# In Vitro Induction and Characterization of Polyploid *Hydrangea macrophylla* and *H. serrata*

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***Abstract. Hydrangea macrophylla* (Thunb.) Ser. and *H. serrata* (Thunb.) Ser. are popular and commercially important landscape and floriculture crops. Although both species are typically diploid, induced polyploids often exhibit horticulturally valuable traits. Procedures for inducing polyploidy vary by species and often have low or inconsistent efficacy. In this study, oryzalin and nitrotyrosine were investigated as in vitro mitotic inhibitors for inducing polyploidy in *H. macrophylla* 'Robert' and *H. serrata* 'MAK20'. First, shoot apices of 'MAK20' were treated with 15 μM oryzalin for 0, 2, 4, 6, or 8 days, and the ploidy of shoots was determined after 8 weeks. A regression analysis showed that the proportion of polyploids (tetraploid plus mixoploid shoots) increased with the exposure duration. During a follow-up experiment, 'MAK20' and 'Robert' were treated with oryzalin (0 or 15 μM) and nitrotyrosine (0, 25, 50, and 100 μM for 'MAK20' and 0, 12.5, 25, 50, and 100 μM for 'Robert') in a factorial treatment arrangement. Oryzalin, nitrotyrosine, and their interaction influenced polyploid frequency for 'Robert', whereby the combination of oryzalin (15 μM) and nitrotyrosine (50 μM) resulted in the highest polyploid induction of 50%. Oryzalin influenced polyploid frequency for 'MAK20' ($\bar{X}$ = 30.4%), but not nitrotyrosine or the interaction between nitrotyrosine and oryzalin. Morphology and pollen germination of these autotetraploid 'Robert', 'MAK20', and previously developed autotetraploid *H. macrophylla* 'David Ramsey' plants were compared with their diploid counterparts 1 year after plants were moved ex vitro. Compared with diploids, tetraploid hydrangeas had larger leaves, thicker stems, lower leaf area/fresh weight ratios, and longer internodes. Although all tetraploids exhibited fewer inflorescences per plant, both *H. macrophylla* cultivars had larger inflorescence diameters and 'David Ramsey' had a greater number of showy florets (sterile florets with enlarged, decorative sepals) per inflorescence. Sepal colors were compared using International Commission on Illumination L*a*b* color space. Tetraploid 'MAK20' had lower L* values (darker sepals), and tetraploid 'Robert' and 'MAK20' both had higher a* values (redder sepals). Pollen germination rates were greatly reduced in all tetraploid lines, but they retained some viability. These results provide an effective protocol for in vitro polyploid induction of *Hydrangea* sp. and documented certain desirable traits associated with tetraploid phenotypes.**

*Hydrangea macrophylla*, commonly known as bigleaf hydrangea, is endemic to Japan, where it thrives in moist, coastal, temperate regions as a deciduous understory shrub of medium size (Dirr, 2004). An important landscape and floriculture crop, *H. macrophylla* has wide cultivation because of its attractive inflorescences that feature a mix of small perfect flowers and showy peripheral florets with enlarged sepals. A distinctive characteristic of the species is its pH-/aluminum-dependent flower color, which can shift from pink in alkaline soils to blue in acidic soil conditions with available aluminum (Schreiber et al., 2011). *Hydrangea macrophylla* have been selected, bred, and distributed throughout the world for centuries, resulting in hundreds of named cultivars of mophead and lacecap inflorescence types (McClintock, 1957; Rinehart et al., 2006; van Gelderen and van Gelderen, 2004). Hydrangeas comprise the second most valuable deciduous shrub class in the United States after roses, with annual sales exceeding $91 million/year, with additional revenue from cut flowers and pot plants (USDA-NASS, 2014).

*Hydrangea serrata* is closely related to *H. macrophylla* and is sometimes considered a subspecies (Granados Mendoza et al., 2013; Reed and Rinehart, 2006; Rinehart and Reed, 2007). Although the two are similar morphologically, *H. serrata* is distinguishable by its smaller, less glossy leaves, increased red coloration in its vegetative parts, and smaller flowers that bloom on both primary and lateral shoots (Uemachi et al., 2014). Commonly called mountain hydrangea, *H. serrata* originates from mountainous regions across Japan and Korea and is often more cold-hardy than *H. macrophylla*. *Hydrangea serrata* can also be distinguished from *H. macrophylla* by its smaller genome size (Cerbah et al., 2001). *Hydrangea serrata* is much less popular as a commercial crop; however, the species is more genetically diverse (Uemachi et al., 2014), and desirable traits (e.g., cold-hardiness) could be introgressed into *H. macrophylla* because the two species are known to hybridize (Reed and Rinehart, 2006).

Both *H. macrophylla* and *H. serrata* are typically diploid, with $2n = 2x = 36$ (Cerbah et al., 2001). Some triploid *H. macrophylla*, assumed to have been formed from unreduced gametes, have been found in cultivation (Jones et al., 2007). Zonneveld (2004) noted that triploid cultivars exhibit "luxuriant growth" and more deeply serrated leaves and produce seed that engenders progeny with poor growth. Alexander (2017) compared half-sibling diploid and triploid families and found that triploid hydrangeas have fewer inflorescences, larger leaves and stems, and stomata that were larger although fewer in number. Although developing polyploids from unreduced gametes can result in limited triploid and possibly tetraploid populations, the direct development of tetraploids from modern elite cultivars would help advance breeding goals more efficiently.

The potential advantages of incorporating polyploid plants into a breeding program have been well-documented. Tetraploid plants may be more self-fertile from the disruption of self-incompatibility systems and may allow for functional chromosome pairing and fertility in wide hybrids (Ranney, 2006). Polyploidy can also confer horticultural benefits to plants, such as larger leaves and flowers, increased pest and stress tolerance, and enhanced vigor (Tamayo-Ordóñez et al., 2016). However, such advantages cannot be assumed because polyploidy can also result in plants with abnormal growth, poor flowering, sterility, and other undesirable phenotypes (Dhooghe et al., 2011, Tamayo-Ordóñez et al., 2016).

In vitro polyploidy can be induced using mitotic inhibitors (Touchell et al., 2020). Chemical agents such as colchicine, oryzalin, and trifluralin cause the depolymerization of microtubules that migrate chromosomes to daughter cells during anaphase, the failure of which results in somatic cells with whole genome duplications. Colchicine has been used in this capacity since the 1930s. However, its greater affinity for animal microtubules requires that it be used in high concentrations to induce polyploidy in plants and poses a human health hazard. Oryzalin (3,5-dinitro-N4,N4-dipropyl sulfanilamide), sold as the herbicide Surflan (United Phosphorus, Inc., Mumbai, India), is a metaphase inhibitor with a high affinity for plant microtubules, thus making it safer and more efficient at inducing polyploidy in plants (Morejohn et al., 1987).

Despite the decades of research of polyploid induction, efficacy rates are highly variable, and it would be desirable to develop more reliable protocols for recalcitrant

species, including hydrangea. One possible approach is to augment commonly used mitotic inhibitors with nitrotyrosine during the induction phase. Nitric oxide is an important signaling molecule implicated in cell growth and development processes that can trigger the creation of various nitrosylated products, including nitrotyrosine. It has been suggested that nitrotyrosine may replace the C-terminal tyrosine of α-tubulins and trigger an irreversible post-translational modification that results in less stable microtubules, which could hinder normal cell division (Lipka and Müller, 2014). Jovanović et al. (2010) tested the combination of oryzalin and nitrotyrosine in rice and found a synergistic interaction with oryzalin when inducing polyploidy. Lipka and Müller (2014) found that nitrotyrosine alone influenced microtubule depolymerization but was protective against the effects of oryzalin in *Arabidopsis thaliana*, and they suggested that nitrotyrosine may alter oryzalin binding sites in tubulin.

The objectives of this study were to investigate the effects of oryzalin and nitrotyrosine on in vitro polyploid induction in *H. macrophylla* and *H. serrata* and to evaluate the effects of induced polyploidy on morphology and fertility.

## Materials and Methods

*Germplasm.* To evaluate the efficiency of polyploidy induction, *Hydrangea macrophylla* 'Robert' (Let's Dance® Moonlight) and *H. serrata* 'MAK20' (Tuff Stuff™) were chosen as commercially available representatives of their respective species. Additionally, a previously developed tetraploid of *H. macrophylla* 'David Ramsey' was also included to compare morphological characters between ploidy levels. 'Robert' and 'David Ramsey' have mophead blooms; 'MAK20' is a lacecap. All cultivars are purportedly remontant.

*In vitro establishment.* During Spring 2017, shoot apices were collected from greenhouse-maintained plants and used to initiate in vitro cultures of 'Robert' and 'MAK20'. Explants were washed under running water for 4 h, surface-disinfested with a 100-mL solution of 20% commercial bleach (6.25% sodium hypochlorite solution) with a drop of Tween 20 (Fisher Scientific, Fair Lawn, NC) for 20 min, and rinsed three times for 5 min each with sterile distilled water. Shoot apices were cultured in 180-mL glass jars containing 25 mL of media. Shoot induction and maintenance medium consisted of full-strength B5 salts and vitamins (Gamborg et al., 1968), 15 μM 6-Benzylaminopurine, 0.1 $g \cdot L^{-1}$ 2-(N-morpholino) ethanesulfonic acid, 0.1 $g \cdot L^{-1}$ myo-inositol, 0.75 $g \cdot L^{-1}$ magnesium sulfate, and 3% sucrose that was pH-adjusted to 5.75 ± 0.03 and solidified with 2.5 $g \cdot L^{-1}$ Gelzan (Phytotechnology Laboratories, Lenexa, KS). Filter-sterilized cefotaxime was added to cooled autoclaved media for a final concentration of 418 μM. Cultures were maintained by transferring shoots to fresh maintenance medium (25 mL in 180-mL glass jars) every 4 to 6 weeks for 12 months and incubated under standard culture conditions [23 ± 2 °C and a 16-h photoperiod of 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (400–700 nm) provided by cool-white fluorescent lamps].

*Oryzalin experiment.* Shoot apices were excised from 3- to 4-week-old 'MAK20' shoots and cultured in 90-mm petri dishes containing 25 mL of pretreatment media consisting of B5 salts and vitamins, 4 μM 6-Benzylaminopurine, 1 μM indole-3-acetic acid, 0.1 $g \cdot L^{-1}$ 2-(N-morpholino) ethanesulfonic acid, 0.1 $g \cdot L^{-1}$ myo-inositol, 0.75 $g \cdot L^{-1}$ magnesium sulfate, and 3% sucrose, pH-adjusted to 5.75 ± 0.03, and solidified with 2.5 $g \cdot L^{-1}$ Gelzan for 7 d. An aliquot of stock solution of 3 mM oryzalin (Supelco, Bellefonte, PA) dissolved in 95% ethanol was added to cooled autoclaved liquid B5 media to achieve a final saturated concentration of 15 μm oryzalin. Shoot apices were then transferred to the liquid media and incubated for 0, 2, 4, 6, or 8 d in the dark on an orbital shaker (60 rpm). After oryzalin treatment, the explants were rinsed with liquid B5 maintenance media for 24 h to remove residual oryzalin and then cultured onto fresh solidified media in reduced light (15 $\mu mol \cdot m^{-2} \cdot s^{-1}$) conditions for 6 weeks until new shoots began to form.

Each treatment consisted of six replications, with each replicate consisting of a petri plate containing five explants (subsamples). Petri plates were randomized within the growth chamber. Data regarding the overall shoot survival and ploidy of surviving shoots were obtained and subjected to an analysis of variance (ANOVA) and regression analyses using PROC GLM (SAS, Cary, NC).

*Nitrotyrosine and oryzalin experiments.* The second set of experiments was performed using both 'MAK20' and 'Robert' to determine the effects of nitrotyrosine, oryzalin, and their potential interaction on polyploid induction. Shoot apices were pretreated as previously described and then moved to liquid B5 treatment media. The experiments were completely randomized, with a factorial arrangement with four levels of nitrotyrosine (0, 25, 50, and 100 μM) for 'MAK20', five levels (0, 12.5, 25, 50, and 100 μM) for 'Robert', and two levels of oryzalin (0 and 15 μM). Treatments had six replications of one jar with five shoots (subsamples) per jar. Plantlets were treated for 6 d, washed for 24 h in liquid B5 media, and transferred to maintenance media under standard culture conditions. Data regarding shoot survival and ploidy of surviving plantlets 8 weeks after treatment were obtained and subjected to an ANOVA and regression analyses with PROC GLM (SAS).

*Flow cytometry.* The ploidy of regenerated shoots was evaluated using flow cytometry according to the methods of Ranney et al. (2018). Approximately 0.5 $cm^2$ of freshly expanded leaf material was sampled and finely chopped with 400 μL of extraction buffer (CyStain ultraviolet Precise P Nuclei Extraction Buffer; Sysmex Partec GmbH, Görlitz, Germany) and passed through a 50-μm nylon mesh filter into a 3.5-mL tube. Then, 1600 μL of 4′,6-diamidino-2-phenylindole (DAPI) staining buffer (Cystain ultraviolet Precise P Staining Buffer; Sysmex Partec) was added to the sample and analyzed immediately using the flow cytometer (Partec PA I; Partec GmbH, Münster, Germany). Tetraploids were identified when DNA contents of the sample were approximately twice that of an untreated diploid ($2n = 2x = 36$) hydrangea of the same cultivar used as an external standard. If samples had ≥20% nuclei in both the diploid and expected tetraploid 2C DNA contents, then they were identified as mixoploids. Samples from three to five randomly selected shoots from separate explants from each replicate were analyzed. Diploid, tetraploid, and mixoploid (cytochimeras) shoots were separated upon identification, and each polyploidization event was maintained separately.

Genome size was determined for each diploid cultivar and polyploidization event by using leaf tissue from *Deutzia calycosa* (2C DNA 6.76 pg) (Hembree et al., 2020) as an internal standard. Combined flow cytometry samples contained leaf tissue from the standard, and the hydrangea being measured were analyzed after a minimum of 5000 nuclei counts was performed. Genome size was calculated as: 2C DNA content of tissue = (mean fluorescence value of sample ÷ mean fluorescence value of standard) × 2C DNA content of the standard. Three subsamples were measured per plant.

*Growing environment.* Diploid and autotetraploid 'Robert' (diploid, n = 9; tetraploid, n = 24), 'David Ramsey' (diploid, n = 10; tetraploid, n = 7), and 'MAK20' (diploid, n = 11; tetraploid, n = 10) plants were transitioned to the greenhouse. In vitro grown shoots ≈25 mm in height were transferred to a 50-cell tray of peat:perlite (50:50) propagation mix and kept under intermittent mist for 4 weeks. Then, rooted plants were potted into 0.8-L containers with pine bark media supplemented with 1.04 $kg \cdot m^{-3}$ dolomitic lime and 0.74 $kg \cdot m^{-3}$ granulated micronutrients (Micromax; ICL Specialty Fertilizers, Tel Aviv, Israel) in Spring 2018 and grown in a greenhouse. Plants were potted in 2.8-L containers using the same media and top-dressed with 12 g of 5- to 6-month slow-release fertilizer (Osmocote Plus 15–9–12; ICL Specialty Fertilizers) during Summer 2018 and moved

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to a 50% shade polyhouse at the Mountain Crop Research and Extension Center in Mills River, NC. Then, plants were overwintered in a polyhouse maintained with a minimum temperature of 6 °C. During Spring 2019, plants were transplanted in 11.3-L containers with the same media with 48 g of slow-release fertilizer (Osmocote Plus 15–9–12) and grown under 50% shade for the remainder of the study. At the end of the study, media pH was determined (≈4.14) by taking two subsamples from 10 randomly selected containers using a pour-through method (LeBude and Bilderback, 2009) and a Cole-Parmer pH/conductivity/temperature meter (Vernon Hills, IL).

*Plant characterization.* Ploidy of all plants was reverified in late Summer 2018 by flow cytometry as previously described. Morphological data regarding inflorescence number and width, showy floret diameter, number of florets per inflorescence, flower color, male fertility, leaf area and mass (fresh and dried), stem thickness, and internode length were collected in May and June 2019.

*Leaf and stem measurements.* Three fully expanded leaves per plant were randomly collected during the morning from well-irrigated plants. Leaf area measurements were performed with an area meter (LI-3100; LI-COR, Lincoln, NE) and the fresh weight was recorded. Leaves were dried at 80 °C for 25 h to obtain dry weights. Stem thickness and internode length were measured for up to five randomly selected flowering stems per plant. The lengths of the first and second internodes beneath the newest fully expanded leaf pair were measured; stem caliper data were determined for these same internodes.

*Floral measurements.* Between 6 and 12 (n ≥6) inflorescences per plant were measured. The diameter of each inflorescence was measured at the widest point when most sterile florets were fully expanded and no longer chartreuse. On each inflorescence, the total number of showy florets was counted. The diameter of a subsample (n = 12) of showy florets was measured across the widest point of the sepals. Inflorescences were assigned matching color chips from the Royal Horticultural Society (RHS) Color Chart (Royal Horticulture Society, 2015) when fertile flowers began to dehisce. The RHS color values were converted to International Commission on Illumination [Commission Internationale de l'Eclairage (CIE)] L*a*b* color space using a conversion method developed by Ryan Contreras (personal communication). The total number of inflorescences per plant was recorded at the end of flowering.

*Fertility evaluation.* Male fertility was assessed via pollen germination using the hanging-drop technique (Brewbaker and Kwack, 1964). Pollen from each plant was collected in a small petri dish during the morning as the fertile flowers began to dehisce. Pollen was dried in a desiccator at 4 °C and stored overnight. A petroleum jelly ring was placed on a microscope slide, and a drop of liquid Brewbaker and Kwack (1964) medium with 10% sucrose and pH-adjusted to 5.5 (Alexander, 2019) was pipetted inside the ring. Pollen was placed on the media and the slide was inverted over a petri dish and placed in a plastic container with a moistened paper towel for 6 h. Then, the slide was removed and a cover slip was placed over the drop. Each sample was examined at 100×, and the

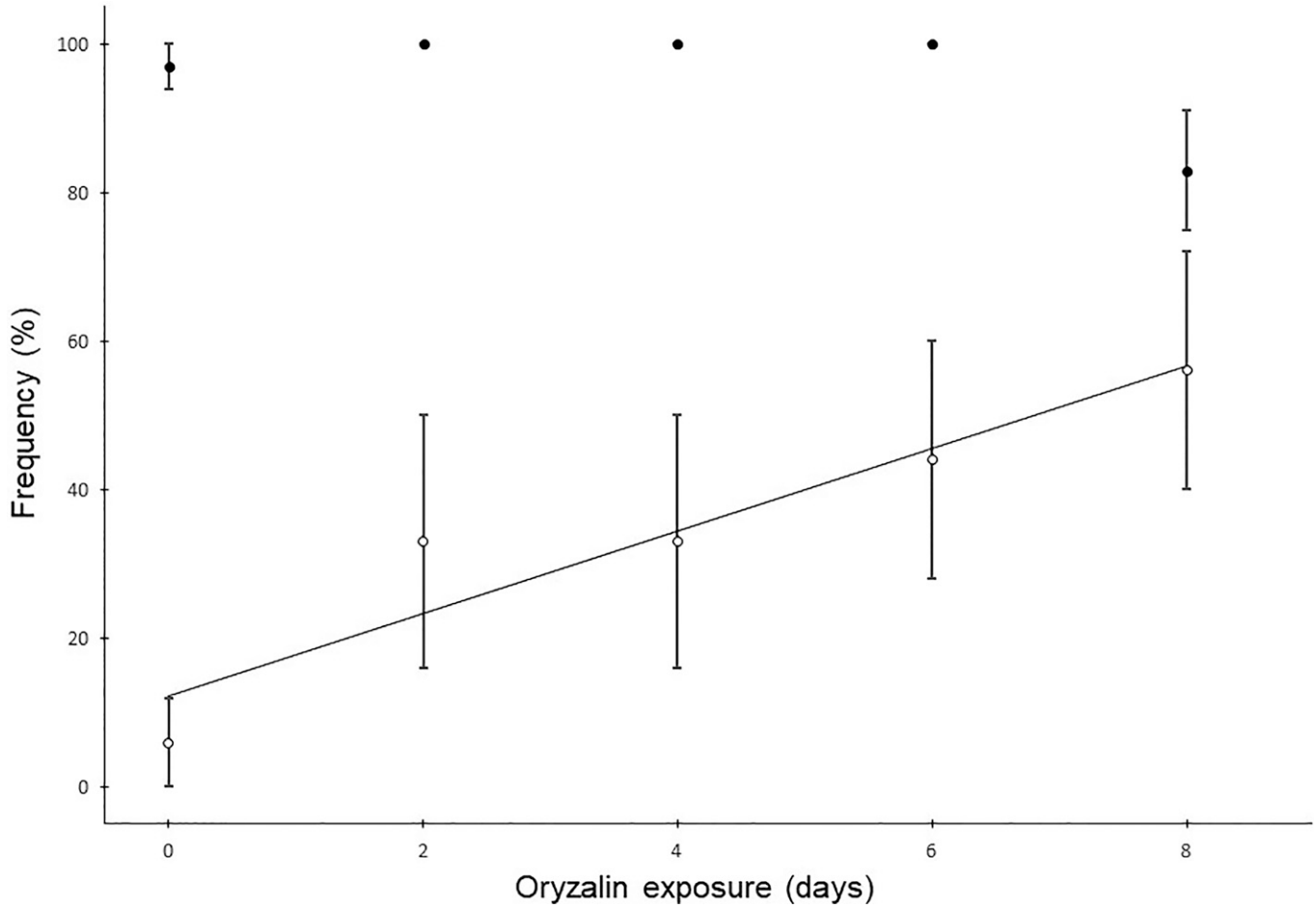


Fig. 1. Shoot survival and frequency of polyploids of *Hydrangea serrata* 'MAK20' plantlets treated with 15 μM of oryzalin for 0, 2, 4, 6, and 8 d and maintained for 8 weeks. Solid circles indicate shoot survival (%); open circles indicate polyploid (tetraploid plus mixoploid) shoots (%). A significant linear trend ($P < 0.05$) for polyploid induction (%) as a function of oryzalin exposure (d) was found: $y = 0.12 + 5.6x$. $P \leq 0.02$. $R^2 = 0.94$. Values are means (n = 6) ± SEM.

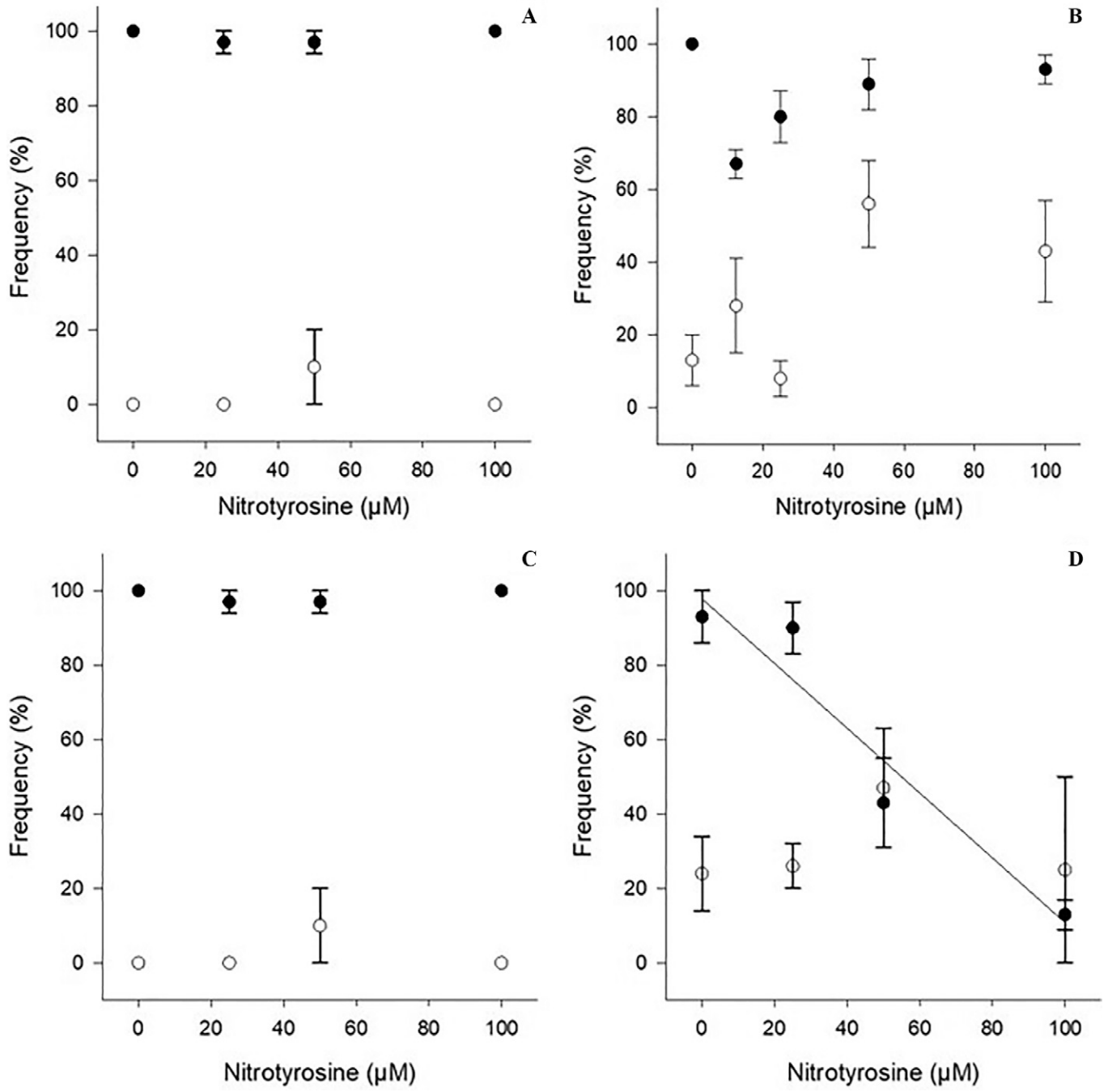


Fig. 2. Shoot survival and frequency of polyploids of *Hydrangea macrophylla* 'Robert' without oryzalin (**A**) and with 15 μM oryzalin (**B**). *Hydrangea serrata* 'MAK20' without oryzalin (**C**) and with 15 μM oryzalin (**D**). Solid circles indicate shoot survival (%) as a function of the nitrotyrosine concentration; open circles indicate polyploid (tetraploid plus mixoploid) shoots (%) confirmed as a function of the nitrotyrosine concentration. The trend line in (**D**) is a linear regression ($P < 0.05$) for shoot survival of 'MAK20': Frequency (%) = 97.8 − 0.87 × nitrotyrosine concentration; $R^2 = 0.92$. Values are means (n = 6 to 24) ± SEM. Means separated at a frequency of 15 (**B**) and 20 (**D**) were significantly different at $P < 0.05$ according to the least significant difference test.

numbers of germinated and nongerminated pollen grains were documented in five view fields to determine the germination rate per sample.

*Statistical analysis.* Plants were completely randomized. Multiple measurements (subsamples) obtained for each plant were averaged and subjected to an ANOVA using PROC GLM (SAS).

## Results and Discussion

*Oryzalin experiment.* The length of exposure to oryzalin influenced shoot survival and the number of polyploid events (mixoploids plus tetraploids) ($P < 0.05$) in 'MAK20' (Fig. 1). Survival remained near 100% for 0 to 6 d of oryzalin treatment; then, it declined to 83% when treated for 8 d. The frequency of polyploids increased with the number of days of oryzalin exposure in a linear fashion ($P < 0.05$), reaching a maximum of 56% at 8 d. Of the polyploid shoots, four were homogeneous tetraploids (one from each at 2, 4, 6, and 8 d) and the remaining were mixoploids. A lone mixoploid shoot was identified in the 0-d control group and had the lowest average rate of polyploid induction (6%). Spontaneous polyploid induction can sometimes occur in response to tissue culture conditions (Meyer et al., 2009).

*Nitrotyrosine and oryzalin experiments.* Oryzalin, nitrotyrosine, and their interaction influenced shoot survival and frequency of polyploids for 'Robert' (Fig. 2A and B). When treated with nitrotyrosine alone, shoot survival ranged from 90% to 100%, with no significant response to nitrotyrosine concentration and no polyploids produced (Fig. 2A). When paired with oryzalin, the frequency of

Table 1. Comparison of DNA contents and vegetative characteristics of diploid and autotetraploid hydrangeas.

| Ploidy | 2C DNA content (pg) | Area per leaf ($mm^2$) | Specific leaf area ($mm^2 \cdot g^{-1}$) | Leaf area/Fresh wt ($mm^2 \cdot g^{-1}$) | Internode length (mm) | Stem diam (mm) |
|---|---|---|---|---|---|---|
| *Hydrangea macrophylla* 'David Ramsey' | | | | | | |
| 2x | 4.51 | 130.8 ± 8.5 | 175.5 ± 10.4 | 35.7 ± 1.6 | 66.2 ± 4.2 | 5.5 ± 1.1 |
| 4x | 9.12* | 167.3 ± 13.8* | 207.7 ± 10.7* | 31.2 ± 0.8* | 84.4 ± 6.9* | 8.9 ± 0.3* |
| *H. macrophylla* 'Robert' (Let's Dance® Moonlight) | | | | | | |
| 2x | 4.55 | 83.9 ± 8.3 | 145.7 ± 7.1 | 29.7 ± 0.7 | 78.9 ± 4.2 | 5.4 ± 0.1 |
| 4x | 8.90* | 107.8 ± 4.0* | 130.1 ± 5.2 NS | 22.9 ± 0.5* | 94.5 ± 2.9* | 8.0 ± 0.1* |
| *H. serrata* 'MAK20' (Tuff Stuff™) | | | | | | |
| 2x | 4.22 | 82.9 ± 4.9 | 138.7 ± 4.8 | 33.0 ± 0.6 | 69.0 ± 4.9 | 4.6 ± 0.2 |
| 4x | 8.35* | 108.0 ± 6.6* | 133.8 ± 4.9 NS | 26.8 ± 1.2* | 90.9 ± 5.2* | 6.2 ± 0.3* |

[z]Values are means ± SEM. Samples sizes were as follows: 'Robert', diploid n = 9 and tetraploid n = 24; 'David Ramsey', diploid n = 10 and tetraploid n = 7; and 'MAK20', diploid n = 11 and tetraploid n = 10.

NS, *Nonsignificant or significant differences from the diploid of the same cultivar according to the least significant difference test. $P < 0.05$.

polyploid events in 'Robert' increased with a concentration of 50 μM nitrotyrosine, although linear and quadratic regression analyses results were not significant (Fig. 2B). Shoot survival of 'Robert' was lower when treated with both agents; however, regression analyses did not identify a significant trend with increasing nitrotyrosine concentrations.

Oryzalin increased polyploid frequency for 'MAK20', but nitrotyrosine and the interaction between nitrotyrosine and oryzalin did not. The frequency of induced polyploidy was higher with treatments that included 15 μM oryzalin, both with and without nitrotyrosine (Fig. 2D). Although the effect of nitrotyrosine on polyploid frequency was not statistically significant, 10% of the shoots treated with 50 μM nitrotyrosine alone were polyploid (Fig. 2C); the combination of 15 μM oryzalin and 50 μM nitrotyrosine had the highest polyploid induction (47%). There was an interaction between oryzalin and nitrotyrosine for shoot survival: increasing nitrotyrosine caused a linear decrease in survival, but only when oryzalin was present. Although 'MAK20' plantlets treated with nitrotyrosine alone demonstrated shoot survival with all treatments between 97% and 100% (Fig. 2C), the plantlet survival rate decreased with the increasing nitrotyrosine concentration to as low as 11% with the 15 μM oryzalin with 100 μM nitrotyrosine treatments (Fig. 2D).

The potential to use nitrotyrosine in combination with oryzalin for polyploid induction has some promise. Although nitrotyrosine alone had little effect, the combination of both agents increased polyploid induction, sometimes substantially. The combination of 15 μM oryzalin and 50 μM to 100 μM nitrotyrosine more than doubled the number of polyploids recovered compared with 0 μM nitrotyrosine in some cases (Fig. 2B and D). Overall, 62 polyploids were recovered from the nitrotyrosine and oryzalin experiments, including 56 mixoploids and 6 homogeneous tetraploids.

*Plant characterization.* Average DNA contents for the induced tetraploids varied from 8.35 to 9.12 pg, which was approximately double that of their diploid counterparts (range, 4.22–4.55 pg) (Table 1). Several vegetative features were larger at the tetraploid level for all tested cultivars (Table 1). The area of individual leaves was larger in tetraploid plants compared with diploids (28% to 29% increase) (Table 1). The specific leaf area was greater for tetraploid 'David Ramsey' than for the diploid counterparts, demonstrating more area per unit of dry weight. Ploidy did not affect this ratio of the other two cultivars. The ratio of the leaf area to the fresh weight was lower for tetraploids than for diploids. The stem diameter of tetraploids was 35% to 63% greater than that of the diploid counterparts, which may provide more support for large inflorescences because flopping/lodging can be problematic for some *H. macrophylla* cultivars. Internodes of tetraploids were 20% to 32% longer. An increased internode length is a potentially negative characteristic because current trends favor shorter, more compact plants. However, tetraploid plants did not appear to be larger than diploid accessions (data not collected) and had compact habits (Fig. 3). It is possible that tetraploid plants had fewer nodes, thereby achieving a similar overall size, with thicker stems, larger leaves, and larger inflorescences (in some cases), thus contributing to a more desirable appearance.

A comparison of floral characteristics showed that induced tetraploidy caused a significant decrease in inflorescence number for all cultivars ($P < 0.05$), with an average 46% reduction in flowering stems for 'MAK20', a 58% reduction for 'Robert', and 66% fewer for 'David Ramsey' (Table 2). However, tetraploid *H. macrophylla* plants had larger inflorescence widths, with average increases of 3.3 cm for 'David Ramsey' and 4.3 cm for 'Robert'. The inflorescence width did not vary between the two ploidy levels for 'MAK20'. Tetraploid 'David Ramsey' was the only cultivar with a greater number of showy flowers per inflorescence than diploids. Ploidy did not affect the diameter of the showy florets of any of the tested cultivars. An analysis of flower color using the three CIELAB color coordinates revealed that 'Robert' and 'MAK20' exhibited color differences between the diploid and tetraploid plants. Color differences were not different between 'David Ramsey' diploids and tetraploids. The L* value, which represents relative lightness (0 = black; 100 = white), showed that tetraploid 'MAK20' was darker than the diploid. The a* CIELAB parameter is the red/green axis (0 is neutral gray, with positive values representing relative redness and negative values representing relative greenness); it shifted toward red for both 'MAK20' and 'Robert' tetraploids. This shift toward slightly darker, redder flowers may be attributable to increased anthocyanin in the sepals. Schreiber et al. (2011) found evidence that *H. macrophylla* with more vibrant flower color had more anthocyanins and required more available aluminum in the soil to change from pink to blue. Our plants were not supplemented with aluminum; therefore, additional pigment would result in a deeper red color. The b* CIELAB parameter is the yellow/blue axis; it was not different for any ploidy or cultivar.

Male fertility was reduced ($P < 0.05$) in tetraploids of all cultivars. Pollen germination for diploid cultivars ranged from 19.9% to 47.9%, and it was reduced to 10.5% to 15.4% for tetraploids (Table 2). Nevertheless, the tetraploids from all three cultivars have been used in crosses

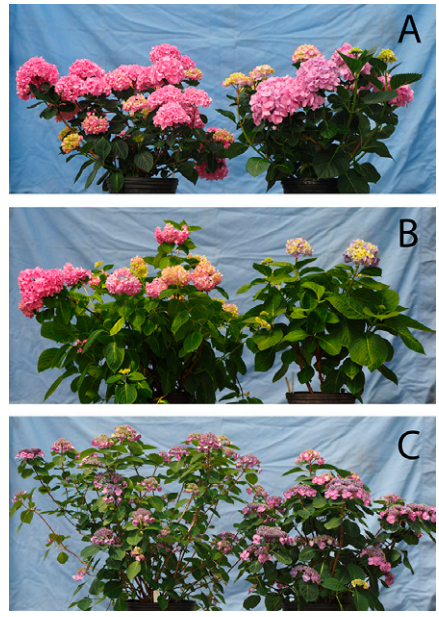


Fig. 3. Sample diploid (left) and tetraploid (right) cultivar pairs. *Hydrangea macrophylla* 'Robert' (**A**), 'David Ramsey' (**B**), and *H. serrata* 'MAK20' (**C**) plants during their third ex vitro growing season.

Table 2. Comparison of floral characteristics of diploid and autotetraploid hydrangea.

| Ploidy | Inflorescences per plant (no.) | Width of inflorescence (cm) | Showy florets per inflorescence (no.) | Diam of showy flower (mm) | Flower color (CIELAB[y]) | CIELAB parameters | | | Pollen germination (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | L* | a* | b* | |
| *Hydrangea macrophylla* 'David Ramsey' | | | | | | | | | |
| 2x | 13.4 ± 0.7[z] | 20.6 ± 0.9 | 457 ± 54 | 38 ± 2 | 76.0 6.1 –14.5 | 76.0 ± 1.3 | 6.1 ± 1.6 | −14.5 ± 1.3 | 33.2 ± 3.5 |
| 4x | 4.4 ± 0.6* | 23.9 ± 1.3* | 663 ± 106* | 42 ± 2 NS | 74.8 4.1 –15.9 | 74.8 ± 2.9 NS | 4.1 ± 1.8 NS | −15.9 ± 2.3 NS | 11.7 ± 2.9* |
| *H. macrophylla* 'Robert' (Let's Dance® Moonlight) | | | | | | | | | |
| 2x | 18.6 ± 2.1 | 19.3 ± 0.9 | 198 ± 14 | 45 ± 2 | 72.5 21.4 –11.9 | 72.5 ± 2.3 | 21.4 ± 1.3 | −11.9 ± 1.2 | 47.9 ± 3.8 |
| 4x | 7.9 ± 0.4* | 23.6 ± 0.8* | 214 ± 9 NS | 46 ± 1 NS | 69.8 27.5 –12.0 | 69.8 ± 0.7 NS | 27.5 ± 0.9* | −12.0 ± 0.5 NS | 15.4 ± 2.0* |
| *H. serrata* 'MAK20' (Tuff Stuff™) | | | | | | | | | |
| 2x | 20.7 ± 2.1 | 12.2 ± 0.5 | 34 ± 25 | 46 ± 3 | 58.4 30.2 –20.5 | 58.4 ± 2.5 | 30.2 ± 2.6 | −20.5 ± 1.2 | 19.9 ± 2.1 |
| 4x | 11.1 ± 1.3* | 13.7 ± 0.7 NS | 36 ± 29 NS | 48 ± 2 NS | 52.2 41.9 –18.2 | 52.2 ± 2.5* | 41.9 ± 2.6* | −18.2 ± 0.9 NS | 10.5 ± 3.8* |

[z]Values are means ± SEM. Samples sizes were as follows: 'Robert', diploid n = 9 and tetraploid n = 24; 'David Ramsey', diploid n = 10 and tetraploid n = 7; and 'MAK20', diploid n = 11 and tetraploid n = 10.
[y]Cells containing CIELAB color values are colored with those color designations.
NS, *Nonsignificant or significant differences from the diploid of the same cultivar according to the least significant difference test. $P < 0.05$.

as both males and females and produced viable progeny (Palmer, personal observation).

The development and use of polyploids in breeding programs can provide multiple benefits. In vitro chromosome doubling was successful, and the combination of oryzalin (15 μM) with nitrotyrosine (50 μM) yielded the greatest frequency of induced polyploids for both 'Robert' and 'MAK20'. Although the effect of induced autotetraploidy varied by cultivar, the sometimes-wider inflorescences, increase in showy floret number, larger leaves, thicker stems, and darker/redder sepals can be more desirable phenotypes (Fig. 3). However, the simple development of autotetraploids may provide limited improvements. Greater benefits may be achieved by using autopolyploids as breeding lines to increase heterozygosity, develop allopolyploids, and select for a broader range of desirable traits in diverse populations of triploids and tetraploids (Ranney, 2006).

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