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Production of Triploid *Hydrangea macrophylla* via Unreduced Gamete Breeding

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***Abstract. Hydrangea macrophylla* (Thunb.) Ser., florist's or bigleaf hydrangea, is the most economically important member of the *Hydrangea* genus, which accounted for over $120,000,000 in U.S. nursery sales in 2014. Both diploid and triploid *H. macrophylla* cultivars exist and there is some evidence that triploidy leads to larger plant and floral structures. The diploid cultivar, *H. macrophylla* 'Trophee', was previously shown to have a bimodal pollen size distribution which may be indicative of unreduced gametes. We used *H. macrophylla* '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 phenotype, determine the impact of ploidy on phenotype, and determine the efficacy of unreduced gamete breeding. Diploids and triploids were found in the offspring pool with mean 2C genome sizes of 4.5 and 6.7 pg, respectively. All offspring from crosses with 'Trophee' as the female parent were diploid as expected. The full-sibling 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 male gametes. Triploids had fewer, wider inflorescences than diploids. The stems of triploids were 16% thicker and their leaves were 20% larger than those of diploid full and half-siblings. Triploids had significantly larger stomata (9.0 μM^2) than diploids (5.9 μM^2). These results establish a link between ploidy and phenotype in plants of similar genetic background and support the efficacy of unreduced gametes in polyploidy breeding.**

Hydrangea macrophylla is one of the most economically important nursery crops in the United States, with sales of *Hydrangea* species topping $120,000,000 in 2014 (USDA-NASS, 2014). Although hydrangeas—prized for their large showy flowers and lush green foliage—have a breeding history stretching hundreds of years, improvement has focused on novel floral traits rather than disease resistance or environmental stress tolerance. Major diseases that may have a detrimental impact on plants in the landscape or on salability of container-grown plants include powdery mildew, incited by the fungus *Erysiphe polygoni* DC, and cercospora leaf spot caused by the fungus *Cercospora arborescentis* Tehone & E.Y. Daniels. Other pests like botrytis leaf blight, phytophthora root rot, mites, and aphids may also be problematic in *Hydrangea* production, especially, where containers are tightly spaced (Halcomb et al., 2013), and preventative fungicide and insecticide treatments are nearly always required to consistently produce quality container-grown *Hydrangeas* (Hagan et al., 2005; Hudson et al., 1996). *Hydrangea* varieties with improved resistance to pests, diseases, and drought would significantly reduce the environmental and budgetary footprint associated with *Hydrangea* production.

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 (Kumari and George, 2008; Van Huylenbroeck and van Laere, 2010). Some effects of increasing ploidy level in woody ornamental species include darker green, thicker leaves and increased number of petals per flower in *Rosa* (Kermani et al., 2003), thicker leaves and larger, more persistent flowers in *Magnolia* (Kehr, 1985), and compact size in *Buddleia* (Rose et al., 2000).

Pollen and egg cells of diploid plants normally contain a single set of chromosomes that unite at fertilization to restore the 2*n* 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 (Bretangolle and Thompson, 1995). 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. For example, a large, unreduced pollen cell (2*n*) and a normal egg cell (*n*) may combine to produce a triploid (3*n*) 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 (Jones et al., 2007).

All *H. macrophylla* cultivars tested to date are diploid (2*n* = 2*x* = 36) or triploid (2*n* = 3*x* = 54; Cerbah et al., 2001; Demilly et al., 2000; Jones et al., 2007; Zonneveld, 2004). Several desirable traits seem to be associated with triploidy in *H. macrophylla* including dark green foliage, strong stems, and large inflorescences (Jones et al., 2007). 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. 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.

Materials and Methods

Controlled pollinations in 2010 produced a series of five reciprocal *H. macrophylla* full-sibling families. The *H. macrophylla* varieties 'Princess Juliana', 'Trophee', and 'Zaunkoenig' were used in the following crosses: 'Princess Juliana' × 'Trophee', 'Trophee' × 'Princess Juliana', 'Princess Juliana' × 'Zaunkoenig', 'Zaunkoenig' × 'Princess Juliana', and 'Trophee' × 'Zaunkoenig' (Table 1). Controlled pollinations were made following the method of Reed (2005). Plants were grown in 3-gallon containers under 56% shade and microirrigated using spray stakes. Growing media consisted of pine bark amended with 6.6 kg·m^{-3} 19N–2.1P–7.4K Osmocote Pro fertilizer (Scotts-Sierra Horticultural Products Co., Maryville, OH); 0.6 kg·m^{-3} Micromax (Scotts-Sierra Horticultural Products Co.); 0.6 kg·m^{-3} iron sulfate; and 0.2 kg·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). About 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

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

		2*n*			3*n*		
Cultivar/cross	n[z]	No. (%)	Center[y]	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 × Zaunkoenig	21	21 (100)	51.3 ± 1.2	4.5 ± 0.04	0	-	-
Zaunkoenig × Princess Juliana	50	47 (94)	51.6 ± 1.2	4.5 ± 0.05	2 (4)	73.6 ± 1.7	6.7 ± 0.05
Princess Juliana × Trophee	18	1 (6)	48.5	4.5 ± 0.07	17 (94)	72.5 ± 1.8	6.7 ± 0.05
Trophee × Princess Juliana	8	8 (100)	51.4 ± 0.95	4.6 ± 0.05	0	—	—
Trophee × Zaunkoenig	15	15 (100)	49.8 ± 1.4	4.6 ± 0.03	0	—	—

GS = genome size.
For each sample, at least 3000 nuclei were analyzed revealing a single peak with a CV less than 4.9%. Peak center and genome size values for individual plants were based on an average of two subsamples per plant.
[z]Number of plants sampled per cultivar or cross.
[y]Peak of relative fluorescence intensity curve, which is proportional to DNA content.

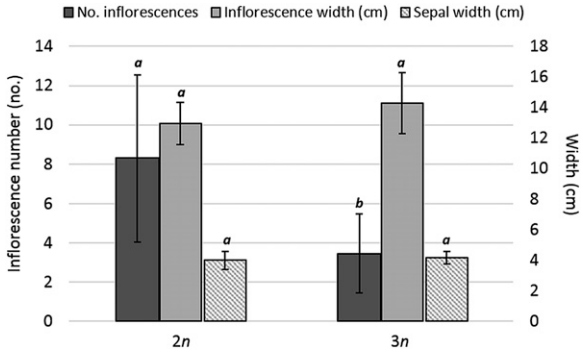

Fig. 1. Mean number of inflorescences, mean inflorescence width, and mean sepal width for container-grown 2*n* (n = 54) and 3*n* (n = 18) *Hydrangea macrophylla* siblings. Mean inflorescence number is measured on the primary axis; inflorescence and sepal width are measured on the secondary axis. Data were pooled over 2 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 $\alpha = 0.05$ level.

(Partec CyStain ultraviolet precise P Nuclei Extraction Buffer; Partec GMBH, Muenster, Germany). The resulting extract was passed through a 30-µM 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 4′,6-diamidino-2-phenylindole (DAPI). The relative fluorescence of the total DNA was measured for each nucleus using a Partec PA-1 ploidy analyzer (Partec GMBH). 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 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. 'Ctirad' with a 2C content of 9.09 pg was used as the internal standard (Doležel and Bartoš, 2005). Ploidy and genome sizes are the averages of two subsamples per plant.

Phenotypic data collection. At the time of the study, three of the full-sibling families ('Princess Juliana' × 'Trophee', 'Zaunkoenig' × 'Princess Juliana', and 'Trophee' × 'Zaunkoenig') were of sufficient age to flower reliably (3 years old). The following variables were used to describe plant inflorescence and size: Number of inflorescences, inflorescence width (mean of tallest three on each plant), sepal width (mean of three on each of three tallest inflorescences), stem width (measured between second and third node of tallest stem), and leaf area (mean of three topmost fully expanded leaves). Leaf area was calculated using ImageJ analysis software v.1.51 (Schneider et al., 2012) through the open-source platform Fiji (Schindelin et al., 2012). All variables were measured on 8 June 2015 and 14 June 2016, except leaf area which was measured in 2016 only.

Stomata and chloroplast counts. For stomatal measurements, the top-most fully expanded healthy leaf from each plant in three full-sibling *H. macrophylla* families (n = 72 plants) was collected between 14 and 18 Sept., 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 5.5–6 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. Three peels per plant were evaluated. Stomata were counted in one field of view (5940.75 µm^2) per peel (n = 3 counts per plant). Length and width of six 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 × half-width × π.

Number of chloroplasts per guard cell was counted in six diploid and six triploid plants. Three leaves were collected per plant. Each leaf was torn or teased apart gently so that a section of thin, abaxial epidermal layer became apparent. This opaque layer was cut away from the other leaf tissue and placed on a microscope slide in a drop of water. A coverslip was applied, pressed to remove air bubbles, and secured with clear fingernail polish to limit water evaporation. The number of chloroplasts per guard cell was counted for six guard cells per leaf. Stomata and chloroplasts were visualized at ×20 magnification with an Olympus BX50 compound microscope (Olympus Corp., Tokyo, Japan) with an Olympus Q Color 5 digital camera for image capture. Measurements and counts were made using Q-Capture Pro 7 software.

Pollen stainability. Six diploid and six triploid plants were used to estimate pollen viability. Fresh pollen from a single flower was placed on a microscope slide using a camel-hair brush (n = 3 flowers per plant). A 30 µL drop of stain [5% aqueous aniline blue solution (1% solution), 2.28 M phenol, 2.67 M lactic acid, and 5.47 M glycerin] was pipetted onto the slide and a coverslip was applied. Slides were observed after 30 min at ×20 magnification using an Olympus BX50 (Olympus Corp.) compound microscope with an Olympus Q Color 5 digital camera for image capture. Percent stained pollen was calculated as: (number of stained pollen grains/total number of pollen grains) × 100%.

Data analysis. Data analysis was performed using SAS® software, Version 9.4, of the SAS system for Microsoft (Copyright© 2013, SAS Institute Inc., Cary, NC). The general linear model procedure was used to partition variance in inflorescence, stomata, and plant size means into sources attributable to year (if applicable), family, ploidy level, and environment (error). Percent stained pollen and chloroplast counts were analyzed using single-factor analysis of variance with ploidy level as the source of variation. 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 *H. 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 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 (Jones et al., 2007). Mean genome size of triploid offspring was 6.7 ± 0.05 pg. These genome sizes are consistent with previously reported genome sizes, though DAPI staining yields a slightly larger genome size than PI staining used in some previous works (Cerbah et al., 2001; Demilly et al., 2000; Jones et al., 2007; Zonneveld, 2004).

The cross 'Princess Juliana' × 'Zaunkoenig' produced all diploid offspring as expected. However, its reciprocal, 'Zaunkoenig' × 'Princess Juliana', produced a small number of triploid offspring (Table 1). This was unexpected as all pollen examined from these two cultivars appeared of average size. Diploid and triploid full-sibling offspring from the cross 'Zaunkoenig' × 'Princess Juliana' showed mean genome sizes of 4.5 ± 0.05 and 6.7 ± 0.05, respectively. Although these spontaneous sexual autopolyploids are likely the result of undetected 2*n* gamete formation (Bretangolle and Thompson, 1995), molecular marker analysis is necessary to verify the origin of these unexpected polyploids.

Influence of ploidy on floral characteristics. A total of 86 plants in three *H. macrophylla* full-sibling families were measured for leaf area, stem width, and stomata size; 72 of these flowered in both 2015 and 2016 and were used for floral data collection. Ploidy level significantly influenced the number of inflorescences ($P < 0.001$); family and year did not influence the number of inflorescences ($P = 0.15$ and 0.68, respectively). The diploid and triploid plants averaged 8.3 and 3.5 inflorescences per plant (Fig. 1). Triploid plants had wider inflorescences than diploids, though the difference was not statistically significant. There was no difference in sepal width between ploidy levels.

Both diploid and triploid plants produced stainable pollen grains with a normal appearance. Diploid plants had significantly more stainable pollen than triploids ($P = 0.002$; Fig. 2). Percent stained pollen ranged from 84% to 92% in diploids and from 25% to 60% in triploids with means of 87.6% ± 0.03% and 50.9% ± 10.7% for diploids and triploids, respectively. Experiments are underway to determine the efficacy of using pollen from these triploids for future breeding efforts.

Influence of ploidy on stomata, chloroplasts, 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 *H. macrophylla* families, whereas family did not influence size ($P = 0.33$) or number ($P = 0.85$) of stomata. Stomatal density was higher in diploids than triploids ($P < 0.001$), whereas stomatal area was significantly larger in triploid plants (8.3 μM^2) than diploid plants (6.0 μM^2; $P < 0.001$; Fig. 2). The mean number of chloroplasts per guard

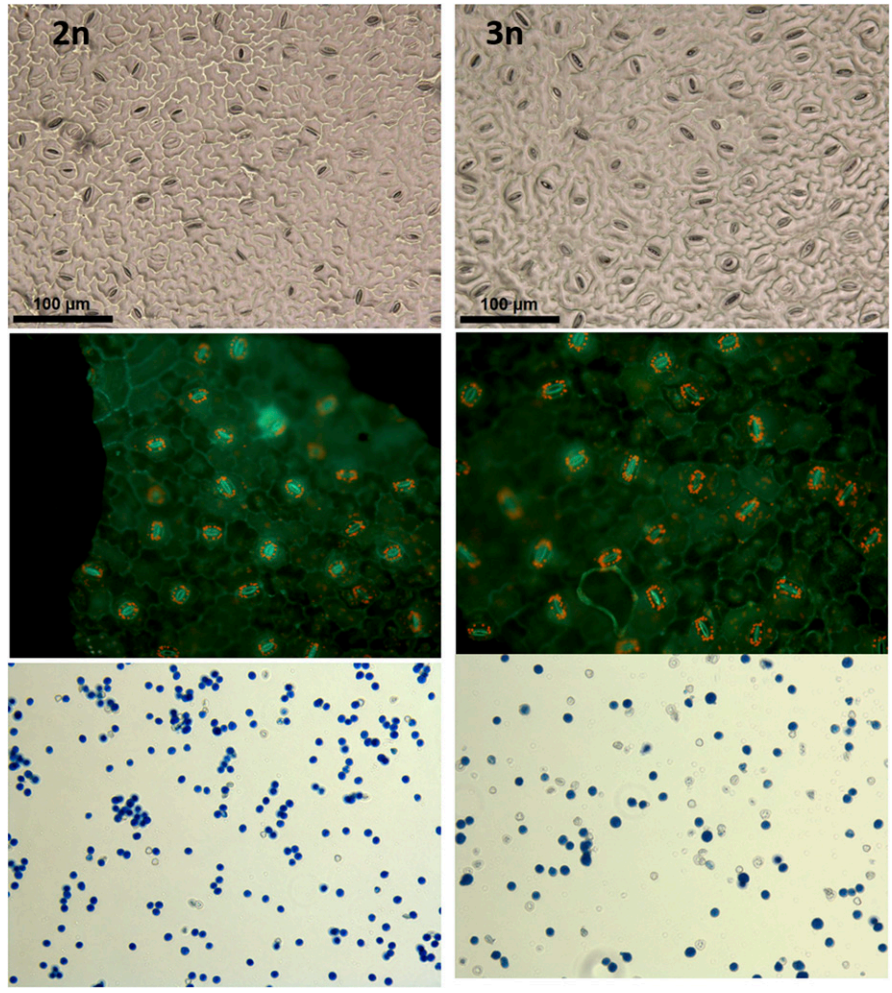

Fig. 2. Stomata, chloroplasts, and stained pollen from 2*n* (left) and 3*n* (right) *Hydrangea macrophylla* full-siblings. Pollen was stained for 30 min using 30 μL stain [5% aqueous aniline blue solution (1% solution), 2.28 M phenol, 2.67 M lactic acid, and 5.47 M glycerin]. Stomata, chloroplasts, and pollen were visualized at ×20 magnification with an Olympus BX50 compound microscope with an Olympus Q Color 5 digital camera for image capture.

Table 2. Comparison of diploid (2*n*) and triploid (3*n*) *Hydrangea macrophylla* siblings.

	2*n*			3*n*		
Variable	n	Range[z]	Mean ± SD	n	Range	Mean ± SD
Stem width (mm)[y]	54	2.6–9.4	5.9 ± 1.3	18	3.7–12.6	7.0 ± 1.4*
Leaf area (cm^2)	54	59.1–121.7	89.3 ± 16.2	18	93.5–124.3	111.2 ± 9.9**
Stomata (number)	54	52.6–95.1	77.4 ± 27.9	18	31.9–75.5	50.9 ± 31.4**
Stomate area (μM^2)	54	2.3–11.6	5.9 ± 3.1	18	2.5–16.3	9.0 ± 2.9**
Chloroplasts per stomate (number)	6	12.9–17.5	16.1 ± 2.9	6	20.5–29.7	24.9 ± 3.5**
Stained pollen (%)	6	84.1–92.5	87.6 ± 0.03	6	25.6–60.1	50.9 ± 10.7**

Ploidy level means were compared using Tukey's studentized range test.
[z]Ranges are based on individual plant means.
[y]Stem width is pooled over 2 years.
*, **Significantly different at $\alpha = 0.05$ or 0.01, respectively.

Fig. 3. Diploid (left) and triploid (right) *Hydrangea macrophylla* full-siblings.

cell ranged from 12.9 to 17.5 for diploids and from 20.5 to 29.7 for triploids. On average, stomata of triploid plants were ringed by 50% more chloroplasts than stomata of diploid plants (Fig. 2). As in other studies, the distribution of stomate length and area overlapped for 2*n* and 3*n* plants, reaffirming that stomate size alone cannot distinguish between diploid and triploid plants (Jones et al., 2007). However, there was no overlap between ploidy levels for mean number of chloroplasts per guard cell. Chloroplast counts for more diploid and triploid individuals are needed to verify whether this measure may reliably distinguish between ploidy levels.

Stem width was significantly affected by year, as expected for a growth trait that increases yearly. Because yearly trends were similar and there were no interactions between year and other variables, data for each year were pooled. Ploidy level was a significant source of variation in stem width, with stems of triploid plants averaging 16% thicker than diploids ($P = 0.032$). Leaves of triploid plants were 20% larger than those of diploid siblings ($P < 0.001$; Table 2). Leaves of triploids were also wider and darker colored compared with diploid plants (Fig. 3). Family was not a significant source of variation in stem width ($P = 0.29$).

Increasing ploidy level from diploid to tetraploid has been shown to decrease stomatal density and increase stomatal size in many species including crape myrtle (Zhang et al., 2010), *Solanum* (Sakhanokho and Islam-Faridi, 2014), and ornamental ginger (Sakhanokho et al., 2009). Few studies have analyzed the effect of triploidy; however, as triploids are less frequent than tetraploids when ploidoidization is induced via chemicals or other mutagens. In the current study, triploid plants had fewer, larger structures (inflorescences, leaves, and stomata) compared with diploids. Increased drought resistance has been imparted by lowered stomatal density in both maize and *Cucumis*, though not all studies have shown a link between stomatal characteristics and drought tolerance (Gharun et al., 2015; Kusvuran et al., 2010; Liu et al., 2015). 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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