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Pre- and Postzygotic Barriers Associated with Intergeneric Hybridization between *Aronia melanocarpa* (Michx.) Elliott x *Pyrus communis* L. and ×*Sorbaronia dippelii* (Zabel) CK Schneid. x *Pyrus communis*

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***Abstract*. Intergeneric hybridization between *Aronia* and *Pyrus* may provide a pathway for developing novel fruit types with larger, sweeter fruits, while maintaining the high levels of biologically health-promoting compounds present in *Aronia* fruits. Here we describe a deleterious genetic incompatibility, known as hybrid necrosis or hybrid lethality, that occurs in intergeneric F1 hybrids of *Aronia melanocarpa* x *Pyrus communis* and ×*Sorbaronia dippelii* x *Pyrus communis*. Pollination experiments revealed that maternal *A. melanocarpa* and ×*S. dippelii* pistils are compatible with pollen from *P. communis*. Controlled pollinations using different mating combinations resulted in varying levels of fruit and seed set. Because every combination produced at least some viable seeds, prezygotic incompatibility does not appear to be present. We attempted to recover putative intergeneric progeny via either in vitro germination or in vitro shoot organogenesis from cotyledons. Progeny of putative hybrids from *A. melanocarpa* x *P. communis* only survived for a maximum of 14 days before succumbing to hybrid lethality. Regeneration of ×*S. dippelii* x *P. communis* was successful for two seedlings that have been maintained for an extended time in tissue culture. These two seedlings have leaf morphologies intermediate between the two parental genotypes. We also confirmed their hybrid status by using AFLPs and flow cytometry. Putative intergeneric hybrids were grown out ex vitro before showing symptoms of hybrid necrosis and dying after 3 months. Eventually micrografts failed, ultimately showing the same symptoms of hybrid necrosis. These results show that intergeneric hybridization is possible between *Aronia* and related genera in the Rosaceae, but there are postzygotic barriers to hybridity that can prevent the normal growth and development of the progeny.**

Intergeneric hybridization in the Rosaceae, subtribe Malinae (Sun et al., 2018), is an important reproductive mechanism that has facilitated plant speciation and domestication of novel plants (Postman, 2011); however, these wide hybridization events can often be hindered by pre- and postzygotic reproductive barriers. Prezygotic barriers can involve spatial isolation of parental plants, asynchrony in flowering, environmental conditions, and self-incompatibility (De Franceschi et al., 2012; Hiscock and McInnis, 2003; Morgan et al., 2011). Postzygotic barriers include failure of the embryo/seed to fully develop, abiotic and biotic stresses on seed and fruit development (e.g., drought, wind, and animal feeding), and hybrid necrosis (Bomblies and Weigel, 2007; Chen et al., 2016). Hybrid necrosis is the reduced viability of a hybrid, characterized by cell death, tissue necrosis, reduced growth rate and in some cases lethality (Bomblies, 2009).

The molecular mechanisms of hybrid necrosis and lethality are reasonably well understood, and the Bateson-Dobzhansky-Muller (BDM) model which provides a plausible genetic explanation (Chen et al., 2016). The BDM model suggests that as ancestral species diverge, each lineage evolves mutations that are not harmful in their native genome, but interact negatively in a hybrid when two divergent genomes recombine (Bomblies, 2006). Hybrid lethality is induced by these deleterious epistatic interactions, which result in spontaneous activation of plant defense genes, primarily nucleotide-binding site leucine-rich repeat (NB-LRR) type resistance proteins (Bomblies et al., 2007; Deng et al., 2019; Jeuken et al., 2009; Montanari et al., 2016).

Aronia fruits contain high levels of antioxidants and polyphenols that are beneficial for human nutrition (Brand et al., 2017; Wu et al., 2004); however, the fruits are astringent and require processing for improved palatability. Intergeneric hybridization between *A. melanocarpa* and *P. communis* may provide a pathway for developing new fruits with desirable traits for both growers and consumers. Fruits of *Pyrus* and *Aronia* hybrids may be large and sweet, while still maintaining the high levels of health-promoting compounds present in *Aronia* fruits. New sources of hybrid germplasm also could be a valuable asset to *Pyrus* breeding programs and may offer novel traits for disease and pest resistance, improved cultivation, and enhanced fruit quality (Bell and Itai, 2011). The Malinae subtribe has a base chromosome count of 17, and hybridization between species and genera is common (Campbell et al., 2007; Persson Hovmalm et al., 2005; Postman, 2011). Previous reports have confirmed intergeneric hybrids between *Aronia* x *Sorbus* (Leonard et al., 2013), *Pyrus* x *Malus* (Gonai et al., 2006), and *Pyrus* x *Sorbus* (Bell and Itai, 2011; Sax, 1929).

The initial objectives of this study were to create novel sources of pome fruit germplasm via intergeneric hybridization and investigate the mating systems and compatibility involving these wide hybridizations and, later, to document evidence for hybrid lethality. The mating systems associated with the plants chosen as parents are critical for determining the likelihood of hybrid formation. Therefore, the results from this study are essential for making informed breeding decisions involving hybridization among *Aronia*, *Pyrus,* and closely related taxa.

Materials and Methods

Plant material and controlled pollinations. Detailed accession information for the plants used in this research is listed in Table 1. Flowering shoots of *Pyrus communis* were sent from the US Department of Agriculture–Agricultural Research Service–National Clonal Germplasm Repository in Corvallis, OR, and were used as paternal genotypes in pollinations (Table 1). Container-grown plants were overwintered in a minimally heated hoop greenhouse (8 ± 5 °C) and a dark cooler (4 ± 2 °C) for ≈6 months. To induce flowering, plants were transferred to a heated greenhouse with set points of 22 °C day and 18 °C night. Pollinations were conducted in a pollinator-free greenhouse. Maternal flowers were emasculated when anthers were pink, before anthesis. Yellow anthers were collected from paternal

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Table 1. Germplasm used for controlled pollinations, amplified fragment length polymorphism analysis, and micrografting.

Accession[z]	Species	Ploidy	Germplasm origin
UC130	*Sorbus aucuparia*	2*x*	Cultivated origin; Field-grown in Storrs, CT
SA001	*Sorbus aria*	2*x*	Cultivated origin; Field-grown in Storrs, CT
UC123 (759-78C[y])	×*Sorbaronia dippelii*	2*x*	Cultivated origin; Field-grown in Jamaica Plain, MA
UC023 (Ames33073)	*Aronia melanocarpa*	2*x*	New Hampshire; Container-grown in Storrs, CT
UC007 (Ames30000)	*A. melanocarpa*	2*x*	Connecticut; Container-grown in Storrs, CT
PC001	*Pyrus communis* 'Bartlett'	2*x*	Cultivated origin; Container-grown in Storrs, CT
PC002	*P. communis* 'Bosc'	2*x*	Cultivated origin; Container-grown in Storrs, CT
NIC20822	*P. communis*	2*x*	India; Flowering shoots from Corvallis, OR
PI183962	*P. communis*	2*x*	Portugal; Flowering shoots from Corvallis, OR
PI437060	*P. communis*	2*x*	United Kingdom; Flowering shoots from Corvallis, OR
PI541391	*P. communis*	2*x*	Turkey; Flowering shoots from Corvallis, OR
PI617640	*P. communis*	2*x*	Cultivated origin; Flowering shoots from Corvallis, OR

[z]U.S. Department of Agriculture accession numbers represented by 'Ames', 'NIC', and 'PI' prefixes.
[y]Arnold Arboretum accession number.

flowers and their pollen was immediately deposited on the maternal stigmas. Pollinations conducted in greenhouses were not covered to prevent unwanted pollinations because insects were excluded. Field pollinations were conducted at the University of Connecticut Research Farm in Storrs, CT, and The Arnold Arboretum in Jamaica Plain, MA. Field pollinations were emasculated before flowers fully opened and were pollinated as described for greenhouse plants. Field pollinations were covered for a period of 2 weeks with dry, waxed paper pollination bags. Control seed was collected from open pollinated maternal plants at the University of Connecticut *Aronia* germplasm collection. Fruits were harvested at peak maturity and seeds cleaned from the fruits and dried before placement into cool, dark storage (13 ± 2 °C, relative humidity 55% ± 5%).

Pollen-tube analysis. Flowers from a subset of mating combinations were collected 48 h after pollination. Styles were fixed in FPA fixative (70% ethyl alcohol: formaldehyde: propionic acid in a ratio of 80:10:10) and stored at 4 °C until further use. Fixed pistils were softened for 24 h in 8 N NaOH and then rinsed for 4 h in distilled water. Pistils were stained with an aqueous solution of 0.1% aniline blue and 0.1 N K_3PO_4 for 24 h. Stained pistil samples were covered with a cover slip and softly squashed and observed under a ultraviolet-light microscope (Zeiss Axiophot, Jena, Germany). Pollen compatibility with each pistil was scored by the extent of pollen-tube growth toward the basal end of the style. Mating combinations were determined to be compatible when pollen tubes reached the base of the style. Mating combinations were incompatible if pollen tubes failed to reach the stylar base. Five styles were evaluated for each mating combination.

In vitro seedling regeneration and microshoot rooting. Mature seeds from putative hybrids and parental genotypes (control group) were used for regeneration. Seeds were surface sterilized for 30 min in 3% (v/v) sodium hypochlorite, rinsed five times in sterile distilled water and then imbibed in sterile distilled water for 48 h at 26 ± 2 °C. Once imbibed, seeds were again surface sterilized in 1% (v/v) sodium hypochlorite for 30 min and rinsed three times in sterile distilled water. Seed coats were aseptically removed in a laminar flow hood with the use of a stereomicroscope.

Seeds with their seedcoats removed were either germinated in vitro, or used as a source of cotyledons for in vitro shoot organogenesis. For in vitro germination, the excised embryo was placed on a petri dish (60 × 16 cm) in direct contact with the medium, which consisted of MS basal medium (Murashige and Skoog, 1962) supplemented with 3% sucrose (w/v) and 0.8% agar (w/v; A1296, Sigma-Aldrich, St. Louis, MO), adjusted to pH 5.8 before autoclaving at 120 °C for 20 min. Cultures were incubated at 26 ± 2 °C under 16-h light photoperiod at ≈50 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After 4 weeks, seeds that germinated in vitro had their epicotyl removed and placed on shoot multiplication medium, which consisted of MS basal medium supplemented with 3 μM 6-benzylaminopurine (BAP), 3% sucrose (w/v), 0.8% agar (w/v), and pH of 5.8 before autoclaving at 120 °C for 20 min. Culture environment for epicotyls was as described for in vitro seed germination. Shoot multiplying cultures resulting from plumules were subcultured every 6 weeks.

Attempts to regenerate some of the progeny via shoot organogenesis from cotyledon explants followed the protocol developed by Mahoney et al. (2018). Briefly, the distal two-thirds of the seed were excised and cotyledons were separated and transferred with the abaxial surface in contact with the medium in petri dishes (60 × 16 cm) with 10 mL of the medium. The remaining portions of the seed (embryo axis and radicle) were not discarded, but instead placed into seed germination medium as described previously. For shoot organogenesis, MS basal medium was supplemented with 5 μM 1-naphthaleneacetic acid (NAA) in combination with 10 μM BAP. MS basal medium was supplemented with 3% sucrose (w/v) and 0.8% agar (w/v) with pH 5.8 before autoclaving at 120 °C for 20 min. Cultures were incubated at 26 ± 2 °C in darkness and transferred onto fresh media after 4 weeks. Eight weeks after incubation the quality of calli and number of explants producing shoots were evaluated and recorded.

Microshoots from crosses that were successfully regenerated and multiplied in vitro were rooted ex vitro. Microshoots were dipped in indole-3-butyric acid rooting hormone (Hormidin 1; OHP Inc., Mainland, PA) and rooted in clear plastic salad trays containing a mix of 1 peat: 1 vermiculite. A completely randomized design with three replicates, each containing five cuttings, was used for rooting. Microcuttings were ≈20 mm long, spaced 10 mm apart. Plants were grown at 26 ± 2 °C under 16-h light photoperiod at ≈50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and fertilized once a week with 100 ppm nitrogen (Peters 20–10–20; Scotts, Marysville, OH). After a 21-d rooting period in salad trays, individual rooted microcuttings were transferred to 7.5-cm pots with the same growing mix and grown under the same conditions with a dome lid covering to maintain humidity. Significance of rooting was determined by analysis of variance, and the differences between the rooting percentage were compared by Fisher's least significance difference test using the R package *agricolae* (de Mendiburu, 2019).

Phenotypic observations. Phenotypic observations were collected on the population of plants that successfully rooted. Plants were evaluated every 7 d for 6 weeks following transfer to individual pots. Plants were observed for tissue necrosis, wilting, and shoot elongation.

Micrografting. Micrografting was used in an attempt to recover and grow out shoots of a putative hybrid (AA15-2) between ×*Sorbaronia dippelii* (UC123) x *Pyrus communis* (PC001). Micrografts were made with scions from AA15-2 shoot cultures, cultured as described previously. Scion shoots were micrografted onto rootstocks of *Sorbus aucuparia*, *Pyrus communis*, and ×*Sorbaronia dippelii*. Rootstock shoots were multiplied on the same shoot multiplication medium as were scions. Individual rootstock shoots were transferred to a liquid root initiation medium consisting of MS basal medium supplemented with 3 μM indole-3-butyric acid (IBA). Root initiation cultures were incubated at 26 ± 2 °C under 16-h light photoperiod at ≈50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 3 weeks. To make the micrografts, individual rootstock shoots were removed from the rooting medium, the top decapitated, and all axillary buds carefully removed. A vertical incision (3 mm) was made centrally at the top of the rootstock. Scion shoot bases were cut at a 45° angle on one side and slipped into the vertical

Table 2. Summary of pollen-tube growth, fruit and seed set, and regeneration of progeny from controlled pollinations.

Maternal	Paternal	Pollination location	No. of flowers	No. of pollen tubes reaching ovary[z]	No. of fruit	No. of seed	No. of germinating progeny	No. of surviving progeny[y]
Aronia melanocarpa	*Pyrus communis*							
UC007	PC001	Greenhouse	70	nt	6	16	16	0
UC023	NIC20822	Greenhouse	50	nt	19	92	78	0
UC023	PI183962	Greenhouse	40	nt	9	22	20	0
UC023	PC001	Greenhouse	85	5/5	29	120	106	0
UC023	PI43706	Greenhouse	15	nt	1	1	1	0
UC023	PI541391	Greenhouse	40	nt	4	6	2	0
UC023	PI61740	Greenhouse	20	nt	4	19	2	0
×*Sorbaronia dippelii*	*P. communis*							
UC123	PC001	Field	150	5/5	5	8	8	2
UC123	PI61740	Field	80	nt	4	6	6	0
P. communis	*P. communis*							
PC001	PC001	Greenhouse	50	0/5	0	0	0	0
PC001	PC002	Greenhouse	50	5/5	0	0	0	0
P. communis	×*S. dippelii*							
PC001	UC123	Greenhouse	25	5/5	0	0	0	0

[z]Number of pistils out of five pistils in which pollen tube reached the ovary; nt = not tested
[y]Number of progeny that survived indefinitely in vitro.

incisions of the rootstocks. Each micrograft was placed into a 50-mL culture vessel with 30 mL of medium consisting of MS basal medium supplemented with 3% sucrose (w/v) and 0.8% agar (w/v) with pH 5.8 before autoclaving at 120 °C for 20 min. Cultures were incubated at 26 ± 2 °C under 16-h light photoperiod at ≈50 μmol·m^{-2}·s^{-1} for 4 weeks. After the graft union formed and roots developed, micrografts were transferred to salad trays containing 1 peat:1 vermiculite mix. Plants were grown at 26 ± 2 °C under 16-h light photoperiod at ≈50 μmol·m^{-2}·s^{-1}. After a 21-d rooting period in salad trays, individual rooted microcuttings were transferred to 7.5-cm pots with the same growing mix and grown under the same conditions with a dome lid covering to maintain humidity. Micrografts were evaluated every 7 d for 4 weeks.

To test if a scion genotype (AA15-2) that displays symptoms of hybrid necrosis would translocate deleterious compounds to a rootstock and result in hybrid necrosis and lethality of the rootstock, 10 additional micrografts were made with AA15-2 grafted onto *S. aucuparia* as described above. In this set of micrografts, the axillary buds were not removed from the rootstocks so the rootstock shoot meristems could grow out if they were unaffected by hybrid lethality. Micrografts to test for translocation of lethality were transferred ex vitro and grown out for 4 weeks. Phenotypic observations of the scion and rootstock were collected every 7 d to document any necroses.

Flow cytometry. Two or three newly emerged leaves (≈50 mg) were thinly sliced with a fresh razor blade in a 55-mm petri dish on a freeze pack with a nuclei suspending solution (Lehrer et al., 2008). The suspending solution was filtered, centrifuged to form a pellet, and resuspended in nuclei staining solution containing propidium iodide. Relative fluorescence of total DNA was measured using a BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA), which quantified 10,000 to 20,000 particles for each sample. Data were analyzed and displayed in histograms by FlowJo Software (Tree Star, Inc., San Carlos, CA) designed to compare standard parental-sample histogram peaks to samples of putative hybrids.

DNA extraction and amplified fragment length polymorphism (AFLP) procedure. Genomic DNA was extracted following the protocol outlined in Mahoney et al. (2019). The quality and concentration of extracted DNA were determined using a NanoDrop-1000 spectrophotometer (Thermo Scientific, Willington, DE). The AFLP steps, including restriction digestion, adaptor ligation, and preselective and selective amplification reactions, were carried out as outlined in the Applied Biosystems (Foster City, CA) AFLP protocol (Anonymous, 2007). Preselective primers had one selective nucleotide (*Eco*RI-A + *Mse*I-C). Four primer combinations were used for selective amplification (*Eco*RI-ACT + *Mse*I-CAC, *Eco*RI-ACT + *Mse*I-CTA, *Eco*RI-ACT + *Mse*I-CAT and *Eco*RI-ACT + *Mse*I-CTG), with fluorescently labeled *Eco*RI and unlabeled *Mse*I probes. The DNA fragments from selective polymerase chain reaction were visualized by capillary electrophoresis on an ABI3730xl DNA analyzer (Applied Biosystems) with a 500 LIZ size standard. AFLP fragment files were processed into binary matrices with GeneMarker Version 1.95 software (SoftGenetics, State College, PA). Peaks were first binary scored (1 for presence and 0 for absence) using automatic settings followed by visual inspection and manual peak adjustment to ensure accurate scoring. Principal component analysis (PCA) was conducted on the binary matrix with the *prcomp* function in R (version 3.5.1).

Results

Pollen-tube analysis and seed set. All outcrossed pollinations had compatible pollen tubes, with pollen tubes reaching the bottom the stylar base, and most of the crosses produced seed (Fig. 1A–D, Table 2). Self-pollinations of *P. communis* (PC001) resulted in incompatible pollen tubes and no seed set (Table 2). Pollen tubes were not observed in the bottom half of the style of the self-pollinated PC001 pistil (Fig. 1E). The percent fruit set of intergeneric crosses between *A. melanocarpa* x *P. communis* ranged from 6% to 38%, with an average of three seeds per fruit. Crosses between ×*S. dippelii* x *P. communis* had a relatively low percent fruit set (between 3% and 5%) and an average of 1.5 seeds per fruit. The other controlled crosses between *P. communis* x ×*S. dippelii*, and *P. communis* selfed and outcrossed produced no fruit (Table 2).

In vitro seed regeneration and microshoot rooting. The percentage of *A. melanocarpa* x *P. communis* seeds germinating ranged from 10% to 100% (Table 2), depending on the parental combination. Of the seeds that successfully germinated, none survived beyond 14 d after in vitro germination began. Parental genotypes did not show symptoms of hybrid necrosis (Fig. 2A), whereas putative hybrid seedlings began showing necrotic lesions between 3 and 14 d after germinating in vitro (Fig. 2B). Hybrid seedlings that had little necrotic lesioning detected on the radicle or cotyledons were observed to exhibit a necrotic plumule, which led to their failure in growth and development (Fig. 2B).

Cotyledons from 50 seeds of *A. melanocarpa* x *P. communis* were used to attempt to regenerate progeny via shoot organogenesis. Cotyledons placed on shoot organogenesis medium began to form callus ≈7 d after culture initiation. The control, *A. melanocarpa* (UC007), produced healthy calli throughout the duration of this experiment. After 8 weeks of culture, *A. melanocarpa* (UC007) produced an average of seven shoots per explant (Fig. 2C), which was similar to previous results by Mahoney et al. (2018). Cotyledon explants from the *A. melanocarpa* x *P. communis* crosses initially formed healthy calli, but at a slightly slower rate than *A. melanocarpa* (UC007) cotyledon explants. After 7 d in vitro, the *Aronia* x *Pyrus* explants began to turn

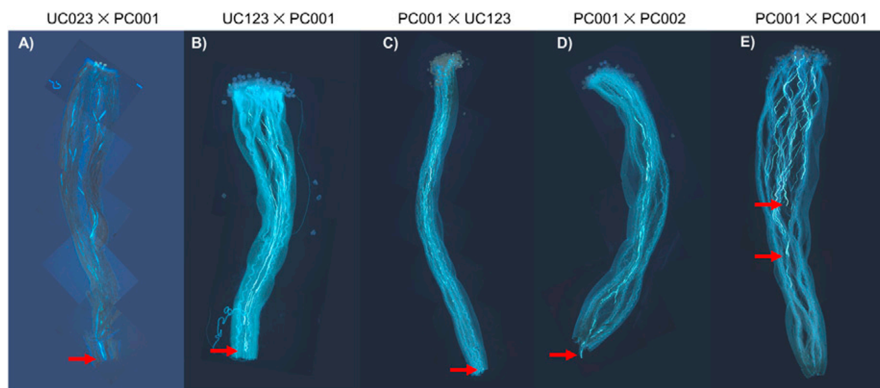


Fig. 1. Pollen-tube–pistil analysis used to determine if intergeneric hybrids exhibit a self-incompatibility system. Intergeneric crosses between (**A**) *Aronia melanocarpa* (UC023) x *Pyrus communis* (PC001); (**B**) ×*Sorbaronia dippelii* (UC123) x *P. communis* (PC001); (**C**) *P. communis* (PC001) x ×*S. dippelii* (UC123); (**D**) *P. communis* (PC001) x *P. communis* (PC002); and self-crossed between (**E**) *P. communis* (PC001) x *P. communis* (PC001). Red arrows indicate terminus of pollen-tube development.

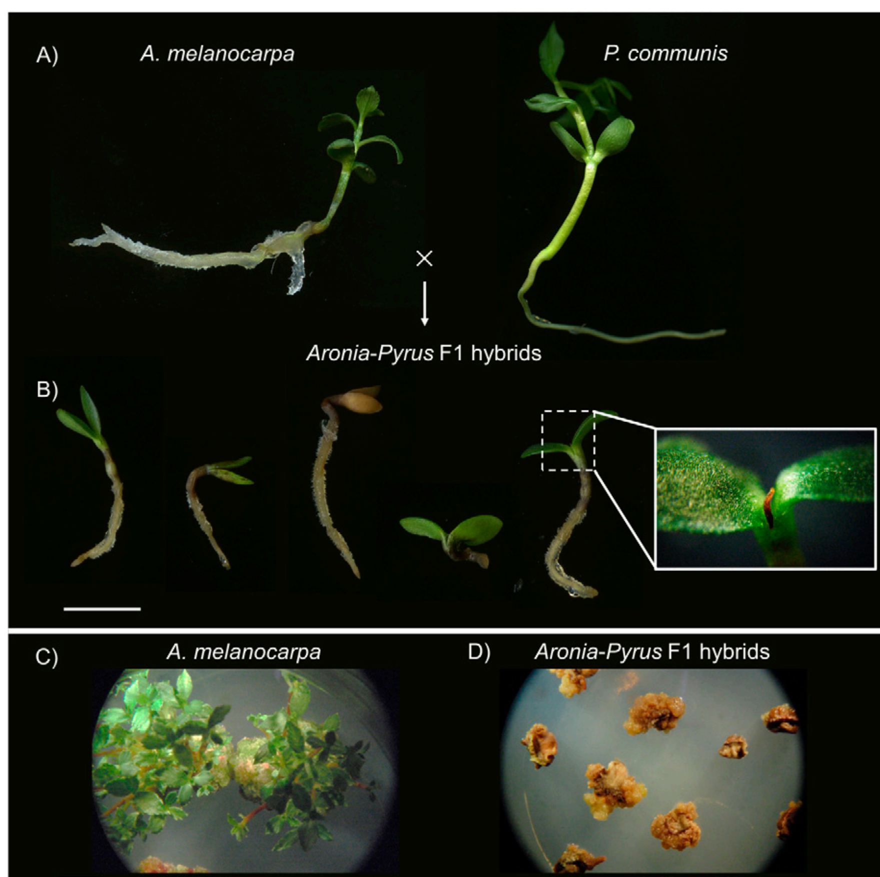


Fig. 2. Regeneration of *Aronia-Pyrus* putative F1 hybrids and parental controls. (**A**) Parental genotypes [*A. melanocarpa* (UC023) and *P. communis* (PC001)] germinate in vitro and continue growth and development (bar = 5 mm); (**B**) *Aronia-Pyrus* putative F1 hybrids showing symptoms of hybrid necrosis 14 d after in vitro germination (bar = 5 mm); (**C**) shoot organogenesis on paternal genotype [*A. melanocarpa* (UC023)] cotyledons after 2 months of culture (bar = 5 mm); and (**D**) necrotic callus development on *Aronia-Pyrus* putative F1 hybrid cotyledons (bar = 5 mm).

necrotic, and callus development slowed significantly (Fig. 2D). The cotyledons from the intergeneric crosses failed to form shoots (Fig. 2D). As a result, none of the *Aronia* x *Pyrus* hybrids was successfully regenerated through in vitro germination or shoot organogenesis.

The ×*S. dippelii* x *P. communis* crosses (UC123 x PC001 and UC123 x PI61740) formed relatively few seeds, so all of the seeds were used for in vitro germination, not shoot organogenesis (Table 2). All seeds turned necrotic and died soon after germination (Table 2), except for two from UC123 x PC001, which remained green and continued growth. The two seedlings that did germinate in vitro, AA15-2 and AA15-3, had their epicotyls transferred to, and multiplied on, shoot multiplication medium (Fig. 3A). Shoot multiplication cultures have been maintained for more than 4 years by subculturing every other month. There have been no symptoms of hybrid necrosis during this process. Both hybrids produce an adequate number of shoots in tissue culture; however, AA15-3 shoots do not elongate well and produce compact shoot rosettes.

Putative intergeneric hybrids were successfully rooted ex vitro from microcuttings (Fig. 3B). There was no significant difference in percent rooting (80% to 100%) among AA15-2, UC123, and PC001 (Fig. 3C). AA15-3 had an average rooting of 27%, which was significantly less than the other genotypes ($P < 0.0001$).

Confirmation of hybrid status. The two putative hybrids (AA15-2 and AA15-3) were analyzed for their hybrid status based on leaf morphological characteristics, AFLPs, and flow cytometry. The morphological characteristics observed on AA15-2 and AA15-3 suggest that both are intergeneric hybrids. As seen in Fig. 4A, leaves of the two putative hybrids (AA15-2 and AA15-3) and the paternal genotype, *P. communis* (PC001), tend to be more broadly elliptical, whereas the maternal parent, ×*S. dippelii* (UC123), has narrowly elliptical leaves. The leaf margins for AA15-2 and PC001 are serrulate, suggesting that this was inherited from the father. The maternal parent (UC123) produces serrate leaf margins, and AA15-3 displays irregular, large serrations at its leaf apices (Fig. 4A).

AFLP markers were a rapid and efficient tool for confirmation of intergeneric hybrids. Four primer pairs generated 84 total bands, of which 96% were polymorphic among parents and putative hybrids. The PCA partitioned the hybrids between the two parental genotypes, as would be expected (Fig. 4B). We also show that ×*S. dippelii* (UC123) is an intergeneric hybrid between *A. melanocarpa* x *S. aria*, which supports the findings of Leonard et al. (2013).

Flow cytometry provided additional insights into the hybrid status of the two putative intergeneric hybrids. The average relative fluorescence values for PC001, UC123, AA15-2, and AA15-3 were 81,000, 96,000, 85,000, and 75,000, respectively (Fig. 4C). One of the putative hybrids, AA15-2, had a mean fluorescence value that was in-between those of its two parents, UC123 and PC001. AA15-3 produced a mean fluorescence that was less than all other samples, suggesting a loss of genetic material.

Phenotypic observations. After 21 d of rooting, plants had no symptoms of hybrid necrosis in the rooting trays and were moved to individual pots. AA15-2 started to show symptoms of hybrid necrosis following day 28, with some necrotic lesions occurring at the shoot growing tip. By day 35, both AA15-

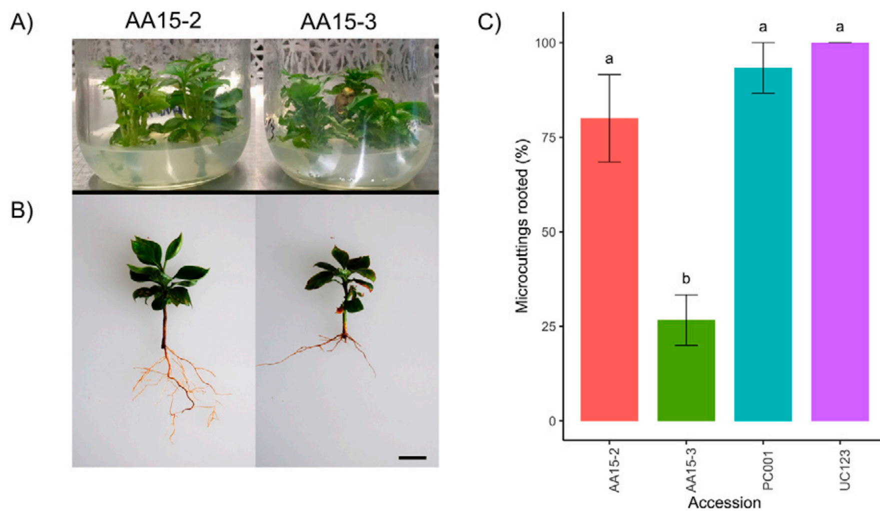
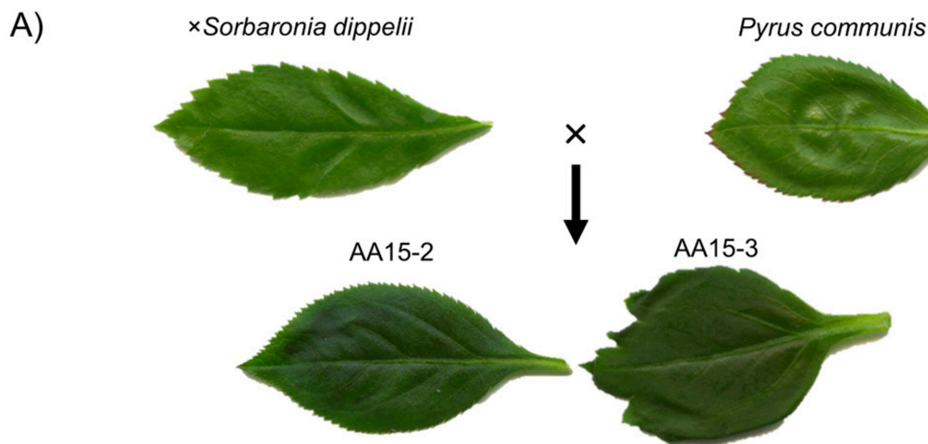


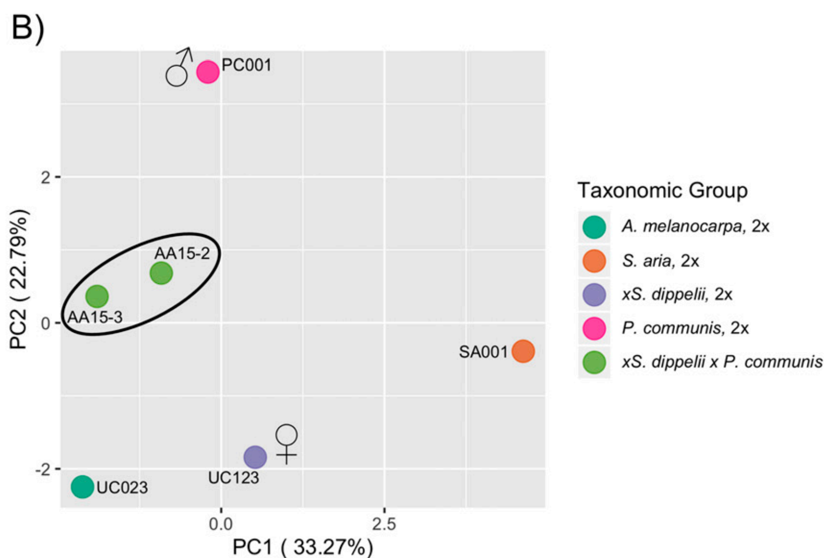
Fig. 3. Putative hybrids (AA15-2 and AA15-3) from ×*S. dippelii* (UC123) x *P. communis* (PC001) (**A**) multiplied in vitro; (**B**) microcuttings rooted ex vitro (bar = 10mm); (**C**) microcutting rooting for putative hybrids and parental genotypes. Bars followed by the same letter did not differ significantly according to Fisher's least significant difference test ($P < 0.05$).

2 and AA15-3 were alive, but their shoot tips started to become necrotic and growth and development ceased (Fig. 5A). UC123 and PC001 continued to grow vigorously without forming necrotic lesions. In most instances, after 2 months of growing ex vitro, AA15-2 started to show severe symptoms of necrosis and hybrid lethality, which resulted in death (Fig. 5B). AA15-3 showed less severe symptoms than did AA15-2, but was stunted and died after 3 months.

Micrografting. Callus formation was visible on micrografts at the graft junction ≈10 d after grafting. Grafted AA15-2 did not survive on any of the rootstocks used in this experiment. The graft union appears to completely fail between 42 to 49 d after grafting resulting in hybrid necrosis and a rapid decline of the scion (Fig. 6A).

AA15-2 scions grafted onto *S. aucuparia* rootstocks with axillary buds left intact for potential grow out, had little effect on the viability of the rootstock. The rootstocks broke bud and continued to grow and develop





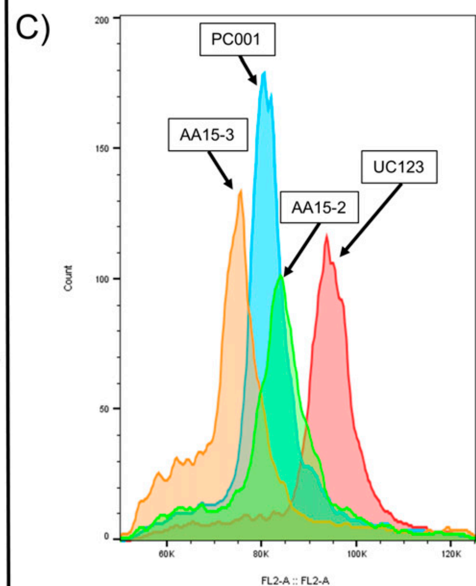


Fig. 4. Confirmation of putative hybrid status using (**A**) leaf morphological features (bar = 5 mm); (**B**) PCA of AFLP marker analysis of putative hybrids and their parental genotypes. Putative hybrids are circled; and (**C**) flow cytometry showing nuclei relative fluorescence values for the maternal genotype [×*S. dippelii* (UC123)] and paternal genotype [*P. communis* (PC001)] and the two putative hybrids (AA15-2 and AA15-3).

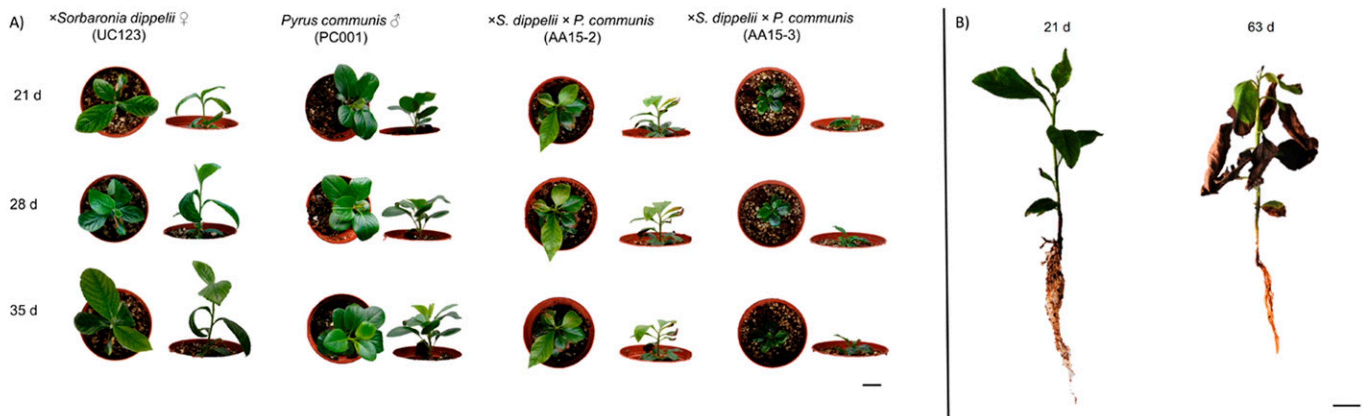


Fig. 5. Phenotypic observations and progression of hybrid necrosis in (**A**) maternal ×*S. dippelii* (UC123), paternal *P. communis* (PC001) and intergeneric hybrid progeny (AA15-2 and AA15-3) over a 3-week period (bar = 20 mm). (**B**) Intergeneric hybrid AA15-2 at 21 and 63 d (bar = 10 mm).

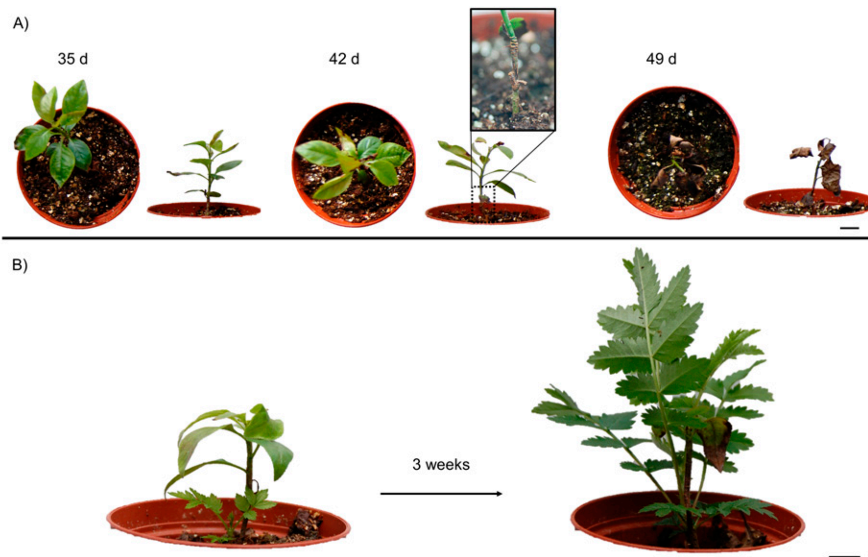


Fig. 6. Micrograft between AA15-2 intergeneric hybrid scion [×*S. dippelii* (UC123) x *P. communis* (PC001)] and *S. aucuparia* (UC130) rootstock. (**A**) Progression of graft union and hybrid necrosis (bar = 10 mm). (**B**) Growth and development of rootstock axillary bud over a 3-week period (bar = 10 mm).

as the scion developed symptoms of hybrid necrosis 42 d after grafting. By 49 d, scion leaves and stem were exhibiting severe symptoms of tissue necrosis, while the rootstock was growing rapidly without necrosis (Fig. 6B). All 10 replicates developed hybrid necrosis in the scion and normal, vigorous growth and development in the rootstock.

Discussion

Our results demonstrate that postzygotic incompatibilities of F1 intergeneric Malinae hybrids resemble cases previously reported (Bomblies et al., 2007; Montanari et al., 2016). Controlled pollinations provide important insights into intergeneric hybridization among *A. melanocarpa*, *P. communis*, and ×*S. dippelii*. Our study was consistent with others showing that *P. communis* is self-incompatible (Claessen et al., 2019). Previous studies have suggested that *Aronia* and closely related intergeneric hybrids are compatible (Connolly, 2014; Leonard, 2011). Compatibility among *A. melanocarpa*, *P. communis*, and ×*S. dippelii* was successful based on stylar pollen-tube germination. Morimoto et al. (2020) found *P. communis* pollen to be noncompatible with *Malus* ×*domestica* pistils, whereas *P. pyrifolia* was compatible. However, there have been other reports showing hybridization and compatibility between *M.* ×*domestica* and *P. communis* (Fischer et al., 2014). These discrepancies suggest that some genotype-specific mating combinations are more successful than are others. In this study, we observed significant variation in the number of fruits and seeds produced among different intergeneric mating combinations.

The lack of fruit and seed set in outcrossed *P. communis* maternal plants may be the result of environmental stressors from growing plants in containers and in a greenhouse where temperatures could not always be limited. Similarly, other crosses conducted in the greenhouse exhibited low fruit and seed set which could have been caused by similar events or hybrid barriers during fertilization. Although most of these crosses were conducted in a climate-controlled greenhouse, the temperature during the spring and summer months can be difficult to manage. High temperatures may have been a contributing factor to a reduction in pollen germination, fertilization, and embryo development.

AFLP markers have previously been used in *Aronia* because of their high frequency of polymorphisms and reproducibility (Leonard et al., 2013; Mahoney et al., 2019). In addition, Leonard et al. (2013) demonstrated that AFLP markers can be used across *Aronia*, *Pyrus,* and other closely related taxa. AFLP marker analysis was used to confirm the hybrid status of two putative intergeneric hybrids, AA15-2 and AA15-3. The results here are the first to demonstrate the hybrid status between ×*S. dippelii* and *P. communis*. Flow cytometry analysis was used to confirm the hybrid status of intergeneric hybrids between apple and Japanese pear (Gonai et al., 2006). Flow cytometry conducted in this study showed that AA15-2 had a DNA content that was intermediate between the two parents. AA15-3 had a DNA content that was less than both parents, which may reflect a loss of genetic material. Nunes et al. (2013) found significant reductions in DNA content in interspecific *Pennisetum* hybrids in comparison with the average DNA content of the parental plants. The reduction in DNA content may act as a mechanism to form genome stability in hybrids (Leitch and Bennett, 2004).

Hybrid necrosis in tobacco has been linked to activation of genes associated with pathogen responses (Masuda et al., 2007). Bomblies et al. (2007) showed that tandem duplication of two *R* genes in *Arabidopsis thaliana* was responsible for overexpression of defense-related pathway genes. They also demonstrated that hybrid necrosis in *A. thaliana* was caused by *DM1*, an allele of an NB-LRR gene. In our study, hybrids maintained in tissue culture did not display symptoms of hybrid necrosis until removed from the microclimate and rooted ex vitro. In vitro,

warmer temperatures and higher humidity may be contributing to a reduction in hybrid necrosis. Higher temperatures have been suggested to interact with the genetic control of plant immune responses (Alcázar and Parker, 2011; Muralidharan et al., 2014; Traw and Bergelson, 2010). Hybrid necrosis-like incompatibilities are dependent on the activation of the salicylic acid stress signaling pathway (Alcázar et al., 2009). Increasing temperatures reduce the salicylic acid pathway, which likely suppress salicylic acid activation (Traw and Bergelson, 2010).

Observing varying degrees of hybrid necrosis is not an unusual biological phenomenon and has been observed in other cases. Montanari et al. (2016) were able to classify seedlings into different phenotypic classes of hybrid necrosis. Similarly, we classified *A. melanocarpa* × *P. communis* hybrids into early-stage hybrid necrosis and ×*S. dippelii* x *P. communis* hybrids into a late-stage hybrid necrosis. One possible explanation for hybrid necrosis appearing at a later stage is the genetic pedigree of the parental genotypes. Previous studies have documented that *S. aria* is compatible with *A. melanocarpa* to form ×*S. dippelii* and with *P. communis* to form ×*Sorbopyrus* spp. (Leonard, 2011; Sax, 1929). It may act as a genetic bridge for intergeneric hybridization between *Aronia* and *Pyrus*. Nevertheless, our two intergeneric hybrids between ×*S. dippelii* and *A. melanocarpa* did not survive past 3 months after removal from the in vitro environment.

Because *Pyrus*-*Sorbus*-*Aronia* intergeneric hybrids did not survive outside of in vitro culture, we attempted micrografting as a method to alleviate symptoms of hybrid necrosis and lethality. A previous study reported that grafting was required to grow out F1 hybrids between *Malus* and *Pyrus* (Fischer et al., 2014). Grafting was necessary for *Malus* x *Pyrus* hybrids because the intergeneric hybrid could not produce a root system sufficient to transport water and nutrients. Previous success has been obtained with micrografting *Aronia mitschurinii* onto *Sorbus aucuparia* and ×*Sorbaronia fallax* (Mahoney and Brand, unpublished data) and chip-bud grafting *A. mitschurinii* onto different Malinae genera (Brand et al., 2018). We did not find that micrografting could reduce hybrid lethality. Instead we observed hybrid necrosis and lethality to occur at a faster rate after grafting. The severity of the micrograft scion succumbing to hybrid lethality may have been accelerated by failure of the graft union. With failure of the graft union, we would expect a rapid reduction of water and nutrients to the scion. In addition, we found that any putative deleterious compounds produced in the scion remain localized there and are not translocated to the rootstock.

In summary, this is the first report of hybrid necrosis in intergeneric hybrids involving *A. melanocarpa*, ×*S. dippelii*, and *P. communis*. In this study, a limited selection of genotypes were used for intergeneric crosses. Future breeding efforts may have greater success if additional genotypes are used in mating combinations. We have shown that, although intergeneric hybridization between these genera is possible, there are postzygotic barriers that result in loss of hybrid offspring. Deleterious epistatic interactions between these divergent lineages follow the BDM model of genetic incompatibility. Transcriptomics and metabolomics could provide further insights into the molecular mechanisms associated with hybrid necrosis in *Aronia*, *Pyrus*, and closely related genera. Future studies could attempt chromosome doubling of intergeneric hybrid seedlings, mutagenesis or genome editing of lethal genes to alleviate the symptoms of hybrid necrosis. In addition, exposing the hybrid plants to high temperatures may reduce lethality. Suppression of hybrid necrosis and lethality would allow for further investigation into the molecular function of hybrid necrosis and enable breeders to recover these intergeneric hybrids for crop improvement.

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