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Adventitious shoot regeneration from *in vitro* leaves of *Aronia mitschurinii* and cotyledons of closely related Pyrinae taxa

Jonathan D. Mahoney, Peter V. Apicella, Mark H. Brand*

Department of Plant Science and Landscape Architecture, University of Connecticut, Storrs, CT, USA

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ABSTRACT

The objective of this study was to develop an *in vitro* shoot regeneration procedure and to evaluate the frequency of adventitious shoot regeneration from: (1) *in vitro* leaves of a commercial cultivar of *Aronia mitschurinii* on various media treatments; (2) cotyledons of closely related Pyrinae taxa; and (3) 21 wild *Aronia* genotypes. Optimum regeneration of leaf explants occurred when they were wounded with two transverse cuts along the midrib and placed on Murashige and Skoog (MS) basal media containing 5 µM indole-3-butyric acid (IBA) and 10 µM thidiazuron (TDZ). TDZ was more effective than 6-benzylaminopurine (BAP) as a cytokinin, and IBA was more effective than the no auxin control, 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA). Regeneration from cotyledons of seven Pyrinae taxa was evaluated using 10 µM BAP in combination with 0.1, 1 and 5 µM NAA. Adventitious shoot formation for *A. melanocarpa* and *P. communis* responded best to 1 µM NAA, whereas all other taxa formed a greater number of adventitious shoots on 5 µM NAA. *A. mitschurinii* cotyledon explants produced a significantly greater number of shoots compared with *in vitro* leaf explants. The number of shoots forming per cotyledon explant and the percent of explants forming shoots were both significantly different among the 21 *Aronia* genotypes. Significant differences were observed between the six *Aronia* taxonomic groups for the number of shoots forming per explant. Diploid and tetraploid *Aronia* genotypes produced a significantly greater number of shoots per explant than did triploid genotypes. Regenerated shoots were rooted *in vitro* and plants grew normally in the greenhouse. These results will be useful for future studies using leaf and cotyledon explants for genetic transformation, genome editing and mutation breeding with *Aronia* and related taxa.

1. Introduction

Plants in the Rosaceae family, subtribe Pyrinae, include a number of economically important fruit crops that are beneficial for human nutrition (Hummer and Janick, 2009). Common pome fruits include *Malus* Mill. (apple), *Pyrus* L. (pear) and *Cydonia* Mill. (quince) along with less commonly known fruits including *Sorbus* L. (mountain ash), *Aronia* Med. (chokeberry), *Amelanchier* Med. (serviceberry), *Crataegus* L. (hawthorn), and several other woody plants (Campbell et al., 2007). Interest in aronia fruit has increased because of their high levels of antioxidants and polyphenols (Zheng and Wang, 2003; Wu et al., 2004; Brand et al., 2017) and wide adaptability to various geographic regions with few disease and pest issues (Dirr, 2009; McKay, 2001). However, aronia fruit production uses germplasm that possesses very little genetic diversity (Persson Hovmalm et al., 2004; Leonard et al., 2013; Connolly, 2014) and to protect fruit growers from adverse biological and economical impacts, it will be essential to create novel types of *Aronia* fruits to reduce monoculture.

Traditional breeding approaches typically require long regeneration periods and significant resources for hybridization and selections to introgress genes for desirable traits. Genetic transformation provides an opportunity to transfer desirable target genes and generate unique breeding materials, and this method of breeding has become important, especially for woody fruit species (Gambino and Gribaudo, 2012). Shoot organogenesis from morphogenic explants, including leaf explants and cotyledons have been used for genetic transformation in pear (Gao et al., 2002; Kaneyoshi et al., 2001), apple (Holefors et al., 1998) almond (Miguel and Oliveira, 1999), and apricot (Petri et al., 2008). With recent advances in genetic transformation and genome editing technologies, sufficient protocols for shoot regeneration are necessary for utilization of these methods for the improvement of these economically important fruit crops.

In vitro shoot organogenesis has been reported from *Sorbus aucuparia* leaf and stem explants (Lall et al., 2006), pear cotyledons (Kaneyoshi et al., 2001; Nakajima et al., 2012) and pear leaves (Hennayake et al., 2003; Tang et al., 2008; Bell et al., 2012). There have

* Corresponding author.
E-mail address: mark.brand@uconn.edu (M.H. Brand).

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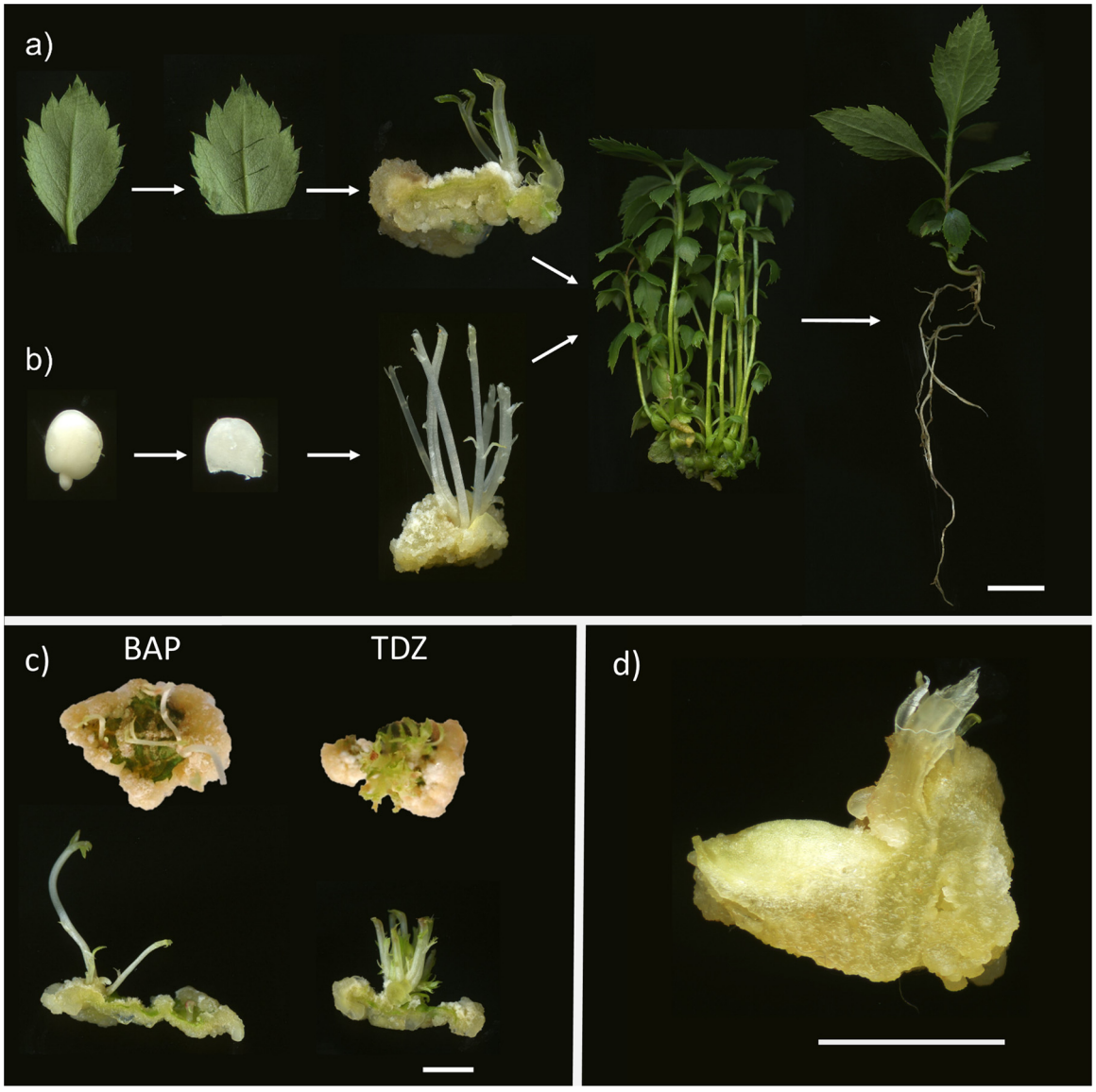

Fig. 1. Explant preparation and process for *Aronia mitschurinii* shoot organogenesis of (a) leaf explant (Experiment I) and (b) cotyledon (Experiment II & III) (*bar 5* mm). (c) Leaf explants after 2 months of culture on media containing 5 μM NAA with either 10 μM BAP or 10 μM TDZ (*bar 5* mm). (e) Cotyledon explants after 5 wk of culture beginning to form adventitious shoots from dedifferentiated callus tissue (*bar 2* mm).

Table 1
Seven Pyrinae accessions used in experiment II.

Species	Parentage	Ploidy	Accession/cultivar	Germplasm source	Germplasm origin
Aronia melanocarpa	–	2*x*	PI 613016	USDA, Ames, IA	Massachusetts
Aronia mitschurinii	×*S. fallax* × *A. melanocarpa*	4*x*	Viking	University of Connecticut, Storrs, CT	Cultivated origin
×*Sorbaronia fallax*	*S. aucuparia* × *A. melanocarpa*	2*x*	None	University of Connecticut, Storrs, CT	Massachusetts
Sorbus aucuparia	–	2*x*	None	University of Connecticut, Storrs, CT	England
×*Sorbaronia dippelii*	*S. aria* × *A. melanocarpa*	2*x*	759-78	Arnold Arboretum, Boston, MA	Germany
Sorbus aria	–	2*x*	None	Sheffield's Seed Co., Locke, NY	Unknown
Pyrus communis	–	2*x*	Bartlett	Sheffield's Seed Co., Locke, NY	Unknown

been no reports for adventitious shoot regeneration from leaves and cotyledons of *Aronia* or cotyledons of *Sorbus*. One objective of this study was to evaluate the frequency of adventitious shoot regeneration from leaves of a commercial cultivar of *Aronia*. A second objective was to examine shoot organogenesis on cotyledons of closely related Pyrinae taxa on media containing various ratios of auxin and cytokinin. The third objective was to examine the regeneration frequency from cotyledons of 21 *Aronia* genotypes comprising six taxonomic groups. The development of improved and more efficient *in vitro* culture protocols for regeneration will be a valuable asset for continued efforts in evaluating and breeding novel genotypes through hybridization, genetic transformation and gene editing.

Table 2
Aronia genotypes used in experiment III.

Accession	Species	Ploidy	Germplasm origin
UC058	*A. arbutifolia*	4*x*	New Jersey
UC014	*A. arbutifolia*	4*x*	Texas
UC111	*A. arbutifolia*	4*x*	Alabama
UC105	*A. arbutifolia*	4*x*	North Carolina
UC017	*A. melanocarpa*	2*x*	Maine
UC160	*A. melanocarpa*	2*x*	Vermont
UC020	*A. melanocarpa*	2*x*	Maine
UC010	*A. melanocarpa*	2*x*	Maine
UC007	*A. melanocarpa*	2*x*	Connecticut
Ames 27615	*A. melanocarpa*	4*x*	Minnesota
PI 586591	*A. melanocarpa*	4*x*	Japan
PI 545686	*A. melanocarpa*	4*x*	Ontario, Canada
UC110a	*A. melanocarpa*	4*x*	Tennessee
UC003	*A. mitschurinii*	4*x*	Cultivated origin
UC141	*A. prunifolia*	3*x*	Cultivated origin
UC145	*A. prunifolia*	3*x*	Cultivated origin
UC043	*A. prunifolia*	4*x*	Massachusetts
UC070	*A. prunifolia*	4*x*	New York
UC114	*A. prunifolia*	4*x*	Rhode Island
UC085	*A. prunifolia*	4*x*	Connecticut
UC040	*A. prunifolia*	4*x*	Rhode Island

Table 3
Effect of different plant growth regulator treatments on shoot regeneration from *in vitro* leaf explants of *A. mitschurinii*.

Treatment	Auxin (μM)	Cytokinin (μM)	Midrib wound	% Explants[z]	No. of shoots per explant[y]
1	NAA 1	BAP 10	n	5 defg	0.1 ± 0.1 de
2	NAA 5	BAP 10	n	5 defg	0.1 ± 0.05 de
3	NAA 1	BAP 20	n	10 defg	0.2 ± 0.12 cde
4	NAA 5	BAP 20	n	10 defg	0.2 ± 0.15 cde
5	NAA 1	TDZ 10	n	0 fg	0 ± 0 de
6	NAA 5	TDZ 10	n	45 ab	1.3 ± 0.51 bcd
7	NAA 1	TDZ 20	n	10 defg	0.3 ± 0.16 cde
8	NAA 5	TDZ 20	n	25 bcdef	1.3 ± 1.07 bcd
9	NAA 1	BAP 10	y	5 efg	0.1 ± 0.05 e
10	NAA 5	BAP 10	y	25 bcd	0.6 ± 0.18 cde
11	NAA 1	TDZ 10	y	18 cdef	0.9 ± 0.35 bcde
12	NAA 5	TDZ 10	y	33 bc	1.8 ± 0.4 b
13	IBA 1	TDZ 10	y	20 cde	1.3 ± 0.51 bc
14	IBA 5	TDZ 10	y	60 a	2.7 ± 0.59 a
15	2,4-D 1	TDZ 10	y	0 g	0 ± 0 e

1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP), thidiazuron (TDZ), indole-3-butyric acid (IBA), and 2,4-dichlorophenoxyacetic acid (2,4-D).

[z] Values followed by the same letter did not differ significantly according to Fisher's LSD test ($P < 0.05$). Percentage data were arcsine-transformed before statistical analysis. Non-transformed data presented.

[y] Mean values ± standard error. Values followed by the same letter in each column are not significant according to Fisher's LSD test ($P < 0.05$).

2. Materials and methods

2.1. Experiment I – shoot organogenesis from leaves

2.1.1. Plant material

Shoot cultures of *Aronia mitschurinii* 'Viking' were maintained on MS basal medium (Murashige and Skoog, 1962) supplemented with 3 μM 6-benzylaminopurine (BAP), 3% sucrose (*w/v*), 0.8% agar (*w/v*; A1296, Sigma-Aldrich, St. Louis, MO), and pH of 5.8 prior to autoclaving at 120 °C for 20 min. Cultures were incubated at 26 ± 2 °C under 16-h light photoperiod at approximately 50 μmol $m^{-2} s^{-1}$ and subcultured every six weeks. Recently fully expanded leaves were excised from the top and mid-portions of 5-week-old *in vitro* shoots and 1 mm of the proximal end was removed on all explants. Leaf explants were either wounded with two transverse cuts across the midrib, or left intact (Fig. 1a), and then placed with their abaxial side in contact with the media in petri dishes (60 × 16 cm).

2.1.2. Media and culture conditions

To study the effect of different types and concentrations of plant growth regulators (PGRs), we tested 1 and 5 μM 1-naphthaleneacetic acid (NAA) in combination with 10 and 20 μM BAP or 10 and 20 μM thidiazuron (TDZ). We also tested 1 and 5 μM indole-3-butyric acid (IBA) in combination with 10 μM TDZ and 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 10 μM TDZ. For all treatments, MS basal medium supplemented with 3% sucrose (*w/v*) and 0.6% agar (*w/v*) was used with pH 5.8 prior to autoclaving at 120 °C for 20 min. Cultures were incubated at 26 ± 2 °C in darkness and transferred onto fresh media after one month. Two months after incubation the number of explants producing shoots and the number of shoots per explant were recorded.

2.2. Experiment II & III – shoot organogenesis from cotyledons

2.2.1. Plant material

Mature seed was used to excise cotyledonary tissues. For experiment II, seeds of *A. mitschurinii*, ×*Sorbaronia fallax*, and *S. aucuparia* were collected in Storrs, CT during late-September 2015, ×*S. dippelii* (759-78) was collected from The Arnold Arboretum of Harvard University (Boston, MA), *S. aria* and *Pyrus communis* were purchased from Sheffield's Seed Co. (Locke, NY), and seeds of *A. melanocarpa* (PI 613016) were acquired from the USDA-NPGS in Ames, IA (Table 1). For experiment III, seeds of 21 *Aronia* accessions, representing six taxonomic groups, were collected from the *Aronia* germplasm collection at the University of Connecticut Research Farm in Storrs, CT from July to November 2015 (Table 2). Seeds were cleaned from the fruits and dried before placing into cool, dark storage (13 ± 2 °C, relative humidity 55 ± 5%).

Seeds were surface sterilized for 30 min in 3% NaOCl, 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% NaOCl for 30 min and rinsed in sterile distilled water. Seed coats were aseptically removed in a laminar flow hood under a stereomicroscope. The distal two thirds of the embryo were excised and cotyledons were separated (Fig. 1b) and transferred with the abaxial surface in contact with the medium in baby food jars (175 ml) with 30 ml of media.

2.2.2. Media and culture conditions

For experiment II, MS basal medium was supplemented with 0.1, 1 or 5 μM NAA in combination with 10 μM BAP. For experiment III, MS basal medium was supplemented 1 μM NAA in combination with 10 μM BAP. MS basal medium used in experiments II and III was supplemented with 3% sucrose (*w/v*) and 0.8% agar (*w/v*) with pH 5.8 prior to autoclaving at 120 °C for 20 min. Cultures were incubated at 26 ± 2 °C in darkness and transferred onto fresh media after one month. Two months after incubation the number of explants producing shoots and the number of shoots per explant were recorded. Regenerated shoots were sub-cultured on shoot proliferation media and rooted *in vitro* as described by Brand and Cullina (1992).

2.2.3. Statistical analysis

A completely randomized design with five replicates, each containing 4 explants, was used for all experiments. Experiments were conducted twice and results were analyzed as one data set. Significance was determined by analysis of variance (ANOVA) and the differences between the means were compared by Fisher's Least Significance Difference test using SAS for Windows 9.3 (SAS Institute Inc., Cary, N.C.). Percentage data was arcsine transformed before statistical analysis.

Table 4
Effect of 1-naphthaleneacetic acid (NAA) concentration (μM) in combination with 10 μM 6-benzylaminopurine (BAP) on shoot organogenesis from excised cotyledons of seven different Pyrinae taxa.

NAA (μM)	Taxon	% cotyledons with shoots[z]	No. of shoots per cotyledon[y]
0.1	*A. melanocarpa*	0.1 a	0 ± 0 a
	A. mitschurinii	10 a	0.4 ± 0.2 a
	×*S. fallax*	0.1 a	0 ± 0 a
	S. aucuparia	0.1 a	0 ± 0 a
	×*S. dippelii*	7.6 a	0.4 ± 0.3 a
	S. aria	7.6 a	0.1 ± 0.1 a
	P. communis	0.1 a	0 ± 0 a
1	*A. melanocarpa*	67.5 a	3.1 ± 0.6 a
	A. mitschurinii	45.0 b	1.7 ± 0.5 ab
	×*S. fallax*	10.0 de	1 ± 0.7 bc
	S. aucuparia	0 e	0 ± 0 c
	×*S. dippelii*	20.0 cd	0.7 ± 0.3 bc
	S. aria	30.0 bcd	1.1 ± 0.6 bc
	P. communis	40.0 bcd	1.1 ± 0.3 bc
5	*A. melanocarpa*	27.5 bc	1.5 ± 0.4 bc
	A. mitschurinii	70.0 a	4 ± 1.2 a
	×*S. fallax*	10.1 d	1.1 ± 0.8 bc
	S. aucuparia	0 d	0 ± 0 c
	×*S. dippelii*	20.0 cd	1.8 ± 1.2 b
	S. aria	53.6 ab	1.6 ± 0.6 bc
	P. communis	19.5 cd	0.9 ± 0.5 bc

[z] Values followed by the same letter in each NAA treatment did not differ significantly according to Fisher's LSD test ($P < 0.05$). Percentage data were arcsine-transformed before statistical analysis. Non-transformed data presented.

[y] Mean values ± standard error. Values followed by the same letter in each NAA treatment group and column are not significant according to Fisher's LSD test ($P < 0.05$).

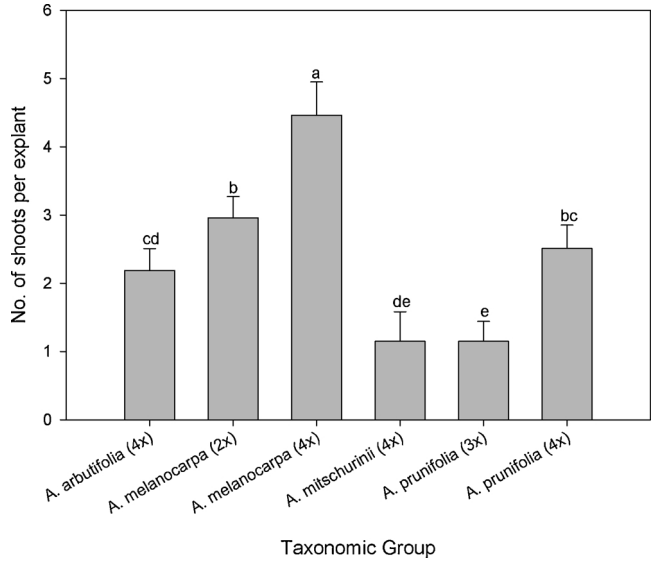

Fig. 2. Effect of taxonomic grouping on number of adventitious shoot per cotyledon in response to 10 μM BAP and 1 μM NAA. Bars followed by the same letter did not differ significantly according to Fisher's LSD test ($P < 0.05$).

3. Results and discussion

3.1. Experiment I – shoot organogenesis from leaves

Leaf explants began to enlarge and form callus after 7 days of culture and adventitious shoot formation was observed after 6 weeks of culture. There was a significant increase in the number of shoots forming per explant when the midrib was wounded (Pr > F = 0.048). Callus appeared along the wounded edges of the proximal end of the explant and at cuts in the midrib vein of the wounded treatment. The 15 different treatments were significantly different (Pr > F < 0.0001), with 5 μM IBA + 10 μM TDZ and 5 μM NAA + 10 μM TDZ resulting in the highest frequency of explants forming shoots. 5 μM IBA + 10 μM TDZ produced the greatest number of shoots forming per explant (Table 3). TDZ produced a significantly greater number of shoots than BAP (Pr > F = 0.0012). TDZ tended to produce shoots that were shorter and wider (Fig. 1c), than BAP produced shoots, which were slender and more elongated (Fig. 1c). Explants on media containing IBA produced a significantly greater number of shoots than NAA and 2,4-D (Pr > F < 0.0001). 2,4-D was ineffective at stimulating adventitious shoot initiation and produced brittle calli formation.

The synthetic cytokinin BAP is commonly used to induce shoot organogenesis and shoot multiplication of Rosaceous taxa in tissue culture, however in this study we found TDZ to be more effective in adventitious shoot regeneration for leaf explants. Similar studies have found TDZ to be effective in shoot regeneration from leaf explants of *Malus domestica* (Zhang et al., 2014), *P. pyrifolia* (Lane et al., 1998) and *P. communis* (Leblay et al., 1991). We found the type of auxin to have a significant role in the ability of *Aronia* leaf explants to produce adventitious shoots. Yancheva et al. (2003) reported that the type of auxin is a critical factor for the determination of shoot organogenesis using apple leaf explants.

3.2. Experiment II – shoot organogenesis from Pyrinae taxa cotyledons

After 4 weeks of culture, adventitious shoots began to emerge from callus tissue which largely formed at the proximal end of cotyledons (Fig. 1d). Significant differences were observed for the number of shoots forming per cotyledon for media treatment (Pr > F < 0.0001), taxon (Pr > F = 0.0003) and media x taxon interaction (Pr > F = 0.0189). Two of the taxa were significant for the number of shoots forming per explant on the three media treatments; *A. melanocarpa* (Pr > F = 0.0003) and *A. mitschurinii* (Pr > F < 0.0001). *A. mitschurinii* produced the greatest number of shoots (4.0 ± 1.2) per explant on 5 μM NAA + 10 μM BAP, while *A. melanocarpa* (3.1 ± 0.6) produced the most shoots on 1 μM NAA + 10 μM BAP. In general, all other taxa produced more shoots on either 1 or 5 μM NAA (Table 4).

The frequency of shoot regeneration per explant was significant for media, taxon and media x taxon interaction (Pr > F < 0.0001). Shoot regeneration was significantly less on 0.1 μM NAA + 10 μM BAP when compared with 1 μM NAA + 10 μM BAP and 5 μM NAA + 10 μM BAP. Media x taxon interaction for frequency of shoot regeneration per explant was significant for *A. melanocarpa* (Pr > F < 0.0001), *A. mitschurinii* (Pr > F < 0.0001), *S. aria* (Pr > F = 0.0007), and *P. communis* (Pr > F = 0.0014). *A. melanocarpa* and *P. communis* had a higher frequency of shoot regeneration on 1 μM NAA + 10 μM BAP, than on the 0.1 μM NAA + 10 μM BAP and 5 μM NAA + 10 μM BAP treatments.

Generally, the number of adventitious shoots per explant increased with increasing percentage of explants forming adventitious shoots. The media and environmental conditions did not induce rhizogenic activity. These results showed that *A. mitschurinii* and *A. melanocarpa* were easy-to-regenerate species using cotyledon explants.

Earlier reports have shown that shoots can be regenerated from *Pyrus* spp. cotyledons using BAP with NAA (Nakajima et al., 2012) or leaves using TDZ with IBA (Hennayake et al., 2003). TDZ and BAP are growth regulators used most frequently in tissue culture to induce shoot organogenesis, while kinetin and isopentenyl adenine (2iP) tend to be used less (Arab et al., 2014). In a preliminary experiment we conducted using cotyledons of ×*S. dippelii* and *P. communis* cultured on media containing either BAP or TDZ, adventitious shoots failed to develop with TDZ (data not shown). Previous studies have indicated that TDZ does not have cytokinin activity, but rather it inhibits endogenous cytokinin oxidases to produce a cytokinin effect (Hare and Van Staden, 1994; Bilyeu et al., 2001). Excised cotyledons presumably produce only

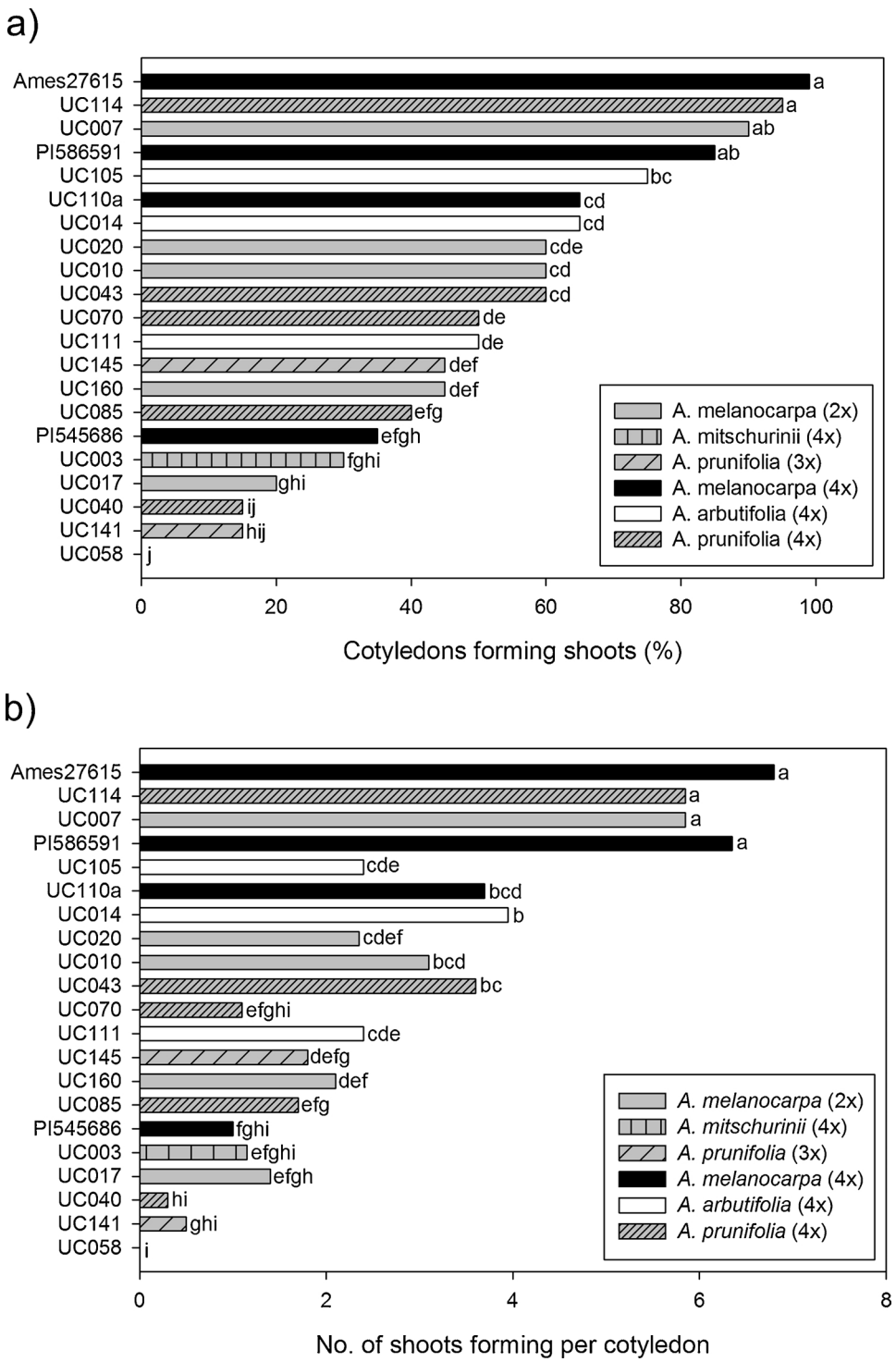

Fig. 3. Effect of *Aronia* taxonomic group and genotype on (a) percent of cotyledons forming adventitious shoots and (b) number of shoots forming per explant on 10 µM BAP and 1 µM NAA. Bars followed by the same letter did not differ significantly according to Fisher's LSD test ($P < 0.05$). Percentage data were arcsine-transformed before statistical analysis. Non-transformed data presented.

low levels of endogenous cytokinin, therefore, TDZ may be less effective at increasing endogenous cotyledon cytokinin content that would stimulate shoot regeneration.

In experiment II, we show that three media treatments, containing various auxin:cytokinin ratios have a significant influence on a cotyledon's ability to become organogenic. In general, *A. mitschurinii* cotyledon explants had a higher frequency of shoot regeneration and number of shoots forming per explant than leaf explants on the same media and optimal media. In most instances, it appears that adventitious shoots were regenerated from dedifferentiated callus tissue, indicating that shoots are forming *via* indirect organogenesis. Nakajima et al. (2012) reported shoot initials forming from the inner part of the wounded site of pear cotyledon explants.

Our results were similar to those reported for cotyledons of *P. communis* 'Bartlett' on media containing 10 µM BAP with 5 µM NAA (Nakajima et al., 2012). However, Gao et al. (2002) reported adventitious shoot regeneration on *Pyrus* cotyledons at higher rates on media containing 20 µM BAP with 0.5 µM NAA and even higher with

30 µM TDZ with 8 µM NAA. TDZ was more effective than BAP in adventitious shoot regeneration of sweet cherry (Canli and Tian, 2008), and TDZ in combination with IBA was effective in regenerating cotyledons of peach (Pooler and Scorza, 1995). Ainsley et al. (2001) reported adventitious shoot regeneration on almond cotyledons to be highest on 10 µM TDZ, however, the presence of auxin (IBA) significantly reduced adventitious shoot formation. The effectiveness of TDZ or BAP with Rosaceous taxa may be the result of different factors such as genotype, explant tissue stage, and other endogenous or exogenous PGRs present.

3.3. Experiment III – shoot organogenesis from cotyledons of Aronia taxonomic groups

The number of shoots forming per cotyledon and the percent of cotyledons forming shoots were both significantly different among the 21 genotypes ($Pr > F > 0.0001$). The most shoot organogenic genotypes were Ames 27615 (*A. melanocarpa,* 4*x*), PI 586591 (*A. melanocarpa,* 4*x*), UC007 (*A. melanocarpa,* 2*x*) and UC114 (*A. prunifolia,* 4*x*) and the least organogenic was UC058 (*A. arbutifolia*, 4*x*), UC040 (*A. prunifolia,* 4*x*) and UC141 (*A. prunifolia,* 3*x*) (Fig. 3).

Significant differences were observed between the six taxonomic groups of *Aronia* for the number of shoots forming per explant (Fig. 2). *A. melanocarpa* tetraploids as a group were most responsive. However, for nearly all taxonomic groups individual accessions that exist fall at the top and bottom of the regeneration spectrum. Within *A. melanocarpa*, ploidy had a significant effect on shoot organogenic capacity, with tetraploid genotypes producing 4.46 shoots per cotyledon, while diploid genotypes only produced 2.96 shoots per cotyledon. Ploidy also influenced shoot organogenesis in *A. prunifolia*, where triploid genotypes produced significantly fewer shoots per explant compared to tetraploid *A. prunifolia.*

Shoot regeneration frequency was noticeably different among the 21 *Aronia* genotypes (Fig. 3). Differences in the ability of callus to become morphogenic from different genotypes has been reported with pear (Caboni et al., 1999; Bell et al., 2012), apple (Sun et al., 2016) and almond (Ainsley et al., 2001). The difference in shoot regeneration of various *Aronia* genotypes confirmed that genotype is a key determinant for shoot organogenesis in this genus. Other factors such as basal medium and growth regulator combinations would presumably have an effect on the regeneration of 21 different genotypes, however, this was not evaluated in the third experiment. The number of shoots forming per explant for *A. mitschurinii* on media containing 10 µM BAP and 1 µM NAA was consistent between experiment one and two, 1.7 ± 0.5 and 1.15 ± 0.4, respectively.

Plantlets regenerated from zygotic embryo tissue will, in most instances, be genetically different from the maternal genotype and therefore the clonal integrity will be lost. However, polyploids of *Aronia* have been documented to reproduce apomictically, resulting in embryos that are identical or nearly identical to the maternal plant (Brand, 2010).

4. Conclusions

This study reports the development of a shoot regeneration system from leaves of *Aronia mitschurinii* 'Viking' and cotyledons of *Aronia* spp. and several Pyrinae taxa. These results will be useful for future studies using leaf and cotyledon explants for genetic transformation, genome editing and mutation breeding with these and similar taxa. Future studies should focus on improving regeneration efficiency and the number of shoots forming per explant.

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References

Ainsley, P.J., Hammerschlag, F.A., Bertozzi, T., Collins, G.G., Sedgley, M., 2001. Regeneration of almond from immature seed cotyledons. Plant Cell Tissue Organ Cult. 67, 221–226.

Arab, M.M., Yadollahi, A., Shojaeiyan, A., Shokri, S., Ghojah, S.M., 2014. Effects of nutrient media, different cytokinin types and their concentrations on in vitro multiplication of G× N15 (hybrid of almond× peach) vegetative rootstock. J. Genet. Eng. Biotechnol. 12, 81–87.

Bell, R.L., Scorza, R., Lomberk, D., 2012. Adventitious shoot regeneration of pear (*Pyrus* spp.) genotypes. Plant Cell Tissue Organ Cult. 108, 229–236.

Bilyeu, K.D., Cole, J.L., Laskey, J.G., Riekhof, W.R., Esparza, T.J., Kramer, M.D., Morris, R.O., 2001. Molecular and biochemical characterization of a cytokinin oxidase from maize. Plant Physiol. (Rockv.) 125, 378–386.

Brand, M.H., 2010. *Aronia*: native shrubs with untapped potential. Arnoldia 67, 14–25.

Brand, M.H., Cullina, W.G., 1992. Micropropagation of red and black chokeberry (*Aronia* spp.). HortScience 27, 81.

Brand, M.H., Connolly, B.A., Levine, L.H., Richards, J.T., Shine, S.M., Spencer, L.E., 2017. Anthocyanins, total phenolics, ORAC and moisture content of wild and cultivated dark-fruited *Aronia* species. Sci. Hortic. (Amst.) 224, 332–342.

Caboni, E., Tonelli, M.G., Lauri, P., D'angeli, S., Damiano, C., 1999. In vitro shoot regeneration from leaves of wild pear. Plant Cell Tissue Organ Cult. 59, 1–7.

Campbell, C.S., Evans, R.C., Morgan, D.R., Dickinson, T.A., Arsenault, M.P., 2007. Phylogeny of the subtribe Pyrinae (formerly the Maloideae, Rosaceae): limited resolution of a complex evolutionary history. Plant Syst. Evol. 266, 119–145.

Canli, F.A., Tian, L., 2008. In vitro shoot regeneration from stored mature cotyledons of sweet cherry (*Prunus avium* L.) cultivars. Sci. Hortic. (Amst.) 116, 34–40.

Connolly, B.A., 2014. Collection, Description, Taxonomic Relationships, Fruit Biochemistry, and Utilization of *Aronia melanocarpa*, *A. arbutifolia*, *A. prunifolia*, and *A. Mitschurinii.* Doctoral Dissertations. Paper 342. .

Dirr, M., 2009. Manual of Woody Landscape Plants, 6th ed. Stipes Publishing, Champaign, Illinois, USA.

Gambino, G., Gribaudo, I., 2012. Genetic transformation of fruit trees: current status and remaining challenges. Transgenic Res. 21, 1163–1181.

Gao, M., Murayama, H., Matsuda, N., Isuzugawa, K., Dandekar, A.M., Nakano, H., 2002. Development of Agrobacterium-mediated transformation of pear (*Pyrus communis* L.) with cotyledon explants and production of transgenic pears using ACC oxidase cDNA. Plant Biotechnol. 19, 319–327.

Hare, P.D., Van Staden, J., 1994. Inhibitory effect of thidiazuron on the activity of cytokinin oxidase isolated from soybean callus. Plant Cell Physiol. 35, 1121–1125.

Hennayake, C.K., Dissanayake, K., Matsuda, N., Takasaki, T., Nakanishi, T., 2003. An efficient and reproducible *in vitro* plant regeneration from leaf discs in pear cultivars (*Pyrus* spp.). Plant Biotechnol. 20, 283–289.

Holefors, A., Xue, Z.T., Welander, M., 1998. Transformation of the apple rootstock M26 with the rolA gene and its influence on growth. Plant Sci. 136, 69–78.

Hummer, K.E., Janick, J., 2009. Rosaceae: taxonomy, economic importance, genomics. Genetics and Genomics of Rosaceae. Springer, New York, NY, pp. 1–17.

Kaneyoshi, J., Wabiko, H., Kobayashi, S., Tsuchiya, T., 2001. Agrobacterium tumefaciens AKE10-mediated transformation of an Asian pea pear, *Pyrus betulaefolia* Bunge: host specificity of bacterial strains. Plant Cell Rep. 20, 622–628.

Lall, S., Mandegaran, Z., Roberts, A.V., 2006. Shoot multiplication and adventitious regeneration in *Sorbus aucuparia.* Plant Cell Tissue Organ Cult. 85, 23–29.

Lane, W.D., Iketani, H., Hayashi, T., 1998. Shoot regeneration from cultured leaves of Japanese pear (*Pyrus pyrifolia*). Plant Cell Tissue Organ Cult. 54, 9–14.

Leblay, C., Chevreau, E., Raboin, L.M., 1991. Adventitious shoot regeneration from in vitro leaves of several pear cultivars (*Pyrus communis* L.). Plant Cell Tissue Organ Cult. 25, 99–105.

Leonard, P.J., Brand, M.H., Connolly, B.A., Obae, S.G., 2013. Investigation of the origin of *Aronia mitschurinii* using amplified fragment length polymorphism analysis. HortScience 48, 520–524.

McKay, S.A., 2001. Demand increasing for aronia and elderberry in North America. N. Y. Fruit Q. 9, 2–3.

Miguel, C.M., Oliveira, M.M., 1999. Transgenic almond (*Prunus dulcis* Mill.) plants obtained by Agrobacterium-mediated transformation of leaf explants. Plant. Cell Rep. 18, 387–393.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15, 473–479.

Nakajima, I., Ito, A., Moriya, S., Saito, T., Moriguchi, T., Yamamoto, T., 2012. Adventitious shoot regeneration in cotyledons from Japanese pear and related species. In Vitro Cell Dev. Biol. Plant 48, 396–402.

Persson Hovmalm, H.A., Jeppsson, N., Bartish, I.V., Nybom, H., 2004. RAPD analysis of diploid and tetraploid populations of *Aronia* points to different reproductive strategies within the genus. Hereditas (Lund) 141, 301–312.

Petri, C., Wang, H., Alburquerque, N., Faize, M., Burgos, L., 2008. Agrobacterium-mediated transformation of apricot (*Prunus armeniaca* L.) leaf explants. Plant Cell Rep. 27, 1317–1324.

Pooler, M.R., Scorza, R., 1995. Regeneration of peach *Prunus persica* (L.) Batsch rootstock

cultivars from cotyledons of mature stored seed. HortScience 30, 355–356.
Sun, Q., Sun, M., Sun, H., Bell, R.L., Li, L., Zhang, W., Tao, J., 2016. Comparative organogenic response of six clonal apple rootstock cultivars. HortScience 51, 272–278.
Tang, H., Luo, Y., Liu, C., 2008. Plant regeneration from in vitro leaves of four commercial *Pyrus* species. Plant Soil Environ. 54, 140.
Wu, X., Gu, L., Prior, R.L., McKay, S., 2004. Characterization of anthocyanins and proanthocyanidins in some cultivars of *Ribes*, *Aronia*, and *Sambucus* and their antioxidant capacity. J. Agric. Food Chem. 52, 7846–7856.
Yancheva, S.D., Golubowicz, S., Fisher, E., Lev-Yadun, S., Flaishman, M.A., 2003. Auxin type and timing of application determine the activation of the developmental program during in vitro organogenesis in apple. Plant Sci. 165, 299–309.
Zhang, X., Qin, Y., Liang, D., Zou, Y., Ma, F., 2014. Enhancement of in vitro shoot regeneration from leaf explants of apple rootstock G. 41. In Vito Cell Dev. Biol. Plant 50, 263–270.
Zheng, W., Wang, S.Y., 2003. Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. J. Agric. Food Chem. 51, 502–509.