

# Production and Verification of *Hydrangea arborescens* ‘Dardom’ × *H. involucrata* Hybrids

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**Abstract.** Previous attempts to use interspecific hybridization to combine flower color and cold hardiness in *Hydrangea* have not produced the desired results, with confirmed hybrids being weak, sterile or aneuploid. In all cases, *H. macrophylla* (Thumb.) Ser. was used as the source of flower color. This work investigates the use of *H. involucrata* Sieb. as an alternative source of flower color in *Hydrangea* interspecific hybridizations. Controlled reciprocal pollinations of *H. involucrata* with two cultivars of *H. arborescens* L. and three cultivars of *H. paniculata* Sieb. were made. Hybridity of progeny was verified using RAPD markers and confirmed with chromosome counts and morphological comparisons of hybrids and parents. Plants were obtained only when *H. involucrata* was used as the pollen parent. No hybrids between *H. paniculata* or *H. arborescens* ‘Annabelle’ and *H. involucrata* were produced. Seven *H. arborescens* ‘Dardom’ × *H. involucrata* progeny showed either a sum of the RAPD bands of both parents or banding patterns that matched those of *H. involucrata*. Leaf blade length and length/width ratio of the hybrid were intermediate to its parents. Chromosome number in the hybrid ( $2n = 34$ ) was also intermediate between *H. arborescens* ( $2n = 38$ ) and *H. involucrata* ( $2n = 30$ ). One ‘Dardom’ × *H. involucrata* plant flowered in 2005. While pollen staining indicated a very low level of fertility, we will continue to evaluate the possibility of using the hybrid for producing advanced filial or backcross progeny.

The genus *Hydrangea* includes at least 23 species and has an Asian-American distribution (McClintock, 1957). The genus is divided into section *Hydrangea*, which contains the temperate climate species, and section *Cornidia*, which consists of vining species from tropical and subtropical climates. As described by McClintock (1957), the cultivated species are all members of section *Hydrangea* and have been placed into subsections *Americanae* (*H. arborescens* and *H. quercifolia* Bartr.), *Asperae* (*H. involucrata* and *H. aspera* D. Don), *Calytranthae* (*H. anomala* D. Don), *Heteromallae* (*H. paniculata*) and *Macrophyllae* (*H. macrophylla*). The Mountain hydrangea (*H. macrophylla* subsp. *serrata*) is considered by some to be a separate species (*H. serrata*) closely related to *H. macrophylla*.

On a worldwide basis, *H. macrophylla*, or bigleaf hydrangea, is the most popular member of the genus. Use of *H. macrophylla* as a garden plant in the U.S. is generally limited to USDA Cold Hardiness Zone 6 and warmer (Dirr, 1998). Because this species primarily sets flowers on previous year’s growth, it is susceptible to damage from early fall and late spring frosts and from cold winter temperatures. Independent projects to investigate the use of interspecific hybridization to improve the cold hardiness of *H. macrophylla* were initiated a few years ago. Hybrids between

*H. macrophylla* and *H. paniculata* were produced using embryo rescue, but the resulting plants were sterile and lacked vigor (Reed et al., 2001; Reed, 2004). *Hydrangea macrophylla* × *H. arborescens* hybrids were also produced using embryo rescue, but only plants that were regenerated from callus derived from cotyledonary tissue survived (Kudo and Niimi, 1999a). Resulting progeny were shown to be aneuploid (Kudo and Niimi, 1999b). These researchers also reported producing putative *H. macrophylla* × *H. quercifolia* hybrids using embryo rescue (Kudo et al., 2002), but no details are available about the vigor or fertility of this interspecific hybrid.

Recently, a few *H. macrophylla* plants termed remontant, or reflowering, have been identified (Dirr, 2004; Adkins and Dirr, 2003). These plants, which were released under the names ‘Bailmer’ (Endless Summer), ‘Oak Hill’, ‘Penny Mac’, ‘Decatur Blue’, ‘David Ramsey’ and ‘Forever and Ever’, appear to be genetically and phenotypically similar (Dirr, 2004; Lindstrom et al., 2003; T.A. Rinehart, personal communication). Floral initiation studies indicated that while nonremontant cultivars have photoperiod and temperature requirements that must be satisfied before flower buds are set, neither ‘Balimer’ nor ‘Penny Mac’ have such requirements (Orozco-Obando and Wetzstein, 2004). Because they flower on current year’s growth, remontant *H. macrophylla* cultivars should flower reliably every year.

Although the discovery of remontancy in *H. macrophylla* helps satisfy the need for more reliable flowering in this species, *Hydrangea* interspecific hybridization offers opportunities for further improvements within the genus. No

evidence has been presented that remontant *H. macrophylla* cultivars have any greater root hardiness than nonremontant cultivars, which may limit their use in the coldest parts of the country. *Hydrangea paniculata* and *H. arborescens* are the most cold hardy members of the species (Dirr, 1998). While the flowers of some cultivars of *H. paniculata* age to pale pink, at maturity the flowers of both of these species are white. Incorporation of blue flower color into either *H. paniculata* or *H. arborescens* would greatly improve the appeal of these species. *Hydrangea macrophylla* is the only member of the genus to produce deep blue flowers, although lavender-blue flowers are found in *H. involucrata* (Dirr, 2004; van Gelderen and van Gelderen, 2004). *Hydrangea involucrata* is rated as hardy to USDA Cold Hardiness Zone 6 or 7 and grows to 1 m in height. In addition to flower color, its large velvety leaves have ornamental appeal. An interspecific hybrid between *H. involucrata* and its close relative *H. aspera* is available in the trade (Dirr, 2004), but no other wide hybrids involving *H. involucrata* have been reported. The objective of this study was to determine if *H. involucrata* could be hybridized with *H. paniculata* or *H. arborescens* for the purpose of combining cold hardiness and flower color. Hybrids were verified using molecular markers, somatic chromosome counts and morphological comparisons of hybrids and parents.

## Materials and Methods

**Pollinations.** The following plants were used in this study: *H. involucrata*; *H. paniculata* ‘Burgundy Lace’, ‘Pink Diamond’, and ‘Tardiva’; and *H. arborescens* ‘Dardom’ (White Dome) and ‘Annabelle’. Plants were grown in 26.4-L containers in full sun (*H. paniculata* and ‘Annabelle’) or under 60% shade (‘Dardom’ and *H. involucrata*) and were irrigated using spray stakes. Growing medium consisted of pine bark amended with 6.6 kg·m<sup>-3</sup> 19N–2.1P–7.4K Osmocote Pro fertilizer (Scotts-Sierra Horticultural Products Co., Maryville, Ohio), 0.6 kg·m<sup>-3</sup> Micromax (Scotts-Sierra Horticultural Products Co.), 0.6 kg·m<sup>-3</sup> iron sulfate, and 0.2 kg·m<sup>-3</sup> Epsom salts. Plants were brought into a greenhouse 4 to 5 d before making pollinations. About 3 weeks after pollinations were completed, all plants were moved outside to a 60% shade structure.

Reciprocal crosses were made between *H. involucrata* and the other two species during Summer 2003. Additional crosses between *H. arborescens* ‘Annabelle’ and *H. involucrata* were made in Summer 2004. Opened and immature flowers were removed from the inflorescence of the female parent. Flowers estimated to open the next day were emasculated. Inflorescences designated as male and female were covered with breathable plastic bags (DelStar Technologies Inc., Middletown, Del). Flowers were pollinated 1 to 3 d following emasculation by touching newly dehiscent anthers to stigmas. Seed capsules were collected in October 2003 and 2004 after they had begun to dry. Seed were stored in glassine bags in a 5 °C refrigerator for 2 months, and then sown on a commercial seed propagation mix (Grow Mix #1, Morton’s Horticultural Products, Inc., McMinnville, Tenn.) in shallow (3 cm) seedling

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flats. Seed trays were placed in a heated (26 °C day/18 °C night) greenhouse under mist. Bottom heat was provided during germination. Seedlings were transplanted to individual 5.7 × 4.9 cm cell packs containing Pro Mix BX (Premier Horticulture, Quakertown, Pa.) when the second set of true leaves had developed. Seedlings were later transplanted to square 12.7 cm pots and then to 11.4 L containers using the pine bark growing medium described above. Plants were grown in a greenhouse throughout the study, but during Winter 2004–05 they were placed in an unheated greenhouse and allowed to go dormant.

**Molecular analysis.** Leaf tissue from progeny and parents was collected, freeze-dried, and maintained at -70 °C until needed. DNA was extracted using a Qiagen DNeasy Plant Mini Kit (Valencia, CA) and diluted to 10 ng·μL<sup>-1</sup> after estimating concentration by comparing with known standards on an agarose gel. Primers were obtained from the University of British Columbia (UBC, Vancouver, British Columbia, Canada) and selected based on results from Reed et al. (2001). From UBC set 100/4, 11 primers (302, 308, 319, 335, 336, 337, 338, 341, 345, 349, and 358) were chosen to verify hybridity of putative interspecific *Hydrangea* plants because these primers produced consistent clarity and reproducibility of polymorphic bands among samples tested. Amplification reactions were carried out in 25-μL volumes containing 1× Eppendorf AG MasterMix (Hamburg, Germany), 0.2 μM primer, and 10 ng DNA template. Amplification involved an initial denaturation step of 95 °C for 5 min and then 45 cycles of 95 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min. This process was followed by a final extension step of 72 °C for 8 min. PCR products were separated in 1% (w/v) agarose gels in 1× TBE by electrophoresis at 120V for 35 min. Gels were stained with ethidium bromide and reaction products were viewed using an AlphaImager (Alpha Innotech Corp., Alameda, Calif.). RAPD reactions were repeated at least once to ensure reproducibility of the amplification products.

**Morphological comparisons.** In Summer 2004, cuttings were made from parents of plants that had been molecularly confirmed to be hybrids. Cuttings were dipped for 5 s in 4.9 mM indole-3-butyric acid in 50% ethanol, stuck into 1 peat : 1 perlite (by volume), and placed under mist. The following spring the rooted cuttings were moved to 11.4-L containers and placed under the same greenhouse conditions as the hybrids so that parents and progeny would be exposed to similar day-lengths, light intensities and temperatures. Blade length, blade width and petiole length were measured from the third node on five shoots of each hybrid and parent in Summer 2005.

**Mitotic cytology.** Root tips collected from hybrids and parents were immersed in 0.1 mM colchicine for 3 h at room temperature (20 °C). Root tips were then rinsed in distilled water, and fixed in a solution of 95% ethanol : chloroform : acetic acid (6:3:1) for 24 h at room temperature. Finally, root tips were transferred to 70% ethanol at -20 °C until needed. Before examination, root tips were hydrolyzed in 5 N HCl for 7 min, rinsed with distilled water, and

Table 1. Results of controlled pollinations of *Hydrangea involucrata* with *H. arborescens* and *H. paniculata*.

	Flowers pollinated (no.)	Seeds collected (no.)	Seeds germinated (no.)	Verified hybrids (no.)
<i>H. involucrata</i> × <i>H. arborescens</i> ‘Annabelle’	496	0	---	---
<i>H. involucrata</i> × <i>H. arborescens</i> ‘Dardom’	78	0	---	---
<i>H. involucrata</i> × <i>H. paniculata</i> ‘Burgundy Lace’	249	0	---	---
<i>H. involucrata</i> × <i>H. paniculata</i> ‘Pink Diamond’	298	0	---	---
<i>H. involucrata</i> × <i>H. paniculata</i> ‘Tardiva’	130	≈100	0	---
<i>H. arborescens</i> ‘Annabelle’ × <i>H. involucrata</i>	206	≈1100	2	0
<i>H. arborescens</i> ‘Dardom’ × <i>H. involucrata</i>	74	≈500	36	7
<i>H. paniculata</i> ‘Burgundy Lace’ × <i>H. involucrata</i>	249	0	---	---
<i>H. paniculata</i> ‘Pink Diamond’ × <i>H. involucrata</i>	298	35	3	0
<i>H. paniculata</i> ‘Tardiva’ × <i>H. involucrata</i>	130	≈100	4	0

soaked in 1% acetocarmine for 20 min. The meristematic region of the root tip was squashed in acetocarmine and chromosomes counted. Ten metaphase cells from each parent and at least two cells from each of three hybrids were examined. Lack of quality roots in the progeny precluded availability of sufficient material for additional chromosome counts.

**Pollen viability.** Flowers were collected on the day of anthesis from the one confirmed hybrid that flowered and from its parents. Three anthers from each flower were placed on a microscope slide and squashed in a drop of 1% aceto-carmine stain. Specimens were examined using a light microscope and pollen scored as stained or unstained. Five fields of 100 pollen grains each were counted and a mean number of stained grains calculated for each slide.

**Statistical analysis.** Analysis of variance (ANOVA) and mean separations of leaf measurements and pollen viability were performed using the General Linear Model procedure of SAS (version 9.1; SAS Institute, Cary, N.C.). Mean separations were based on the least significant difference (LSD) after a significant F test ( $P \leq 0.05$ ) in the ANOVA.

## Results and Discussion

**Pollinations.** Viable seed were collected from hybridizations of *H. arborescens* and *H. involucrata*, but only when *H. arborescens* was used as the maternal parent (Table 1). About 7% of the ‘Dardom’ × *H. involucrata* seed germinated. While 14 of the seedlings died before transplanting and several others died within a few months of germination, seven plants obtained from ‘Dardom’ × *H. involucrata* hybridizations currently remain alive. One seed obtained from 2003 hybridizations of ‘Annabelle’ and *H. involucrata* germinated. While initially slow-growing, the plant survived to flowering. One seed from the 2004 hybridizations of ‘Annabelle’ and *H. involucrata* also germinated, but the seedling died before producing the first set of true leaves.

Table 2. Leaf measurements of *Hydrangea arborescens* ‘Dardom’, *H. involucrata* and their hybrid.

Taxon	Mean blade length (cm) <sup>a</sup>	Mean blade width (cm)	Blade length to width ratio	Mean petiole length (cm)
<i>H. arborescens</i> ‘Dardom’	12.8 a	9.5 a	1.3 a	4.8 a
<i>H. arborescens</i> ‘Dardom’ × <i>H. involucrata</i>	16.0 b	7.5 b	2.1 b	3.1 b
<i>H. involucrata</i>	19.0 c	7.5 b	2.5 c	2.9 b

<sup>a</sup>Mean separation based on least significant difference (LSD) after a significant F test ( $P \leq 0.05$ ) in the ANOVA.

<sup>b</sup>Leaf measurements taken from the third node on five shoots of progeny and parents.

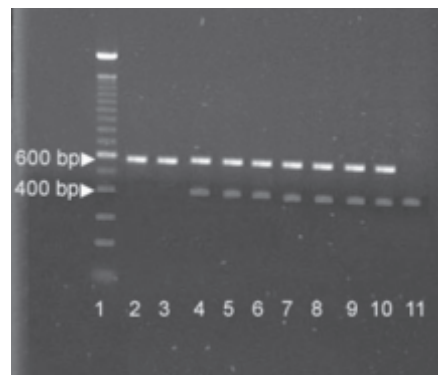


Fig. 1. RAPD banding pattern (primer UBC-349) of two *Hydrangea* species and eight progeny. Lane 1 = molecular weight standard, 2 = *H. arborescens* ‘Dardom’, 3 = *H. arborescens* ‘Annabelle’ × *H. involucrata* progeny, 4–10 = *H. arborescens* ‘Dardom’ × *H. involucrata* hybrids (#735-2, 735-4, 735-5, 735-7, 735-8, 735-10, 735-13), 11 = *H. involucrata*. Arrows indicate polymorphic markers (size in base pairs) which identify the ‘Dardom’ × *H. involucrata* progeny as hybrids.

Viable seeds were obtained from crosses of *H. paniculata* and *H. involucrata*, but only when *H. paniculata* ‘Tardiva’ or ‘Pink Diamond’ was used as the maternal parent. About 5% of the seed from the *H. paniculata* × *H. involucrata* hybridizations germinated, and all seven seedlings survived.

**Molecular analysis.** Eleven RAPD primers each produced one to five polymorphic bands that distinguished between *Hydrangea* species and ranged in size from 300 to 2000 base pairs. The seven surviving ‘Dardom’ × *H. involucrata* progeny showed either a sum of the bands of both parents (Fig. 1), or banding patterns that matched those of *H. involucrata*. Based on this evidence, all the ‘Dardom’ × *H. involucrata* progeny appear to be hybrids.

The banding patterns of the one plant obtained from *H. arborescens* ‘Annabelle’ × *H. involucrata* hybridizations were identical





Fig. 2. *Hydrangea arborescens* 'Dardom' × *H. involucrata* (#735-2) hybrid inflorescence.

to those of 'Annabelle'. All banding profiles from the seven progeny of the *H. paniculata* × *H. involucrata* crosses were also identical to their maternal parent; none of the RAPD markers were paternal-specific. The morphological similarity between these plants and their maternal parent provides additional support that the plants we obtained from crosses of 'Annabelle' and *H. paniculata* with *H. involucrata* are not interspecific hybrids.

**Morphological comparisons.** Average blade length and blade length/width ratio were intermediate between the two parents in the 'Dardom' × *H. involucrata* hybrids (Table 2). Average blade width and petiole length were similar in the hybrids and *H. involucrata*. These results provide additional evidence of hybridity.

One of the seven 'Dardom' × *H. involucrata* hybrids produced one inflorescence in Summer 2005 (#735-2, Fig. 2). The inflorescence, which was 4 cm wide, consisted primarily of small, inconspicuous flowers, but a few flowers with slightly larger sepals were present in the outer whorl of flowers. Sepal color was white. This inflorescence may not be a true indicator of the floral characteristics of the hybrid as the plant that flowered was the smallest and weakest of the hybrids. Pollen viability was estimated in the hybrid and its parents. The parents had statistically similar pollen stainability, with 88% in 'Dardom' and 90% in *H. involucrata*, while only 1% of the pollen in the hybrid was stainable.

**Mitotic cytology.** Examination of mitotic root tip cells revealed 38 chromosomes in *H. arborescens* 'Dardom' (Fig. 3). Previous studies have reported 36 (Sax, 1931; Cerbah et al., 2001) or 38 (Kudo and Niimi, 1999b) chromosomes in *H. arborescens*. Our results support those of Kudo and Niimi (1999b), who studied 'Annabelle'. We observed 30 chromosomes in *H. involucrata* (Fig. 3), which is consistent with previous studies of this species (Funamoto and Tanaka, 1988; Cerbah et al., 2001). *Hydrangea arborescens* chromosomes appeared to be about half the size of *H. involucrata* chromosomes. Flow cytometric measurement of both species supports this difference in size. Zonneveld (2004) reported a nuclear DNA content of 5.36 pg in *H. involucrata* and 2.64 pg in *H. arborescens*. Similar results were reported by Cerbah et al. (2001) with 5.00 pg in *H. involucrata* and 2.31 pg in *H. arborescens*.

The 'Dardom' × *H. involucrata* hybrids had



Fig. 3. Chromosomes of (A) *Hydrangea arborescens* 'Dardom' ( $2n = 38$ ), (B) *H. arborescens* 'Dardom' × *H. involucrata* hybrid #735-10 ( $2n = 34$ ), and (C) *H. involucrata* ( $2n = 30$ ) from root tip cells. Bar = 2.5  $\mu$ m. All images taken at same magnification.

34 chromosomes (Fig. 3). A complete complement of parental chromosomes was present in all three of the progeny observed, including the hybrid that flowered. The chromosomal size difference in the parents could be distinguished in the hybrids; 19 of the chromosomes appeared very similar in size to *H. arborescens*, and the remaining 15 had a strong resemblance in size to *H. involucrata* chromosomes.

Molecular markers, morphological features and mitotic cytology all confirmed the hybrid nature of the plants obtained from crosses of *H. arborescens* 'Dardom' with *H. involucrata*. In contrast, molecular data and morphological comparisons indicated that plants obtained from hybridizations of *H. involucrata* with *H. arborescens* 'Annabelle' or *H. paniculata* were not of hybrid origin. Dirr (2004) has stated that 'Dardom' belongs to *H. arborescens* subspecies *radiata* or *discolor*; this may be the reason that it reacted differently than 'Annabelle' when hybridized to *H. involucrata*. Because the inflorescence of 'Annabelle' is much showier than that of 'Dardom', 'Annabelle' × *H. involucrata* hybrids might be more attractive than 'Dardom' × *H. involucrata* hybrids and have greater breeding potential. Many nonviable and a single weak seedling were produced from 'Annabelle' × *H. involucrata* hybridizations. Therefore, embryo rescue may be useful in recovering this hybrid.

Because previous efforts to use *H. macrophylla* as a source of flower color in interspecific crosses did not produce the desired results, the possibility of using *H. involucrata* as an alternative to *H. macrophylla* was investigated. Unlike hybrids between *H. macrophylla* and *H. paniculata*, *H. arborescens* and *H. quercifolia* (Kudo and Niimi, 1999a; Kudo et al., 2002; Reed, 2000), *H. arborescens* 'Dardom' × *H. involucrata* hybrids did not require embryo rescue. Difficulty in creating interspecific hybrids using *H. macrophylla* may be partially explained by recent phylogenetic research using simple sequence repeat (SSR) markers, which indicates that *H. paniculata*, *H. involucrata*, *H. arborescens*, and *H. quercifolia* are all more closely related to each other than to *H. macrophylla* (T. Rinehart, personal communication).

The one 'Dardom' × *H. involucrata* hybrid that flowered produced a small, unattractive inflorescence with white sepals. Fertility, as estimated by pollen staining, was low. However, unlike the *H. macrophylla* × *H. paniculata* hybrid, which produced petaloid anthers (Reed, 2004), no floral modifications were noted in the 'Dardom' × *H. involucrata* hybrid. As additional hybrids flower, efforts to utilize this hybrid in a breeding program will be made by

attempting full-sib crosses and backcrosses to both parents. Chromosome doubling also offers an opportunity to increase fertility and allow the production of advanced generation progeny. If 'Dardom' × *H. involucrata* hybrids can be used as parents in crosses, segregation may eventually provide individuals exhibiting both blue flower color and cold hardiness.

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