Plant Breeding and Evaluation

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Section Editor

Induction of Tetraploid *Hibiscus moscheutos* through Soaking of Seedlings

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Significance to Industry As a widely favored ornamental plant, hibiscus has been bred for a series of cultivars on the market. Varieties with compact form and reduced fertility would be popular for gardens and private yards for their convenience in landscape design and reduced production of fruits. Polyploidization could yield plants with reduced height and fertility [2]. Inducing polyploids was attempted to enhance plant compactness and potential morphological characteristics of native hardy hibiscus and to simultaneously redistribute the energy from producing seeds to constant blooming.

Nature of Work *Hibiscus moscheutos* (2n=2x=38) is an herbaceous plant native to wetland areas in the eastern United States [3, 6]. It has long been bred for its large and showy flowers during summertime, but in recent years interest has been raised for its compactness and reduced fertility for the purpose of cultivar improvement. We attempted to further improve morphological characteristics of hibiscus through polyploidy induction, with the possibility that tetraploids would be superior to the original diploids [1, 5]. In this research project, we seek to determine an optimal method to induce tetraploid hardy hibiscus via soaking germinated seedlings in mitosis-inhibiting chemicals.

A popular inbred cultivar *Hibiscus moscheutos* cultivar 'Luna Red' was used in this experiment. Pre-scarified seeds geminated, and were ready for treatment after reaching 'cotyledon stage' [2] when cotyledons were fully expanded and the first true leaf was not fully formed.

The experiment was designed in a three-way factorial linear model with two doubling agents, three chemical concentrations, and three incubation periods. Colchicine and Surflan® (oryzalin 40.4%) suspension were used to treat the seedlings. A control group was added with a water soaking treatment. After chemical soaking, seedlings were rinsed thoroughly under water to remove any residue.

Seedlings were immediately relocated to the greenhouse and sown in Pro-Mix® potting soil in flats. Divided by per replicate group, containers of seedlings were placed in a randomized complete block design.

Seedling survival was evaluated after eight weeks of growth. A ploidy level analysis took place after treated seedlings started developing regular leaves but before setting flower buds. Ploidy levels were analyzed via flow cytometry. By comparing putative plants with a known diploid control plant, diploid, mixaploid or tetraploid plants were determined.

Under greenhouse and field conditions, diploid and tetraploid plants were evaluated and compared on morphological characteristics such as flower diameter, flower redness, leaf thickness and leaf stomata size.

Results and Discussion Growth of treated seedlings was expected to stagnate during early growing stages [4]. When seedlings were planted in the greenhouse after treatment, plants in the control group started shoot and root growth quickly while treated plants remained stunted. After 4-6 weeks, some seedlings started regenerating shoots while some seedlings started showing signs of necrosis. Colchicine-treated seedlings died back after moderate growth due to a rupture at the base of stems from colchicine shock, while oryzalin treated seedlings showed no growth and gradually became necrotic (Figure 1).

Both colchicine and oryzalin proved effective at inducing tetraploids from diploid seedlings, considering the overall efficiency combing survival rate and tetraploid conversion performances (Table 1, Table 2). Colchicine contributed higher survival percentage than oryzalin, and it also induced a higher percentage of tetraploids (Table 1, Table 2). Colchicine was most effective at 0.025% colchicine solution soaking for 24 h, producing 35.5% tetraploids with a survival rate of 48.8% (Table 1). Though some other treatment groups yielded higher rates of survival, our ultimate goal was to maximize the probability of obtaining surviving tetraploids.

In oryzalin treatments, both concentration ($p<0.01$) and treatment period ($p<0.03$) were significant with an interaction effect ($p<0.03$) (Table 2). Seedlings treated for longer periods of time had a tetraploid conversion percentage lower than 1%, along with low rates of survival. Seedlings treated for six hours yielded tetraploids in a range between 24.8% and 33.4% (Table 2).

On each plant identified as a tetraploid, ten samples were taken for assessment of morphological characteristics and comparison to diploid plants. Results of a two-sample t-test indicated tetraploid leaves were significantly thicker than diploids, with an increase of 24.8% (Table 3). Tetraploid leaves also have an almost leathery texture while diploid leaves are much finer. Tetraploid plants had larger leaf stomata size ($p=0.01$), while there was no significant difference with diploid plants for flower diameter and flower anthocyanin content (Table 3).

Overall, tetraploid hardy hibiscus were successfully obtained through soaking germinated seedlings in colchicine and oryzalin. Exposure to low concentrations of colchicine solution for a longer time or to a medium concentration of oryzalin for a short period were considered to be efficient in yielding a high number of tetraploids with a low rate of mortality. The study indicated an increase on leaf thickness and stomata size between tetraploid and diploid hardy hibiscus, and irrelevance of ploidy level on flower diameter and redness. More foliage, floral and fruit data will be recorded this summer for a full estimation of tetraploid hibiscus performance.

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Figure 1. Representative dying plants after treatment with chemicals. Two seedlings on the left were colchicine treated with swelling stems at the base, and three seedlings on the right were oryzalin treated with wilting roots.

Table 1. (Survival rate, tetraploid conversion rate) of colchicine treated plants.

Conc. \ Time	6 hours	12 hours	24 hours
0.025%	(70.9%, 30.4%)	(64.0%, 32.1%)	(48.8%, 35.5%)
0.050%	(65.7%, 27.2%)	(58.7%, 28.6%)	(43.8%, 31.5%)
0.100%	(54.4%, 21.5%)	(47.5%, 22.5%)	(34.3%, 24.4%)

Table 2. (Survival rate, tetraploid conversion rate) of oryzalin treated plants.

Conc. \ Time	6 hours	12 hours	24 hours
100 uM	(52.2%, 33.4%)	(7.9%, 1.0%)	(2.4%, 0.3%)
125 uM	(59.2%, 30.4%)	(10.2%, 0.8%)	(3.1%, 0.3%)
150 uM	(48.6%, 24.8%)	(6.9%, 0.2%)	(2.0%, 0.4%)

Table 3. Comparison of morphological characteristics between tetraploid and diploid hardy hibiscus plants.

	Tetraploid Increase or Decrease	Significance
Leaf Thickness	+ 24.8%	***
Leaf Stomata Size	+ 30.9%	*
Flower Diameter	- 3.9%	NS
Flower Anthocyanin Content Index	+ 5.6%	NS

Production of Polyploid *Hydrangea macrophylla* via Unreduced Gametes

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Significance to Industry *Hydrangea macrophylla* is one of the most economically important nursery crops in the U.S, with sales of *Hydrangea* species topping $120,000,000 in 2014[1]. *Hydrangea* varieties with resistance to pests, disease, and drought would significantly reduce the environmental and budgetary footprint associated with *Hydrangea* production. In an effort to improve these traits, we have produced diploid, triploid, and a putative tetraploid *H*. *macrophylla* from controlled crossed of diploid parents. Triploid *H. macrophylla* plants were significantly more compact with thicker stems, darker foliage, and larger leaves than diploid plants. We established a link between ploidy and stomate size, showing that triploids have significantly fewer, larger stomata than diploids or tetraploids while the putative tetraploid has more, smaller stomata per leaf area. As in other species, changes in plant form and stomatal density may lead to increased disease and drought resistance in *Hydrangea*. These results have wide-ranging impacts on *Hydrangea* breeding improvement such as the ability to create a "bridge" for breeding cultivars and species of differing ploidy levels, and the ability to change the ploidy level of popular cultivars. Superior plants from this population will be released as cultivars that will provide nursery growers an effective way to reduce time, cost, and environmental impact of production.

Nature of Work Polyploidy – also known as whole genome duplication – has long been associated with changes to ornamental traits in plants. Increasing the number of chromosome sets in plant cells often leads to thicker stems and leaves, a deeper green foliage color, wider leaves, larger and more textured flowers, a longer flowering period, more compact growth habit, and increased resistance to diseases and environmental stress[2,3]. Some effects of changing ploidy level from two sets of chromosomes (i.e., diploid or 2n) to four sets of chromosomes (tetraploid or 4n) in woody ornamental species include darker green, thicker leaves and increased number of petals per flower in *Rosa*[4], thicker leaves and larger, more persistent flowers in *Magnolia*[5], and compact size in *Buddleia*[6].

Pollen and egg cells of diploid plants normally contain a single set of chromosomes that unite at fertilization to restore the 2n chromosome number. Problems during meiosis, however, may lead to some pollen or egg cells that contain no chromosomes and some that contain two sets of chromosomes[7]. Pollen or egg cells that contain a double set of

chromosomes are termed “unreduced gametes”. The presence of large pollen grains is often used as an indicator of unreduced gamete formation, and offspring of various ploidy levels may be recovered when one parent in a controlled cross produces a substantial proportion of unreduced gametes[7]. For example, a large, unreduced pollen cell (2n) and a normal egg cell (n) may combine to produce a triploid (3n) offspring. An examination of the frequency distribution of pollen grain size in *H. macrophylla* revealed a single cultivar, ‘Trophee’, with a significant portion of large pollen grains[8].

All *H. macrophylla* cultivars tested to date are diploid (2n=2x=36) or triploid (2n=3x=54)[8, 9, 10, 11]. Several desirable traits seem to be associated with triploidy in *H. macrophylla* including dark green foliage, strong stems and sturdy inflorescences[8]. However, the influence of ploidy level on these traits is difficult to determine due to genetic differences between cultivars, and there are no reports on the influence of ploidy on ornamental traits in closely related individuals. In order to produce ploidy variation in a closely related group of plants, we used the diploid *H. macrophylla* cultivar ‘Trophee’ as a parent in a series of crosses with other diploid *H. macrophylla* cultivars. The objective of this study was to evaluate reciprocal full-sibling *H. macrophylla* families for ploidy and ornamental traits, determine the impact of ploidy on ornamental traits, and determine the efficacy of unreduced gamete breeding for producing genomic and phenotypic variation. Results will determine the effectiveness of introducing polyploidy into other favorable *Hydrangea* species and cultivars and provide germplasm for further species improvement.

Controlled pollinations in 2010 produced a series of five reciprocal *Hydrangea macrophylla* full-sibling families. The *H. macrophylla* varieties ‘Princess Juliana’, ‘Trophee’, and ‘Zaunkoenig’ were used in the following crosses: ‘Princess Juliana’ x ‘Trophee’, ‘Trophee’ x ‘Princess Juliana’, ‘Princess Juliana’ x ‘Zaunkoenig’ ‘Zaunkoenig’ x ‘Princess Juliana’, and ‘Trophee’ x ‘Zaunkoenig’ (Table 1). Plants were grown in 3 gallon containers under 60% shade and micro-irrigated using spray stakes. Growing media consisted of pine bark amended with 6.6 $kg \cdot m^{-3}$ 19N-2.1P-7.4K Osmocote Pro fertilizer (Scotts-Sierra Horticultural Products Co., Maryville, Ohio), 0.6 $kg \cdot m^{-3}$ Micromax (Scotts-Sierra Horticultural Products Co.), 0.6 $kg \cdot m^{-3}$ iron sulfate, and 0.2 $kg \cdot m^{-3}$ Epsom salts.

Flow cytometry

Ploidy levels were determined using young, fully expanded leaves from five *H. macrophylla* full-sibling families (n = 112 plants). Approximately 0.5 cm^2 of growing leaf tissue of sample and standard were chopped for 30 to 60 s in a plastic petri dish containing 0.4 mL extraction buffer (Partec CyStain ultraviolet precise P Nuclei Extraction Buffer; Partec GMBH Muenster, Germany). The resulting extract was passed through a 30-mL filter into a 3.5-mL plastic tube to which was added 1.6 mL Partec CyStain ultraviolet precise P Staining Buffer containing the fluorochrome DAPI. The relative fluorescence of the total DNA was measured for each nucleus using a Partec PA-1 ploidy analyzer (Partec GMBH, Muenster, Germany). Results were displayed as histograms showing the number of nuclei grouped in peaks of relative fluorescence intensity. For each sample, at least 3000 nuclei were analyzed revealing a single peak with a coefficient of variation (CV) less than 4.9%. The peak of fluorescence intensity for the diploid parents

was set to 50; thus, peaks of fluorescence intensity representing nuclei from full-sibling offspring were expected at 50 (diploid) or 75 (triploid). Genome sizes were calculated as nuclear DNA content for unreduced tissue (2C) as: 2C DNA content of tissue = (mean fluorescence value of sample ÷ mean fluorescence value of standard) × 2C DNA content of standard. *Pisum sativum* L. 'Citrad' with a 2C content of 9.09 pg was used as the internal standard[12]. Ploidy and genome sizes are the averages of two subsamples per plant.

Stomata counts

The top-most fully expanded healthy leaf from each plant in three full-sibling *H. macrophylla* families (n = 85 plants) was collected between September 14 and 18, 2015. All leaves sampled were mature leaves that were average in size for the plant. Clear nail polish was applied to three areas on the abaxial side of a freshly sampled leaf and allowed to dry completely. A 5 ½ - 6 cm piece of transparent tape was firmly pressed over each area of dried polish and peeled off gently. The tape with affixed peels was applied to a previously labeled microscope slide, sticky side down, as flat as possible to avoid bubbling which can cause visualization distortion. Stomata were visualized at 20x magnification with an Olympus BX50 compound microscope (Olympus Corp., Tokyo, Japan) with an Olympus Q Color 5 digital camera for image capture. Three peels per plant were evaluated. Number of stomata were counted in one field of view (5,940.75 μm^2) per peel (n = 3 counts per plant). Length and width of 6 stomata were measured per peel (n = 18 stomata per plant). Stomatal area was calculated using the formula for the area of an ellipse: half-length x half-width x π. The stomatal measurements and counts were made using Q-Capture Pro 7 software (Q Imaging, 2010).

Phenotypic data collection

At the time of the study, three of the full-sibling families (n = 85 plants) were of sufficient age to flower reliably. The following variables were used to describe plant inflorescence and size: Number of inflorescences, inflorescence width (mean of three on each plant), sepal width (mean of three on each of three inflorescences), height, and stem width. All variables were measured on 6/8/2015 and 6/14/2016. Data analysis was performed using SAS® software, Version 9.4 of the SAS system for Microsoft (Copyright © 2013, SAS Institute Inc., Cary, NC, USA). The general linear model procedure (PROC GLM) was used to partition variance in inflorescence, stomata, and plant size means into sources attributable to year, family, ploidy level, and environment (error). Means for each ploidy level were compared using Tukey's studentized range test with an $\alpha = 0.05$ significance level.

Results and Discussion

Flow cytometry

Flow cytometric analysis was performed on five related full-sibling *Hydrangea macrophylla* families (n = 112 plants). The three parents used to produce the full-sibling families were diploid, with an average peak center of 50.9± 0.8 and an average genome size of 4.55 pg (Table 1). Three of five full-sibling families, including those with 'Trophee' as the female parent, consisted of all diploid offspring as expected. Diploid offspring had an average

peak center of 50.7 ± 1.2 and an average genome size of 4.54 ± 0.05 pg. The family with 'Trophee' as the male parent contained 94% triploids, supporting the hypothesis that the bimodal pollen size distribution of 'Trophee' reflects the presence of unreduced gametes. Mean peak center and mean genome size of triploid offspring were 73.1 ± 0.78 and 6.7 ± 0.05 pg, respectively.

The cross 'Princess Juliana' x 'Zaunkoenig' produced all diploid offspring as expected. However, its reciprocal, 'Zaunkoenig' x 'Princess Juliana', produced a small number of polyploid offspring including a putative tetraploid (Table 1). This was unexpected as all pollen examined from these two cultivars appeared of average size[8]. Diploid, triploid, and the putative tetraploid full-sibling offspring from the cross 'Zaunkoenig' x 'Princess Juliana' showed mean peak sizes of 51.6±1.2, 73.6±1.7, and 88.5 and genome sizes of 4.5 ± 0.05, 6.7 ± 0.05, and 8.3, respectively. These spontaneous sexual autopolyploids are likely the result of undetected 2n gamete formation, as chromosome doubling is much less common than unreduced gamete formation in plants[7]. Molecular marker analysis is necessary to determine the origin of these unexpected polyploids.

Ploidy levels and genome sizes of the *H*. *macrophylla* full-siblings in the current study are very similar to previous studies, where mean diploid and triploid genome sizes ranged from 4.5 to 4.8 pg and from 6.7 to 7.1 pg, respectively[8, 11]. There are no data to compare with tetraploid values as this is the first report of sexually produced putative tetraploid *H. macrophylla*. The expectation is that tetraploid peak centers and genome sizes will be double (i.e. 100% larger) that of a related diploid[3, 13]. In our study, the tetraploid peak center was only 77% larger than the diploid peak center, and the genome size was only 84% larger than the mean diploid genome size. Many examples exist where tetraploid values are not exactly double that of the diploid, such as *Solanum*, where tetraploids were 91% larger than diploids[14], and *Lagerstroemia* where tetraploids were 110% larger than diploids[15]. However, the large discrepancy in this case may indicate the putative tetraploid is an aneuploid; that is, it may have three sets of some chromosomes and four sets of other chromosomes. Chromosome counts are needed to confirm the ploidy level of this putative tetraploid.

Influence of ploidy on floral characteristics

Three of the five full-sibling families were of sufficient age to phenotype during the study period. A total of 86 plants in three *H*. *macrophylla* full-sibling families were measured for plant height, stem width, and stomata size; 63 of these flowered in both 2015 and 2016 and were used for floral data collection. Ploidy level significantly influenced the number of inflorescences; family and year did not influence the number of inflorescences. The tetraploid individual averaged 57.6 inflorescences each year while the diploid and triploid plants averaged 8.3 and 3.5 inflorescences per plant, respectively (Figure 1). Triploid plants had the widest inflorescences, followed by diploids and the tetraploid. There was no difference in sepal width between diploids and triploids. The tetraploid inflorescences did not develop showy sepals.

Influence of ploidy on stomata and plant size

Stomata, also called guard cells, play an important role in plant water use as they open to allow respiration and close to prevent water loss. In the current study, ploidy level significantly impacted the size of stomata measured in three full-sibling *Hydrangea macrophylla* families. The putative tetraploid had higher numbers of stomata than diploids and triploids which had similar numbers of stomata (Figures 2 and 3). Stomatal area was largest in triploid plants (8.3 uM^2) and similarly sized in 2n and 4n plants (6.0 and 5.5 uM^2, respectively). As in other studies, the distribution of stomate length and area overlapped for 2n and 3n plants, reaffirming that stomate size alone cannot distinguish between diploid and triploid plants[8]. However, the number of stomata per unit area was very high for the putative tetraploid – double and triple the number of stomata found in diploids and triploids, respectively.

Plant height and stem width were significantly affected by year, as expected for traits that increase yearly. Separate analyses of variance for each year revealed that ploidy level influenced height each year. In both 2015 and 2016, 2n plants were tallest, followed by 3n plants. The putative 4n plant was the shortest among the 85 measured offspring. Triploids had the widest stems each year, averaging 16% thicker than diploids and 40% thicker than the putative tetraploid (Figures 4 and 5). Leaf size followed a similar pattern: triploids had the widest leaves and the putative tetraploid had the smallest leaves (Figure 6). Leaves of triploid plants were darker colored, thicker, and wider than leaves of other ploidy levels.

Increasing ploidy level from diploid to tetraploid has been shown to decrease stomatal density and increase stomatal size in many species including crape myrtle[15], *Solanum*[14], and ornamental ginger[13]. However, none of these studies included triploids in the analysis. In the current study, triploid plants had fewer, larger structures (inflorescences, leaves, stomata) whereas the putative tetraploid had more, smaller structures compared to diploids. Increased drought resistance has been imparted by lowered stomatal density in both maize[16] and *Cucumis*[17], though not all studies have shown a link between stomatal characteristics and drought resistance[18]. Replicated trials of abiotic and disease resistance are ongoing, and superior plants will be evaluated for cultivar release and used as parents for further breeding improvement. Genetic tolerance to stress and pests in popular *Hydrangea* cultivars would lower both the environmental and operational cost of producing these beautiful and popular plants.

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Table 1. Number and percent of diploid (2n), triploid (3n), and tetraploid (4n) plants in three cultivars and five full-sibling families of *Hydrangea macrophylla* as determined by flow cytometry.

		2n			3n			4n‡		
Cultivar/Cross	n*	No. (*%*)	Center**	GS† (pg)	No. (*%*)	Center	GS (pg)	No. (*%*)	Center	GS (pg)
Princess Juliana	3	3 (*100*)	50.1 ± 1.6	4.5 ± 0.05						
Trophee	3	3 (*100*)	50.9 ± 1.3	4.6 ± 0.03						
Zaunkoenig	3	3 (*100*)	51.7 ± 1.7	4.6 ± 0.05						
Princess Juliana x Zaunkoenig	21	21 (*100*)	51.3 ± 1.2	4.5 ± 0.04	0	-	-	0	-	-
Zaunkoenig x Princess Juliana	50	47 (*94*)	51.6 ± 1.2	4.5 ± 0.05	2 (*4*)	73.6 ± 1.7	6.7 ± 0.05	1 (*2*)	88.5	8.3 ± 0.07
Princess Juliana x Trophee	18	1 (*6*)	48.5	4.5 ± 0.07	17 (*94*)	72.5 ± 1.8	6.7 ± 0.05	0	-	-
Trophee x Princess Juliana	8	8 (*100*)	51.4 ± 0.95	4.6 ± 0.05	0	-	-	0	-	-
Trophee x Zaunkoenig	15	15 (*100*)	49.8 ± 1.4	4.6 ± 0.03	0	-	-	0	-	-

*n=number of plants sampled per cultivar or cross.

**Center = peak of relative fluorescence intensity curve which is proportional to DNA content. For each sample, at least 3000 nuclei were analyzed revealing a single peak with a coefficient of variation (CV) less than 4.9%.

† = Genome size. Peak center and genome size values for individual plants were based on an average of two subsamples per plant.

‡ = 4n, putative tetraploid.

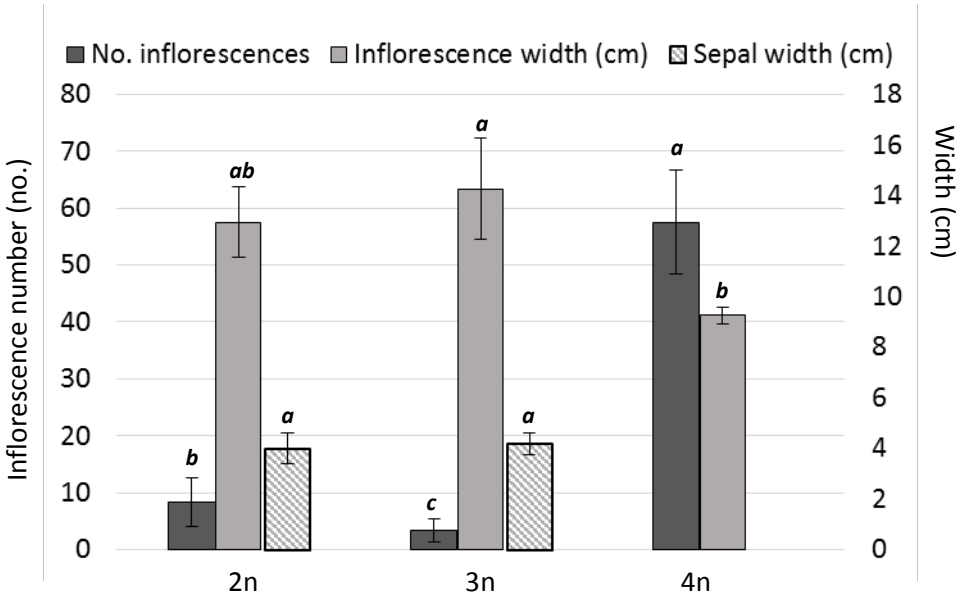

Figure 1. Mean number of inflorescences, mean inflorescence width, and mean sepal width for container-grown 2n (*n* = 54), 3n (*n* = 18), and 4n (*n* = 1) *Hydrangea macrophylla* siblings. Mean inflorescence number is measured on the primary axis; inflorescence and sepal width are measured on the secondary axis. 4n plants did not develop showy sepals. Data were pooled over two years (2015 and 2016). Error bars represent standard deviation. Tukey's mean separation is shown in lower-case letters; for each variable, means with the same letter are not significantly different at the α=0.05 level.

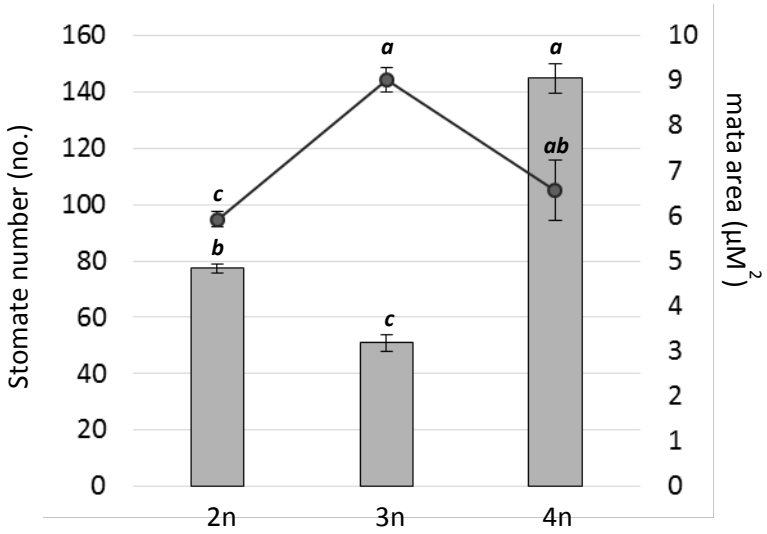

Figure 2. Mean number of stomata (bars, primary axis) and mean stomate width (line, secondary axis) for container-grown 2n (*n* = 54), 3n (*n* = 18), and 4n (*n* = 1) *Hydrangea macrophylla* siblings. Error bars represent standard deviation. Tukey's mean separation is shown in lower-case letters; for each variable, means with the same letter are not significantly different at the α=0.05 level.

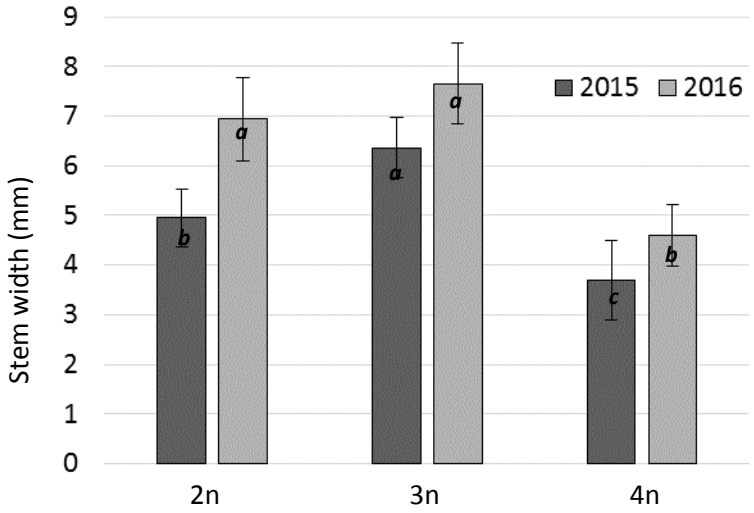

Figure 3. Mean stem width for container-grown 2n (*n* = 54), 3n (*n* = 18), and 4n (*n* = 1) *Hydrangea macrophylla* siblings in 2015 and 2016. Stem width was measured halfway between the second and third node of the tallest stem of each plant. Error bars represent standard deviation. Tukey's mean separation is shown in lower-case letters; for each year, means with the same letter are not significantly different at the α=0.05 level.

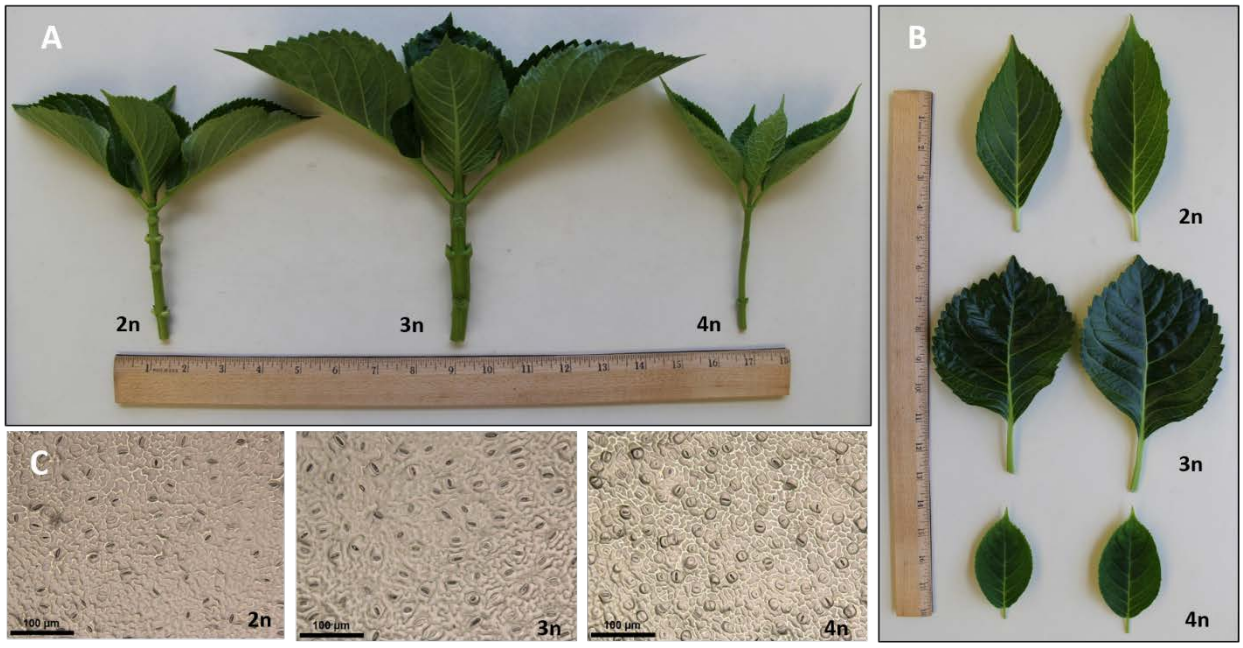

Figure 4. Representative stems (A), leaves (B), and stomata (C) from 2n, 3n, and 4n *Hydrangea macrophylla* full-siblings.