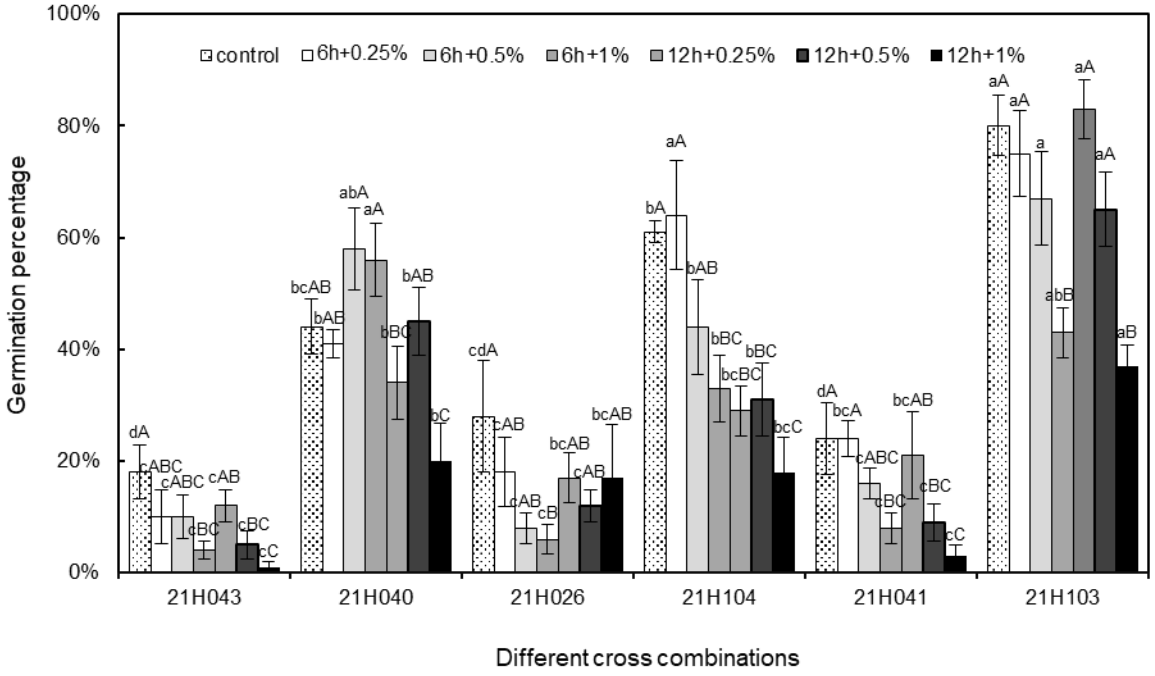


Figure 4.2. Survival percentage of elite crosses under different time and EMS concentration treatments. The data are the means ± SEs (n=4). The different lowercase letters indicate significant differences among elite crosses based on Duncan's test at $P < 0.05$. The different upper letters indicate significant differences among elite crosses based on Duncan's test at $P < 0.05$.

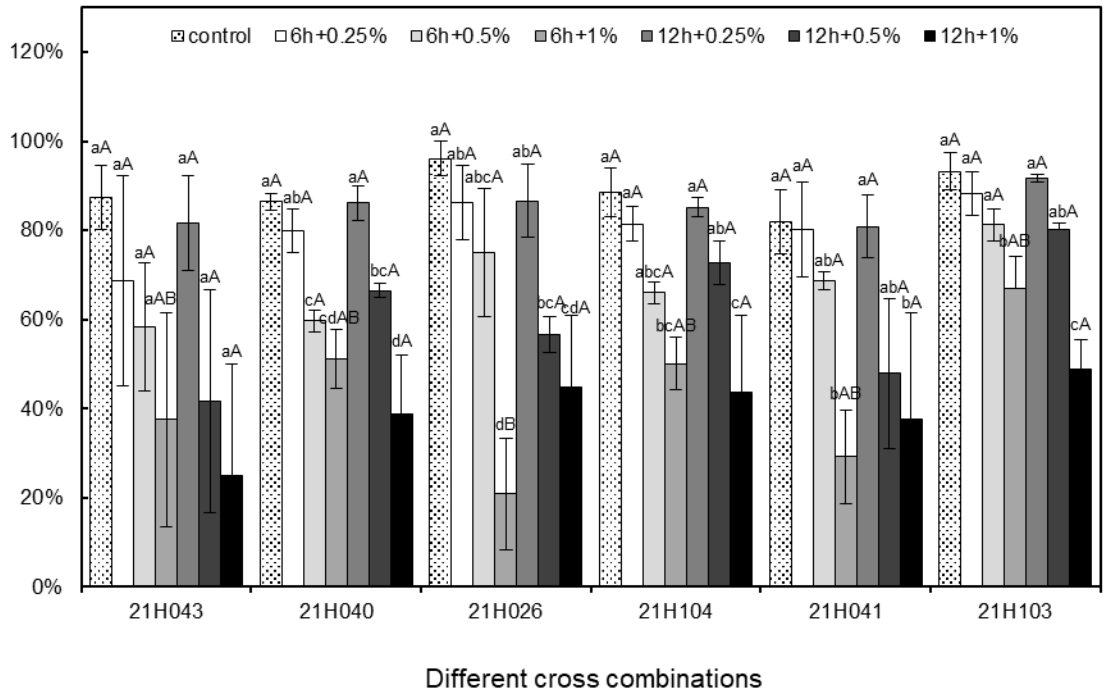


Figure 4.3. Survival rate and median lethal dose under 6 hours EMS soaking treatment.

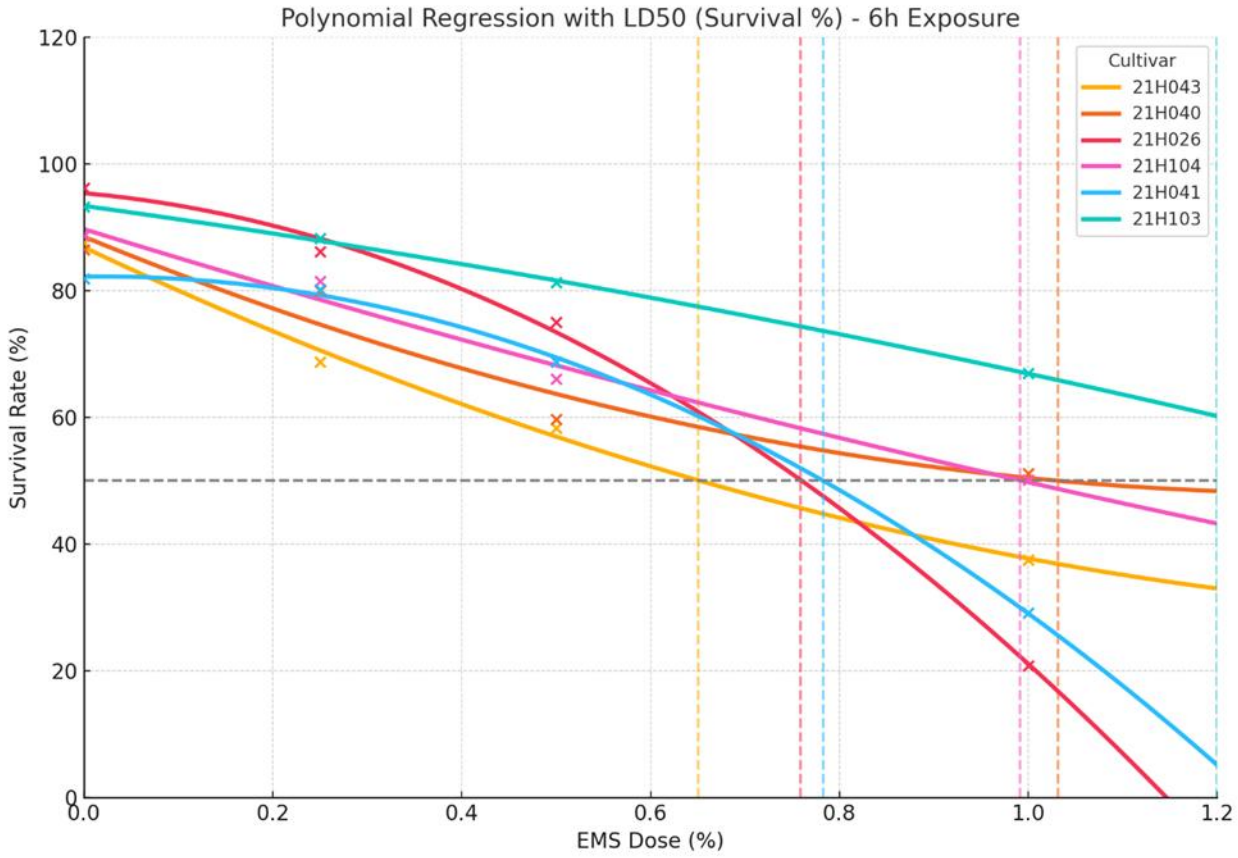
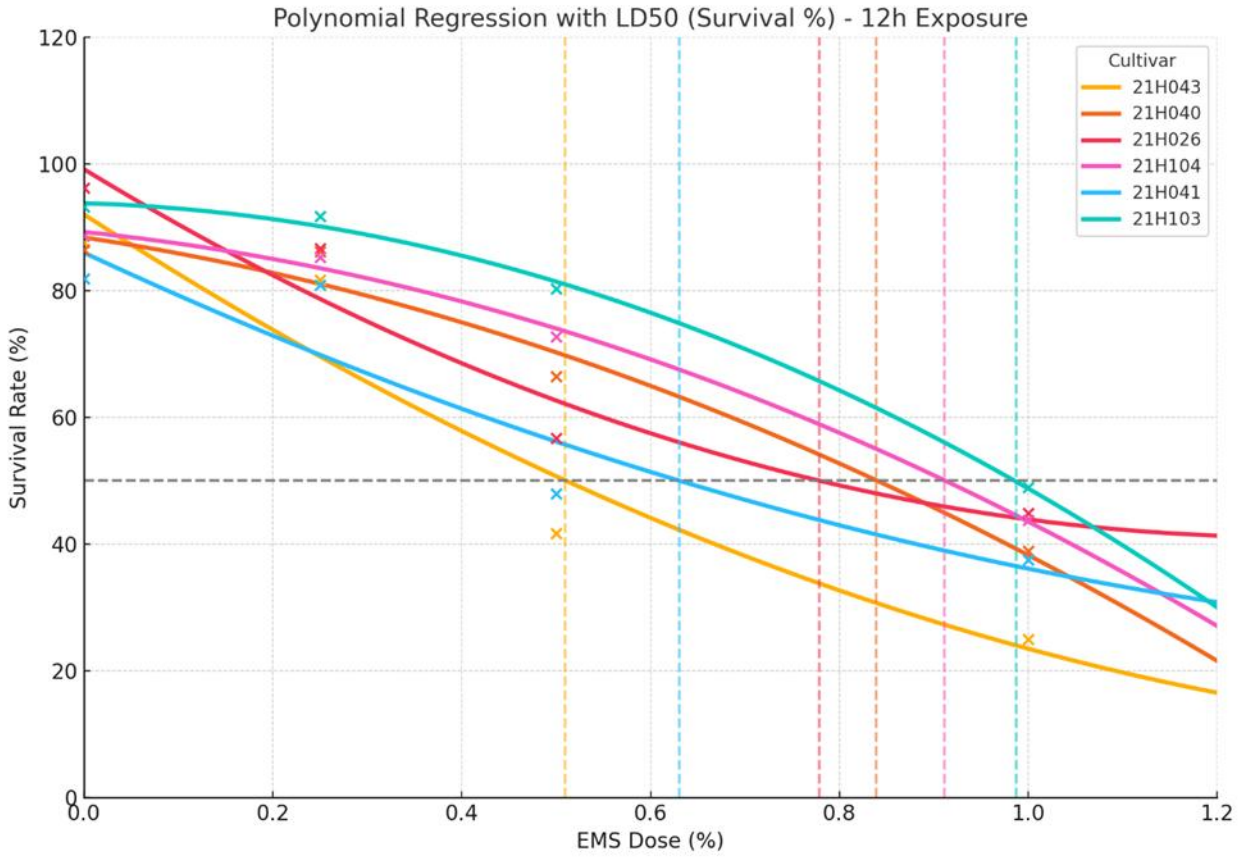


Figure 4.4. Survival rate and median lethal dose under 12 hours EMS soaking treatment.



CHAPTER 6

Whole Genome Sequencing and Genome Assembly of *Lagerstroemia speciosa*

Yue Y, Zhang D. To be submitted to *HortScience*.

Additional index words. new cultivars; *Lagerstroemia*; crape myrtle; whole genome sequencing; genome assembly

Abstract.

Crape myrtle (*Lagerstroemia* spp.) is a highly valued ornamental plant renowned for its vibrant flower displays, attractive bark, and broad adaptability across mild-climate regions. Despite its extensive cultivation history and economic importance, genomic resources for Lagerstroemia remain limited, hindering molecular breeding efforts. In this study, we present a comprehensive, haplotype-resolved genome assembly of *Lagerstroemia speciosa*, a species noted for its large leaves, showy inflorescences, and potential disease resistance. High-quality genomic DNA was extracted from newly emerged leaves, and whole-genome sequencing was performed using PacBio Revio technology. Supplementary Hi-C and RNA sequencing data were integrated to facilitate scaffolding and gene annotation. Initial k-mer based genome size estimations via GenomeScope suggested a genome of ~348 Mb which is close to the previous flow cytometry and assembly metrics converged on a haploid genome size of approximately 366.75 Mb. The final assembly yielded two haplotypes with sizes of 343.38 Mb and 317.00 Mb, exhibiting scaffold N50 values of 13.95 Mb and 12.75 Mb, respectively, and high BUSCO completeness scores (95.80% and 93.60%). Repeat analysis revealed that approximately 40% of the genome comprises repetitive sequences, with LTR elements predominating. This high-quality genomic resource provides a robust scaffold for the identification of candidate genes underlying key ornamental traits, disease resistance, and stress tolerance. Our findings not only enhance the genomic understanding of *Lagerstroemia speciosa* but also lay the groundwork for accelerated breeding programs aimed at developing superior crape myrtle cultivars.

Introduction

Crape myrtle (*Lagerstroemia* spp.) ranks among the most popular landscape ornamentals in mild-climate regions, owing to its long blooming period, diverse flower colors, ornamental bark features, and ease of propagation (Pooler, 2007). Originating in southern China, Southeast Asia, and Japan, *Lagerstroemia* has been cultivated for timber and ornamental use for over 1,500 years (Huxley, 1992; Pooler, 2006; Pounders et al., 2013; Wang et al., 2014). Introduced to Europe in the mid-1600s and later to the southeastern United States in the late-1700s (Egolf and Andrick, 1978), crape myrtle now thrives across at least 33 U.S. states and contributes significantly to the green industry. Current estimates place their market value at approximately $70 million annually in wholesale and retail sales (USDA, 2019)—nearly double the reported value in 1988 (USDA, 2001, 2009, 2014).

Despite the genus's economic and horticultural prominence, fundamental genomic and genetic data remain limited. *Lagerstroemia* is a member of the loosestrife family (Lythraceae), which contains an estimated 53–80 species (Furtado and Montien, 1969; Cabrera, 2002). Although most modern crape myrtle cultivars derive from *L. indica* × *L. fauriei* crosses that confer improved powdery mildew resistance (Pooler, 2006, 2007), chromosome numbers and genome sizes have been elucidated for only a few species, hindered in part by their small genome and high chromosome count. Historically, *Lagerstroemia* breeding has also employed mutation and polyploid approaches, yielding over 200 named cultivars—roughly half of which are widely commercialized in North America, Europe, Asia, and Australia (Pooler, 2007).

In addition to ongoing breeding for extended color palettes and plant architecture, disease and pest management is a critical concern. Powdery mildew poses a significant threat to crape myrtle's ornamental quality by producing white fungal growth on leaves, shoots, and flowers, while

Cercospora leaf spot further challenges growers in the southern United States (Hagan, 2001; Chappell et al., 2012; Thurn et al., 2019). More recently, crape myrtle bark scale (*Acanthococcus lagerstroemiae*) has proliferated in the Southeast, causing black sooty mold and reducing overall vigor (Gu et al., 2014; Xie et al., 2021). *L. speciosa* has shown relative resistance to this pest (Wu et al., 2021), highlighting the potential of interspecific crosses for enhanced pest and disease tolerance.

Modern genomics offers powerful means to accelerate breeding solutions for challenges like disease resistance, novel plant architecture, and improved ornamental traits. In other crops, whole genome sequencing has enabled the identification of large numbers of molecular markers (e.g., SNPs, indels, SSRs) and provided insights into key genetic pathways for phenotypic traits (Shirasawa et al., 2017; Niu et al., 2023). As of 2020, 69 ornamental plant genomes have been published (Zheng et al., 2021), revealing genes involved in floral color biosynthesis, scent production, stress tolerance, and other complex phenotypes. However, *Lagerstroemia* lacks a high-quality reference genome, limiting the pace of molecular marker development and functional gene discovery. In this study, we focus on *L. speciosa*—noted for its large leaves, showy inflorescences, and potential disease resistance—to generate a comprehensive whole-genome assembly. This reference will provide a foundation for comparative genomic analyses within *Lagerstroemia*, facilitating marker-assisted breeding and enabling more targeted approaches to developing crape myrtle cultivars with enhanced ornamental and disease-resistance traits.

Materials and Methods

Newly emerged leaves from *Lagerstroemia speciosa* (USDA-GRIN Accession MIA 36605) were collected in March 2024 at the University of Georgia Horticulture Farm. Immediately after harvesting, leaf samples were flash-frozen in liquid nitrogen and shipped to the Genomic and

Bioinformatics Center at Brigham Young University for PacBio Revio (Menlo Park, CA, USA) whole-genome sequencing. In June 2024, leaf samples from the same individual were sampled again and sent to HudsonAlpha Genome Sequencing Center (Huntsville, AL, USA) for Hi-C and RNA sequencing analyses.

Library Preparation and Sequencing

Hi-C library construction was carried out using the Dovetail® Omni-C® Kit (Scotts Valley, CA, USA), and sequencing was performed on an Illumina NovaSeq X platform (paired-end 150 bp). For RNA sequencing, total RNA was extracted using a Qiagen RNeasy kit (Germantown, MD, USA), followed by library preparation and Illumina sequencing on the same NovaSeq X system (PE150).

Data Filtering and Quality Control

All Illumina reads underwent adapter trimming and low-quality read removal using Trimmomatic (version 0.39-Java-13) (Bolger et al., 2014). PacBio Revio raw data were screened for potential contaminants with Kraken2 (version 2.1.3-gompi-2022a) (Wood et al., 2019), retaining only reads longer than 10 kb. K-mer frequency distributions (k = 17) were then computed with Jellyfish (version 2.3.1-GCC-12.3.0) (Marçais and Kingsford, 2012) for genome size estimation.

Genome Assembly and Scaffolding

High-quality filtered PacBio HiFi reads were de novo assembled into contigs using hifiasm (version 0.24.0-GCCcore-12.3.0) (Cheng et al., 2021), incorporating Hi-C data for phasing. The resulting haplotype-resolved draft assembly was aligned and scaffolded with RagTag (version 2.1.0) (Alonge et al., 2022) using a reference genome from NCBI (GCA_037672795.1) to assess assembly completeness. Additional scaffolding with Juicer (version 1.6-foss-2022a-CUDA-11.7.0)

(Durand et al., 2016) and 3D-DNA (version 201008-foss-2021b-Python-2.7.18) (Dudchenko et al., 2017) further leveraged the Hi-C data to order and orient contigs. Following these steps, a second RagTag run was performed to refine scaffolding. Fragments deemed extraneous or misassembled by 3D-DNA were removed from the final assembly, resulting in a high-quality *L. speciosa* genome sequence.

Genome annotation

Both homology-based and de novo methods were used to identify repetitive elements. RepeatModeler (version 2.0.4-foss-2022a) (Flynn et al., 2020) and LTR-Finder (version 1.0.7-GCCcore-11.3.0) (Xu and Wang, 2007) conducted de novo searches for long terminal repeats (LTRs) and other classes of repetitive elements, while RepeatMasker (version 4.1.5-foss-2022a) (Smit et al.) and TRF (version 4.09.1-GCCcore-11.3.0) (Benson, 1999) aligned sequences to known repeat databases and identified tandem repeats, respectively. The results from these approaches were integrated into a comprehensive repeat library for *L. speciosa*. Gene models were generated using a combination of *ab initio* prediction and evidence-based alignment. Three software packages—Augustus (version 3.5.0-foss-2023a) (Stanke et al., 2008), GeneMark-ET (version 4.72-GCCcore-12.3.0) (Lomsadze et al., 2014), and GlimmerHMM (version 3.0.4c-GCC-11.3.0) (Majoros et al., 2004)—performed *de novo* predictions. RNA-seq data, protein evidence, and known gene models were then aligned against the assembled genome. Annotation Maker (MAKER, version 3.01.04-foss-2022a) (Cantarel et al., 2008) integrated these *ab initio* predictions with external evidence (e.g., ESTs and protein homologies) to produce the final, consensus gene model set.

Results

The PacBio HiFi sequencing results in 71908423599 bp data from 4040729 HiFi reads with the average read length to 17795 bp (Table 5.1). After the contamination check, a big portion of the PacBio HiFi sequencing data was contaminated by human genome (Fig 5.1). Contaminated sequences and sequences shorter than 10 kb have been filtered, and 124.56 Gb of sequencing data were used for further analysis. GenomeScope profile indicates that the genome under investigation is approximately 348 Mb in size, with a significant majority of unique sequences and a moderate level of heterozygosity (Fig. 5.2) (Ranallo-Benavidez et al., 2020). The sequencing coverage is sufficiently high, and the low error rate supports the overall quality of the sequencing data. These insights are crucial for guiding subsequent genomic analyses, including assembly and annotation, as well as for understanding the genetic complexity of the organism.

The genome assembly with hifiasm with the filtered PacBio HiFi reads had big trouble since the data was around 400x coverage in depth and the huge amount of data might complicate read alignment, especially in repetitive regions. Therefore, the hifiasm output had two haplotypes around 650 Mb in size which more than doubled compared with GenomeScope's prediction. Later, the filtered reads were subsampled to 50x-200x for further tests. The results showed that subsample with 80x had the most balanced two haplotypes. The two haplotypes were about 439.6 Mb and 370.5 Mb in size. To further remove duplications in the genome, purge dup was applied, and the cutoff was manually set to a better threshold. The purged two haplotypes were 372.5 Mb and 343 Mb respectively. With the assistance of Juicer and 3D-DNA, fragments and debris from the haplotypes were successfully discovered and removed.

Genome assembly statistics summarize the key assembly metrics for the haplotype-resolved *L. speciosa* genome. Haplotype 1 (hap1) spans 343.38 Mb, split across 101 scaffolds, while Haplotype 2 (hap2) encompasses 317.00 Mb in 76 scaffolds (Table 5.2). The largest scaffold

lengths were 22.39 Mb for hap1 and 19.70 Mb for hap2. Both assemblies exhibited similar GC contents, measuring 40.11% (hap1) and 40.23% (hap2). Contiguity metrics indicated a relatively high degree of assembly completeness. The N50 values, representing the scaffold length at which half of the total assembly size is contained in scaffolds of equal or greater length, were 13.95 Mb in hap1 and 12.75 Mb in hap2. Likewise, N90 values were 9.84 Mb (hap1) and 10.02 Mb (hap2). Both haplotypes had an L50 of 11 and an L90 of 22, suggesting comparable scaffold distributions and indicating that a modest number of larger scaffolds capture most of each genome.

Both haplotypes were evaluated using BUSCO to estimate genome completeness based on evolutionarily conserved single copy orthologs (Table 5.3). Hap1 contained 95.80% complete BUSCOs (73.60% single-copy and 22.20% duplicated), whereas hap2 featured 93.60% complete BUSCOs (76.50% single-copy and 17.00% duplicated). Fragmented BUSCOs were low and nearly identical between haplotypes (0.70% each). Missing BUSCOs accounted for 3.50% of the ortholog set in hap1 and 5.80% in hap2. Overall, these scores indicate that both haplotypes capture most conserved genes expected for this taxonomic group, further underscoring the assembly's high completeness and quality. The final haplotypes were compared against the reference genome using D-GENIES (Cabanettes and Klopp, 2018) in a dot-plot analysis (Fig. 5.3–5.4). The resulting diagonal alignment indicates that both haplotypes are structurally consistent with reference, exhibiting minimal large-scale rearrangements or discrepancies. The relative absence of off-diagonal signals further suggests that the assemblies are largely free from major misassemblies. Together, these observations highlight the high quality of the *L. speciosa* genome reconstruction.

Repeat annotation revealed that repetitive sequences constitute roughly 39.56% of the haplotype 1 (hap1) assembly and 40.51% of the haplotype 2 (hap2) assembly (Table 5.4). Among the classified repeat families, LTR elements were notably abundant, accounting for 8.10% and

8.19% of hap1 and hap2, respectively. LINEs (1.57% in hap1; 1.49% in hap2) and DNA transposons (0.87% in hap1; 0.85% in hap2) contributed more modest proportions, whereas SINE elements comprised a minor share of each haplotype (~0.19% in hap1 and 0.08% in hap2). Smaller repeat types, such as Penelope (~0.02% in both haplotypes) and rolling-circle elements (~0.22–0.24%) represented only a small fraction of the genome. A substantial portion of the repeats (~25.60% in hap1; ~26.31% in hap2) remained unclassified, indicating regions with repetitive sequences that could not be confidently assigned to known families. The "Other" category, which grouped additional lower-abundance elements, comprised 3.02–3.36%. Overall, these data suggest that while *L. speciosa* features a diverse assortment of transposable elements, a considerable portion of the repetitive content remains to be further characterized.

Discussion

The whole genome sequencing offers transformative potential for plant breeding but also poses significant interpretive challenges, particularly when reconciling different estimates of genome size. In our study, a notable discrepancy emerged between the *L. speciosa* hap 2 genome size estimated by GenomeScope and the assembled genome. Flow cytometry measurements indicated that *L. speciosa* has a 2C genome size of approximately 0.75 pg. Converting this figure to a 1C value places the haploid genome around 366.75 Mb (1 pg = 978 Mbp), which is a little bigger than the GenomeScope estimation. Comparable trends are evident in a recent *L. speciosa* reference genome (Wan et al., 2024), where the reported genome size (349.8 Mb) contrasts with a final assembly of only 306.76 Mb.

Several factors may contribute to these discrepancies. First, repetitive elements (e.g., transposable elements, tandem repeats, segmental duplications) can complicate k-mer analyses by inflating the apparent incidence of certain kmers. In diploid or polyploid species, substantial

heterozygosity can further split k-mer profiles, generating separate peaks for homozygous and heterozygous segments. Additionally, uneven coverage across the genome—including low-coverage regions or high-coverage artifacts (e.g., PCR duplicates)—can skew k-mer distributions, resulting in under- or overestimation of total genome size. Consequently, while GenomeScope and related tools remain valuable for preliminary assessments, integrating flow cytometry results, reference genome comparisons, and assembly-based evidence provides a more reliable overall picture. Our findings highlight the importance of a multifaceted approach to genome size estimation, especially in species with complex or highly repetitive genomes like *L. speciosa*.

Comparable genome sizes have been reported in other closely related *Lagerstroemia* species, such as *L. indica* (329.14 Mb) and *L. exselsa* (330.4 Mb) (Qiao et al., 2025; Zhou et al., 2023), reinforcing the notion that *Lagerstroemia* genomes hover around 300–350 Mb. Notably, with expanding genome-sequencing efforts—ranging from whole-genome sequencing (WGS) to targeted resequencing and transcriptome profiling—researchers have begun linking specific genes and pathways to key ornamental traits. For instance, differential gene expression analyses in red-, purple-, and white-flowered crape myrtles have implicated multiple genes in the flavonoid pathway that drive flower color variations, while *MYB35*, *NCED*, and *KAS1* loci on chromosomes 12 and 17 have been associated with leaf color phenotypes in *L. indica*. Likewise, *L. exselsa* studies suggest that bark color differences arise from altered flavonoid metabolism in the stem tissues.

These emerging insights spotlight the power of coupling genomic and transcriptomic data to dissect trait architecture in *Lagerstroemia*. High-resolution genome assemblies—such as those described for *L. speciosa*—can provide a scaffold for identifying candidate genes, exploring allele-specific expression patterns, and mapping quantitative trait loci (QTLs) underlying features like flower color, bark coloration, growth habit, and disease resistance. By revealing the genomic basis

for important ornamental traits, WGS and RNA-seq findings enable breeders to prioritize promising gene targets and pathways, accelerating the development of novel cultivars. This information is especially valuable in a genus where new introductions can capitalize on diverse aesthetic attributes, including foliage coloration, bloom duration, and unique bark characteristics—to capture new market niches.

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Table 5.1. PacBio HiFi sequencing raw data summary.

PacBio HiFi sequencing summary	
Unique Barcodes	1
Barcoded HiFi Reads	4,040,729
Unbarcoded HiFi Reads	13,635
Barcoded HiFi Reads (%)	99.66%
Barcoded HiFi yield (Gb)	71,908,423,599
Unbarcoded HiFi yield (Gb)	233,106,119
Barcoded HiFi Yield (%)	0.996768767
Mean HiFi Reads per Barcode	4,040,729
Max. HiFi Reads per Barcode	4,040,729
Min. HiFi Reads per Barcode	4,040,729
Barcoded HiFi read length (mean, kb)	17,795
Unbarcoded HiFi read length (mean, kb)	17,096

Table 5.2. Genome assembly statistics of hap1 and hap2.

genome assembly	hap1	hap2
Total scaffold number	101	76
Largest scaffold	22,385,829	19,701,704
Total length (bp)	343,376,716	317,000,314
GC (%)	40.11	40.23
N50 length (bp)	13,954,461	12,746,183
N90 length (bp)	9,843,800	10,016,566
L50	11	11
L90	22	22

Table 5.3. Genome BUSCO evaluation results of hap1 and hap2.

	hap1	hap2
Complete BUSCOs	95.80%	93.60%
Complete Single-Copy BUSCOs	73.60%	76.50%
Complete Duplicated BUSCOs	22.20%	17.00%
Fragmented BUSCOs	0.70%	0.70%
Missing BUSCOs	3.50%	5.80%

Table 5.4. Classification of repetitive sequence.

	hap1	hap2	hap1	hap2
	Length (bp)		Proportion in genome (%)	
DNA transposon	2,976,771	2,687,675	0.87%	0.85%
LINE	5,377,975	4,729,179	1.57%	1.49%
SINE	662,392	266,784	0.19%	0.08%
LTR	27,826,042	25,948,988	8.10%	8.19%
Penelope	57,360	56,136	0.02%	0.02%
Rolling-circles	756,339	756,280	0.22%	0.24%
Unclassified	87,893,670	83,413,118	25.60%	26.31%
Other	10,372,401	10,628,045	3.02%	3.36%
Total	135,851,015	128,422,685	39.56%	40.51%

Figure 5.1. Kranken2 result of the raw PacBio HiFi genome sequencing data.

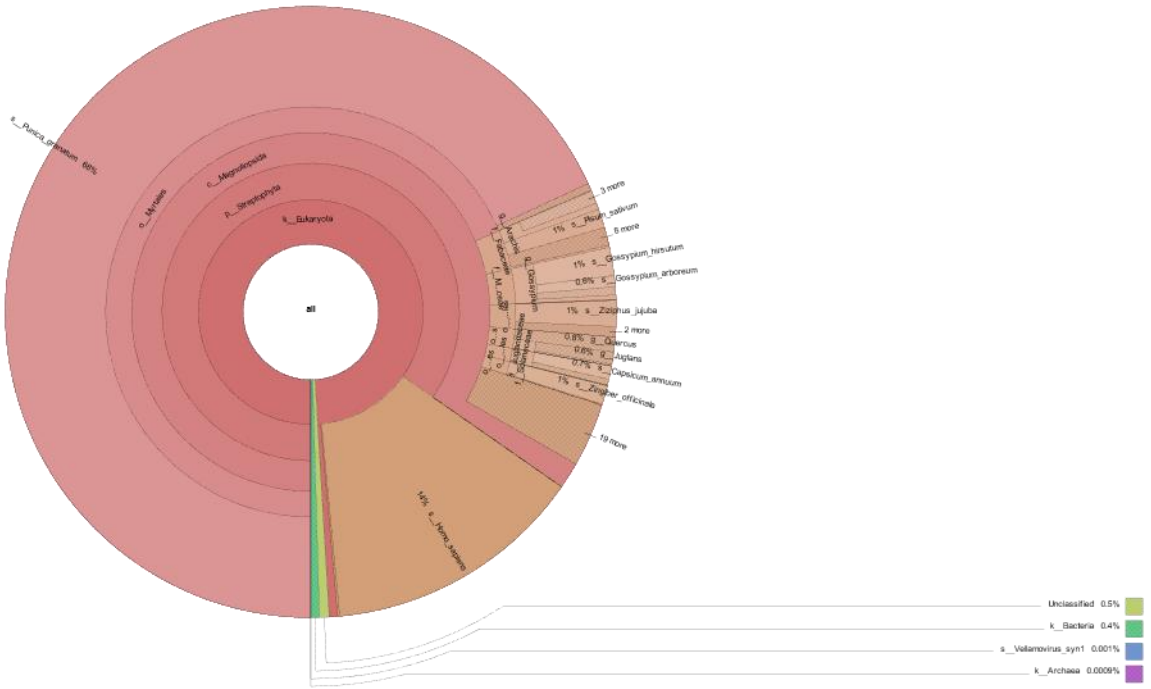


Figure 5.2. Genome size and sequencing depth estimation via 17-mer Analysis.

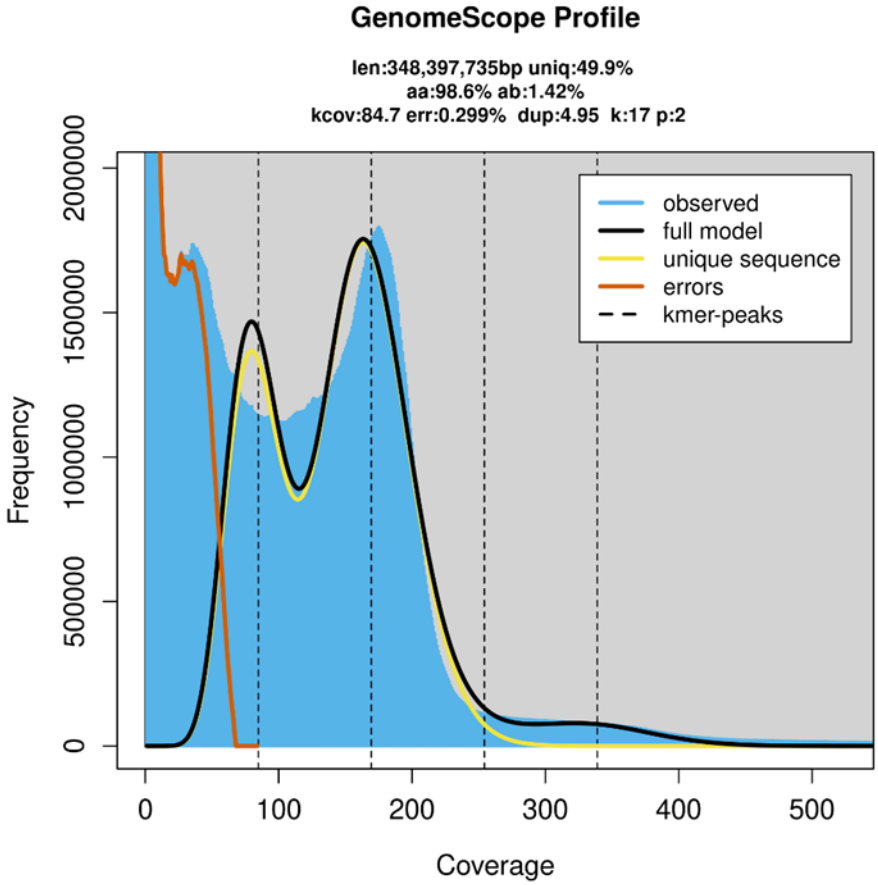


Figure 5.3. D-GENIES dot plot compared haplotype 1 with reference genome.

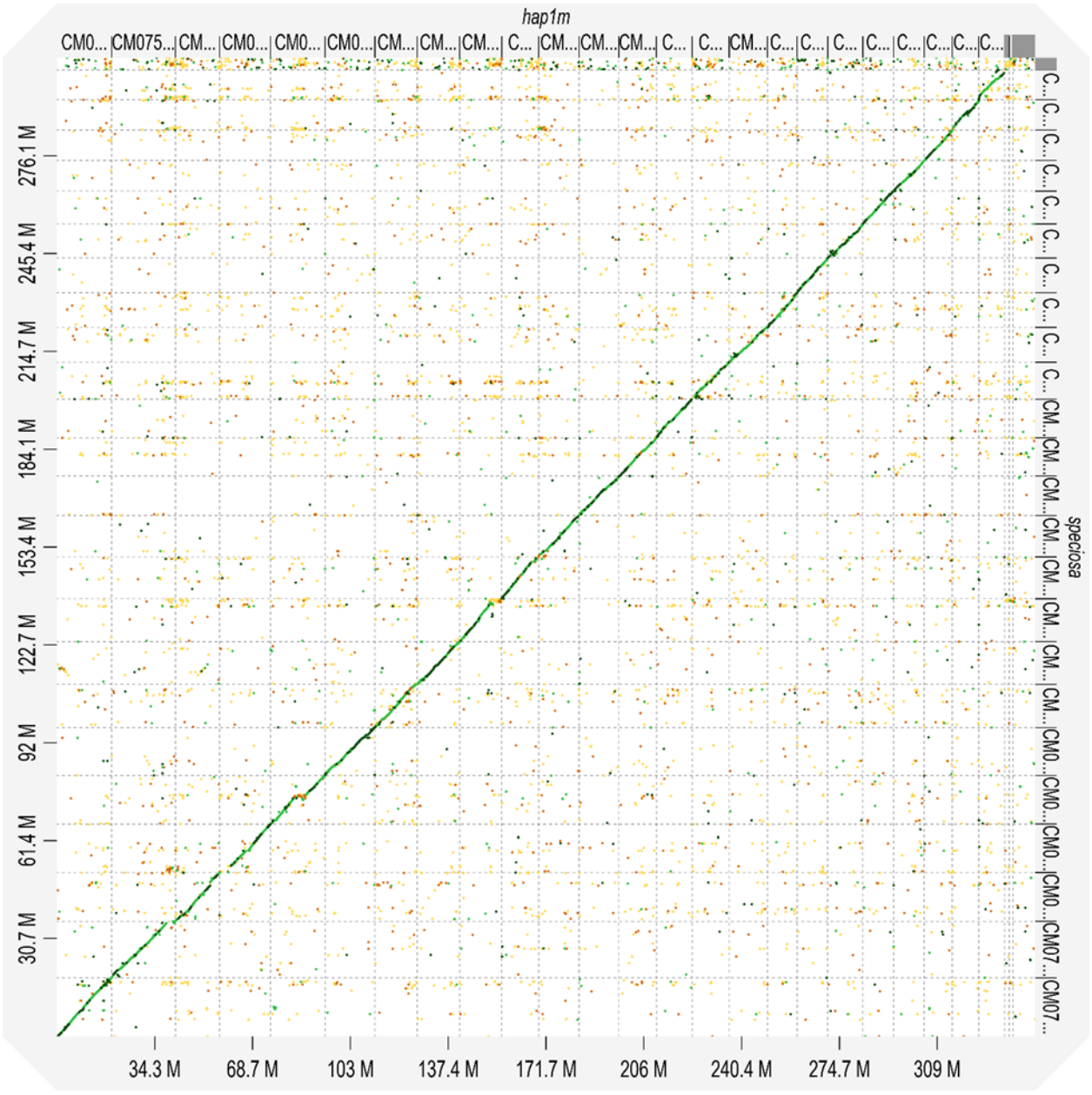
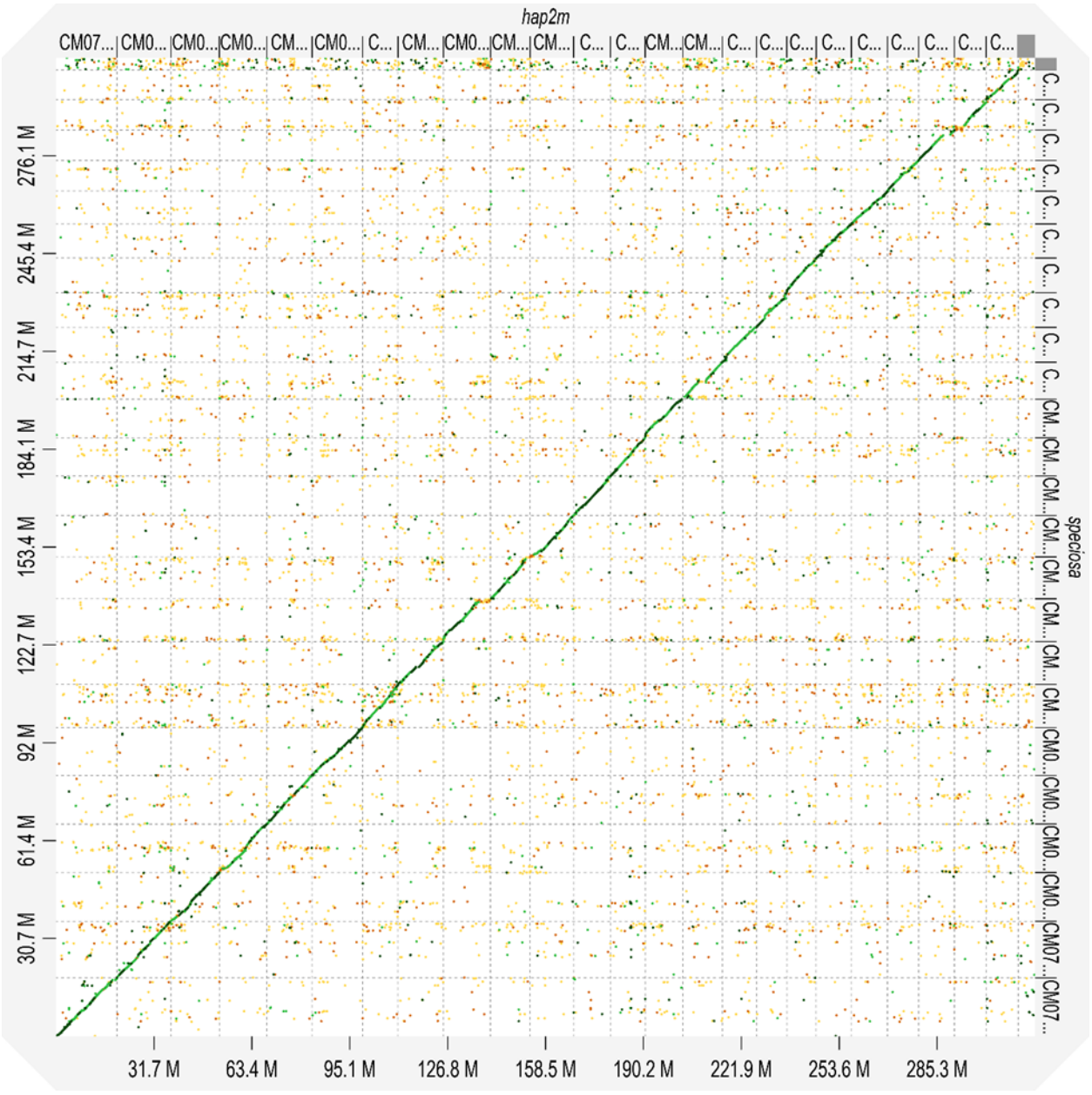


Figure 5.4. D-GENIES dot plot compared haplotype 2 with reference genome.



CHAPTER 1

Three New Crape Myrtle (*Lagerstroemia*) Cultivars for Southern Landscape

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Keywords: new cultivars; *Lagerstroemia*; crape myrtle; breeding series

Introduction

Crape myrtle (*Lagerstroemia)* is a genus in the *Lythraceae* (loosestrife family) that comprises around 50 to 80 species (Cabrera, 2002). The origins of crape myrtle vary, including the Indian subcontinent, Southeast Asia, central and southern China, Korea, Japan, and parts of Australia (Zhou et al., 2023). Due to their colorful flowers, attractive bark, and long blooming duration, they are widely cultivated in tropical and subtropical regions worldwide. Among all the species, the most popular ones are *Lagerstroemia indica* L. and *L. indica* × *L. fauriei* Koehne hybrids (Pounders and Sakhanokho, 2015) because of the various flower colors from *L. indica* and the disease resistance from *L. fauriei*. Crape myrtles were first introduced to the United States in the late 1700s (Egolf and Andrick, 1978; Pooler, 2006) and later became popular for landscape use in subtropical and tropical regions of the United States. Depending on their use in landscapes, crape myrtles have been categorized into three types based on plant height: semi-dwarf cultivars, intermediate cultivars, and tree-type cultivars. In the United States, the commercial production of crape myrtles relies mostly on asexual propagation, particularly the cloning of named cultivars (Byers, 1997). This ensures consistency in characteristics such as flower color, size, and growth habits. Although modern crape myrtle cultivars often possess a genetic background from *L. fauriei*, their disease and pest resistance vary due to physical and chemical mutations as well as genetic recombination. Despite the U.S. National Arboretum releasing many powdery mildew-resistant cultivars, the market and growers continue to seek new cultivars with novel traits, such as dark leaves, which may not have strong disease resistance. While many dwarf cultivars are available today, most develop water sprouts a few years after establishment and revert to their normal growth habit. Our new cultivars, however, are easy to propagate and manage, have a long-lasting blooming

period, exhibit various flower colors, maintain great dark green foliage throughout entire growing season, and semi-dwarf, fountain-like habit.

Origin

Since 2012, the Woody Ornamental Lab at the University of Georgia has embarked on a project to develop new crape myrtle cultivars. With objectives centered around extending the blooming period, diversifying flower and foliage hues, and enhancing disease resistance, numerous hybridization attempts were undertaken, leading to a wide selection of seedlings being planted for evaluation. The initial crosses were made in 2016 for all the three cultivars. By 2019, the most promising seedlings were selected for rigorous field trials at the Durham Horticulture Farm in Watkinsville, GA, and all the three cultivars were assigned accession numbers (C14-35 for ‘Pristine Crystal’, C14-39 for ‘Pristine Lilac’, and D03-29 for ‘Pristine Ruby’) for potential new cultivars and future breeding work. After a comprehensive four-year assessment, three standout seedlings, notable for their clean foliage and flower colors, were selected as new cultivars. Remarkably, two of these seedlings (C14-35 and C14-39) emerged from a cross between PM-8 (a powdery mildew resistant seedling of Delta Jazz®) and PM-58 (the gamma-ray irradiated seedling of Dazzle® Me Pink dwarf round leaf pink selection), showcasing the innovative use of physical mutation in plant breeding, while D03-29 resulted from a hybridization between the 'Crescent Moon' and 'Ebony Fire' cultivars. ‘Crescent Moon’ is an intermediate-sized, round-shaped cultivar known for its excellent disease resistance and long blooming period, producing an abundance of attractive white flowers. ‘Ebony Fire’, on the other hand, is an intermediate-sized, upright-shaped cultivar characterized by its dark purple leaves and red flowers. These three cultivars were distinguished by their superior resistance to powdery mildew, maintaining vibrant, green foliage throughout the summer season. In recognition of their clean foliage year-round and captivating

floral displays, these cultivars were named ‘Pristine Crystal’, ‘Pristine Lilac’, and ‘Pristine Ruby’. Each name reflects the unique aesthetic characteristics that these plants bring to landscapes and gardens, promising not only visual beauty but also improved health and longevity. This initiative underscores the lab's commitment to enhancing the diversity and resilience of crape myrtles, offering gardeners and landscapers new options for their green spaces.

Description

Habit

The three cultivars, characterized as semi-dwarf, fountain-like shrubs, have demonstrated remarkable growth over four years at the UGA Horticultural Farm. These cultivars, named ‘Pristine Crystal’, ‘Pristine lilac’, and ‘Pristine Ruby’, have reached impressive heights of 2.8 meters, 2.7 meters, and 3.3 meters, respectively. Their vertical growth is complemented by substantial horizontal expansion, with the north-south and east-west dimensions measuring 3.3 × 3.4 meters for ‘Pristine Crystal’ (Fig. 6.1A), 3.2 × 3.1 meters for ‘Pristine Lilac’ (Fig. 6.1B), and 3.9 × 3.7 meters for ‘Pristine Ruby’ (Fig. 6.1C). These measurements underscore not only the robust nature of their growth but also the meticulous cultivation and selection process that has led to their development.

The dimensions of each cultivar reflect their unique genetic makeup. ‘Pristine Crystal’, with its slightly more compact form, demonstrates a balanced growth that could be advantageous for certain landscaping applications where space is a consideration. Meanwhile, ‘Pristine Lilac’, with its slightly smaller stature, still shows significant growth potential and may offer unique aesthetic qualities with its likely vibrant coloration, hinted at by its name. Their semi-dwarf, fountain-shaped structure makes them versatile, suitable for use in borders, hedges, and containers. ‘Pristine Ruby’ exhibiting the most substantial growth in both height and spread, suggesting a potentially superior

adaptation to the local environment. This vigorous growth pattern is indicative of a strong root system and optimal nutrient uptake, likely a result of selective breeding practices aimed at enhancing these traits.

The potential impact of environmental factors on these growth patterns cannot be overstated. Soil composition, water availability, and microclimatic conditions at the UGA Horticultural Farm have undoubtedly played a role in shaping the growth characteristics of these cultivars. Continued observation and study of these factors will provide valuable insights into optimizing growth conditions for future cultivars. These findings highlight the interplay between genetics and environment in horticultural success and pave the way for further advancements in ornamental plant breeding. The growth of the three new cultivars was very fast during the first three years after being planted in the field. By 2022, 2023, and 2024, the size of the three cultivars had stabilized with little change. This fast growth habit is advantageous for new homeowners looking to quickly establish their yards.

Foliage

Beginning in late March to early April, all three cultivars initiate leafing, each showcasing uniquely attractive young foliage. ‘Pristine Crystal’ features young leaves of a greyish brown color (RHS 166A, Table1), which mature into a moderate olive-green shade (RHS 137A, Table 6.1), providing a subtle yet elegant foliage display. Meanwhile, ‘Pristine Lilac’ stands out with its vibrant strong yellow-green young leaves (RHS 144A, Table 6.1) that evolve into a greyish olive green as they mature, creating a bold and eye-catching contrast in any garden setting. ‘Pristine Ruby’ presents young leaves with a striking brown hue (RHS 172A, Table 6.1) that transitions to a moderate olive green (RHS 146A, Table 6.1) upon maturity, offering a dynamic visual change throughout the growing season.

The foliage of these new cultivars measures 5-7 cm in length and 2.5-4 cm in width, all characterized by an entire leaf margin and an apiculate tip, adding to their refined appearance. Notably, ‘Pristine Ruby’ boasts an ovate leaf shape, distinguishing it from the elliptical form seen in both ‘Pristine Crystal’ and ‘Pristine Lilac’ (Fig. 6.1H). This variation in leaf shape provides consumers with more choices and indicates the genetic diversity among crape myrtle cultivars achieved through breeding.

From spring through fall, the leaves of these cultivars remain remarkably clean, a stark contrast to other seedlings that often suffer from moderate to severe powdery mildew infections. We also have other cultivars on our farm for breeding work, including the Dazzle series and Ebony series. However, none of the existing cultivars surpass our new cultivars in terms of leaf cleanliness. This resistance to common foliar diseases significantly reduces the need for chemical treatments, making these cultivars more sustainable and environmentally friendly options for gardeners and landscapers. Additionally, all three cultivars feature very short petioles, ranging from 2 to 4 mm in length, which contribute to their compact and tidy growth habit, further emphasizing their distinctiveness and appeal as superior selections for cultivation and ornamental use.

These unique characteristics make ‘Pristine Crystal’, ‘Pristine Lilac’, and ‘Pristine Ruby’ highly desirable for a variety of landscape applications. Their robust growth, disease resistance, and visually appealing foliage ensure that they will be standout additions to any ornamental planting. Their introduction represents a significant advancement in horticultural breeding, providing gardeners with new options that combine beauty, resilience, and low maintenance. As these cultivars continue to be evaluated, their full potential in diverse environmental settings will be better understood, paving the way for their broader adoption and appreciation.

Flower

The three new cultivars, 'Pristine Crystal', 'Pristine Lilac', and 'Pristine Ruby', exhibit an extended blooming season from the end of May or early June through late August and early September, aligning perfectly with the climatic conditions of USDA Cold Hardiness Zone 8a (Table 1). This characteristic, coupled with their vibrant colors and disease resistance, positions them as exceptional choices for landscaping in the southern United States.

'Pristine Crystal' captivates with its pristine white blossoms (RHS NN155D, Table 6.1), slightly smaller in size, ranging from 2.9 cm to 3.4 cm in diameter, offering a clean and elegant aesthetic. Meanwhile, 'Pristine Lilac' enchants with its light purple flowers (RHS 77C, Table 6.1), with diameters spanning from 3.5 cm to 3.9 cm, adding a delicate touch of color to the landscape. 'Pristine Ruby' is distinguished by its striking strong purplish-red flowers (RHS 58C, Table 6.1), with individual blooms measuring between 3 cm and 4 cm in diameter, making a bold statement in any garden. Each flower across the cultivars is composed of six segments, featuring ruffled, claw-like petals, and distinctive style-like staminodes (Fig. 6.1G). The blooms are organized in terminal panicle arrangements, contributing significantly to their visual appeal and garden presence.

Remarkably, all three cultivars share a similar blooming timeline, initiating in early June and continuing through the end of August, with July marking the peak of their floral display. Reblooming may occur with deadheading. This synchronized blooming period, alongside their aesthetic attributes and robustness, underscores their potential to enhance southern landscapes with continuous and colorful blooms throughout the summer months.

The petal lengths of the three new cultivars, 'Pristine Crystal', 'Pristine Lilac', and 'Pristine Ruby', vary distinctly, measuring 1.0-1.3 cm, 1.5-1.8 cm, and 1.3-1.5 cm, respectively. Similarly, their sepals exhibit varying lengths: 0.4-0.6 cm for 'Pristine Crystal', 0.3-0.5 cm for 'Pristine Lilac',

and 0.5-0.6 cm for 'Pristine Ruby'. Although the individual flowers and petals of these cultivars are relatively small compared to other species, they compensate with their impressive panicle size and density. The panicle lengths are particularly noteworthy, with 'Pristine Crystal' (Fig. 6.1D) measuring 11-15 cm, 'Pristine Lilac' (Fig. 6.1E) extending from 12-22 cm, and 'Pristine Ruby' (Fig. 6.1F) showcasing panicles of about 14-17 cm.

These dimensions not only highlight the unique characteristics of each cultivar but also suggest their potential for creating visually striking displays in gardens and landscapes. The extended blooming period and substantial aesthetic appeal of their panicles, despite the smaller size of individual floral components, make these cultivars highly desirable for enhancing outdoor spaces with vibrant, long-lasting floral arrangements. The robustness and disease resistance of 'Pristine Crystal', 'Pristine Lilac', and 'Pristine Ruby' further ensure their suitability for a wide range of landscaping applications, promising to bring continuous beauty and color to southern landscapes throughout the summer months.

Fruit

Fruits became visible one week after the flowers bloomed. The initial color of the fruits for all three cultivars was light green, which gradually darkened to dark brown or black over time. The fruits were ready for harvest from late September to late October. When mature, the capsule-like fruits split open into six channels, dispersing seeds onto the ground. Each fruit could bear 30-50 seeds, though some seeds were defective and lacked an embryo. Despite the large number of seeds produced annually, no seedlings emerged around the three cultivars, making them easy to manage for landscape use.

Additional note

Despite Georgia experiencing severe winter weather conditions occasionally, these plants demonstrate remarkable resilience, easily overwintering due to their robust cold hardiness. However, the blooming period of the three cultivars—'Pristine Crystal', 'Pristine Lilac', and 'Pristine Ruby'—is significantly affected by late frosts. Such frost can cause damage to new leaves and result in a delay of the blooming time by approximately 2-3 weeks. This susceptibility highlights the importance of considering climatic challenges in their cultivation and underscores the need for protective measures or strategic planting to mitigate the impact of late frosts on these otherwise resilient and beautiful cultivars.

Propagation

Upon identifying promising cultivars, developing an efficient propagation method is crucial for their multiplication. For the three new cultivars, 'Pristine Crystal', 'Pristine Lilac', and 'Pristine Ruby', softwood cuttings collected on May 31st and June 7th, 2023, and treated with Hormodin #1 (OHP, Inc., Mainland, PA) have shown successful rooting. The rooting percentages for 'Pristine Crystal', 'Pristine Lilac', and 'Pristine Ruby' were 86.5%, 87.5%, and 80.2%, respectively. These cuttings were nurtured under a shade cloth equipped with an adequate misting system. The misting was programmed to activate for 10 seconds every 10 minutes over a period of 4 weeks. Once rooted, the cuttings were transplanted into 2.8 L pots complemented with 1 tsp/gal of slow-release fertilizer (14-4-14) to support optimal plant growth. Typically, cuttings reach a height of approximately 1 meter by the end of their first growing season and will start to bloom from late summer until the first frost. This propagation strategy underscores the balance between creating a conducive environment for root development and ensuring the young plants have the necessary nutrients to flourish and reach their blooming potential within their first year.

Availability

Information about the three new cultivars can be obtained from Dr. Donglin Zhang (e-mail: donglin@uga.edu). Right now, three new cultivars are available for restricted test agreement only.

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Table 6.1. Distinguished flower and foliage characteristics of the three new cultivars. The data are the means ± SEs (n=10).

	Pristine Crystal	Pristine Lilac	Pristine Ruby
Flower color	RHS NN155D white	RHS 77C light purple/77C strong purple	RHS 58B/RHS 58C strong purplish red
Young leaf color	RHS 166A greyish brown	RHS 144A strong yellow green	RHS 172A strong brown
Matured leaf color	RHS 137A moderate olive green	RHS NN137 greyish olive green	RHS 146A moderate olive green
Flower diameter (cm)	3.14 ± 0.15	3.81 ± 0.23	3.65 ± 0.14
Spal length (cm)	0.54 ± 0.07	0.53 ± 0.05	0.40 ± 0.07
Bud length (cm)	0.73 ± 0.05	0.92 ± 0.06	0.67 ± 0.07
Bud width (cm)	0.61 ± 0.06	0.74 ± 0.05	0.66 ± 0.07
Pedicel length (cm)	0.50 ± 0.08	0.58 ± 0.06	0.43 ± 0.07
Petiole length (cm)	0.12 ± 0.04	0.32 ± 0.06	0.17 ± 0.05
Leaf length (cm)	5.51 ± 0.49	6.59 ± 0.31	6.24 ± 0.39
Leaf width (cm)	3.16 ± 0.33	3.10 ± 0.25	4.19 ± 0.33
Panicle length (cm)	13.18 ± 1.48	16.23 ± 3.51	15.97 ± 0.86
Petal length (cm)	1.15 ± 0.10	1.64 ± 0.10	1.44 ± 0.07
Petal width (cm)	1.01 ± 0.07	1.10 ± 0.08	1.05 ± 0.05
Leaf out	late March/early April	late March/early April	late March/early April
Bud out	early June	early June	late May/early June

Figure 6.1. Habit, blossom, and foliage of the three new cultivars. A. Growth habit of ‘Pristine Crystal’. B. Growth habit of ‘Pristine Lilac’. C. Growth habit of ‘Pristine Ruby’. D. Panicles of ‘Pristine Crystal’. E. Panicles of ‘Pristine Lilac’. F. Panicles of ‘Pristine Ruby’. G. Flower characters of the three cultivars. H. Leaf characters of the three cultivars.