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Verification and Establishment of *Hydrangea macrophylla* 'Kardinal' x *H. paniculata* 'Brussels Lace' Interspecific Hybrids[1]

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Abstract

An interspecific hydrangea breeding project with the goal of producing cold-hardy hydrangeas with brightly colored flowers was initiated in 1997. The objective of the current study was to transfer *Hydrangea macrophylla* x *H. paniculata* plants obtained using ovule culture to *in vivo* conditions and to verify their hybrid nature. Putative hybrids, representing five *H. macrophylla* x *H. paniculata* cultivar combinations, were propagated and rooted *in vitro*. 'Kardinal' x 'Brussels Lace' putative hybrids were the only plants that produced roots and survived transfer to the greenhouse. RAPD markers were used to verify hybridity in 13 of these plants, only 5 of which survived. Four of the 'Kardinal' x 'Brussels Lace' hybrids were greatly reduced in size and slow-growing, having an average height of only 6.4 cm (2.5 in) 8 months after being removed from *in vitro* conditions. Height, internode length, leaf length and leaf width were approximately six times greater in the remaining 'Kardinal' x 'Brussels Lace' hybrid than in the four small hybrids. All hybrids resembled *H. paniculata* in leaf shape and pubescence, and appeared to be less susceptible than *H. macrophylla* to powdery mildew. Intercrosses between hybrids and backcrosses to parental species will be made when the hybrids flower.

Index words: interspecific hybridization, RAPDs, breeding, hydrangea, embryo culture.

Species used in this study: bigleaf hydrangea (*H. macrophylla* (Thunb. Ex J.A. Murr.) Ser.); panicle hydrangea (*H. paniculata* Sieb.).

Growth regulators used in this study: benzyladenine (BA); indolebutryric acid (IBA).

Significance to the Nursery Industry

Hydrangea macrophylla is a popular summer-flowering shrub that is valued for its large brightly colored inflorescences. Many cultivars are available and are rated cold hardy to USDA cold hardiness zones 6 or 7. Because flowers occur primarily on the previous season's growth, flowering is often reduced by an unusually cold winter or a late spring freeze. In colder areas of the country, entire plants are killed by winter weather. An interspecific hybridization project designed to combine flower color from *H. macrophylla* with cold hardiness from *H. paniculata* was initiated in 1997. An embryo rescue procedure was used to obtain seedlings from *H. macrophylla* x *H. paniculata* hybridizations. This study reports efforts to transfer putative hybrids from *in vitro* to greenhouse conditions and to verify their hybridity. Most plants died either while growing in culture or after being transferred from *in vitro* conditions. However, five hybrids, all of which were derived from *H. macrophylla* 'Kardinal' x *H. paniculata* 'Brussels Lace' hybridizations, were successfully transferred to the greenhouse. Molecular markers verified that these five plants were interspecific hybrids. Four of the hybrids were very small and slow growing, while the fifth hybrid was larger and more vigorous. Future work will focus on intercrossing these hybrids and in using them in backcrosses to the parental species with the continuing goal of developing cold-hardy hydrangeas with vividly colored inflorescences.

Introduction

An interspecific hybridization project was initiated in 1997 for the purpose of combining cold hardiness and flower color in *Hydrangea*. *Hydrangea macrophylla* (Thumb. Ex J.A. Murr.) Ser. is a popular summer-flowering shrub with showy inflorescences that range in color from blue to pink, depending on soil pH and cultivar. Many cultivars are available and are rated cold hardy to USDA hardiness zones 6 or 7 (2, 6, 7). While a few cultivars are reported to produce flowers on the current year's growth, *H. macrophylla* flowers generally develop from buds that were produced during the previous growing season (2). Consequently, an unusually cold winter or a late spring freeze in zones 6 and 7 often results in greatly reduced flowering.

Three cold hardy *Hydrangea* species were selected for hybridization with *H. macrophylla*. Panicle hydrangea (*H. paniculata* Sieb.) is a tall shrub that produces large white to pale pink panicles in mid- to late summer. It is rated hardy to zone 3 (2) and, like *H. macrophylla*, is indigenous to eastern Asia. Smooth hydrangea (*H. arborescens* L.), produces delicate corymbs of white flowers in early summer and is hardy to zone 4 (2). These two species flower on the current year's growth. Oakleaf hydrangea (*H. quercifolia* Bartr.) is valued for its large panicles of white flowers, mahogany-red autumn foliage and exfoliating bark. It flowers on the previous year's wood, and is hardy to zone 5 (2). Both *H. arborescens* and *H. quercifolia* are native to the eastern United States.

Reciprocal crosses were made between *H. macrophylla* and *H. paniculata*, *H. arborescens* and *H. quercifolia*, using many different combinations of cultivars (8). A few viable

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seed were produced when *H. macrophylla* was used as the maternal parent; however, most of the seedlings died at the cotyledonary stage, and the remainder died before the first set of true leaves had fully expanded. An embryo rescue procedure in which intact ovules were placed into culture (ovule culture) was utilized with the *H. macrophylla* x *H. paniculata* hybridizations (9). Plants obtained from ovules cultured 3 to 6 weeks after pollination either developed callus or died in culture. An endogenous bacterial contaminant initially interfered with efforts to culture older ovules, but the inclusion of a biocide in the medium alleviated this problem. Over 140 plants were obtained from *H. macrophylla* x *H. paniculata* ovules cultured 9 to 10 weeks after pollination. At the time of that report (9), the plants were still growing in culture and no tests had been conducted to confirm their hybridity. This report describes the successful transfer of some of those plants from *in vitro* conditions to the greenhouse, and the verification of hybridity using Randomly Amplified Polymorphic DNA (RAPD).

Materials and Methods

Rooting and transfer of plants from in vitro conditions. Plants that were previously recovered (9) from ovule cultures were utilized in this study. The plants represented the following *H. macrophylla* x *H. paniculata* cultivar combinations: 'Blaumeise' x 'Pink Diamond'; 'Kardinal' x 'Brussels Lace'; 'Kardinal' x 'Pink Diamond'; 'Kardinal' x 'Unique; and, 'Pia' x 'Tardiva'. These plants developed on ovule culture medium that consisted of Gamborg's B-5 medium (3) supplemented with 0, 0.05 or 0.10% (v:v) Plant Preservation Medium (PPM™; Plant Cell Technology, Inc., Washington, DC), a biocide that was found to control endogenous bacterial contamination in these cultures.

Germinated seedlings were transferred from the ovule culture medium to biocide-free media of the same composition 6 to 8 weeks after the cultures were initiated. Four to six weeks later, the plants were placed on agar-solidified B-5 medium supplemented with 1mg/liter benzyladenine (BA) for 6 to 12 weeks. Plants that formed adventious shoots on this medium were separated into individual plantlets, which were transferred to liquid or agar-solidified half-strength B-5 medium supplemented with 0 or 1 mg/liter indolebutyric acid (IBA). After 6 weeks on these rooting media, the root system of each plant was evaluated. Plants with roots were transferred to potting soil. Plants that had not rooted were transferred to fresh rooting medium of the same composition for another 6 weeks.

A steam-sterilized pinebark:peat (4:1 by vol) mixture was used as the potting soil for all plants removed from *in vitro* conditions. Plants were placed in 6 cm (2.5 in) square plastic pots and were kept in a 25C (77F) incubator under a 16-hour photoperiod for 1 month, after which they were transferred to a 25–27C (77–80F) greenhouse under 60% shade. When roots began to reach the edges of the potting soil, plants were transferred to 10 cm (4 in) plastic pots containing 100% pinebark. Plants were watered three times a week with a water-soluble fertilizer (fertilizer 7N–3.8P–4.1K) with trace elements at the rate of 150 ppm N.

RAPD markers. Fresh, newly expanding leaves were collected, freeze-dried, and stored at –70C until used. For each sample, leaves were placed in a lysing matrix (Bio101, Vista, CA) with 500 µl CTAB buffer and processed in a FastPrep FP120 machine (Bio101) on speed 4 for 12 seconds. The resulting homogenate was incubated at 65C for 15 minutes, then extracted with 500 µl of chloroform:isoamyl alcohol, 24:1. The DNA from this crude first extraction was then isolated using the QIAamp Tissue Kit (Qiagen, Inc., Valencia, CA). DNA purity and quantity was estimated by visual comparison with known standards on a 1% agarose gel. All samples were diluted to 10 ng/µl.

PCR was performed in 25 µl volumes containing PCR buffer (20 mM NaCl, 50 mM Tris pH 9.0, 1% Triton X-100 (1)), 3 mM $MgCl_2$, 200 µM dNTP, 0.2 µM primer (primers with 70–90% G+C content were selected from UBC set 100/4; UBC Nucleic Acid-Protein Service Unit, Vancouver, British Columbia, Canada), 0.25 U of Taq DNA polymerase, and 10 ng DNA template. DNA amplification was carried out in a Biometra T3 Thermocycler (Biometra Gottingen, Germany) programmed for 45 cycles of 30 sec at 95C, 30 sec at 48C and 45 sec at 72C. RAPD reactions were analyzed on 1.4% agarose TBE gels stained with ethidium bromide. Gels were visualized and documented using an AlphaImager 2000 (Alpha Innotech Corp., Alameda, CA). Reactions were repeated at least once to insure reproducibility of scored amplification products.

Characterization of hybrids. Morphological measurements were made approximately 9 months after plants were removed from *in vitro* conditions on plants for which molecular evidence of hybridity had been obtained. Plant height, number of leaf pairs, and length and width of the most recently fully expanded leaf pair were recorded. Mean internode length was calculated by dividing height by number of leaf pairs. Leaf length to width ratio was also calculated. Upper leaf surfaces were examined for the presence of trichomes using a stereomicroscope. Plants were rated for naturally occurring powdery mildew infection on a scale of 0 to 4, where 0

Table 1. Summary of events leading to establishment of embryo-rescued *H. macrophylla* x *H. paniculata* plants and number of plants surviving each stage.

	Number of plants				
Cross	**Transferred from ovule cultures**	**Transferred to shoot multiplication medium**	**Transferred to rooting medium**	**Transferred to soil**	**Surviving to present**
'Blaumeise' x 'Pink Diamond'	22	6	2	0	0
'Pia' x 'Tardiva'	27	1	2	0	0
'Kardinal' x 'Brussels Lace'	40	40	79	46	20
'Kardinal' x 'Pink Diamond'	28	6	16	0	0
'Kardinal' x 'Unique'	26	8	0	0	0

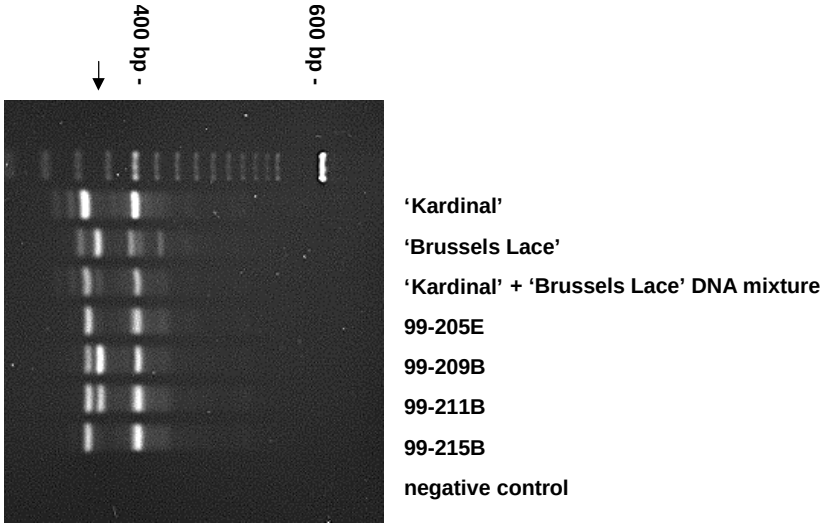

Fig 1. Agarose gel electrophoresis of DNA fragments obtained by RAPD amplification of *H. macrophylla* 'Kardinal', *H. paniculata* 'Brussels Lace' and four 'Kardinal' x 'Brussels Lace' progeny using primer 345. The segregating 'Brussels Lace'-specific marker is indicated by an arrow, and is present in progeny 99–209B and 99–211B.

= no disease, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100% of the leaves showing disease symptoms. The same morphological measurements and disease evaluations were made on rooted cuttings of parental cultivars growing under the same greenhouse conditions as the hybrids.

Results and Discussion

Rooting and transfer of plants from in vitro conditions. While all of the 'Kardinal' x 'Brussels Lace' plants survived the transfer to fresh ovule culture medium, many of the plants of the other four cultivar combinations died before they could be transferred to shoot multiplication medium (Table 1). Consequently, approximately two-thirds of the plants that were placed on the shoot multiplication medium were from 'Kardinal' x 'Teller Red' crosses. Over 100 plants were recovered from this medium and subsequently transferred to rooting media; the number of clones obtained from each original plant ranged from 1 to 20. Rooting was achieved only with 'Kardinal' x 'Brussels Lace', but approximately half of rooted 'Kardinal' x 'Brussels Lace' plants died after being transferred to soil. The surviving 20 plants represented only five unique genotypes, as 15 of the plants were clones that were produced *in vitro*.

Verification of hybridity. RAPD primers 302 and 345 produced clear polymorphisms between *H. macrophylla* 'Kardinal' and *H. paniculata* 'Brussels Lace'. When primer 345 was used for RAPD analysis of 13 'Kardinal' x 'Brussels Lace' putative hybrids, a single marker that was unique to 'Brussels Lace' was found in 10 of the plants (Fig. 1). The three plants that did not produce this marker produced a 'Brussels Lace'-specific marker when primer 302 was used for RAPD analysis (Table 2). Thus, these 13 plants appeared to be hybrids based on molecular evidence. RAPD data also confirmed that progeny from two other *H. macrophylla* x *H.*

Table 2. Morphological and molecular characteristics of five sets of *H. macrophylla* 'Kardinal' x *H. paniculata* 'Brussels Lace' hybrids and parental cultivars.

Plant	No. of clones	Presence (+) or absence (–) of RAPD marker: 345	302	Height (cm)[z]	Internode length (mm)	Leaf length (cm)	Leaf width (cm)	Ratio of leaf length to width	Leaf pubescence	Powdery mildew rating[y]
'Kardinal' x 'Brussels Lace' hybrids										
99–205E	3	–	+	5.2 ± 0.8	3.5 ± 0.4	3.9 ± 0.3	1.8 ± 0.1	2.2 ± 0.1	yes	1
99–207C	1	–	+	2.0	2.5	1.7	1.0	1.7	yes	1
99–209B	1	+		40.6	22.2	13.8	7.0	2.0	yes	1
99–211B	5	+		5.7 ± 0.8	3.8 ± 0.2	2.3 ± 0.2	1.0 ± 0.1	2.3 ± 0.2	yes	1
99–215B	10	–	+	7.1 ± 0.8	3.6 ± 0.4	2.1 ± 0.2	1.1 ± 0.1	1.9 ± 0.1	yes	1
Parental cultivars										
'Kardinal'	3	–	–	n/a	31.3 ± 3.7	15.4 ± 0.7	11.5 ± 0.4	1.3 ± 0.1	no	4
'Brussels Lace'	3	+	+	n/a	40.0 ± 2.3	12.3 ± 0.7	6.1 ± 0.2	2.0 ± 0.1	yes	0

[z]For those plants in which more than one clone was available, values represent mean ± s.e. of all clones.

[y]Based on scale of 0 to 4 where 0 = no disease, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100% of the leaves infected with powdery mildew.

paniculata crosses were hybrids. Unfortunately, these 'Kardinal' x 'Pink Diamond' and 'Pia' x 'Tardiva' hybrids did not survive.

Eight of the plants for which hybridity was verified using RAPD markers died after being transferred to the greenhouse. Four of the five surviving 'Kardinal' x 'Brussels Lace' hybrids were very similar in appearance (Table 2). They were small, with an average height of only 6.4 cm (2.5 in). Leaves averaged 2.4 cm (1 in) long and 1.2 cm (0.5 in) wide, and were numerous and spaced closely together along the stem, with a mean internode length of only 3.6 mm (0.14 in). The remaining 'Kardinal' x 'Brussels Lace' hybrid (99–209B) was much larger than the other plants obtained from this hybridization. Height, internode length, leaf length and leaf width of plant 99–209B were approximately six times greater than comparable measurements from the other 'Kardinal' x 'Brussels Lace' hybrids.

Leaf dimensions and internode lengths of 'Kardinal' and 'Brussels Lace' rooted cuttings growing in the same greenhouse with the putative hybrids were substantially larger than those of the four small 'Kardinal' x 'Brussels Lace' hybrids, but the leaves of plant 99–209B were similar in size to those of 'Brussels Lace'. While no seed-produced *H. macrophylla* or *H. paniculata* plants were available to directly compare height and growth rate to the hybrids, our previous experience indicates that seed-grown plants of both species will fill a #3 container in a single growing season. None of the 'Kardinal' x 'Brussels Lace' hybrids grew this vigorously.

All of the 'Kardinal' x 'Brussels Lace' hybrids resembled *H. paniculata* in several characteristics. The leaves of 'Brussels Lace' and the hybrids were approximately twice as long as they were wide. In contrast, the leaves of 'Kardinal' had a length to width ratio of 1.3. 'Kardinal' leaves were glabrous, while the upper leaf surface of 'Brussels Lace' and all the 'Kardinal' x 'Brussels Lace' hybrids were moderately pubescent. Finally, the hybrids did not appear to be nearly as susceptible to powdery mildew as was *H. macrophylla*. While greenhouse-grown 'Kardinal' plants became heavily infested with powdery mildew, 'Brussels Lace' showed no evidence of powdery mildew. An occasional leaf of the 'Kardinal' x 'Brussels Lace' hybrids developed powdery mildew symptoms but, in the absence of any fungicide treatment, the plants were generally disease-free.

Along with the plants utilized in this study, an additional 578 plants were obtained via ovule culture of *H. macrophylla* x *H. paniculata* (data not presented). These plants represented 18 combinations of parents, involving eight *H. macrophylla* and six *H. paniculata* cultivars. Only 36 of these plants produced roots in culture and only one of the rooted plants survived transfer to the greenhouse. Therefore, the lack of viability of most of the plants obtained from *H. macrophylla* x *H. paniculata* ovule cultures appears to be due to genetic or cytogenetic incompatibility between the two species. The incompatibility is also reflected in the very small size and slow growth rate of most of the viable *H. macrophylla* x *H. paniculata* hybrids.

Interspecific hybridizations in *Hydrangea* are difficult. Researchers attempting to produce hybrids between *H. macrophylla* and *H. arborescens* (4, 5) encountered difficulties similar to what we experienced with the *H. macrophylla* x *H. paniculata* hybrids. Seeds were produced only when *H. macrophylla* was used as the maternal parent, but these failed to germinate. Ovule culture was successful in producing plants, but they died as young seedlings. Finally, cotyledonary tissue of the hybrids was used to develop callus, from which plants were regenerated. Only one of the callus lines produced plants that survived when removed from *in vitro* conditions. Hybridity was verified in that line using RAPD markers.

We will continue to monitor the growth of our *H. macrophylla* x *H. paniculata* hybrids. While it is possible that plant 99–209B may have some ornamental value, it appears highly unlikely that any of the other hybrids obtained in this study will be commercially useful. We are hopeful, however, that the hybrids will eventually flower so they can be intercrossed and backcrossed to the parental species. Gene segregation in advanced generations of materials derived from the *H. macrophylla* x *H. paniculata* hybrid may allow the recovery of vigorous plants with the desired combination of cold hardiness and flower color.

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