Dormancy and Germination *In Vitro* Response of *Hydrangea macrophylla* and *Hydrangea paniculata* Seed to Light, Cold-Treatment and Gibberellic Acid[1]

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Abstract

Seed germination was optimized for ten *Hydrangea macrophylla* (Thunb.) Ser. and two *Hydrangea paniculata* Siebold cultivars *in vitro*. Methods were also developed to assay seed physiology. Best results for *in vitro* study were obtained with 0.5× Gamborgs solid media in conjunction with Plant Preservative Mixture (PPM), and by sterilizing seed with trichloro-s-triazinetrione (Trichlor). Assays of physiology were conducted by sterilizing seed and treating with combinations of white and red light, cold-treatment, gibberellic acid and potassium nitrate, and light cycles. Estimates of seed viability/dormancy, germination of non-dormant seed, and germination overall were calculated for each treatment combination. The most favorable conditions for overall *Hydrangea* seed germination were cold-treatment for 6 weeks, imbibition with GA_3 + KNO_3, and plating on half-strength Gamborgs media supplemented with GA_3 in the presence of white light.

Index words: 2,3,5-Triphenyl-2H-tetrazolium chloride; Ryan-Einot-Gabriel-Welsch Range test (REGWQ); stratification; ornamental; plant breeding.

Chemicals used in this study: trichloro-s-triazinetrione (Trichlor); Plant Preservative Mixture (PPM); gibberellic acid (GA_3); phytoblend agar; Gamborgs B5 + vitamins media; 2,3,5-triphenyl-2H-tetrazolium chloride (TTC); potassium nitrate (KNO_3).

Species used in this study: *H. macrophylla* (Thunb.) Ser. subsp. *serrata* (Thunb.) Makino cultivars 'Blue Bird', 'Beni Gaku', 'Intermedia', 'Omacha'; *H. macrophylla* (Thunb.) Ser. subsp. *macrophylla* cultivars 'Coerulea', 'Lady in Red', 'Nikko Blue', 'Seafoam', 'Tokyo Delight', and 'Veitchii'; *H. paniculata* Siebold cultivars 'Big Ben' and 'Pink Diamond'.

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Significance to the Nursery Industry

We have developed and optimized methods for the cultivation and assay of *Hydrangea* seed *in vitro*. This study describes the physiological effects of light, cold-treatment, and chemical treatment with GA_3 + KNO_3 upon seed germination and dormancy. Results and methods detailed in this study should prove useful to *Hydrangea* breeders seeking optimal recovery of viable mutant and cross progeny.

Introduction

Hydrangea cultivars are among the top selling deciduous flowering shrubs in the United States, and current breeding programs for this genus are using traditional methods to develop varieties of desirable and divergent ornamental traits (3, 15, 16, 20). For over three centuries, traditional breeding of *Hydrangea* has relied heavily upon observed occurrences of spontaneous mutation and chance random assortment events; more recent work has sought to increase phenotypic diversity through influx of new germplasm and wide crosses (3, 11, 17, 18). Limitations innate to these traditional methods include the relative rareness of spontaneous mutation, difficulty in obtaining and discovering wild germplasm, incompatibility of cultivars, species, and subspecies, and sterility. These limitations have been amplified as a result of only anecdotal knowledge of *Hydrangea* seed physiology and the lack of *in vitro* methods to optimize and track seed germination.

Traditional breeding methods have been used extensively to improve *H. macrophylla* cultivars and produced a range of phenotypes but these efforts are largely based on a relatively narrow germplasm base initially imported from Asia to Europe (19, 20). To date, there are no published reports of attempts to increase allelic diversity through random (chemical or radiation) or targeted (transformation) mutagenesis of *Hydrangea*. Successful application of these approaches to *Hydrangea* breeding requires established methods for growing and assessing seed *in vitro*, which is the goal of this study. Development of these methods provides an ideal framework to optimize germination rates, allow more precise physiological monitoring of viable seed produced by crosses, keep pathogen interference to a minimum, and allow weaker progeny with useful breeding potential to survive under favorable conditions that cannot be easily reproduced in soil or outside the lab. Thus, to facilitate progress in understanding *Hydrangea* seed physiology and the eventual successful application of mutagenesis, we developed *in vitro* methods to assay *Hydrangea* seed viability, dormancy, and germination. We then subjected open-pollinated *H. macrophylla* and *H. paniculata* seed from 12 cultivars to series of sterilization, media, environmental conditions, chemical, and light treatments, assaying them individually and at times in combination with one another, in order to establish optimal conditions for their initial propagation *in vitro*.

Materials and Methods

Seed collection. Commercial cultivars of *H. macrophylla* (Thunb.) Ser. subsp. *serrata* (Thunb.) Makino 'Blue Bird', 'Beni Gaku', 'Intermedia', 'Omacha' and *H. macrophylla* (Thunb.) Ser. subsp. *macrophylla*, 'Lady in Red', 'Nikko Blue', 'Seafoam', and 'Veitchii', and possible hybrids between the two subspecies, 'Coerulea' and 'Tokyo Delight' were obtained from Amethyst Hill Nursery, Aurora, OR, and grown outside under 50% shade at the USDA-ARS Southern Horticultural Laboratory in Poplarville, MS, or at

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the Mississippi State Truck Crops Branch Experiment Station in Crystal Springs, MS. Whole infructescences from these open pollinated *H. macrophylla* plants were collected in October and November of 2007, and seed were initially harvested by crushing seed pods manually between fingers or mechanically disrupting them with the aid of a one-touch electric food chopper processor (Black and Decker Corp., Towson, MD). Following harvesting, seed were initially separated from larger particulates by repeated passage through a tea strainer. Seed and smaller particulates were then rolled around on loose paper to separate seed from the finer particulates (which adhered to the paper). Open pollinated seed from *H. paniculata* 'Big Ben' and 'Pink Diamond' were obtained from Dr. Sandy Reed at the Tennessee State University Nursery Research Center in McMinnville, TN. All seed were stored at 24C (75F) in ambient relative humidity (ranging from 55 to 70%) for one to five months until their use in experiments.

Cold treatment. Empirical studies looking at different approaches to stratification revealed that most forms of moist stratification at 4C (39F) for periods of over 1 month exposed seeds to large amounts of bacterial and fungal pathogen stress, causing seed to germinate in lower numbers. Additionally, batches of moist stratified hydrangea seed were appreciably more difficult to sterilize than seed stored in cold, dry conditions as their associated pathogens seemed to have had an adequate environment (in spite of the cold temperature) to establish themselves in the seed coat at the cost of the embryo. Consequently, all cold-treated seed in our studies were kept in dry conditions within polypropylene microtubes at 4C for 6 weeks in darkness.

Seed sterilization. Sterilization methods were modeled after those detailed in Greer and Rinehart (8), Parkinson et al. (14), and Sarasan et al. (21). Prior to plating, seed were imbibed with agitation in solution containing 300 ppm of Trichlor (trichloro-s-triazinetrione, Pool Time Products, Buford, GA) for 24 hours. The resultant supernatant was decanted and a second wash of 2500 ppm Trichlor was applied to the seed with agitation for 10–15 minutes. This wash was decanted and seeds were then suspended in a solution containing appropriate volumes (1–3 ml, enough to allow even seed dispersal across the plate surface) of 0.2% agar and 1000 ppm Trichlor for immediate plating. Sterilized seed were sown in petri dishes (100–500 seed per dish) containing solid media.

Media and chemicals used for experimental treatments. Control *Hydrangea* seed were imbibed in the presence of 300 ppm Trichlor for 24 hours. Experimental 'treated' seed were imbibed with 1300 ppm gibberellic acid (GA_3, PhytoTechnology Laboratories) and 1000 ppm KNO_3 (Acros Organics, Morris Plains, NJ) in addition to 300 ppm Trichlor for 24 hours as part of the standard sterilization regimen (prior to plating). All *Hydrangea* seed were plated and allowed to germinate in 100 × 15 mm (3.9 × 0.6 in) petri dishes (VWR International, West Chester, PA) on solid media that contained 0.5% phytoblend agar (Caisson Laboratories, North Logan, UT), 0.5× Gamborgs B5 + vitamins (PhytoTechnology Laboratories; 6), and 0.2% Plant Preservative Mixture (PPM, Plant Cell Technology, Inc., Washington, DC) a broad-spectrum biocide/fungicide for plant tissue culture containing a proprietary active ingredient mixture of methylchloroisothiazolinone, methylisothiazolinone, magnesium chloride, magnesium nitrate, potassium sorbate and sodium benzoate (9). Experimental, or treated seed plates were additionally supplemented with GA_3 at 130 ppm final concentration.

Light conditions for experimental treatments. For all white light and red light seed response comparisons, seeds sown in petri dish plates were placed on shelving under 40W Sylvania Gro-Lux lights for 21 days at 24C (75F) with or without red cellophane completely covering the lights. The range of visible light emitted from the red cellophane was from 610–750 nm as measured by a Perkin Elmer (Waltham, MA) Lambda3B spectrophotometer. Photosynthetic photon flux (measured with an Apogee QMSW-5S quantum meter, Apogee Instruments Inc., Logan, UT) reaching the plates was 32 μmol quanta·m^{-2}·s^{-1} for uncovered lights and 15 μmol quanta·m^{-2}·s^{-1} levels for lights covered with red cellophane. Illuminance, as measured by a Control Company Model #4332 light meter (Control Company, Friendswood, TX) was approximately 2200 lux for white (uncovered) light reaching the plates and 650 lux for red (cellophane-covered) light reaching the plates. For all experiments except those denoted as 24 hr light, seeds were subject to a 16:8 (light:dark) hour photoperiod. To minimize additional light artifacts, all plates were placed under their respective light sources in a random design where they remained until germination data was collected.

Three day dormancy controls. To better understand change in dormancy states over the course of 21 day incubation, 3 day controls were done to estimate initial dormancy states of imbibed seed. For comparison with experimental treatments, batch replicates of seed from each cultivar were cold-treated, sterilized, imbibed in GA_3 + KNO_3 and plated on 0.5× Gamborg plates supplemented with GA_3 (exactly like cold-treated and GA_3 + KNO_3 imbibed seed destined for 21 day incubation). Controls were then incubated under 24 hr continuous red light (650 lux) at 24C (75F) within a random design placement for three days, and then were removed for TTC staining and processing.

Seed staining and image acquisition. After 21 days of incubation for treatment plates (3 days for dormancy controls), the total number of germinated seed per plate were manually quantified with the aid of an Olympus CO-11 Stereo microscope (Olympus America Inc., Center Valley, PA). Seed were counted as germinated if the seed coat had been clearly broken and a radicle of any size had emerged. After germination counts were recorded for each plate, 10 ml of freshly made 0.2% 2,3,5-triphenyl-2H-tetrazolium chloride (TTC; Alfa Aesar, Ward Hill, MA) was added to each plate (enough to completely cover and submerge all the seed) and each plate was immediately put in complete darkness for 48 hours. After 48 hours, all the material on each plate was photographed at 7.1 megapixel resolution under bright white light conditions with a Canon Powershot A560 digital camera (Canon USA Inc., Lake Success, NY) and each image digitally processed with the aid of Picasa (Google Inc., Mountain View, CA).

Image processing and seed quantification. Stained plate images were all processed and analyzed as reported in Greer

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and Rinehart (8). Briefly, three respective color filtered pictures were generated for each plate image, with the first picture exposing all seeds and contaminating particulate matter on the plate, the second picture exposing only TTC-stained (non-dormant/viable) seed and contaminating particulates, and a third image exposing only contaminating particulates on the plate. Visible particles in each filtered picture were quantified with the aid of the 'Analyze Particles' in ImageJ (1). Average area ranged from 0.25 to 0.40 mm^2 for *H. macrophylla* seed and from 0.65 to 1.0 mm^2 for *H. paniculata* seed. Within our pictures, particles that fell within the appropriate range were counted; particles that did not fall within our established area ranges were not quantified. Total numbers of (yellow, unstained) dormant or dead seeds for each sample was calculated subtracting the number of total particles in image two from image one, and the total number of non-dormant (red, TTC-stained) seeds for each sample was calculated subtracting the number of particles in image three from the particles counted in image two.

Calculations and statistical analyses. Percentage of viable/non-dormant seed (%ND) for each plate was calculated by adding the numbers of germinated and non-dormant (red, TTC-stained) seed and dividing the sum by the total of non-dormant (R), dormant (Y) and germinated (G) seed: (G + R) / (G + R + Y). Percent germination of viable/non-dormant (%GND) seed on a plate was calculated by dividing the number of germinated seed by the sum of the germinated seed and the non-dormant seed on a plate: G / (G + R). The germination index (GINDX, a measure of total germination expected per amount of dormant and non-dormant seed) was calculated by multiplying %ND by %GND.

Univariate Analysis of Variance (ANOVA, $\alpha = 0.05$) and Ryan-Einot-Gabriel-Welsch Range (REGWQ) posthoc tests ($\alpha = 0.05$) were conducted with SPSS software (SPSS Incorporated, Chicago, IL) to analyze significance. When transformation was required to meet assumptions of normality or equal variances, data sets were Tukey normal scored using the formula (r – 1/3) / (w + 1/3), where r is the rank and w is the sum of the case weights (see 24). One set of ANOVA were conducted to ascertain treatment affects within individual cultivars and a separate set of larger (full factor) ANOVA were also conducted which proportionally ranked factor contributions to overall variance in terms of partial eta-square (η^2). Values for η^2 range from 0 to 1 with 0 indicating no relationship and 1 indicating the strongest relationship between an individual factor and the variance of a dependent variable. Traditionally, η^2 values of 0.01, 0.06, and 0.14 represent small, medium, and large effect sizes, respectively (7). Type II error statistics (β) were also calculated. Values for β range from 0 to 1 and correspond directly to the chance of committing Type II error in an ANOVA F test. The power of an ANOVA F test can be calculated as $1 - \beta$, yielding the probability that the F test will detect the differences between groups equal to those implied by sample differences (23).

Results and Discussion

Cultivar differences. Substantial maternal cultivar differences of dormancy and germination were evident early on in all our experiments. Of all the potential factors affecting these aspects of seed physiology, maternal cultivar was the by far the largest contributor to variance (%ND: $F_{11, 168} = 42.143$, $P < 0.0005$, $\eta^2 = 0.734$, $\beta < 0.0005$; %GND: $F_{11, 168} = 29.557$, $P < 0.0005$, $\eta^2 = 0.659$, $\beta < 0.0005$; GINDX: $F_{11, 168} = 71.136$, $P < 0.0005$, $\eta^2 = 0.823$, $\beta < 0.0005$). However, our main objective was to investigate differences in treatments and not evaluate germination across all hydrangea cultivars. Thus, to better ascertain treatment effects within cultivar, we replicated each individual cultivar treatment in triplicate (*n = 3)* and only reported respective means and effects of treatments upon %ND, %GND, and GINDX within cultivar in Tables 1, 2, and 3.

Time of in vitro incubation. Germination rates for all *Hydrangea* seed had peaked by day 21 in all of our experiments. Three day control values for percent non-dormant seed were as follows: 'Blue Bird' 6.0 ± 5.2; 'Beni Gaku' 25.6 ± 6.5; 'Coerulea' 14.9 ± 3.5; 'Intermedia' 11.6 ± 1.0; 'Lady in Red' 12.0 ± 2.3; 'Nikko Blue' 28.8 ± 8.7; 'Seafoam' 22.6 ± 1.9; 'Omacha' 26.3 ± 2.6; 'Tokyo Delight' 17.4 ± 9.0; 'Veitchii' 19.1 ± 4.4; 'Big Ben' 55.7 ± 21.7; 'Pink Diamond' 78.4 ± 12.3. Three day %ND values were consistently lower than overall %ND values calculated at 21 days (Tables 1, 2 and 3)

Defining seed dormancy in terms that are distinctly separate from the absence of germination is a relatively recent development in seed physiology study (see ref. 5 for a recent review) and to date there are few species-specific seed physiology studies that address this issue. Typically, seed staining procedures incorporating TTC are used to estimate viability in terms of whether a seed is alive or dead. TTC staining relies heavily on the biochemistry involved. The reduction of TTC is catalyzed by dehydrogenases of metabolically active tissue, particularly those dehydrogenases found in the malic and alcohol systems (22) and the final product of the reaction is formazan (12), an insoluble red dye that remains in the respective tissue. Effects of dormancy upon TTC staining have rarely been accounted for in seed physiology studies; however, our prior study has illustrated that in any particular pool of *Hydrangea* seed there is a portion that are dormant (but alive) and incapable of being discriminately stained with TTC (8). In this study, identical TTC staining methods were applied to *Hydrangea* seed incubated upon plates for 3 days versus 21 days. The consistent use of digital means to assess the red color signal provided by TTC in our experiments allowed us to minimize bias when determining TTC staining results. When using these methods, there were consistently lower percentages of red stained seed present on plates incubated for 3 days than there were on plates that were incubated for 21 days. Since such results would be impossible if our TTC methods were applicable only to the estimation of seed viability, the best explanation for these staining differences is that our procedure allowed us to demarcate elevated seed coat permeability, the induction or increase of energy-dependent metabolic processes needed for proliferation of the embryo (producing larger amounts of discernable red color), or both. Similar to a prior study of *Hydrangea* seed (8), indirect estimation of some type of dormancy within the seed was obtained, although the type of dormancy (either physiological or physical, or a combination of the two) has yet to be determined.

Overall effects of red and white light treatments. As a whole, red light and white light (16:8 hour day:night photoperiod) treatment had substantial effects on dormancy ($\eta^2 = 0.088$), insignificant effects upon germination of non-dormant seed, and substantial effects on overall germina-

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Table 1. *In vitro* non-dormancy assessments of open-pollinated seed planted under 16:8 (day:night photoperiod) white or red light cycle conditions. Calculated non-dormancy for each cultivar and treatment is reported as mean percentage (%ND) ± SE (n = 3).

Cultivar	White light				
	NC/NT[z]	NC/T	C/NT	C/T	C/T/24
H. macrophylla subsp. serrata					
Bluebird	30.8 ± 1.2 (A)[x]	19.7 ± 1.1 (B)	28.3 ± 3.1 (AB)	24.0 ± 1.4 (AB)	28.0 ± 1.2 (AB)
Beni Gaku	63.3 ± 7.1 (A)	39.9 ± 3.8 (AB)	36.0 ± 4.7 (BC)	32.0 ± 2.8 (BC)	34.5 ± 2.3 (BC)
Intermedia	50.3 ± 3.9 (A)	34.9 ± 3.2 (AB)	30.9 ± 3.9 (AB)	23.9 ± 1.1 (B)	25.6 ± 1.8 (B)
Omacha	34.7 ± 4.4 (A)	20.9 ± 4.1 (A)	22.3 ± 2.8 (A)	30.1 ± 2.1 (A)	21.7 ± 5.9 (A)
H. macrophylla subsp. macrophylla					
Coerulea	40.5 ± 9.3 (AB)	38.2 ± 3.2 (AB)	46.0 ± 1.6 (A)	33.0 ± 2.7 (AB)	35.0 ± 1.1 (AB)
Lady in Red	27.9 ± 5.1 (A)	19.8 ± 3.5 (A)	31.6 ± 1.3 (A)	24.5 ± 1.0 (A)	21.9 ± 4.6 (A)
Nikko Blue	72.7 ± 5.8 (A)	44.2 ± 4.4 (BC)	69.6 ± 5.1 (AC)	42.4 ± 1.2 (BC)	42.4 ± 2.3 (BC)
Seafoam	41.3 ± 4.4 (A)	27.6 ± 3.0 (A)	46.5 ± 3.4 (A)	33.7 ± 5.2 (A)	37.5 ± 6.1 (A)
Tokyo Delight	17.6 ± 2.5 (ABC)	14.6 ± 0.7 (ABC)	28.1 ± 3.6 (A)	20.3 ± 1.7 (ABC)	13.2 ± 0.6 (B)
Veitchii	65.0 ± 9.5 (A)	23.9 ± 2.3 (B)	50.8 ± 3.9 (A)	35.8 ± 0.9 (AC)	28.8 ± 1.0 (BC)
H. paniculata:					
Big Ben	95.4 ± 1.3 (A)	96.1 ± 1.3 (A)	87.0 ± 5.8 (AB)	78.9 ± 2.3 (BC)	48.7 ± 0.9 (BC)
Pink Diamond	87.7 ± 3.1 (AB)	90.4 ± 1.3 (AB)	89.0 ± 6.8 (A)	67.0 ± 11.1 (ABC)	31.3 ± 5.0 (C)

Cultivar	Red light			
	NC/T	C/NT	C/T	C/T/24
H. macrophylla subsp. serrata				
Bluebird	25.1 ± 2.1 (AB)	32.2 ± 5.1 (AB)	26.9 ± 2.0 (AB)	20.3 ± 3.3 (AB)
Beni Gaku	37.1 ± 3.1 (ABC)	47.6 ± 3.2 (AB)	27.1 ± 2.1 (C)	26.7 ± 2.1 (C)
Intermedia	30.8 ± 6.3 (AB)	29.3 ± 0.7 (AB)	25.7 ± 3.8 (B)	26.3 ± 0.9 (AB)
Omacha	23.1 ± 5.8 (A)	19.7 ± 2.9 (A)	25.0 ± 0.9 (A)	24.2 ± 5.5 (A)
H. macrophylla subsp. macrophylla				
Coerulea	34.2 ± 3.1 (AB)	40.7 ± 2.5 (AB)	32.7 ± 0.5 (AB)	28.6 ± 4.2 (B)
Lady in Red	17.3 ± 3.7 (A)	31.1 ± 2.4 (A)	30.2 ± 1.6 (A)	22.8 ± 1.1 (A)
Nikko Blue	29.1 ± 4.0 (D)	43.2 ± 1.8 (BC)	31.4 ± 4.2 (BD)	32.8 ± 0.4 (BD)
Seafoam	33.3 ± 8.5 (A)	37.5 ± 5.2 (A)	34.0 ± 3.0 (A)	28.9 ± 1.4 (A)
Tokyo Delight	14.3 ± 1.0 (BC)	16.8 ± 1.8 (ABC)	24.1 ± 0.7 (AC)	20.2 ± 5.4 (ABC)
Veitchii	30.4 ± 0.9 (BC)	34.3 ± 2.0 (AC)	28.7 ± 3.3 (BC)	30.9 ± 1.0 (BC)
H. paniculata:				
Big Ben	44.2 ± 5.5 (C)	62.8 ± 13.8 (BC)	81.2 ± 4.5 (BC)	49.7 ± 4.7 (BC)
Pink Diamond	59.6 ± 4.0 (ABC)	68.5 ± 12.7 (ABC)	84.0 ± 6.8 (AB)	44.1 ± 7.4 (BC)

[z]NC = not cold-treated; C = cold-treated; T = treated with GA and KNO_3; NT = not treated with GA and KNO_3; 24 = 24 hour continuous light exposure.

[x]Letters in parentheses that follow after %ND ± SE values are the results of REGWQ posthoc analyses ($P < 0.05$) from Univariate ANOVA ($\alpha = 0.05$) of all the treatments conducted within respective cultivars. Thus, within cultivar, different letters between treatments signify that corresponding values of %ND differed significantly.

tion ($\eta^2 = 0.069$) upon all of the *Hydrangea* cultivars used in this study. Interactive effects of cultivar and light upon germination and dormancy were also quite large (%ND $\eta^2 = 0.186$; %GND $\eta^2 = 0.162$; GINDX $\eta^2 = 0.173$). Other interactive effects of red and white light with (and without) GA_3 + KNO_3 treatment and cold-treatment were relatively small and insignificant.

Broad definitive effects of red versus white light upon *H. macrophylla* seed were not evident for dormancy and overall germination. Red versus white light effects upon *H. paniculata* seed physiology for the most part mirrored that seen in *H, macrophylla,* but there were trends for increased %ND (Table 1) and increase of overall germination (Table 3) under white light in cases where *H. paniculata* seed were not cold-treated but chemically treated, or cold-treated and not chemically treated.

Overall effects of chemical treatment. Although interactive effects of cultivar and chemical treatment upon germination and dormancy were quite large (%ND $\eta^2 = 0.204$; %GND $\eta^2 = 0.136$; GINDX $\eta^2 = 0.313$), other interactive effects of GA_3 + KNO_3 treatment with light and cold-treatment were relatively small and insignificant. Interestingly, all of our observed chemical effects were most likely attributable solely to the presence of GA_3 since 1250 ppm KNO_3 is innate to the 0.5× Gamborgs media used in all of our experiments. GA_3 is commonly used as an exogenous treatment to force seeds to overcome dormancy and reports of GA_3 prolonging seed dormancy, which is evident in our data, are rare (see 10, 25). Increase of germination in response to GA_3 has been proposed to occur because of increases in embryo growth potential and a conferred ability of the embryo to overcome the mechanical restraint conferred by the seed-covering layers by weakening tissues surrounding the radicle (5). When our *Hydrangea* seed were imbibed with GA_3 + KNO_3 and plated on media containing GA_3, the resulting changes in dormancy and non-dormant germination differed substantially from seed that was imbibed only in 300 ppm Trichlor

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Table 2. *In vitro* germination of open-pollinated non-dorma nt seed planted under 16:8 (day:night photoperiod) white or red light cycle conditions. Calculated non-dormancy for each cultivar and treatment is reported as mean percentage (%GND) ± SE (n = 3).

Cultivar	White light				
	NC/NT[z]	NC/T	C/NT	C/T	C/T/24
H. macrophylla subsp. serrata					
Bluebird	39.9 ± 1.9 (A)[x]	55.5 ± 6.3 (A)	42.9 ± 5.4 (A)	45.9 ± 5.6 (A)	53.0 ± 5.7 (A)
Beni Gaku	28.8 ± 2.0 (A)	44.7 ± 4.6 (ABC)	54.3 ± 5.4 (BC)	55.9 ± 7.7 (B)	85.5 ± 3.1 (D)
Intermedia	21.8 ± 2.8 (A)	42.0 ± 4.7 (ABC)	30.2 ± 4.6 (AB)	43.5 ± 8.5 (ABC)	65.7 ± 7.6 (C)
Omacha	4.1 ± 1.2 (A)	27.9 ± 3.3 (B)	23.2 ± 3.3 (AB)	22.2 ± 4.1 (AB)	26.6 ± 2.7 (B)
H. macrophylla subsp. macrophylla					
Coerulea	47.7 ± 5.8 (A)	42.6 ± 2.4 (A)	44.2 ± 1.0 (A)	55.2 ± 0.8 (AB)	66.5 ± 2.4 (B)
Lady in Red	8.2 ± 2.7 (A)	15.6 ± 0.6 (AB)	28.2 ± 3.4 (BC)	31.8 ± 2.4 (BC)	64.4 ± 9.7 (D)
Nikko Blue	62.1 ± 4.6 (A)	56.3 ± 1.8 (A)	50.3 ± 11.6 (A)	63.5 ± 5.6 (A)	75.9 ± 3.8 (A)
Seafoam	37.2 ± 8.5 (A)	75.4 ± 7.8 (B)	57.8 ± 1.5 (AB)	50.2 ± 5.9 (AB)	69.6 ± 4.5 (B)
Tokyo Delight	30.9 ± 3.3 (A)	41.6 ± 6.8 (A)	30.5 ± 4.6 (A)	43.4 ± 4.3 (A)	46.4 ± 3.3 (A)
Veitchii	34.9 ± 2.9 (A)	65.5 ± 9.1 (B)	33.7 ± 4.1 (A)	57.8 ± 2.1 (AB)	66.6 ± 1.5 (B)
H. paniculata:					
Big Ben	30.8 ± 3.6 (A)	43.5 ± 3.6 (AB)	42.9 ± 3.0 (AB)	50.9 ± 2.9 (BC)	63.3 ± 9.9 (BC)
Pink Diamond	30.1 ± 6.2 (A)	44.3 ± 8.0 (A)	26.5 ± 1.8 (A)	44.6 ± 3.8 (A)	50.0 ± 12.7 (A)
	Red light				
Cultivar		NC/T	C/NT	C/T	C/T/24
H. macrophylla subsp. serrata					
Bluebird		53.7 ± 7.4 (A)	44.7 ± 10.0 (A)	53.8 ± 2.0 (A)	55.0 ± 4.7 (A)
Beni Gaku		39.6 ± 5.4 (AB)	53.6 ± 3.8 (BC)	60.8 ± 3.0 (BC)	77.9 ± 2.4 (CD)
Intermedia		32.6 ± 5.8 (AB)	40.3 ± 3.4 (ABC)	45.7 ± 7.3 (BC)	51.6 ± 8.7 (BC)
Omacha		10.8 ± 3.0 (AB)	25.0 ± 5.8 (AB)	20.0 ± 3.2 (AB)	26.3 ± 6.6 (B)
H. macrophylla subsp. macrophylla					
Coerulea		55.7 ± 5.4 (AB)	57.6 ± 1.4 (AB)	56.4 ± 1.3 (AB)	67.3 ± 2.2 (B)
Lady in Red		4.8 ± 1.3 (A)	31.5 ± 5.4 (BC)	32.0 ± 6.7 (BC)	43.1 ± 5.7 (CD)
Nikko Blue		68.5 ± 4.5 (A)	69.2 ± 2.0 (A)	58.5 ± 3.1 (A)	64.9 ± 5.9 (A)
Seafoam		37.6 ± 2.2 (A)	65.6 ± 8.0 (AB)	49.0 ± 4.8 (AB)	59.6 ± 9.9 (AB)
Tokyo Delight		39.1 ± 6.7 (A)	46.5 ± 9.9 (A)	27.6 ± 3.1 (A)	41.7 ± 8.6 (A)
Veitchii		49.8 ± 1.4 (AB)	64.7 ± 2.5 (B)	64.9 ± 7.1 (B)	50.9 ± 3.4 (AB)
H. paniculata:					
Big Ben		65.4 ± 7.1 (BC)	31.4 ± 5.3 (A)	44.5 ± 5.2 (AB)	74.3 ± 6.4 (C)
Pink Diamond		40.7 ± 4.1 (A)	29.2 ± 6.3 (A)	37.3 ± 7.1 (A)	46.9 ± 11.4 (A)

[z]NC = not cold-treated; C = cold-treated; T = treated with GA and KNO_3; NT = not treated with GA and KNO_3; 24 = 24 hour continuous light exposure.

[x]Letters in parentheses that follow after %GND ± SE values are the results of REGWQ posthoc analyses ($P < 0.05$) from Univariate ANOVA ($\alpha = 0.05$) of all the treatments conducted within respective cultivars. Thus, within cultivar, different letters between treatments signify that corresponding values of %GND differed significantly.

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and plated on 0.5% Gamborgs + vitamins / 0.2% PPM (%ND $\eta^2 = 0.138$; %GND $\eta^2 = 0.109$). In spite of these differences, our data suggest that treatment of *Hydrangea* seed with GA_3 and KNO_3 in efforts to increase overall germination will not always meet with success because chemical treatment on overall germination of *Hydrangea* seed (GINDX) proved insignificant. Closer scrutiny of %ND means (Table 1) versus %GND means (Table 2) reveals that chemical treatment actually trended toward prolonging dormancy of seed in cultivars but this proclivity is offset by trends of increased germination of non-dormant seed. The distinctive effects of GA_3 upon *Hydrangea* seed are illustrative of the fact that dormancy, germination of non-dormant need, and overall germination are more discreet indicators for understanding *Hydrangea* seed physiology than many current approaches of assaying seed viability.

Overall effects of dry cold-treatment. As a whole, cold-treatment had insignificant effects on *Hydrangea* seed dormancy even though significant interactive effects of cultivar and cold-treatment (%ND $\eta^2 = 0.204$) indicate that the dormancy responses of individual cultivars were distinct from one another. Cold-treatment did have a substantial effect upon the germination of non-dormant seed ($\eta^2 = 0.084$) as well as overall germination of *Hydrangea* seed ($\eta^2 = 0.112$); and similar to dormancy, there also were large interactive effects of cultivar and cold-treatment upon germination (%GND $\eta^2 = 0.247$; GINDX $\eta^2 = 0.230$) indicating unique responses to cold-treatment from individual cultivars. Interactive (additive) effects of cold-treatment upon other treatments were insignificant. However, individual effects of cold-treatment were evident within GA_3 and KNO_3 imbibed, as well as red versus white light treated cultivars, where in almost all cases, differences caused by GA_3 and KNO_3, or light treatments were minimized when seeds were cold-treated (Tables 1, 2, and 3)

Thus, for *in vitro* study, the effects of cold-treatment upon *Hydrangea* seed were proven to be beneficial for increasing

Table 3. *In vitro* germination indices of open-pollinated seed planted under 16:8 (day:night photoperiod) white or red light cycle conditions. Calculated non-dormancy for each cultivar and treatment is reported as mean percentage (%GND) ± SE (n = 3).

	White light				
Cultivar	**NC/NT**[z]	**NC/T**	**C/NT**	**C/T**	**C/T/24**
H. macrophylla subsp. serrata					
Bluebird	0.123 ± 0.010 (A)[x]	0.108 ± 0.006 (A)	0.118 ± 0.002 (A)	0.110 ± 0.017 (A)	0.150 ± 0.022 (A)
Beni Gaku	0.183 ± 0.028 (AB)	0.175 ± 0.005 (AB)	0.195 ± 0.027 (AB)	0.174 ± 0.009 (AB)	0.297 ± 0.031 (C)
Intermedia	0.108 ± 0.008 (A)	0.146 ± 0.018 (A)	0.090 ± 0.004 (A)	0.102 ± 0.017 (A)	0.170 ± 0.028 (A)
Omacha	0.014 ± 0.003 (A)	0.057 ± 0.009 (ABC)	0.050 ± 0.001 (ABC)	0.065 ± 0.007 (B)	0.055 ± 0.009 (ABC)
H. macrophylla subsp. macrophylla					
Coerulea	0.189 ± 0.037 (A)	0.164 ± 0.021 (A)	0.203 ± 0.010 (A)	0.182 ± 0.015 (A)	0.233 ± 0.015 (A)
Lady in Red	0.026 ± 0.013 (A)	0.031 ± 0.007 (AB)	0.089 ± 0.010 (BC)	0.078 ± 0.008 (BC)	0.136 ± 0.027 (D)
Nikko Blue	0.453 ± 0.060 (A)	0.248 ± 0.018 (BC)	0.340 ± 0.057 (AB)	0.270 ± 0.030 (BC)	0.323 ± 0.028 (AB)
Seafoam	0.152 ± 0.033 (A)	0.204 ± 0.016 (A)	0.270 ± 0.026 (A)	0.168 ± 0.027 (A)	0.263 ± 0.050 (A)
Tokyo Delight	0.054 ± 0.006 (A)	0.060 ± 0.007 (A)	0.084 ± 0.009 (A)	0.088 ± 0.009 (A)	0.061 ± 0.002 (A)
Veitchii	0.222 ± 0.020 (A)	0.158 ± 0.030 (A)	0.169 ± 0.015 (A)	0.207 ± 0.012 (A)	0.192 ± 0.004 (A)
H. paniculata:					
Big Ben	0.294 ± 0.033 (AB)	0.418 ± 0.037 (A)	0.374 ± 0.038 (AB)	0.401 ± 0.021 (A)	0.309 ± 0.051 (AB)
Pink Diamond	0.268 ± 0.062 (A)	0.401 ± 0.073 (A)	0.238 ± 0.033 (A)	0.307 ± 0.078 (A)	0.159 ± 0.046 (A)
	Red light				
Cultivar		**NC/T**	**C/NT**	**C/T**	**C/T/24**
H. macrophylla subsp. serrata					
Bluebird		0.134 ± 0.018 (A)	0.136 ± 0.020 (A)	0.145 ± 0.014 (A)	0.110 ± 0.015 (A)
Beni Gaku		0.144 ± 0.008 (A)	0.253 ± 0.011 (BC)	0.163 ± 0.005 (AB)	0.208 ± 0.017 (ABC)
Intermedia		0.095 ± 0.017 (A)	0.118 ± 0.009 (A)	0.112 ± 0.004 (A)	0.136 ± 0.024 (A)
Omacha		0.021 ± 0.003 (AC)	0.046 ± 0.005 (ABC)	0.050 ± 0.007 (ABC)	0.060 ± 0.013 (BC)
H. macrophylla subsp. macrophylla					
Coerulea		0.188 ± 0.012 (A)	0.235 ± 0.016 (A)	0.184 ± 0.004 (A)	0.193 ± 0.030 (A)
Lady in Red		0.009 ± 0.005 (A)	0.101 ± 0.023 (CD)	0.095 ± 0.017 (CD)	0.097 ± 0.008 (CD)
Nikko Blue		0.203 ± 0.042 (BC)	0.300 ± 0.019 (ABC)	0.186 ± 0.035 (C)	0.213 ± 0.022 (BC)
Seafoam		0.125 ± 0.033 (A)	0.242 ± 0.031 (A)	0.164 ± 0.005 (A)	0.172 ± 0.026 (A)
Tokyo Delight		0.056 ± 0.011 (A)	0.075 ± 0.011 (A)	0.067 ± 0.009 (A)	0.080 ± 0.020 (A)
Veitchii		0.151 ± 0.001 (A)	0.222 ± 0.016 (A)	0.181 ± 0.004 (A)	0.158 ± 0.013 (A)
H. paniculata:					
Big Ben		0.282 ± 0.013 (AB)	0.191 ± 0.046 (B)	0.361 ± 0.042 (AB)	0.365 ± 0.022 (AB)
Pink Diamond		0.239 ± 0.009 (A)	0.208 ± 0.062 (A)	0.304 ± 0.039 (A)	0.192 ± 0.029 (A)

[z]NC = not cold-treated; C = cold-treated; T = treated with GA and KNO3; NT = not treated with GA and KNO3; 24 = 24 hour continuous light exposure.

[x]Letters in parentheses that follow after GINDX ± SE values are the results of REGWQ posthoc analyses (P < 0.05) from Univariate ANOVA ($\alpha = 0.05$) of all the treatments conducted within respective cultivars. Thus, within cultivar, different letters between treatments signify that corresponding values of GINDX differed significantly.

overall numbers of germinated seed within the course of 21 days. In all cases, cold-treatment did not significantly hinder germination and in many cases overall germination increased 10–40% depending on conditions. Although prior suggestion that stratification is not necessary for germination of *H. macrophylla* and *H. paniculata* seed (2, 3, 4, 13) is still valid, our data show that dry cold-treatment of seed for these species should be considered when trying to improve germination rates.

Photoperiod effects. Additional ANOVA conducted to ascertain photoperiod impact (16:8 vs. 24 hr; red vs. white light) upon seed dormancy revealed effects that were statistically significant ($F_{1, 140} = 4.135$, $P = 0.044$) but relatively small ($\eta^2 = 0.029$, $\beta = 0.476$). Observed photoperiod effects were additive to the effects of GA and KNO_3 treatment and cold-treatment. Differences were most pronounced in our *H. paniculata* cultivars, where 24 hour light caused an overall trend for prolonging dormancy (Table 1, S/T vs. S/T/24). In contrast to dormancy, the effects of different light cycle exposures upon germination of non-dormant seed was significant and substantial ($F_{1, 140} = 19.934$, $P < 0.0005$, $\eta^2 = 0.125$, $\beta = 0.007$) with 24 hour light causing an increase in %GND in most cases (Table 2, S/T vs. S/T/24). Effects upon overall germination of *Hydrangea* seed (GINDX) were statistically insignificant, although there was a trend for increased germination under 24 hour light in most of the *H. macrophylla* cultivars (Table 3, S/T vs. S/T/24). This same trend was not seen in *H. paniculata* cultivars. Confounding effects of red versus white light were insignificant and minimal (all $\eta^2 < 0.013$) in all cases, thus seed physiology differences observed within cultivars were due to the extended amount of light to which the seed were exposed. In applied terms, 24 hour light is an effective treatment to use in efforts to increase the overall germination (GINDX) of *H. macrophylla* seed, but these same benefits will not be realized when applied to *H. paniculata* seed as a result of the increased dormancy.

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Benefits of the developed in vitro method. *Hydrangea* seed physiology observations have relied primarily on anecdotal evidence, and prior to this study there have been no published data of *Hydrangea* seed germination rates or germling survival. This may be due to the fact that empirical observations of seed viability are usually done in soil and since most *Hydrangea* seeds are so small and quite hard to recover once sown in soil, initial and final counts of viability/non-dormant seeds in addition to the numbers of very small resultant radicles would be extremely hard, if not impossible to calculate. Our *in-vitro* conditions allowed us to better observe the emergence of radicles and calculate the results of TTC staining in relevant real time. When our *in vitro* soilless substrate conditions were utilized, we observed initial germination of *Hydrangea* seed around 7–10 days post plating; and germlings survived more than 21 days on plates before final transplantation into a soil substrate. Germination indexes of open pollinated seed calculated in this study also show that germination yields for different hydrangea cultivars can range from 6 to over 40% when favorable (soilless substrate) conditions are employed. The highest overall rates of *in vitro* germination of *Hydrangea* seed were obtained with dry cold-treatment for 6 weeks, imbibition with GA_3 + KNO_3, and plating on half-strength Gamborgs media supplemented with GA_3 in the presence of white light (24 hour for *H. macrophylla*, 16:8 (day:night) hour photoperiod for *H. paniculata*). Seed used in this study came from open pollinated plants and some of the variation observed in seed physiological responses is undoubtedly linked to paternal alleles. In spite of this fact, seed response to treatment was remarkably consistent within each open-pollinated maternal cultivar.

We have successfully developed *in vitro* methods for the cultivation and assay of *Hydrangea* seed. Using these methods, we have elucidated physiological effects of light, cold-treatment, and chemical treatment with GA_3 + KNO_3 upon seed germination and dormancy. Results and methods disseminated in this study should prove useful to *Hydrangea* breeders seeking optimal recovery of cross progeny and mutants.

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