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

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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-butryic 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.

**Keywords:**
Rosaceae, Aronia, Sorbus, Shoot organogenesis, 6-Benzylaminopurine, Thidiazuron

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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 common 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 (Miguez 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
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 1**

Seven Pyrinae accessions used in experiment II.

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

**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).
enth condition, we tested 1 and 5μM 1-naphthaleneacetic acid (NAA) in combination with 10 and 20μM BAP or 10 and 20μM thidiazuron (TDZ). We also tested 1 and 5μM indole-3-butyric acid (IBA) in combination with 10μM TDZ and 1μM 2,4-di-chlorophenoxyacetic acid (2,4-D) in combination with 10μM 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 5μM NAA in combination with 10μM BAP. For experiment III, MS basal medium was supplemented 1μM NAA in combination with 10μM 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.

<table>
<thead>
<tr>
<th>NAA (μM)</th>
<th>Taxon</th>
<th>% cotyledons with shoots¹</th>
<th>No. of shoots per cotyledon²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>A. melanocarpa</td>
<td>0.1 a</td>
<td>0 ± 0 a</td>
</tr>
<tr>
<td></td>
<td>A. mitschurinii</td>
<td>10 a</td>
<td>0.4 ± 0.2 a</td>
</tr>
<tr>
<td></td>
<td>× S. fullus</td>
<td>0.1 a</td>
<td>0 ± 0 a</td>
</tr>
<tr>
<td></td>
<td>S. aucuparia</td>
<td>0.1 a</td>
<td>0 ± 0 a</td>
</tr>
<tr>
<td></td>
<td>× S. dippeli</td>
<td>7.6 a</td>
<td>0.4 ± 0.3 a</td>
</tr>
<tr>
<td></td>
<td>S. aria</td>
<td>7.6 a</td>
<td>0.1 ± 0.1 a</td>
</tr>
<tr>
<td></td>
<td>P. communis</td>
<td>0.1 a</td>
<td>0 ± 0 a</td>
</tr>
<tr>
<td>1</td>
<td>A. melanocarpa</td>
<td>67.5 a</td>
<td>3.1 ± 0.6 a</td>
</tr>
<tr>
<td></td>
<td>A. mitschurinii</td>
<td>45.0 b</td>
<td>1.7 ± 0.5 ab</td>
</tr>
<tr>
<td></td>
<td>× S. fullus</td>
<td>10.0 de</td>
<td>1 ± 0.7 bc</td>
</tr>
<tr>
<td></td>
<td>S. aucuparia</td>
<td>0 e</td>
<td>0 ± 0 c</td>
</tr>
<tr>
<td></td>
<td>× S. dippeli</td>
<td>20.0 cd</td>
<td>0.7 ± 0.3 bc</td>
</tr>
<tr>
<td></td>
<td>S. aria</td>
<td>30.0 bed</td>
<td>1.1 ± 0.6 bc</td>
</tr>
<tr>
<td></td>
<td>P. communis</td>
<td>40.0 bed</td>
<td>1.1 ± 0.3 bc</td>
</tr>
<tr>
<td>5</td>
<td>A. melanocarpa</td>
<td>27.5 bc</td>
<td>1.5 ± 0.4 bc</td>
</tr>
<tr>
<td></td>
<td>A. mitschurinii</td>
<td>70.0 a</td>
<td>4 ± 1.2 a</td>
</tr>
<tr>
<td></td>
<td>× S. fullus</td>
<td>10.1 d</td>
<td>1.1 ± 0.8 bc</td>
</tr>
<tr>
<td></td>
<td>S. aucuparia</td>
<td>0 d</td>
<td>0 ± 0 c</td>
</tr>
<tr>
<td></td>
<td>× S. dippeli</td>
<td>20.0 cd</td>
<td>1.8 ± 1.2 b</td>
</tr>
<tr>
<td></td>
<td>S. aria</td>
<td>53.6 ab</td>
<td>1.6 ± 0.6 bc</td>
</tr>
<tr>
<td></td>
<td>P. communis</td>
<td>19.5 cd</td>
<td>0.9 ± 0.5 bc</td>
</tr>
</tbody>
</table>

¹ 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.

² 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).

Table 4 illustrates the 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. The table shows the percentage of cotyledons with shoots and the number of shoots per cotyledon for each treatment. The data were arcsine-transformed before statistical analysis. Non-transformed data presented.

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).

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 < 0.0001) 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 ± 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 < 0.00001). 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), A. 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 less effective at stimulating adventitious shoot initiation and produced brittle callus 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 A. melanocarpa 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.

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).
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 1 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

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**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.
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 ‘A. melanocarpa’, PI 586591 (‘A. melano-nine groups (Fig. 1). 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 M 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 ssp. 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


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