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Keyword:	<i>Betula lenta</i> , cryopreservation, droplet-vitrification, shoot tips, encapsulation-vitrification

Development of cryopreservation methods for cherry birch (*Betula lenta* L.), an endangered tree species in Canada

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Abstract

Cherry birch (*Betula lenta* L.) is an endangered species in Canada with only a single natural population of 18 trees found in the Niagara region of Ontario. The tree was reportedly used as a medicine by First Nations and Native American peoples. The current study describes a cryopreservation method for seeds and *in vitro* shoot tips of *B. lenta* in hopes of conserving available germplasm. Cryopreservation of mature seeds was successful with 24% of seeds germinating in the greenhouse and 12% of seeds germinating *in vitro* on medium supplemented with 10 µM thidiazuron or 6-benzylaminopurine. Post-cryopreservation regrowth of *in vitro* shoot tips was achieved using a droplet-vitrification protocol after preculture in 0.3 M sucrose for 24 h followed by osmoprotection in loading solution for 20 min and treatment with vitrification solution A3 composed of glycerol, sucrose, ethylene glycol and dimethylsulfoxide at 0°C for 60 min. The highest plant regeneration (52%) was obtained after unloading (rehydration) in medium with 0.8 M sucrose for 30 min. Differential scanning calorimetry analysis confirmed the absence of ice crystallization in the shoot tips during cooling and rewarming. This is the first

report for successful cryopreservation of *B. lenta*, which will further promote initiatives for conservation of North American biocultural diversity.

Keywords: *Betula lenta*, cryopreservation, droplet-vitrification, encapsulation-vitrification, conservation, seeds, shoot tips

Introduction

Cherry birch or Sweet Birch, *Betula lenta* L., is an ecologically important tree species native to North America (Fig. 1A) with purported medicinal uses amongst Native American and First Nations peoples to treat fevers, colds and flues, or general malaise (Moerman 2009). Since 1967 the population for *B. lenta* in Canada has declined significantly (72%) as a result of increased pressures from urban development and shoreline erosion. To date it is estimated that only 18 trees remain in the Niagara region of Ontario, Canada (Zoladeski and Hayes 2013). As a result of the above, *B. lenta* is designated as endangered, with N1 (national rank) and S1 (provincial rank) designations in Canada (COSEWIC 2006).

Current strategies to conserve and recover this species in Canada include monitoring (keeping track of trees and their health) and maintaining the current habitat (Zoladeski and Hayes 2013) as well as planting trees in arboretums. Recently an efficient protocol for *in vitro* propagation of *B. lenta* was developed at the Gosling Research Institute for Plant Preservation (GRIPP), University of Guelph (Canada) (Rathwell et al. 2016). However, all these options have

limitations in terms of long-term conservation. Trees in the natural environment and arboreta are susceptible to pests, diseases, and environmental changes, while *in vitro* collections are expensive to maintain and are prone to contamination (Benelli et al. 2013). Conventional methods for seed banking where seeds are stored in both cold (-18°C to 4°C) and dry (3-5% moisture content, MC) environments are not efficient for *Betula* species, as seed germination can decrease significantly over time under these conditions (Heit 1967; Seed Information Database of the Royal Botanic Gardens Kew, SID 2015). Furthermore, there is currently scarce information available with respect to the storage behavior of cherry birch seeds.

In order to further support conservation efforts, this study aimed to develop a protocol for cryopreservation in liquid nitrogen (LN, -196°C) of *B. lenta* seed and *in vitro* shoot tips. Cryopreservation is a highly efficient method for long-term storage of living cells and tissues (Pritchard and Nadarajan 2008). At cryogenic temperatures below -130°C, cell division and nearly all metabolic activities of cells cease, and samples can theoretically be preserved for hundreds of years while requiring limited space and maintenance compared to material grown *in vitro* or in the field (Benelli et al. 2013). The type of material (e.g. seeds, dormant buds, or shoot buds), its moisture content, and the physiological status before cryopreservation are known to significantly influence regrowth after cryopreservation (Sakai and Engelmann 2007). Seeds are the preferred option for cryobanking native plant species, however, as is the case for *B. lenta*, they are difficult to collect due to high annual variation in seed production. Cryopreservation of vegetative plant tissues is challenging, primarily due to the high amount of water that can be converted into ice which in turn may cause irreversible cell damage during cooling and rewarming. Reducing MC in the samples prior to cryopreservation as well as applying cryoprotectant solutions can help minimize such damage (Volk and Walters 2006). The process

of dehydration and cryoprotection may, however, require optimization for every new species in order to obtain regrowth (Sakai and Engelmann 2007).

Two methods were used for cryopreserving *in vitro* shoot tips in this study: (1) encapsulation-dehydration and (2) droplet-vitrification. Encapsulation of tissues in a sodium-alginate matrix followed by dehydration in air or in cryoprotectant solutions (Fabre and Dereuddre 1990; Paul et al. 2000) prior to cooling is preferable when operating with fragile and sensitive materials (Sakai and Engelmann 2007). In droplet-vitrification, shoot tips are dehydrated in highly concentrated cryoprotectant solutions (vitrification solutions, VS) and then cryopreserved in tiny drops of VS attached to aluminum foil strips to ensure high rates of cooling and rewarming (Panis et al. 2005; Teixeira et al. 2014). This method has proven effective for cryopreserving shoot tips of important agricultural crops such as potato, chrysanthemum, garlic, and banana (Panis et al. 2005; Kim et al. 2012), however, high toxicity of many VS may limit their application (Sakai and Engelmann 2007; Kim et al. 2009b). To date, vitrification and encapsulation-based methods have not been applied to *B. lenta* germplasm, however, they have been used for cryopreserving vegetative materials of trees and shrubs in several genera including *Populus*, *Prunus*, *Quercus*, *Malus*, *Picea*, and *Olea* (Benelli et al. 2013; Pritchard et al. 2014).

The main objective of this study was to develop a protocol for cryopreservation of *B. lenta* using seed and *in vitro* shoot tips, thus allowing the use of this approach for the conservation of endangered Canadian cherry birch.

Materials and Methods

Cryopreservation of Seeds: Seeds were collected from mature cherry birch trees maintained in the University of Guelph Arboretum during the winters of 2012/2013 and 2013/2014, and were

92 stored at 4°C. Moisture content was measured by placing seeds in an oven at 100°C and
93 recording change in weight at various time intervals until a constant weight was achieved.
94 Moisture content was calculated using the formula:

$$MC = \frac{fw - dw}{fw}$$

95 where MC is the moisture content, and fw and dw are the weights of fresh and dried seeds,
96 respectively. MC was expressed in percentage. Measurement was performed in three technical
97 replications, each containing at least 30 seeds, and repeated twice.

98 To determine the impact of cryopreservation, seeds were sealed in 2 ml cryovials (Nunc,
99 USA) and stored in LN for a minimum of one hour. Cryovials were then placed in a 40°C water
100 bath for 90 seconds to re-warm. Control and cryopreserved seeds were surface sterilized with 4%
101 commercial bleach (Clorox®, The Clorox company; 5.4% sodium hypochlorite) for one hour,
102 followed by 20% bleach for 15 min, and rinsed with autoclaved deionized water four times, each
103 wash for 3 min.

104 For germination, seeds were plated on semi-solid or liquid media of the same
105 composition. Standard medium (STM) consisted of DKW basal salts (Driver and Kuniyuki
106 1984), 3% sucrose and vitamins (100 mg/l myo-inositol + 2 mg/l glycine + 1 mg/l nicotinic
107 acid), without growth regulators. The semi-solid medium was prepared with 2.2 g/l phytagel
108 (Sigma-Aldrich, Canada). To improve germination, this standard medium was supplemented
109 with 6-benzylaminopurine (BA) (Phytotechnology, Shawnee Mission, Kansas, USA), or
110 thidiazuron (TDZ) (Caisson, Logan, Utah, USA) at 10.0 or 20.0 µM. All treatments were kept in
111 the dark at 24 ± 2°C. Seed germination was recorded at various intervals over 66 days.
112 Germination was determined by the presence of developed cotyledons and roots and expressed

as percentage out of the number of seeds taken for the treatment. Three to five replications were used for each treatment; each replication contained 4 seeds.

Alternatively, control and cryopreserved seeds were planted directly in Sunshine professional growing medium (Sun Gro Horticulture Canada Ltd., Vancouver, Canada) and germinated in the greenhouse (24°C for 16 h light and 20°C for 8 h darkness, light intensity 110 $\mu\text{mol m}^{-2}\text{s}^{-1}$). Germination was recorded at various intervals over 66 days. Both control and cryopreservation treatments were performed in twelve replications, each replication consisting of 30 seeds.

Cryopreservation of *in Vitro* Shoot Tips

In vitro stock plants of *B. lenta* were obtained from the GRIPP (University of Guelph, Guelph) *in vitro* collection and maintained on shoot induction media (SIM) at $25 \pm 2^\circ\text{C}$ under a 16-h photoperiod at light intensity of $40 \text{ mol m}^{-2}\text{s}^{-1}$ by periodic subcultures every 6 weeks (Rathwell et al. 2016). SIM was composed of DKW basal salts (Driver and Kuniyuki 1984), 3% sucrose and vitamins (100 mg/l myo-inositol + 2 mg/l glycine + 1 mg/l nicotinic acid) and was supplemented with 5 μM BA and 1 μM gibberellic acid (GA_3). Shoot tips with an average size of 1.5 - 2.0 mm were dissected from: i) 12-day-old nodal segments or ii) 60-day-old plants. Explants were placed on SIM for 24 h to recover from stress caused by excision. These shoot tips were cryopreserved using encapsulation-vitrification or droplet-vitrification methods.

Encapsulation-vitrification

For encapsulation, shoot tips were excised from 60-day-old plants and suspended in 3% Na-alginate (Sigma-Aldrich, Canada). Alginate droplets, each containing one explant, were transferred into 100 mM CaCl_2 solution using a wide mouth 10 ml serological pipette (Costar®,

USA) and left for 20 min with gentle agitation to form beads ca. 4 mm in diameter (Fabre and Dereuddre 1990; Fig. 1D). Beads were precultured in 0.3 M sucrose solution for 24 h and then transferred into loading solution (2.0 M glycerol + 0.4 M sucrose) (Nishizawa et al. 1993) for 20 min at room temperature. Afterwards, plant vitrification solution 3 (PVS3), composed of 50% glycerol and 50% sucrose, w/v (Nishizawa et al. 1993) was applied to beads for 20, 40 or 60 min at room temperature on a shaker (90 rpm). For cryopreservation, beads were placed in 2 ml cryovials filled with 1 ml fresh PVS3. Cryovials were sealed and quickly immersed in LN for one hour then rewarmed in a 40°C waterbath for 2 min. Beads were transferred to unloading solution containing 1.2 M sucrose for 40 min then placed on SIM for regrowth. Further treatments aimed at the optimization of this technique included: i) using shoot tips without alginate beads as compared to shoot tips encapsulated in alginate beads; and ii) removing shoot tips from beads after rewarming and unloading, before plating them on SIM. All materials were cultured in darkness for the initial five days after rewarming and then moved to low light conditions ($5 \pm 2 \mu\text{mol m}^{-2} \text{s}^{-1}$). Shoot tips that remained green after 10 days were counted for “survival”. Shoot tips that developed into plantlets after 30 days were counted to assess “regeneration”. All treatments were performed with three independent replications, each containing at least 10 shoot tips.

153

154 *Droplet-vitrification*

155 Experiments were performed using shoot tips excised from 12-day-old nodal segments, if
156 not stated otherwise. In the first experiment, the effect of different steps of the droplet-
157 vitrification protocol, named preculture, osmoprotection and dehydration in VS, on shoot tip

regrowth was evaluated without cryopreservation. To do this, shoot tips were collected after each step in a series of sequential treatments including:

- i) preculture in 0.3 M sucrose solution for 24 h;
- ii) preculture and osmoprotection in loading solution (1.9 M glycerol + 0.5 M sucrose, LS) (Kim et al. 2009a) for 20 min at room temperature;
- iii) preculture, osmoprotection and exposure to VS A3 (37.5% glycerol, 22.5% sucrose, 15% ethylene glycol (EG), and 15% dimethyl sulfoxide (DMSO), w/v, (Kim et al. 2009b) at 0°C for 60 min;
- iv) preculture, osmoprotection and exposure to PVS3 for 60 min at room temperature.

Shoot tips after treatments (i) and (ii) were directly transferred to SIM in 6 cm Petri dishes. Shoot tips after treatments (iii) and (iv) were transferred into 1.2 M sucrose solution (unloading solution) and for 30 min on a shaker (90 rpm) to remove cryoprotectants, then placed on SIM in 6 cm Petri dishes. Cultures were kept under low light ($5 \pm 2 \mu\text{mol m}^{-2} \text{s}^{-1}$) for the initial five days then transferred to light intensity of $110 \mu\text{mol m}^{-2} \text{s}^{-1}$. Survival and regeneration were assessed as described above in the encapsulation-vitrification method.

In the second experiment, cryopreservation of shoot tips was performed by preculturing in 0.3 M sucrose for 24 h, then transferring to an osmoprotected solution in LS for 20 min, followed by treatment in VS A3 at 0°C for 60 min. After that, each shoot tip was placed in an individual drop of ice-cold A3 on strips of aluminum foil (Western Plastics, Canada) (6×30 mm) (Fig. 1E). Foil strips with shoot tips were quickly immersed in LN for at least one hour. For rewarming, the foil strips were plunged in preheated ($40 \pm 1^\circ\text{C}$) 1.2 M sucrose solution for 30 seconds; after which an equivalent volume of unloading solution at room temperature was added.

181 Foils were removed with forceps and shoot tips were kept in unloading solution for 30 min on a
182 shaker (90 rpm), blotted dry on sterile filter paper and plated on SIM for regrowth. Control shoot
183 tips were treated equally except they were not immersed in LN.

184 Since regeneration of shoot tips cryopreserved by this protocol remained below 25%,
185 different modifications of the protocol steps were further investigated:

186 Preculture. Shoot tips were precultured in 0.3 M sucrose for 24 h (standard protocol) or
187 step-wise in sucrose solutions with progressively increasing concentrations (0.3 M for 1 day then
188 0.5 M for 16 h followed by 0.7 M for 4 h).

189 Cold hardening of donor plants. Shoot tips were excised from *in vitro* plants that were
190 cold hardened on media supplemented with 3 and 5% sucrose for 6 and 12 days at 5°C in
191 darkness.

192 Vitrification solution type and duration of treatment. In order to reduce toxicity and find
193 the most effective combination of VS type and treatment, various durations of exposure to VS
194 A3 and PVS3 were tested with and without cryopreservation. Shoot tips were precultured and
195 loaded as in standard protocol, then exposed to VS A3 at 0°C for 0, 5, 10, 20, 40, 60, or 80 min,
196 or to PVS3 for 30 or 60 min at room temperature.

197 Growth regulators in regrowth medium. Different combinations of GA₃, BA, zeatin and
198 kinetin in culture media were tested in order to stimulate plant regeneration from cryopreserved
199 shoot tips. The following combinations of growth regulators have been tested: “GA₃”: 1 µM
200 GA₃; “BKG”: 2.5 µM BA + 2.5 µM kinetin + 1 µM GA₃; “BG”: 5µM BA + 1 µM GA₃; “ZG”:
201 5µM zeatin + 1 µM GA₃; “KG”: 5 µM kinetin + 1 µM GA₃.

202 Modification of osmoprotection and unloading treatments. The protocol was further
203 modified by increasing duration of osmoprotection treatment from 20 to 40 min and reducing

concentration of unloading solution and/or duration of unloading step. In this experiment, shoot tips were excised from 60-day-old plants and only plant regeneration was measured. Treatments included: Tr1: standard protocol, Tr2: osmoprotection for 40 min, Tr3: unloading in 0.8 M sucrose for 30 min, Tr4: unloading in 0.8 M sucrose for 15 min, Tr5: unloading in 1.2 M sucrose for 15 min.

All solutions used for preculture, cryoprotection, and unloading were prepared in MS basal medium with MS vitamins (pH 5.7), and sterilized by filtering through 0.2 μ M filters (Falcon, USA).

All experiments in droplet-vitrification method were performed with three independent replications, each containing at least 10 shoot tips. Survival and regeneration were assessed as described in encapsulation-vitrification method.

Differential Scanning Calorimetry Analysis

Phase transitions in shoot tips of cherry birch were investigated using a Differential Scanning Calorimeter (DSC) (DSC1, Mettler Toledo, Leicester, UK), calibrated with zinc (422.81°C, 115.57 J g⁻¹) and indium (156.85°C, 29.03 J g⁻¹) standards. Shoot tips were sealed in 40 μ l aluminum pans. Samples were placed in the DSC at 22°C and cooled at a rate of -10°C min⁻¹ to -80°C, at which temperature the sample was held isothermally for 5 min before rewarming to 25°C at a rate of 10°C min⁻¹. Samples tested included i) control shoot tips without any treatment and ii) shoot tips precultured in 0.3 M sucrose for 24 h and exposed to loading solution for 20 min followed by either PVS3 (30 or 60 min) or A3 at 0°C (20, 40, 60, or 80 min). Each treatment was replicated three times. Thermograms were analyzed using STARE thermal analysis software (Mettler Toledo, Leicester, UK).

Statistical Analysis

Data from experiments were first tested for normality using the D'Agostino-Pearson normality test. Normally distributed data were compared using a one-way ANOVA followed by Duncan's multiple range test (MRT) in cases where the model was significant. If the data were not normally distributed, Kruskal-Wallis test was applied followed by Dunn's multiple comparisons test. All statistical analyses were conducted using JMP for Mac and SAS University edition 3.4 (SAS Institute Inc. Cary, North Carolina, USA). Statistics are presented as means with standard errors, and different letters in figures indicate significant differences at $\alpha = 0.05$.

Results

Cryopreservation of Seeds

The average seed MC before cryopreservation was 10.5%. Seeds collected during the winter 2012/2013 completely lost viability and did not germinate after two years of storage at 4°C. One-year-old seeds from the 2013/2014 collection remained viable: 33-50% of seeds germinated *in vitro* and 31% of seeds germinated in the greenhouse (Fig. 2C). Highest germination of control (non-cryopreserved) seeds *in vitro* (50%) was recorded in semi-solid medium supplemented with 20 μ M TDZ, followed by medium without growth regulators (Fig. 2A, LNC). After cryopreservation, *in vitro* germination above 10% was observed only for seeds cultured on semi-solid media supplemented with 10 μ M TDZ, or liquid media supplemented with 10 μ M BA (Fig. 2, LN). Germination of cryopreserved seeds in the greenhouse was ca. 10% lower than for non-cryopreserved seeds (Fig. 2C). All seedlings that emerged from control and cryopreserved seeds developed into normal plants (Fig. 1B, C).

Cryopreservation of *in Vitro* Shoot Tips

Isolated shoot tips that were placed on SIM without any treatment showed 100% survival and shoot development (regeneration).

Encapsulation-vitrification. Over 80% survival was recorded prior to cryopreservation for shoot tips following all steps of the encapsulation-vitrification protocol. However, regeneration of plantlets from the encapsulated shoot tips was very poor (below 5%). By contrast, 60% regeneration was observed for shoot tips that were manually removed from beads before placing them on culture medium for regrowth. After cryopreservation, shoot tips remained green for 10-15 days but were unable to develop shoots. No regeneration of cryopreserved shoot tips was recorded in any treatment including those when the explants were removed from the beads.

Droplet-vitrification. More than 95% shoot tips survived after the two initial steps of the protocol, i.e. preculture and osmoprotection (Fig. 3). Meanwhile, regeneration was reduced to 92% and 88% after preculture and osmoprotection, respectively (Fig. 3). Both survival and regeneration sharply decreased after applying VS A3 or PVS3 (Fig. 3). The adverse effect of VS exposure was highly significant ($P < 0.0001$) on regeneration which dropped down to 27% and 16% after 60 min treatment with PVS3 and VS A3, respectively (Fig. 3). When shoot tips were exposed to VS A3 and PVS3 for various durations, no significant differences in survival and regeneration were observed between treatments without cryopreservation, mostly due to high variations between replications within each treatment (Fig. 4, LNC). After cryopreservation, treatment with A3 for 5 and 10 min resulted in survival of over 35%, mostly due to callus formation as no plant regeneration was observed. The highest plant regeneration of 13% was

272 obtained when shoot tips were treated with VS A3 for 80 min, and with PVS3 for 60 min (Fig. 4,
273 LN), however the differences between the two solutions were not significant.

274 Shoot tips excised from cold hardened plants did not show any improvement in regrowth
275 after cryopreservation. Similarly, no beneficial effect on regrowth was observed when shoot tips
276 were precultured with step-wise increasing sucrose concentration (data not shown). Regeneration
277 of non-cryopreserved shoot tips was significantly ($P < 0.0082$) higher on media supplemented
278 with GA₃ alone without cytokinins (Fig. 5). Survival of cryopreserved shoot tips was higher on
279 media supplemented with GA₃ alone or in combination with BA compared to media with kinetin
280 or zeatin. However, there was no significant difference in plant regeneration between
281 cryopreserved shoot tips cultured on different media (Fig. 5).

282 The highest regeneration of 52% after cryopreservation was achieved when sucrose
283 concentration in unloading solution was reduced from 1.2 M to 0.8 M (Fig. 6, Tr 3). Reducing
284 the duration of unloading step from 30 min to 15 min (Trs 4 and 5) significantly ($P < 0.0003$)
285 reduced regeneration while increasing osmoprotection step by 20 min (Tr 2) showed no effect.

286

287 **Differential Scanning Calorimetry Analysis**

288 Thermograms were interpreted for critical cryopreservation parameters such as
289 crystallization, melt onset temperatures, and enthalpy of crystallization and melting. Exothermal
290 and endothermal peaks indicating, respectively, ice crystallization during cooling and ice
291 melting during rewarming were observed in shoot tips without any treatment (Fig. 7, Control) in
292 all replicates. By contrast, no crystallization or melting events could be detected if shoot tips
293 were preliminary treated with vitrification solutions A3 or PVS3 for all time periods tested (Fig.

7). These studies provide indirect evidence of the absence of ice in shoot tips cryoprotected before cryopreservation.

Plant development and initiation of cherry birch collection in a cryobank

The majority of shoot tips cryopreserved using the improved protocol (Tr 3 on Fig. 6) showed direct plant regeneration without callus formation (Fig. 1F). During regrowth, these shoot tips produced multiple shoots (Fig. 1G) which were further propagated and rooted following the protocol developed earlier (Rathwell et al. 2016) to obtain healthy looking young plants with roots.

The cryopreservation method developed in this study was used to initiate the long-term storage collection of *B. lenta* germplasm at the GRIPP cryobank by cryopreserving 200 shoot tips. 30 additional shoot tips were simultaneously cryopreserved and rewarmed after one hour as an inner control; these shoot tips showed 50% plant regeneration (data not presented).

Discussion

Cryopreservation of endangered tree species can be achieved by using seeds, pollen, winter buds and *in vitro* cultured materials, however all of these propagules have their own limitations. Storing seeds is an efficient and practical approach as a large diversity of genotypes can be stored using minimal space (Pritchard et al. 2014). Seed cryostorage in LN (-196°C) or in its vapor phase (-160 to -195°C) has been successfully applied to conserve wild plant species at risk (e.g. Pence 1991; Kholina and Voronkova 2008; Chmieraz 2010b; Popova et al. 2013; Michalak et al. 2015). Seeds of *B. lenta* are listed as orthodox in the Seed Information Database of Kew Botanical Garden (SID 2015). The same database referred to Joseph (1929) for the

statement that 77% of *B. lenta* seeds were able to germinate after 1.5 years of hermetic storage at room temperature with 7.4% MC. According to Brinkman (1974), seeds of *Betula* species can be stored at room temperature with 1% MC. The Canadian Forestry Service (Wang 1974) reported that *B. lenta* seeds tolerated desiccation to 1% MC and recommended hermetic storage at temperatures of 3°C to -25°C with 1-3% MC for their long-term conservation. Supporting the data on orthodox behavior of birch seeds, Chmielarz (2010a) reported that seeds of silver birch (*Betula pendula* Roth.) tolerated desiccation over silicagel to 0.02 g g⁻¹, with 77% of seeds germinating after 14 days of sowing. Chmielarz (2010a) also referred to other studies where seeds of *Betula* species with 8 – 10% MC could be stored at -25°C for 10–12 years (Heit 1967; Wang 1974). In our study, mature seeds of cherry birch completely lost their viability after two years of storage at 4°C with 10.5% MC, while one-year-old seeds showed 30-32% germination in the greenhouse. *In vitro* germination rates ranged from 0 to 50% depending on the medium used. Relatively low germination of cherry birch seeds in this study may have resulted from storage conditions at 4°C or may be related to seed dormancy which has been previously reported for *Betula* species (Chmielarz 2010a; De Atrip and O'Reilly 2006).

Cryopreserving seeds within a range of safe MC is crucial for their survival after cryopreservation (Pritchard et al. 2014). While a safe MC window varies among species, it is generally accepted that orthodox seeds with a MC below 13% are sufficiently dry to be cryopreserved without any reduction in germination rates being seen (Pritchard and Nadarajan 2008). For dormant seeds of closely related species such as *B. pendula*, the safe range of MC for cryopreservation was found to be between 2.0 and 18.7% (Chmielarz 2010a). In comparison, safe MC for cryopreservation of orthodox seeds was 7.4 - 19.4% for *Fraxinus excelsior* L. (Chmielarz 2009) and 2.9 - 17.4% for *Ulmus glabra* Huds (Chmielarz 2010b). Thus, it is likely

that *B. lenta* seeds cryopreserved at 10.5% MC should be within their safe MC range. However, this does not guarantee that their germination will be fully retained after cryogenic exposure and rewarming as other factors such as cooling and rewarming velocity, presence of the pericarp and seed provenance may influence seed viability (Kholina and Voronkova 2008; Popova et al. 2013, 2016). Since there is limited information in the literature about the physiology of *B. lenta* seeds, further investigation is required to reveal factors that caused low germination after cryopreservation. Apart from lower germination, cryopreservation had no negative effect on seed development. The plantlets raised from cryopreserved seeds both *in vitro* and in a greenhouse appeared strong and healthy without any abnormalities or delay in growth. Thus, the cryopreservation protocol is expected to provide germination of seeds, which are physiologically competent. In the future, the simplicity of the protocol would allow testing of seeds collected from various trees at various environmental conditions over years to select best material for cryopreservation.

Cryopreservation of *in vitro* shoot tips is a valuable option for conservation of tree genetic resources (Ryynänen and Aronen 2005). Various methods such as vitrification, encapsulation-dehydration, and encapsulation-vitrification have been proven effective for cryopreserving shoot tips of American elm (Uchendu et al. 2013), white poplar (Lambardi et al. 2000), silver birch (Ryynänen and Aronen 2005) and others (Häggman et al. 2008 and Benelli et al. 2013). In this study encapsulation-vitrification did not work, and the cryopreserved shoot tips died within two weeks after rewarming. One of the reasons could be a detrimental effect of the alginate matrix that suppressed the growth of regenerating plantlets out of the beads or insufficient dehydration of alginate-coated shoot tips.

Successful cryopreservation was achieved using a modified droplet-vitrification protocol. As with almost any vegetative material, shoot tips of *in vitro* cultured plants experience severe stress during the cryopreservation process which may arise due to a combination of osmotic, mechanical, temperature, chemical, oxidative, and other detrimental events (Sakai and Engelmann 2007). This stress is intensified by toxic effects of some cryoprotectant chemicals (Volk and Walters 2006). The composition of a cryoprotectant solution and the duration of exposure are the most critical factors in plant cryopreservation (Sakai and Engelmann 2007). The results of the present study showed high sensitivity of cherry birch shoot tips to the toxic effects of both PVS3 and VS A3 solutions as a sharp drop of both survival and regeneration percentage after 60 min treatment (Fig. 3). The most prominent adverse effect was observed with plant regeneration, which dropped from 100% for the untreated shoot tips to 15% after 20 min of treatment with A3 solution and without cryopreservation (Fig. 4). The highest regeneration of cryopreserved shoot tips was observed following treatment with A3 solution for 80 min and with PVS3 solution for 30 min (Fig. 4). In comparison, the optimum duration of exposure to another well-known four-component vitrification solution PVS2 was 60 min at 0°C for white poplar (Lambardi et al. 2000), 120 min at 0°C for silver birch (Ryynänen and Aronen 2005) and 105 min at 25°C for cherry shoot tips (Niino et al. 1997). Recently, Vujović et al. (2015) reported cryopreservation of plum shoot tips using two variations of the aluminum cryo-plate method and A3 solution for cryoprotection with average regrowth between 58-78% in cherry plum and 28-48% in plum cultivar “Požegača”. The results shown in Figs 3 and 4, and the absence of ice crystallization during cryopreservation process confirmed by DSC (Fig. 7) suggest that low regeneration after cryopreservation was likely caused by explant sensitivity to dehydration stress and/or toxicity of VS rather than ice crystallization-induced damage. In other words, the protocol

provided sufficient cryoprotection, but the explants could not tolerate the treatment due to their sensitivity. The two common ways to solve this situation are to increase desiccation tolerance of the initial plant material and to optimize the protocol to help the explants survive through severe dehydration and rehydration stresses.

Cold-hardening at low positive temperatures and preculture with elevated sucrose concentrations is often used for plant materials in order to induce their desiccation and chilling tolerance, help mitigate osmotic stress, and reduce injury during cryopreservation (Sakai and Engelmann 2007). In the present study, however, such treatments did not result in any significant improvement towards regeneration. Also, no beneficial effect was obtained by removal or changing the type of cytokinins in the regrowth media (Fig. 5). The media used was previously optimized for cherry birch (Rathwell et al. 2016) therefore we did not test other combinations and concentrations of growth regulators.

The results of protocol modification (Fig. 6) demonstrate the importance of individual steps of the cryopreservation procedure for plant regrowth. Improvement in regeneration was observed after reducing sucrose concentration (0.8 M) in the unloading solution and its duration. During unloading, cryoprotectants are removed from the shoot tips, and the kinetics of this process is affected by shoot tips size and the composition of VS. For example, concentration of DMSO in PVS2-pretreated garlic shoot tips decreased sharply from 70 to 60 mg/g fresh weight within the first 10 min of unloading followed by further slow decrease to 56 mg/g fresh weight during the next 50 min (Kim et al. 2004). By contrast, the glycerol concentration in PVS3-treated shoot tips decreased slowly and steadily during 90 min of unloading (Kim et al. 2005). It is likely that for shoot tips of cherry birch, which are highly sensitive to VS, it is important to remove the components of VS or significantly reduce their concentration before placing the

explants on medium for regrowth. Reducing the concentration of sucrose in unloading solution may speed up removal of toxic cryoprotectant chemical. Osmotic potential of unloading solution may also influence regeneration of shoot tips by controlling the rehydration process. It has been shown that MC of shoot tips may increase by 20% during the first 10 min of unloading (Kim et al. 2005). Thus it is essential to find the balance between the potentially beneficial and hazardous effects at every step of the procedure, including rehydration. Based on these preliminary findings, further optimization of the protocol can be done in the future to improve post-cryopreservation performance of cherry birch shoot tips. The plants developed from cryopreserved shoot tips grew well, looked healthy and could be further multiplied using the method of *in vitro* propagation developed earlier (Fig. 1G) to produce large number of plants for replenishing habitats.

In conclusion, this is the first report on successful cryopreservation of cherry birch, an endangered species in Canada. Using cryopreserved seeds or shoot tips, vigorous and healthy plants were obtained *in vitro*, and used further for subsequent propagation and acclimatization in the greenhouse. A long-term storage collection for *B. lenta* has been initiated by placing the first 200 shoot tips of cherry birch in the GRIPP cryobank at the University of Guelph (Guelph, Canada). These results also open the door for more detailed studies on *B. lenta* seed storage behavior and the application of cryopreservation protocols in conservation strategies, and may provide a model for conserving other tree species of concern. Furthermore, this study also demonstrates the potential *in vitro* culture technologies for species recovery and a long-term conservation of Canadian plant biodiversity including the species with biocultural, medicinal, and agricultural significance.

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Figure Captions

Fig. 1. **A** –30-years-old *Betula lenta* tree in the Arboretum of the University of Guelph (Ontario, Canada); **B, C** - germination of cryopreserved seeds *in vitro* and in the greenhouse, respectively; **D** - shoot tips encapsulated in alginate beads; **E** - shoot tips in drops of A3 solution on aluminium foil strip before immersion into liquid nitrogen, bar = 5 mm; **F** - regrowth of the shoot tip cryopreserved by droplet-vitrification method; **G** - *In vitro* plants developed from the cryopreserved shoot tips.

Fig. 2. Germination of control (LNC) and cryopreserved (LN) seeds. **A** – semi-solid media; **B** - liquid media. **C** - seeds sown in the greenhouse. BA - benzylaminopurine, TDZ - thidiazuron. Numbers in baskets show the concentrations (in μM) of BA or TDZ in the medium. Basal – basal media without growth regulators. No germination was observed on media supplemented with 20 μM BA in any treatment; this media is not shown for simplicity.

Fig. 3. Effect of different stages of the droplet-vitrification protocol on survival and regeneration of shoot tips (without cryopreservation). Con - control (untreated) shoot tips. PC - preculture with 0.3 M sucrose for 24 h, PC+LS - preculture followed by loading solution for 20 min (osmoprotection), PC+LS+A3 and PC+LS+PVS3 – shoot tips after preculture and osmoprotection were treated for 60 min with vitrification solution A3 at 0°C or with PVS3 at room temperature, respectively. Values followed by different letters are significantly different based on Duncan MRT (Survival) or Dunn's multiple comparisons test (Regeneration) at $\alpha = 0.05$.

Fig. 4. Effect of treatment duration with A3 and PVS3 cryoprotectant solutions on shoot tip survival and regeneration before (LNC) and after (LN) cryopreservation using droplet-vitrification method. VS A3 was applied at 0°C. Values followed by different letters are significantly different based on Dunn's multiple comparisons test at $\alpha = 0.05$.

Fig. 5. Effect of combinations of various growth regulators in the regrowth media on survival and regeneration of shoot tips after treatment with VS A3 at 0°C for 60 min (LNC) and cryopreservation (LN).

The following combinations of growth regulators have been tested: "GA₃": 1 μ M GA₃; "BKG": 2.5 μ M BA + 2.5 μ M kinetin + 1 μ M GA₃; "BG": 5 μ M BA + 1 μ M GA₃; "ZG": 5 μ M zeatin + 1 μ M GA₃; "KG": 5 μ M kinetin + 1 μ M GA₃. Asterisk indicate the data sets where the effect of different media was significant ($P < 0.0082$). Values followed by different letters are significantly different based on Duncan MRT at $\alpha = 0.05$.

Fig. 6. Effect of modifications in osmoprotection and unloading steps on the regeneration of cryopreserved shoot tips. See Materials and Methods for the description of treatments. Values followed by different letters are significantly different based on Duncan MRT at $\alpha = 0.05$.

Fig. 7. DSC thermograms of shoot tips without any treatment (Control) and after treatment with VS A3 and PVS3 for indicated durations.

Fig. 1.

254x190mm (96 x 96 DPI)

Fig. 2

Fig. 3

Fig. 4

Fig. 5.

Fig. 6