Plant Breeding and Evaluation

Matthew Chappell
Section Editor


Pollen Viability and Storage Temperature for Southern Highbush and Rabbiteye Blueberry Breeding

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Index Words: In vitro pollen germination, pollen storage temperature, rabbiteye blueberry, southern highbush blueberry

Significance to Industry: Blueberry plants are becoming more popular as dual purpose ornamentals. Significant correlation has been reported between pollen viability and fruit quality parameters in blueberry (9). Additionally, low blueberry pollen viability has been linked to poor fruit set (2, 8). Therefore, maintaining viability of stored pollen for a determined amount of time can be crucial to crop improvement programs, particularly when the genotypes to be hybridized have asynchronous flowering or are geographically separated (7). Germination percentage of pollen tetrads from four rabbiteye and four southern highbush blueberry genotypes immediately after collection (fresh) and after 2, 4, 6, or 8 weeks of storage at room temperature (22 ºC), in a refrigerator (4 ºC), or in a freezer (-20 ºC) was determined. Germination percentage of fresh blueberry pollen was generally high, but storing blueberry pollen tetrads at room temperature or in a freezer resulted in rapid decline in germination ability. From our results, it can be concluded that germination of rabbiteye and southern highbush blueberry pollen is best maintained when pollen is stored at 4 ºC.

Nature of Work: Pollen viability, as measured by tetrad germination, has been reported (4, 3, 1), but these studies focused on freshly collected pollen and did not address viability of pollen stored at different temperatures over time. Moreover, genetic differences in pollen viability have been reported in blueberry genotypes (4), so knowledge of pollen viability in genotypes at hand for hybridization is desired. Therefore, the objective of this study was to determine appropriate temperature parameters for storage of blueberry pollen, focusing on four rabbiteye (DeSoto, MS1228, MS282, MS454) and four southern highbush (Biloxi, Gupton, MS1377, Rebel) blueberry genotypes. Fresh pollen was collected from field-grown plants of these four genotypes. Germination percentage was determined for fresh pollen and pollen grains stored at 22 ºC, 4 ºC, or -20 ºC after 2, 4, 6, or 8 weeks. An in vitro pollen germination test was performed to evaluate pollen viability using the "hanging drop" technique as described by Sakhanokho and Rajasekaran (5). The liquid germination medium
consisted of 1.2 M sucrose, 0.42 g L\(^{-1}\) calcium nitrate [Ca(NO\(_3\)]\(_2\)], 0.20 g L\(^{-1}\) boric acid (H\(_3\)BO\(_3\)), 0.1 g L\(^{-1}\) potassium nitrate (KNO\(_3\)), 0.22 g L\(^{-1}\) magnesium sulfate (MgSO\(_4\)·7H\(_2\)O), and 15% (w/v) polyethylene glycol (PEG). Data were analyzed using generalized linear models with the GLIMMIX procedure of SAS (version 9.3; SAS Institute Inc., Cary, NC) with the Poisson distribution and log link function. Due to significant two-way interaction terms, data were analyzed by genotype and week. Comparisons of means under the three storage temperatures were carried out using the Schaffer-Simulated method.

**Results and Discussion:** Germination percentage of fresh pollen was high in all genotypes tested, ranging from 67% to 87% in rabbiteye and 89% to 95% in southern highbush blueberries (Table 1). Germination percentages of all genotypes under all storage temperatures declined over time. This decline was particularly pronounced when pollen grains were stored at room temperature (22 °C) or in a freezer (-20 °C) (Table 1), even though the pollen tetrads looked morphologically "normal" (Fig. 1B, 1D). Germination capability over time was best maintained when pollen was refrigerated at 4°C. In general, pollen grains from rabbiteye genotypes appear to better maintain germination capability over time. For example, germination percentages for rabbiteye pollens stored at 4°C ranged from 36% (MS1228) to 84% (MS454) after 4 weeks, whereas those for southern highbush genotypes ranged from 0% (MS1377) to 9% (Gupton and Rebel) for the same storage duration (Table 1). In this study, the viability of a particular pollen refers to its ability to germinate rather than the ability to fertilize, which is better measured by in vivo pollen tube growth or vigor (6). However, the ability of pollen tetrad to germinate more than one pollen tube (Fig. 1A, 1C) could also indicate pollen viability if germination of each grain is independent within the tetrad (4). In conclusion, pollens of most genotypes remained viable for at least 4 weeks and germination of blueberry pollen was best preserved in refrigeration (4°C).

**Literature Cited**


Fig. 1. In vitro blueberry pollen germination. Freshly collected pollens, including those of 'Gupton' (A), had a high germination rate and pollen tube growth. (B): Pollen from 'Gupton' showed very little to no germination when stored in a freezer (-20 ºC) for four weeks. (C): Germination percentage in pollen collected from the blueberry line MS454 and stored in a refrigerator (4 ºC) was still high at four weeks, but it was very low or non-existent for pollens of the same line stored in a freezer (-20 ºC) for four weeks (D).
Table 1. Germination (%) of pollen grains from four rabbiteye and four southern highbush blueberry genotypes immediately after collection (fresh) and after 2, 4, 6, or 8 weeks of storage at room temperature (22 °C), in a refrigerator (4 °C), or in a freezer (-20 °C).

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Rabbiteye</th>
<th>Southern highbush</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DeSoto</td>
<td>MS1228</td>
</tr>
<tr>
<td>0</td>
<td>Fresh</td>
<td>71</td>
</tr>
<tr>
<td>2</td>
<td>22°C</td>
<td>6 b²</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>56 a</td>
</tr>
<tr>
<td></td>
<td>-20°C</td>
<td>7 b</td>
</tr>
<tr>
<td>4</td>
<td>22°C</td>
<td>0 b</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>40 a</td>
</tr>
<tr>
<td></td>
<td>-20°C</td>
<td>3 b</td>
</tr>
<tr>
<td>6</td>
<td>22°C</td>
<td>0 b</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>21 a</td>
</tr>
<tr>
<td></td>
<td>-20°C</td>
<td>0 b</td>
</tr>
<tr>
<td>8</td>
<td>22°C</td>
<td>0 a</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>5 a</td>
</tr>
<tr>
<td></td>
<td>-20°C</td>
<td>0 a</td>
</tr>
</tbody>
</table>

²Means followed by different letters within a genotype and weeks of storage are significantly different according to the Schaffer-Simulated method at α = 0.05.
Relative Fertility and Ploidy Levels of Selected Rose of Sharon Cultivars

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Index words: *Hibiscus syriacus*, sterility, polyploidy

**Significance to Industry:** *Hibiscus syriacus* (rose of sharon) is a popular flowering shrub in the United States. Rose of sharon is widely adaptable and provides vivid flowers late in the season, but unfortunately it produces prolific seeds and can be weedy (2). The U.S. National Arboretum (USNA) attempted to address this issue by releasing four cultivars (‘Aphrodite’, ‘Diana’, ‘Helene’, and ‘Minerva’) that were reported to be sterile (or nearly sterile) triploids (4, 5, 6, 7). However, these cultivars have been observed to produce seed. The fecundity of these cultivars raises questions that may have serious implications for future ornamental plant breeding with regard to reversion of odd-ploidy selections and restored fertility. To answer some of the questions surrounding these cultivars, reciprocal crosses were conducted among nine cultivars readily available in the trade, including three of the USNA cultivars. We also conducted flow cytometry analysis of DAPI-stained nuclei to determine relative genome sizes and infer ploidy levels for 20 cultivars. All cultivars, including the purported triploid cultivars, were both male and female fertile with the exception of ‘Lucy’, which has petaloid stamens. We determined that all cultivars included were tetraploid with the exception of ‘Pink Giant’, which was hexaploid. Our work shows that the USNA cultivars included in our study (‘Aphrodite’, ‘Diana’, and ‘Minerva’) were fertile tetraploids. It remains unclear why they appeared to have low fertility, or near sterility, at the time of evaluation and release but have apparently regained fertility. The cultivar ‘Pink Giant’ appeared to have the lowest number of seedlings per pollinated flower and was the only cultivar in our study with a ploidy level differing from the wild-type. This suggests that ploidy manipulation remains a viable option for developing truly sterile rose of sharon cultivars.

**Nature of Work:** Ploidy manipulation is an important tool in developing sterile forms of nursery crops. Historically, polyploids were identified based on their morphology. Thicker and darker leaves as well as twisted or malformed flowers were often used as indicators of polyploidy. However, relying on gross morphology alone can lead to misidentification. Another method used by plant breeders to identify polyploids is the measurement of stomata. Measuring stomata is useful in identifying the ploidy level of the L-I histogenic layer, but provides no information on the germ layer (L-II) from which pollen and eggs are derived. The results of using either morphology or stomata measurements may lead to erroneously identifying plants that breed as polyploids. Today, breeders have the advantage of using flow cytometry to accurately and quickly identify ploidy levels and genome sizes.
The natural form of rose of sharon is tetraploid \((2n = 4x = 80)\). However, publications on the cultivars ‘Aphrodite’, ‘Diana’, ‘Helene’, and ‘Minerva’ state that they are the result of chromosome doubling of the diploid cultivar ‘William R. Smith’, resulting in a tetraploid, and then crossing with various diploid cultivars to produce sterile triploid progeny \((4, 5, 6, 7)\). If the original treated cultivar was actually doubled, the result would have actually been an octoploid \((2n = 8x = 160)\) and crosses with untreated tetraploid cultivars would have resulted in hexaploid progeny \((2n = 6x = 120)\). It remains unclear if the original polyploids were identified by any other method than gross morphology, which is an unreliable method. Therefore, the ploidy level of the original purported polyploid parent plant is in doubt. One possibility is that they were cytochimeras with their L-I histogenic layer doubled, or perhaps they contained no polyploid cells if morphology alone was used to identify them.

To evaluate male and female fertility, reciprocal crosses were made by hand among nine rose of sharon cultivars grown in a glasshouse (Table 1). There was sufficient distance between the stigmas and anthers at anthesis, eliminating the need for emasculation. Flowers that were not hand pollinated did not set seed, demonstrating that recovered seedlings are the result of cross-pollination. At maturity, seeds were collected, counted, and sown within two days. Seedling numbers were recorded after germination.

Flow cytometry was conducted according to Contreras et al. (1) with the modification that tomato \((Solanum lycopersicum ‘Stupicke’ 2C = 1.96 pg) (3) was used as the internal standard because its genome size was more similar to previous reports for Hibiscus than pea. We used three replications for each cultivar and presented the means ± SEM. We analyzed a minimum of 2,000 particles and CV% for all samples was ≤5%.

**Results and Discussion:** All cultivars were male and female fertile (Table 1). Female fertility ranged from 0.08 to 10.1 seedlings per pollinated flower in ‘Pink Giant’ and ‘Aphrodite’, respectively, with a mean of 3.0 for the nine cultivars. Male fertility ranged from 0.5 to 10.1 seedlings per pollinated flower in ‘Pink Giant’ and ‘Marina’ (Blue Satin®), respectively, with a mean of 2.9 for the nine cultivars. ‘Lucy’ was not evaluated for male fertility because it has double flowers and does not produce pollen. The purported sterile triploid cultivars included in the study, ‘Aphrodite’, ‘Diana’, and ‘Minerva’ were all male and female fertile. Statistical analysis was not conducted. However, the level of fertility of these cultivars appears to be similar to the other six cultivars included. A notable exception is ‘Pink Giant’, which produced only 0.08 and 0.5 seedlings per pollinated flower when used as a female and male parent, respectively. This reduced level of fertility can likely be explained by the fact that ‘Pink Giant’ has a higher ploidy level than other cultivars, which leads to increased autosynthetic pairing of homologous chromosomes.

All cultivars except ‘Pink Giant’ were tetraploids with genome sizes comparable to those previously reported (Table 2) \((9)\). The cultivar ‘Oiseau Bleu’ (syn. ‘Blue Bird’) was found
to have a genome size of $2C = 4.6$ pg, confirming a previous report of its genome size as $2C = 4.68$ pg (9). Other research confirmed that ‘Lucy’, ‘Oiseau Bleu’, ‘Red Heart’, and ‘Woodbridge’ were $2n$ (actually $4x$) and that ‘Diana’, ‘Helene’, and ‘Pink Giant’ were $3n$ (actually $6x$) (9). The history of ploidy levels of ‘Aphrodite’, ‘Diana’, and ‘Minerva’ has been unclear, but the previous report suggests that ‘Diana’ was a homogeneous hexaploid. One possibility is that the original plants were cytochimeras that eventually stabilized at the tetraploid or hexaploid level after repeated cycles of asexual propagation. Related to this possibility, there may be various lines of descent with various ploidy levels; depending on the source, ‘Diana’ and other USNA cultivars may be tetraploid or hexaploid. To investigate this, we plan to test material from original plants of ‘Aphrodite’, ‘Diana’, and ‘Minerva’ at the USNA in addition to testing various cultivars from nurseries, gardens, and arboreta. Additional testing of these plants may elucidate the ploidy level of their composite histogenic layers to indicate why these cultivars are now fertile tetraploids.

**Literature Cited**

Table 1. Results of crossing study conducted during 2012 to estimate the relative fertility of nine rose of sharon (*Hibiscus syriacus*) cultivars.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Flowers pollinated (No.)</th>
<th>Seedlings (No.)</th>
<th>Seedlings per poll. flower (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>As female parent</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Aphrodite’</td>
<td>42</td>
<td>423</td>
<td>10.1</td>
</tr>
<tr>
<td>‘Blue Bird’</td>
<td>36</td>
<td>66</td>
<td>1.8</td>
</tr>
<tr>
<td>‘Marina’</td>
<td>59</td>
<td>318</td>
<td>5.4</td>
</tr>
<tr>
<td>‘Diana’</td>
<td>97</td>
<td>155</td>
<td>1.6</td>
</tr>
<tr>
<td>‘Lucy’</td>
<td>34</td>
<td>27</td>
<td>0.8</td>
</tr>
<tr>
<td>‘Minerva’</td>
<td>24</td>
<td>44</td>
<td>1.8</td>
</tr>
<tr>
<td>‘Pink Giant’</td>
<td>39</td>
<td>3</td>
<td>0.08</td>
</tr>
<tr>
<td>‘Red Heart’</td>
<td>74</td>
<td>212</td>
<td>2.9</td>
</tr>
<tr>
<td>‘Woodbridge’</td>
<td>70</td>
<td>180</td>
<td>2.6</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>52.8</td>
<td>158.7</td>
<td>3.0</td>
</tr>
<tr>
<td><strong>As male parent</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Aphrodite’</td>
<td>74</td>
<td>55</td>
<td>0.7</td>
</tr>
<tr>
<td>‘Blue Bird’</td>
<td>54</td>
<td>212</td>
<td>3.9</td>
</tr>
<tr>
<td>‘Marina’</td>
<td>44</td>
<td>443</td>
<td>10.1</td>
</tr>
<tr>
<td>‘Diana’</td>
<td>55</td>
<td>186</td>
<td>3.4</td>
</tr>
<tr>
<td>‘Lucy(^{\text{z}})’</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>‘Minerva’</td>
<td>66</td>
<td>222</td>
<td>3.4</td>
</tr>
<tr>
<td>‘Pink Giant’</td>
<td>50</td>
<td>25</td>
<td>0.5</td>
</tr>
<tr>
<td>‘Red Heart’</td>
<td>66</td>
<td>154</td>
<td>2.3</td>
</tr>
<tr>
<td>‘Woodbridge’</td>
<td>66</td>
<td>131</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>59.4</td>
<td>178.5</td>
<td>2.9</td>
</tr>
</tbody>
</table>

\(^{\text{z}}\)‘Lucy’ is a double-flowered cultivar that does not produce pollen, therefore could not be assessed as a staminate parent.
Table 2. Mean relative holoploid genome size (2C) estimates ± SEM and inferred ploidy levels of nine cultivars of rose of sharon (*Hibiscus syriacus*). Estimates were performed by analyzing DAPI-stained nuclei using flow cytometry using *Solanum lycopersicum* ‘Stupicke’ (2C = 1.96 pg) as an internal standard.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Trade Name</th>
<th>2C Genome Size</th>
<th>Ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Aphrodite’</td>
<td></td>
<td>4.7 ± 0.04</td>
<td>4x</td>
</tr>
<tr>
<td>‘Diana’</td>
<td></td>
<td>4.7 ± 0.06</td>
<td>4x</td>
</tr>
<tr>
<td>‘Lucy’</td>
<td></td>
<td>4.6 ± 0.01</td>
<td>4x</td>
</tr>
<tr>
<td>‘Minerva’</td>
<td></td>
<td>4.6 ± 0.05</td>
<td>4x</td>
</tr>
<tr>
<td>‘Pink Giant’</td>
<td></td>
<td>6.8 ± 0.05</td>
<td>6x</td>
</tr>
<tr>
<td>‘Red Heart’</td>
<td></td>
<td>4.7 ± 0.00</td>
<td>4x</td>
</tr>
<tr>
<td>‘Woodbridge’</td>
<td></td>
<td>4.6 ± 0.06</td>
<td>4x</td>
</tr>
<tr>
<td>‘Blushing Bride’</td>
<td></td>
<td>4.8 ± 0.00</td>
<td>4x</td>
</tr>
<tr>
<td>‘Ardens’</td>
<td></td>
<td>4.7 ± 0.04</td>
<td>4x</td>
</tr>
<tr>
<td>‘Oiseau Blue’</td>
<td>Blue Bird</td>
<td>4.6 ± 0.04</td>
<td>4x</td>
</tr>
<tr>
<td>‘Marina’</td>
<td>Blue Satin®</td>
<td>4.6 ± 0.03</td>
<td>4x</td>
</tr>
<tr>
<td>‘America Irene Scott’</td>
<td>Sugar Tip™</td>
<td>4.7 ± 0.04</td>
<td>4x</td>
</tr>
<tr>
<td>‘Notwoodone’</td>
<td>Lavender Chiffon™</td>
<td>4.7 ± 0.08</td>
<td>4x</td>
</tr>
<tr>
<td>‘Notwoodtwo’</td>
<td>White Chiffon™</td>
<td>4.7 ± 0.02</td>
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<td>‘Notwoodthree’</td>
<td>Blue Chiffon™</td>
<td>4.6 ± 0.03</td>
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<tr>
<td>‘Antong Two’</td>
<td>Lil’ Kim™</td>
<td>4.7 ± 0.04</td>
<td>4x</td>
</tr>
<tr>
<td>‘Minspot’</td>
<td>Fiji™</td>
<td>4.7 ± 0.06</td>
<td>4x</td>
</tr>
<tr>
<td>‘Mingrand’</td>
<td>Hawaii™</td>
<td>4.7 ± 0.05</td>
<td>4x</td>
</tr>
<tr>
<td>‘Minfren’</td>
<td>Bali™</td>
<td>4.6 ± 0.01</td>
<td>4x</td>
</tr>
<tr>
<td>‘Mineru’</td>
<td>Tahiti™</td>
<td>4.6 ± 0.04</td>
<td>4x</td>
</tr>
</tbody>
</table>
Hybrids Between the U.S. Native *Ruellia caroliniensis* (J.F. Gmel.) Steud. and Invasive *Ruellia simplex* Wright

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Index words. Acanthaceae, Carolina Wild Petunia, Britton’s Wild Petunia, Mexican Petunia, Mexican Bluebell, interspecific hybrid

Significance to Industry The purpose of this study was to conduct manual crosses between U.S. native *Ruellia caroliniensis* and non-native, invasive *R. simplex* and to test whether the two species can be artificially hybridized. Hybrids obtained were analyzed morphologically. Results from this investigation should be of interest to conservation biologists and botanists of the southeastern U.S. Moreover, new hybrids developed could have ornamental potential and could be released as new cultivars.

Nature of Work *Ruellia caroliniensis* (J.F. Gmel.) Steud. (Carolina Wild Petunia) is native to 18 U.S. states, from New Jersey south to Florida, west to Texas and Oklahoma, and northwest to Illinois and Indiana (8). In Florida, *R. caroliniensis* is a common in native woodlands, and plants grow robustly in adverse conditions (4). *Ruellia simplex* Wright (Britton’s Petunia, Mexican Petunia, or Mexican Bluebell) is found in sunny areas of periodically inundated soils in Mexico, the Antilles, and central western South America (1). This species was introduced to Florida sometime before 1940 (5) and is a very popular landscape plant in the Southern U.S. (3). However, it has become naturalized in 31 states of the continental U.S. (from Minnesota south to Florida and west to Arizona) as well as Hawaii, the Virgin Islands, and Puerto Rico (10). In Florida, *R. simplex* has become naturalized in 29 counties (13) and has been recorded in 20 designated conservation areas in South Florida (7). Since 2001, the Florida Exotic Pest Plant Council has labeled this species as a Category I invasive plant, which is described as “plants that are altering native plant communities by displacing native species, changing community structures or ecological functions, or hybridizing with natives” (2).

*Ruellia caroliniensis* and *R. simplex* are sympatric in numerous areas in the U.S., occupying both wetlands and non-wetland environments. A comparison of growth and development of *R. caroliniensis* and *R. simplex* established that under wet conditions in
laboratory experiments, *R. simplex* had several traits that favor efficient use of resources and high growth rates. The conclusion was that under typical wetland conditions in parts of southern Florida, *R. simplex* might be expected to out-grow and out-compete the native species, especially if the nutrient supply is limited (12).

*Ruellia carolinensis* and *R. simplex* are closely related (9), but there have not yet been reports of natural hybridization. However, ample crossability between species of *Ruellia* has been demonstrated experimentally (6). A cross between *R. simplex* and *R. carolinensis* var. *succulenta* (direction unknown) was performed and reportedly produced viable seeds; however, no details on the obtained F₁ hybrids were provided. Until a few years ago, the commercial cultivar *Ruellia* ‘Oh What a Feeling’ was available in the trade. It presumably originated from natural hybridization between *R. simplex* and *R. carolinensis* because the only plants of *Ruellia* in the vicinity were the dwarf *R. simplex* ‘Katie’ and a plant of *R. carolinensis* (A. Advent, pers. comm.).

Two accessions of *Ruellia simplex* were included in this study: wild-type purple-flowered *R. simplex* (sim1), and pink-flowered *R. simplex* ‘Chi Chi’ (sim 2) (as described in (11)). Three different *R. carolinensis* accessions were obtained from different sources in Florida: Fort Pierce (car1), Alachua (car2) and Lee (car3). All plants were propagated by cuttings from stock plants in greenhouses at the University of Florida, Gainesville. Hybridizations were conducted between March and May 2008. The pollinated flower was tagged with a colored plastic string. When the fruit developed, it was enclosed with an empty tea bag secured with a paper clip, to prevent loss of seeds during fruit dehiscence (fruits of Acanthaceae have explosive dehiscence).

For each cross in which a fruit developed, the total number of seeds per capsule was counted. Immature or damaged seeds were separated from mature, apparently viable seeds. On September 2008, normal seeds were sown and after 10-15 days, seedlings were transplanted into 6” (15 cm) pots and maintained in a greenhouse. Plants were grown to maturity (i.e., flowering), and herbarium vouchers were taken for the morphological study. Hybrid plants were studied using light microscopy. Plants of wild-collected *Ruellia carolinensis* and *R. simplex* from different geographic origins were also studied taxonomically for comparison to hybrids.

**Results and Discussion** Hybridizations were performed with three *Ruellia carolinensis* accessions and two *R. simplex* accessions in all possible combinations. Twenty hybridizations were performed for each *R. carolinensis* × *R. simplex* combination, and 10 hybridizations for each reciprocal combination. All *R. carolinensis* × *R. simplex* combinations were successful, and the average fruiting percentage was 47% (Table 1). A total of 84% of the seeds obtained were visually normal, and their average germination was 36%. A total of 45 seedlings were obtained, and when they grew (based on their morphology) it became apparent that 11 of them were *R. carolinensis* selfs, and 34 were of hybrid origin. For the *R. simplex* × *R. carolinensis* combinations, a total of 41 seedlings were obtained, but it became apparent that they were all product of accidental *R. simplex* selfing.
The *Ruellia caroliniensis* x *R. simplex* obtained were very weak and slow growing. Their morphology was intermediate between that of both parents (Fig. 1). Characteristics that more clearly distinguish the parents and hybrids are shown in Table 1. All the hybrids were sterile, with no fruit or pollen production.

Our study indicates that production of interspecific hybrids is possible, particularly in the *Ruellia caroliniensis* [maternal] x *R. simplex* [paternal] direction. The hybrids obtained were very weak and slow growing, and would possibly be outcompeted by other species when growing under natural conditions. Moreover, all these F₁ hybrids were found to be sterile, thus would not be able to self or backcross to either parental species. Therefore the likelihood of interspecific *R. caroliniensis* x *R. simplex* hybrids affecting native *R. caroliniensis* populations appears to be null.

The interspecific hybrids obtained were propagated vegetatively to evaluate them for ornamental potential. In this case, it is advantageous to have sterile plants, which will not pose a risk of becoming invasive though seed dispersal. Due to their prostrate habit, they could potentially be used in pots or hanging baskets, which would constitute a new use for plants in the genus *Ruellia* in the ornamental industry.

**Literature Cited**


Table 1. Hybridizations between *Ruellia caroliniensis* x *R. simplex*

<table>
<thead>
<tr>
<th>Female parent</th>
<th>Male parent</th>
<th>No. crosses</th>
<th>Fruits</th>
<th>% Fruiting</th>
<th>Normal (abnormal) seeds</th>
<th>% normal seeds</th>
<th>Seeds germinated</th>
<th>% germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>car1 sim1</td>
<td>20</td>
<td>12</td>
<td>60</td>
<td>40(5)</td>
<td>89</td>
<td>17</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>car1 sim2</td>
<td>20</td>
<td>11</td>
<td>52</td>
<td>33(11)</td>
<td>75</td>
<td>8</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>car2 sim1</td>
<td>20</td>
<td>5</td>
<td>35</td>
<td>15(7)</td>
<td>68</td>
<td>8</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>car2 sim2</td>
<td>20</td>
<td>7</td>
<td>25</td>
<td>18(1)</td>
<td>95</td>
<td>5</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>car3 sim1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>car3 sim2</td>
<td>20</td>
<td>5</td>
<td>22(2)</td>
<td>92</td>
<td>7</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>47</td>
<td>84</td>
<td></td>
<td>36</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Morphological characteristics of *Ruellia caroliniensis*, *R. simplex* and *R. caroliniensis x R. simplex* hybrids.

<table>
<thead>
<tr>
<th></th>
<th><em>R. caroliniensis</em></th>
<th><em>R. simplex</em></th>
<th><em>R. caroliniensis x R. simplex</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Distribution</strong></td>
<td>Eastern North America</td>
<td>Neotropics</td>
<td>Natural hybrids not known</td>
</tr>
<tr>
<td><strong>Leaf length:width ratio</strong></td>
<td>1.9-3.7</td>
<td>10-22.5</td>
<td>2.9-6.8</td>
</tr>
<tr>
<td><strong>Dichasia</strong></td>
<td>Congested</td>
<td>Expanded</td>
<td>Partially expanded</td>
</tr>
<tr>
<td><strong>Bracts &amp; Bracteoles</strong></td>
<td>Elliptical</td>
<td>Linear</td>
<td>Narrowly elliptical</td>
</tr>
<tr>
<td><strong>Stamens</strong></td>
<td>Weakly didynamous</td>
<td>Strongly didynamous</td>
<td>Didynamous</td>
</tr>
<tr>
<td><strong>Stigma Lobes</strong></td>
<td>Dorsal and ventral equal</td>
<td>Dorsal completely reduced</td>
<td>Dorsal reduced to 1/3 length of ventral</td>
</tr>
</tbody>
</table>

Fig. 1. Flower, leaf and immature fruit of *Ruellia caroliniensis* (top left), *R. simplex* (top right) and *R.caroliniensis x R. simplex* hybrid
Pollen Tube Growth in Interspecific and Intergeneric Crosses in Melastomaceae: Implications for Plant Breeding

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Significance to Industry The Melastomaceae family, a family comprising tropical and subtropical plants, contains many species with potential to be good ornamental plants. Although some Melastomaceae species and cultivars, such as Tibouchina ‘Athens Blue’, are already in the trade, many more remain to be discovered and used in breeding programs. Interspecific and intergeneric crosses have often been used to create new hybrids for the ornamental nursery trade. Pollen tube germination was evaluated in crosses between plants in the genera Dissotis and Tibouchina to assess whether poor pollen tube germination would be a barrier to creating hybrids.

Nature of Work The Melastomaceae family contains approximately 5,000 species in 185 to 190 genera [1]. The two genera used in this study (Dissotis and Tibouchina) originate from Africa and South America, respectively [2]. Flowers are buzz pollinated in the wild. In the greenhouse, buzz pollination is mimicked by striking the anthers with a tuning fork to cause pollen to dehisce [3]. Interspecific and intergeneric crosses were made between species in the two genera in an attempt to create hybrids. The initial crosses produced low or no seed set.

Many interspecific and intergeneric crosses fail to set viable seed due to prezygotic hybridization barriers [4, 5]. The pollen parent may not produce viable pollen, viable pollen may fail to germinate on the stigma of the female parent, or pollen may germinate, but pollen tubes may not grow to the end of the style and into the ovary to fertilize the ovules. In this study, pollen germination and pollen tube growth was examined to assess whether the lack of seed development possibly resulted from prezygotic hybridization barriers.

Intergeneric and interspecific crosses (Table 1) were performed during January and February 2013. The number of repetitions of each cross was dependent on the number of flowers available. Some species were less floriferous in the winter, such as Dissotis princeps, Tibouchina granulosa ‘Gibraltar’, and Tibouchina lepidota. A lower number of crosses were performed using those species compared to crosses between species that flowered more profusely.
Flowers were emasculated before pollination to prevent the possibility of self-pollination. Pollen was then gathered from the male parent by striking the anthers with a tuning fork causing the pollen to dehisce into a glass container. Once collected, the pollen was applied to the stigma of the female parent. Styles were harvested 24 hours after pollination and placed in ethanol:acetic acid (1:2 w/v) for 1 to 24 hours to fix them. Once the styles were fixed, they were placed in 65% ethanol for 20 minutes to clear them, autoclaved in 0.8 mol/L NaOH at 120°C for 20 minutes and stained with 0.1% aniline blue in 0.1M K3PO4 [6, 7]. The styles were allowed to remain in the aniline blue for 4 to 24 hours. After that, they were mounted on slides and examined under a fluorescent microscope.

Results and Discussion
In every sample, pollen tubes grew to the end of the style, although not every pollen tube reached the end of the styles (Table 1) (Figures 1 and 2). Intergeneric crosses and interspecific crosses showed the same pattern. Since at least some of the pollen tubes reached the end of the style in every case, the low seed set between species and genera cannot be entirely due to poor pollen tube growth. Other factors play into low seed set of intergeneric and interspecific crosses. Incompatibility of parents often occurs, especially when the parents are distantly related, preventing fertilization after pollination [8]. Incompatibility may sometimes be overcome by treating the end of the stigma with calcium or boron, or both [9]. Pollen tubes may reach the end of the stigma and the ovum will be fertilized, only to abort before seed can mature. In these cases, embryo rescue can be attempted in order to produce viable seed [9]. However, seeds of Melastomaceae species are so small that embryo rescue was not feasible.

Since seed set and germination of seed from several crosses did occur, results indicate that hybridization between Melastomaceae is possible. Future breeding efforts may require larger populations of species in order to be able to make more crosses to compensate for low seed set.

Literature Cited


Table 1. Interspecific and intergeneric crosses in and *Tibouchina* performed in order to assess pollen tube germination.

<table>
<thead>
<tr>
<th>Female Parent</th>
<th>Male Parent</th>
<th>Repetitions</th>
<th>Styles with Pollen Tube Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. debilis</em></td>
<td><em>D. rotundifolia</em></td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td><em>D. debilis</em></td>
<td><em>T. lepidota</em></td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><em>D. princeps</em></td>
<td><em>D. rotundifolia</em></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>D. princeps</em></td>
<td>*T. granulosa ‘Gibraltar’</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><em>D. princeps</em></td>
<td><em>T. lepidota</em></td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><em>D. rotundifolia</em></td>
<td><em>D. debilis</em></td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td><em>D. rotundifolia</em></td>
<td><em>D. princeps</em></td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td><em>D. rotundifolia</em></td>
<td>*T. granulosa‘Gibraltar’</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td><em>D. rotundifolia</em></td>
<td><em>T. lepidota</em></td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>*T. granulosa‘Gibraltar’</td>
<td><em>D. princeps</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>T. lepidota</em></td>
<td><em>D. princeps</em></td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 1a. Pollen germinating on end of stigma and beginning of pollen tubes in *D. debilis x T. lepidota* cross.

Figure 1b. Pollen tubes growing to end of style in *D. debilis x T. lepidota* cross.

Figure 2a. Pollen germinating on end of stigma and beginning of pollen tubes in *D. rotundifolia x D. debilis* cross.

Figure 2b. Pollen tubes growing to end of style in *D. rotundifolia x D. debilis* cross.
Cytometric and Cytological Analyses of Cultivated Dogwoods (*Cornus* spp.)

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**Index Words:** *Cornus*, dogwoods, DNA content, flow cytometry, genome size, ploidy level, interspecific hybridization

**Significance to Industry:** Dogwoods are an important ornamental nursery crop of the southern region. According to the 2009 USDA Census of Horticultural Specialties, annual sales of dogwoods in southern states exceeded $12 million (1). While breeding, selection and cultivation of dogwoods is widespread, little is known about ploidy and genome size of dogwood cultivars and hybrids. Knowledge of ploidy and genome size is a valuable tool for breeding programs. Ploidy levels can influence fertility, crossability, segregation, and gene expression (2, 3). Relative genome size can be used to determine ploidy among closely related species when calibrated using traditional cytology (4). In this study, flow cytometry and traditional cytology were used to determine relative genome sizes and ploidy levels of 94 accessions of various species, hybrids and cultivars of dogwoods. Although most accessions were found to be diploid, selections of *C. canadensis* were tetraploid and the hybrid cultivar C. ‘KN30-8’ (Venus™) was triploid. A broad range of interspecific hybrids was also documented based on genome sizes being intermediate between their parents. These results provide new insights into the cytogenetics, reproductive biology, crossability, and systematics of dogwoods.

**Nature of Work:** The genus *Cornus* is comprised of a wide range of diverse species that includes shrubs, small trees, and herbaceous perennials (7, 8). Many species of dogwoods are used in the landscape providing four seasons of interest with attractive flowers, fruit, foliage, bark, and form (9). While considerable research has been focused on determining systematic relationships among dogwoods, little has been reported relative to genome size and ploidy (7, 8, 10).

In 2006, a species-level phylogeny (8) divided the dogwoods into several major clades including the big-bracted dogwoods (BB), the cornelian cherries (CC), and the dwarf dogwoods (DW). Cytological studies in the past have shown that the base chromosome number for BB is \( n = 11 \), for CC is \( n = 9 \) or 10, and for DW is \( n = 11 \) (8, 11, 12). For DW, the chromosome counts vary and ploidy has been reported as both diploid \( (2n = 2n = 22) \) (13 – 15) and tetraploid \( (2n = 4n = 44) \) (11, 16). There are no other known reports of polyploidy for other species of dogwoods. The objectives of this study were
to determine ploidy level and genome size of dogwood species, cultivars and hybrids representing the aforementioned clades.

**Flow Cytometry.** Flow cytometry was used to determine relative 2C genome size. Over the course of the summer season, expanding leaf tissue, vegetative buds and floral buds were collected from 94 accessions at the Mountain Horticultural Crop Research and Extension Center of North Carolina State University in Mills River, NC. Additionally, Dr. Thomas Molnar at Rutgers University provided leaf tissue and buds from the original *Cornus* ‘KN30-8’ (Venus ™). Approximately 1 cm² or 20 mg of tissue from each accession was co-chopped with a known standard in a petri dish with 400 µL of nuclei extraction buffer (Cystain ultraviolet Precise P Staining Buffer; Partec, Münster, Germany). Chopped tissue and extraction buffer was then filtered through a 50-µm nylon filter and stained using 1.6 mL 4’, 6-diamidino-2-phenylindole (DAPI) staining buffer (Cystain ultraviolet Precise P Staining Buffer; Partec). The subsample was then analyzed using a flow cytometer with fluorescence excitation provided by a mercury arc lamp (Partec PA-I; Partec). For most accessions, *Pisum sativum* ‘Ctirad’ was used (2C = 8.75 pg)(17) as an internal standard. For *Cornus eydeana* (CC), *Magnolia virginiana* ‘Jim Wilson’ (Moonglow™, 2C = 3.92 pg)(4) was used as an internal standard due to the relatively large genome size of *C. eydeana*. For each replicate, two subsamples were analyzed. 2C DNA contents were calculated as: 2C = DNA content of standard × (mean fluorescence value of sample ― mean fluorescence value of standard). Monoploid genome sizes were calculated as: 2C DNA content ÷ ploidy level.

**Cytology.** Traditional cytology was used to calibrate relative genome size to ploidy and confirm base chromosome counts. Actively growing root tips were collected in the morning prior to 10 am. Root tips were collected from seedlings or rooted stem cuttings and then suspended in a pre-fixative solution of 2mM 8-hydroxyquinoline + 0.24 mM cycloheximide in glass vials. Root tips were incubated in the dark at room temperature for 3 h. Root tips were then refrigerated at 6° C for 3 h. Following pre-fixative treatment, root tips were rinsed four times using refrigerated distilled water and placed into a fixative solution of 3 parts 95% ethanol : one part propionic acid. The following morning, root tips were rinsed four times using 70% ethanol and stored in 70% ethanol until needed. Prior to squashing, cells walls of root tips were hydrolyzed using three parts 95% ethanol: one part 12 M hydrochloric acid for approximately 3 minutes. Root tips were then placed in a staining solution of modified carbol fuschin (18) for approximately 5 minutes. The distal end of the root tip was excised under a dissecting microscope (Leica Stereozoom 6 Photo, Buffalo Grove, IL), placed on a glass slide, and gently squashed with a coverslip using a pencil eraser. Chromosomes were viewed using a light microscope (Nikon Eclipse 80i, Melville, NY). Chromosome counts were determined for *C. florida*, *C. nuttallii*, *C. capitata*, *C. hongkongensis*, *C. ‘KN30-8’*, *C. canadensis*, and *C. officinalis*.

**Data analysis.** Genome size data was subject to analysis of variance by clade, subgenus, and species/grex. Means were separated using Fisher’s least significant difference (Proc GLM; SAS Version 9.2; SAS Inst., Cary, NC).
Results and Discussion: Flow cytometry was found to be an effective and efficient tool for determining relative genome sizes and ploidy levels of *Cornus* (Table 1). There was significant variation in genome size among clades, subgenera, and species with a range from 1.07 pg to 5.16 pg (Table 1). There were also differences within clades. In the BB clade, the *Syncarpea* 1Cx values were higher than that of *Cynoxylon*; and hybrids between the two subgenera displayed an intermediate genome size. Differences in 1Cx values were also found when comparing species within subgenera. An example can be found within the subgenus *Cornus*. *C. eydeana* (5.08 pg) was much larger than *C. mas* (3.31 pg) and *C. officinalis* (3.28 pg). Additionally, within *Cynoxylon*, *C. florida* (1.58 pg) differed from *C. nuttallii* (1.71 pg). Among the *Syncarpea* species, it was noted that the evergreen species (*C. capitata, elliptica, and hongkongensis;* 2.07 to 2.27 pg) had a significantly larger 1Cx value than that of *C. kousa* (1.92 pg). The results of this study provide further support for the taxonomic groupings put forth by Xiang et al. (8) with the range of genome sizes for subgenera and clades being distinct and discontinuous.

Bai et al. (17) reported 2C value of one sample of *C. canadensis* to be 4.4 pg using propidium iodide (PI) stain. This result was close to our findings of 4.2 to 4.3 pg using the DAPI stain. In 2005, Zonneveld et al. (19) found the 2C value of *C. mas* to be 6.8 pg using PI stain. This was consistent with our findings of 6.5 to 6.7 pg (DAPI). While different fluorochrome stains will give slightly different estimates of relative genome sizes, both methods have been found to be effective and consistent for use in determining relative genome size and ploidy levels among closely related species (4). However, the DAPI stain is faster, less expensive, less toxic, and generally produces results with a lower CV for mean nuclei fluorescence.

All but one species tested proved to be diploid, confirming the findings of past cytological studies conducted on dogwoods. Only *C. canadensis* was found to be tetraploid with $2n = 4x = 44$. While this is consistent with Dermen and Löve and Löve (11, 16), this conflicts with other reports stating that only *C. unalaschkensis* is a tetraploid species (15). *C. canadensis* is a circumboreal species with a wide-ranging distribution. It is possible that this species displays a ploidy series over its geographic range. Studies of other species with wide ranging geographic distribution have shown that ploidy series is a commonly encountered phenomenon (21, 22). Still others have found that there is ploidy variation within populations (23).

Although there has been speculation that *C. officinalis* ‘Spring Glow’ might be a triploid due to very low fruit set (personal observations), this study found that its genome size was consistent with other diploids. The only triploid identified in this study was *C. ‘KN30-8’* with $2n = 3x = 33$. It has been found that hybrids may produce unreduced gametes (24). This could be the source of the unlikely polyploidy found within the BB clade.

A valuable and practical use of relative genome size in dogwood breeding was found when comparing genome sizes of hybrids to that of parent species. When genome
sizes of parent species varied considerably, hybrid progeny were shown to have intermediate genome sizes. Examples of such hybrids include: $C$. capitata $\times$ $C$. florida, $C$. hongkongensis $\times$ $C$. florida, $C$. elliptica $\times$ $C$. florida, and $C$. x rutgersensis, $C$. capitata $\times$ $C$. kousa, and $C$. kousa $\times$ $C$. elliptica.

This study provides new and pertinent information about genome sizes and ploidy levels for widely cultivated dogwoods. It was also found that the use of flow cytometry can be an effective and efficient way to confirm hybridization of BB dogwoods and contributes to the larger census of genome sizes of angiosperms.

**Acknowledgements:** This work was funded, in part, by the North Carolina Agricultural Research Service (NCARS), Raleigh, N.C., the North Carolina Biotechnology Center, Research Triangle Park, N.C., the Kenan Institute, Chapel Hill, N.C., and the North Carolina Nursery and Landscape Association, Raleigh, N.C.

**Literature Cited**


Table 1. Base, monoploid genome sizes (1Cx) for *Cornus* spp. grouped by clade, subgenus, and species/grex.

<table>
<thead>
<tr>
<th>Clade</th>
<th>1Cx (pg)</th>
<th>Subgenus</th>
<th>1Cx (pg)</th>
<th>Species/grex</th>
<th>1Cx (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornelian Cherries (CC)</td>
<td>3.88 A</td>
<td><em>Cornus</em></td>
<td>3.89 A</td>
<td><em>C. eydeana</em></td>
<td>5.08 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. mas</em></td>
<td>3.31 B</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. officinalis</em></td>
<td>3.28 B</td>
</tr>
<tr>
<td>Big Bracted Dogwoods (BB)</td>
<td>1.89 B</td>
<td><em>Syncarpea</em></td>
<td>2.00 B</td>
<td><em>C. capitata</em></td>
<td>2.27 C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. elliptica</em></td>
<td>2.14 D</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. hongkongensis</em></td>
<td>2.07 DE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. kousa</em></td>
<td>1.92 HI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. capitata</em> x</td>
<td>2.11 D</td>
</tr>
<tr>
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<td></td>
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<td><em>C. kousa</em></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. hongkongensis</em> x</td>
<td>2.03 EF</td>
</tr>
<tr>
<td></td>
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<td></td>
<td><em>C. kousa</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. hongkongensis</em> x</td>
<td>2.08 DE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. kousa, F2</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. kousa</em> x</td>
<td>2.01 EFG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. elliptica</em></td>
<td></td>
</tr>
<tr>
<td>Syncarpea x Cynoxylon</td>
<td>1.83 BC</td>
<td></td>
<td></td>
<td><em>C. capitata</em> x</td>
<td>1.98 FGH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. florida</em></td>
<td>1.86 I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. hongkongensis</em> x</td>
<td>1.94 GH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. florida</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. elliptica</em> x</td>
<td>1.73 J</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. rutgersensis</em></td>
<td></td>
</tr>
<tr>
<td>Cynoxylon</td>
<td>1.60 C</td>
<td></td>
<td></td>
<td><em>C. florida</em></td>
<td>1.58 K</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>C. nuttallii</em></td>
<td>1.71 J</td>
</tr>
<tr>
<td>Dwarf Dogwoods (DW)</td>
<td>1.07 C</td>
<td><em>Arctocrania</em></td>
<td>1.07 D</td>
<td><em>C. canadensis</em></td>
<td>1.07 L</td>
</tr>
</tbody>
</table>

1Values followed by different letters within a column are significantly different, LSD, $P \leq 0.05$. 

1Values followed by different letters within a column are significantly different, LSD, $P \leq 0.05$. 

---

Plant Breeding and Evaluation Section
Table 2. List cultivars that were evaluated.

<table>
<thead>
<tr>
<th><strong>Cornus florida</strong></th>
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<td>‘Appalachian Spring’</td>
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<tr>
<td>‘Dixie Colonnade’</td>
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<tr>
<td>‘Eternal Dogwood’</td>
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<td>‘Little Princess’</td>
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<tr>
<td>‘Rutnam’ (Red Beauty ®)</td>
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<td>‘Spartanburg’</td>
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<td>‘World’s Fair’</td>
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<td>‘Girard’s Nana’</td>
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<td>‘Greensleeves’</td>
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<td>‘Little Beauty’</td>
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<td>‘Lustgarten Weeping’</td>
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<td>‘Madison’ (Crown Jewel ™)</td>
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<td>‘Radiant Rose’</td>
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<td>‘Rochester’</td>
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<td>‘Snowbird’</td>
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<td>‘Speciosa’</td>
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<td>‘Spinners’</td>
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<td>‘Square Dance’</td>
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<td>‘Temple Jewel’</td>
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<td>‘Wolf Eyes’</td>
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<td>‘Kintoki’</td>
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<td>‘Spring Glow’</td>
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Unfolding the Gene Flow of Powdery Mildew Resistance in *Cornus florida* and Determination of Markers Associated with Resistance/Susceptibility

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**Index Words:** Powdery mildew, Disease resistance, Simple sequence repeat (SSR) Markers, *Cornus florida*.

**Significance of work:** Powdery mildew caused by *Erisyphe pulchra* (Microsphaera pulchra) and *Phyllactinia guttata*, is the most common disease problem causing economic losses in dogwood production. Planting resistant varieties would be best way to control the disease, but only a few cultivars are resistant. Breeding for disease resistance in tree crops take a long time; combining traditional breeding methods with molecular techniques can speed up the process. Information on inheritance of disease resistance along with markers associated with powdery mildew resistance will benefit breeding efforts. Simple sequence repeats (SSRs) in dogwood DNA sequence provide markers of choice due to their ability to be inherited from resistant to susceptible plants and extremely reproducible results. Additionally, identifying SSR markers associated with resistance to powdery mildew in dogwoods would help in Marker assisted breeding (MAB).

**Nature of work:** Flowering dogwoods (*Cornus florida*) is an economically important ornamental tree with 30 million dollars in total US sales; 23.2% of the total US dogwood supplies is from Tennessee. Powdery mildew caused by *Erisyphe pulchra* is the most important disease that limits nursery production of flowering dogwood as compared to 30 years ago. It reduces plant growth, thereby increasing the time needed for infected trees to reach prime size for sale; it also reduces the aesthetic appeal and market value of plants (2, 3, 8).

Effective fungicides for powdery mildew control have been identified, but routine applications are required season-long and this has increased production costs and forced small growers out of business. Powdery mildew resistance is the best method for controlling this disease; it will reduce production costs and is also environmentally friendly. While efforts to breed for powdery mildew resistance are being taken, only a few cultivars have powdery mildew resistance; understanding the pattern of inheritance of powdery mildew resistance from parents to progeny is of utmost importance to facilitate breeding strategies. This study was carried out at Tennessee State University, Otis L. Floyd Nursery Research Center, McMinnville, TN to unfold the inheritance of powdery mildew resistance/susceptibility.
Controlled hand pollinations were made between susceptible cultivars *C. florida* 'Cherokee Princess' and resistant selections R14, MI9 using on an average, 8 inflorescence in sets of 50. The inflorescences were covered with breathable plastic bags prior to opening to eliminate any unknown pollen contaminations. Since dogwood is self-sterile (self-incompatible), flower emasculation was not needed. Reciprocal crosses were between R14 x Cherokee Princess; Cherokee Princess x R14; MI9 x Cherokee princess and Cherokee princess x MI9. Freshly collected pollens from newly dehisced anthers of male parent were rubbed over the stigma of female parents. The newly dehisced anthers held with fine tipped forceps were used for pollination and placed onto exposed stigma of the female parent. Due to the self-sterile condition of flowering dogwood (4), inflorescence of male parent was also rubbed directly onto flowers of female parent. Seeds of the progenies were harvested and vernalized for germination and seedlings were grown in greenhouse environment, exposed to powdery mildew infection. Seedlings were then rated for disease severity using a 0-5 scale where, 0 = no disease, 1 = 1 ≤ 2%, 2 = 2 ≤ 10%, 3 = 3 ≤ 25%, 4 = 4 ≤ 50%, 5 = 5 ≤ 75% (2).

Previous studies on powdery mildew in flowering dogwoods focused mainly on breeding or selecting new varieties with disease or pest resistance (2, 8, 9, 10). However, conventional breeding for resistance may require a long time because of their long generation time. Molecular markers offer promising tools for dogwood improvement via breeding; characterization of plants at genetic level can be done using nucleic acid diagnostic tools (1, 5, 6). Molecular markers will help facilitate the breeding process in achieving powdery mildew host resistance.

Genomic DNA was isolated from the parents and the progeny populations from the controlled crosses. Genomic DNA extraction from young leaves or apical buds of the plant material was carried out using DNeasy plant mini kit protocols. The DNA extracted was visualized on 3% agarose gel, and quantified using Nanodrop (ND-1000 (NanoDrop technologies, Inc, Wilmington, DE, USA). PCR amplification was performed to amplify the microsatellite regions on the genome of parents and progenies from the controlled crosses to determine the polymorphic markers that may be linked to resistance. PCR amplification consisted of 25µl reaction containing 0.4-2.0 ng of total plant genomic DNA and 12.5 µl of Promega PCR master mix (Promega Corporation, Madison, WI, USA). Amplification profile was 94°C for 5 min, followed by 35 cycles of 94°C for 40 seconds, 50-60°C for 40 seconds, 72°C for 30s, and final extension at 72°C for 4 min. PCR products were visualized and separated by Native Gel electrophoresis using Thermoscientific QWL B3, Gel electrophoresis Chamber (Porthmouth, NH, USA) (7). Gel was prepared using a 3g of certified Agarose from BIO-RAD (Hercules, CA) in 100ml of 1X TAE Buffer (3% gel). The Samples were loaded with the 6X loading dye and a 100 bp marker loaded in one of the wells. The gel was allowed to run for 3 hours at 100V and was imaged and observed under Gel logic 200 Imaging System (Rochester, NY, USA) (7).
Results and discussion: The controlled crosses of resistant selections R14 and MI9 with Susceptible cultivar ‘Cherokee Princess’ produced 155 and 97 progeny seedlings respectively. A segregation pattern was identified for each controlled cross. The progeny of both R14 X CP and MI9 X CP seedlings segregation showed that susceptibility was dominant and resistance was recessive. Results also showed that powdery mildew susceptibility inherited from parents to progeny in R14 X CP seemed to be by duplicate genes and additive genes (Table 1). However, in addition to duplicate genes and additive genes inherited from parents to progeny in MI9 X CP crosses, powdery mildew susceptibility seemed to be by dominant epistasis (Table 2).

SSR markers were the best choice to identify polymorphisms between susceptible and resistant progenies of *C. florida* (7). Markers set 1, 4, 8 and 12 showed significant polymorphism for the susceptible and resistant parents CP, R14 and MI9 (Figure 1). However, when markers were used to analyse the polymorphism from the progeny population for the susceptibility, Marker 8 exclusively showed significant difference in the amplification at all levels of disease severity that could be associated to susceptibility (Fig 2). The results look promising to help eliminate susceptibility at genetic level.

Literature Cited


**Table 1:** Inheritance of powdery mildew disease resistance and susceptibility in R14 X CP progeny seedlings.

<table>
<thead>
<tr>
<th>Segregation Model</th>
<th>Mechanism</th>
<th>Disease severity</th>
<th>Disease Scale&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Observed (O)</th>
<th>Expected (E)</th>
<th>(O-E)&lt;sup&gt;2/E&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>15:1</td>
<td>Duplicate genes</td>
<td>Susceptible</td>
<td>&gt;1.5</td>
<td>145</td>
<td>145.3</td>
<td>0.00061</td>
</tr>
<tr>
<td></td>
<td>One dominant Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>from either of two genes is needed for a phenotype</td>
<td>Resistant</td>
<td>≤1.5</td>
<td>10</td>
<td>9.7</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Statistical Significance at 5% : α = 3.841 for df = 1

\[ x^2 = 0.009 \]

<table>
<thead>
<tr>
<th>Segregation Model</th>
<th>Mechanism</th>
<th>Disease severity</th>
<th>Disease Scale&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Observed (O)</th>
<th>Expected (E)</th>
<th>(O-E)&lt;sup&gt;2/E&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>9:3:3:1</td>
<td>Additive Genes Each genotype results in unique phenotype</td>
<td>Susceptible</td>
<td>&gt;3</td>
<td>81</td>
<td>86.4</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moderately</td>
<td>3</td>
<td>27</td>
<td>29.06</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Susceptible</td>
<td>2-2.5</td>
<td>37</td>
<td>29.06</td>
<td>2.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moderately</td>
<td>≤1.5</td>
<td>10</td>
<td>9.6</td>
<td>0.016</td>
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</table>

Statistical Significance at 5% : α = 7.815 for df= 3

\[ x^2 = 2.67 \]

<sup>a</sup>0-5 scale where, 0 = no disease, 1 = 1 ≤ 2%, 2 = 2 ≤ 10%, 3 = 3 ≤ 25%, 4 = 4 ≤ 50%, 5 = 5 ≤ 75% foliage covered with symptoms.
**Table 2:** Inheritance of powdery mildew disease resistance and susceptibility in MI9 X CP progeny seedlings.

<table>
<thead>
<tr>
<th>Segregation Model</th>
<th>Mechanism</th>
<th>Disease severity</th>
<th>Disease Scale&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Observed (O)</th>
<th>Expected (E)</th>
<th>(O-E)&lt;sup&gt;2&lt;/sup&gt;/E</th>
</tr>
</thead>
<tbody>
<tr>
<td>15:1</td>
<td>Duplicate genes One dominant Allele from either of two genes is needed for a phenotype</td>
<td>Susceptible</td>
<td>≥2.5</td>
<td>93</td>
<td>91</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resistant</td>
<td>&lt;2.5</td>
<td>4</td>
<td>6.06</td>
<td>0.699</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:3:1</td>
<td>Dominant Epistasis Dominant allele on one locus masks the expression of Second locus</td>
<td>Susceptible</td>
<td>≥3.5</td>
<td>72</td>
<td>72.75</td>
<td>0.0076</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moderately Susceptible</td>
<td>2.5-3</td>
<td>21</td>
<td>18.18</td>
<td>0.4372</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resistant</td>
<td>≤2.25</td>
<td>4</td>
<td>6.06</td>
<td>0.701</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9:3:3:1</td>
<td>Additive Genes Each genotype results in unique phenotype</td>
<td>Susceptible</td>
<td>≥3.75</td>
<td>47</td>
<td>54.56</td>
<td>1.047</td>
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<td>Moderately Susceptible</td>
<td>3.5</td>
<td>25</td>
<td>18.18</td>
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<td>Moderately Resistant</td>
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<td>21</td>
<td>18.18</td>
<td>0.556</td>
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<td></td>
<td>Resistant</td>
<td>≤2.25</td>
<td>4</td>
<td>6.06</td>
<td>0.701</td>
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</table>

Statistical Significance at 5%: \( \alpha = 3.841 \) for df = 1 \( \chi^2 = 1.13 \)

Statistical Significance (5%): \( \alpha = 5.99 \) for df = 2 \( \chi^2 = 1.14 \)

Statistical Significance (5%): \( \alpha = 7.815 \) for df = 3 \( \chi^2 = 5.13 \)

<sup>a</sup>0-5 scale where, 0 = no disease, 1 = 1 ≤ 2%, 2 = 2 ≤ 10%, 3 = 3 ≤ 25%, 4 = 4 ≤ 50%, 5 = 5 ≤ 75% foliage covered with symptoms
**Figure 1**: Polymorphisms of twelve SSR primer markers in flowering dogwood parents ‘Cherokee Princess’ (CP), and R14 and MI9 selections.
a0-5 disease scale where, 0 = no disease, 1 = 1 ≤ 2%, 2 = 2 ≤ 10%, 3 = 3 ≤ 25%, 4 = 4 ≤ 50%, 5 = 5 ≤ 75% foliage covered with symptoms. M = 100bp ladder.

Figure 2: Polymorphic SSR markers in progeny of R14 X CP cross (a, b), and progeny of MI9 X CP cross (c, d) showing different disease severity ratingsa
Taxodium Research at SFA Gardens

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Index words: Taxodium, hybrid, salinity, alkalinity, nitrogen, propagation, pruning

Significance to Industry: While bald cypress enjoys a modest market share in big tree sales in the U.S., it cannot be considered a huge nursery item. More suited to large landscape settings – public parks, commercial endeavors, and roadsides - there is little market for trees that become giants in only a few decades. Slow housing starts since the 2008 recession have further reduced demand. That said, there remains a large potential market not just in those arenas, but also in coastal windbreak forest projects along the Gulf of Mexico from Texas to Florida and northward into North Carolina (11). Salt tolerant and naturally tolerant of tropical storms, this ancient species enjoys remarkable genetic diversity that allows adaptation to a number of diverse and challenging habitats (9).

Nature of Work: Location: SFA Gardens comprises 52 ha (128 acres) of on-campus property at Stephen F. Austin State University (SFA), Nacogdoches, Texas and includes four main gardens. Representing the oldest plantings, the 17 ha (10 acre) SFA Mast Arboretum was initiated in 1985 and includes the horticulture facility of the Agriculture Department. Second, the Ruby M. Mize Azalea garden is a 3.2 ha (8 acre) garden of primarily azaleas, camellias and Japanese maples and was dedicated in April, 2000. Third, the 17 ha (42 acre) Pineywoods Native Plant Center (PNPC) was dedicated by Lady Bird Johnson in April 2000. Finally, the SFA’s Recreational Trail and Gardens was dedicated in March 2010 and comprises 27.5 ha (68 acres) of mostly undisturbed forest. As the result of a donor with a vision, SFA Gardens is responsible for the development of a 3.2 ha (8 acre) garden in the SW portion of this property, directly across the from the Ruby M. Mize Azalea Garden. SFA Gardens enjoys four full time employees and two half-time employees, all funded by a combination of state and external grant funding. SFA Gardens is a collector’s garden, one that adds hundreds of new plants each year to the plantings. Those that survive, perform well, and impress visitors make their way into propagation and distribution. Some of those plants make their way into the landscape trade. This program has introduced and promoted numerous plants through a wide range of print and electronic media. Many have been documented in past IPPS Proceedings (2, 3, 4).

Climate: Nacogdoches is in Zone 8B of the Pineywoods region East Texas with an average annual rainfall of 1219 mm. (48 in.) June through August is characteristically
hot and dry. In 2010 and 2011, Nacogdoches experienced all-time record drought and heat. In recorded history, 1 Sept. 2000 was the record high, 44.4 °C (112 °F), and 23 Dec. 1989 was the record low -17.8 °C (0 °F). In 2005 and 2008, Nacogdoches was damaged by hurricanes with winds in excess of 139 km/hr (100 mph) that toppled many large trees in our region.

**Taxonomy:** *Taxodium* is in the cypress family, Cupressaceae, one of several ancient genera in the family commonly known as cypresses. Once three separate species under the genus *Taxodium*, we are currently accepting *Taxodium* as one species with three botanical varieties (1). For the purpose of brevity, the three genotypes will be referred to in this article as BC, PC, and MC.

*Taxodium distichum* (L.) Rich.var.* *distichum* (Baldcypress)
*Taxodium distichum* var. *imbricarium* (Nutt.) Croom (Pondcypress)
*Taxodium distichum* var. *mexicana* Gordon (Montezuma cypress)

**Taxodium Collection:** SFA Gardens has collected Taxodium varieties and genotypes since the 1980s. The collection includes single representatives or multiples of over 74 varieties or selections representing the diversity in BC, PC, and MC. SFA Gardens Taxodium bulletins, research reports and updates can be retrieved at the SFA Gardens Webpage (10). The collection database and maps are available online as an Excel file (10). In addition to a wide range of cultivars, the collection includes many seedlings of known provenance. A 2006 genotype planting representing 36 provenances is still underway (8). In the general collection, South Texas and Mexico MC are well represented. In addition, SFA Gardens is evaluating a dozen advanced selections from China. The author has been collaborating with colleagues in the Taxodium Improvement Program at Nanjing Botanical Garden (NBG) since 2001. Directed by Dr. Yin Yunlong, the NBG Taxodium breeding program involves controlled crosses utilizing superior parents, evaluation of seedlings under high salt and high alkalinity conditions, and selection of superior forms. These are cutting propagated, multiplied and distributed into test plantings. The market for Taxodium in China is quite large, encompassing millions of trees, and they are planted in a wide range of provinces from the Yunnan in the southwest to Shandong in the northeast. The NBG breeding program has introduced and registered a number of superior clones. Many are under evaluation at SFA Gardens and at cooperator sites. The first introduction, T302, a BC X MC selection, was given the name 'Nanjing Beauty' by SFA Gardens and NBG and introduced in 2004. It is fast growing, alkaline tolerant, produces no knees, and, in this author’s opinion, has less than acceptable form. Unless pruned and trained properly the first few years, ‘Nanjing Beauty’ becomes MC-like, more prone to dominant side branches and multiple leaders. However, a second generation of introductions (T405, T406, T407, and T502) by NBG is reported to have superior salt tolerance, growth rate and habit (13). These four, and others, are under evaluation at SFA Gardens and in a number of southern USA locations. Most are BC X MC crosses, and they are best characterized as fast growing, salt and alkaline tolerant, of superior form and branching, and they do not produce knees. SFA Gardens has also acquired and is evaluating
‘Dongfangshan’, the purported Cryptomeria X Taxodium hybrid of Shanghai. We have concluded this clone is not a hybrid but certainly an interesting form, perhaps of MC (7).

Salt Tolerance Research: Lijing Zhou’s MS and PhD work at SFA included a series of salinity studies utilizing container grown Taxodium plants (14, 15, 16, 17). A number of general conclusions can be made. Taxodium demonstrates great tolerance for exposure to water with high sea salt concentrations, as long as exposure times are limited and high salt events are followed by leaching within a reasonable amount of time (days). Concentrations as high as 36 parts per thousand (ppt) with twice weekly applications were tolerated in a sixteen week study, when salt treatment exposure was followed by fresh water in 24 to 48 hours. In a total submergence study, MC and the hybrid were slower to demonstrate salt burn symptomology than BC and leaf Na values were lower in MC and the hybrid. In all submergence chronic treatments, irrigation salt concentrations higher than 3 ppt resulted in browning and growth reduction in BC, MC, and hybrid. BC and the hybrid was also more submergence tolerant than MC.

N Source/N rate Study: In a 2012 field study involving 48 Taxodium X ‘T405‘ plants, two sources of Nitrogen (CaNO\textsubscript{3} and Urea) were used at four rates to evaluate the effects on plant growth, as well as determine the preference for a nitrate or ammoniacal form of Nitrogen (12). In the first year, growth was excellent averaging 1.3 m. in height increase. This study is being continued in the current year (2013) and initial results indicate no significant growth or leaf nutrient differences in response to N source or rate at this site. Our initial conclusion is that in this bottomland site, while N levels are classified as Very Low, VL, there is sufficient N available for optimal Taxodium growth. In sandy, droughty locations, the results may have been different. Provided plants are well irrigated, Taxodium efficiently exploits available soil N.

Propagation studies: Four cutting propagation studies were reported in the MS thesis of Lijing Zhou (14). Results indicate that June cuttings subjected to a five second dip of K-IBA at 7500 PPM and slight wounding increased rooting percentages (up to 88%, depending on treatment). More important perhaps is the vigor of the cutting wood. Thin twiggy new growth rooted poorly. Cuttings taken from trees cut back heavily in the winter produce a greater number of pencil-thick cuttings in June and July that root at higher percentages. In one unpublished 2011 study, hardwood cuttings of Taxodium X ‘T406‘ taken in late January rooted in good numbers (up to 88% depending on hormone treatment) and were ready for potting in 12-16 weeks. Cuttings from juvenile trees (chronological age) root easier than cuttings taken from mature trees.

Pruning study: In a 2013 field study, Taxodium X ‘T406‘ were pruned to three forms in February 2013: 1) unpruned, 2) side branches cut back to create a Christmas tree form, and 3) side branches cut back all the way to the trunk. The impetus for this study was derived from the common practice in China to drastically cut back large ball and burlap trees to the central trunk prior to planting. Chinese horticulturists suggest that this strategy produces “a straighter tree” and one “more likely to survive the first year of establishment”, particularly in plantings without irrigation. Growth measurements in this study will include starting and ending plant heights, trunk diameter, and tree width.
Results and Discussion  *Taxodium* are recognized world-wide as durable long-lived landscape trees. The species finds merit in large scale plantings, including coastal windbreak forests, large parks and public landscapes, and along roadsides. Most *Taxodium* sold in the USA are seedlings. While there are many interesting varieties and forms, they are commonly grafted, somewhat difficult to find, and occupy only a small part of the total market share. Chinese scientists recognized the great diversity in the species and through controlled breeding and cutting propagation they are exploiting the genetic diversity in *Taxodium* to find trees suited to specific habitats and landscape uses. The Taxodium collection at SFA Gardens is a unique genetic resource for this special native of the South.

Literature Cited


