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Cryopreservation enables long-term conservation of critically endangered species *Rubus humulifolius*

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Abstract

Ex situ storage plays an important role in the conservation of plant biodiversity. Cryo-preservation at ultra-low temperatures (− 196 °C) is the only long-term *ex situ* preservation method for plant species that cannot be stored in seed banks. In the present study, we developed a cryopreservation protocol for micropropagated *Rubus humulifolius* (Rosaceae) plants representing currently critically endangered population of the species in Finland. Abscisic acid (ABA) has been found to increase the freezing tolerance of several plant species. Thus, we studied the effect of a 10-day pretreatment with 0, 2 or 4 mg/l ABA in comparison to freshly dissected buds. We also studied how the duration of in vitro subculture affects cryopreservation result. The ABA pretreatment had divergent effect on control and cryopreserved buds: the regeneration of non-cryopreserved control buds increased from 51% to 70%, 90% or 87% while the regeneration of cryopreserved buds decreased from 52% to 35%, 6% or 9% after 0, 2 or 4 mg/l ABA pre-treatments, respectively. Buds from plants subcultured for 1 month had 63% survival, which, however, decreased to 29% or nil% after 2 or 4 months subculture. The regenerated plants were successfully transferred from in vitro to in vivo conditions in common garden. Growing in garden is needed for future restoration of the species in wild. Cryostorage and other *ex situ* conservation actions carried out in botanical gardens may be of increasing importance as a tool to maintain plant biodiversity in the future.

Keywords In vitro cultivation · Abscisic acid · Threatened plant species · *Ex situ* conservation

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Abbreviations

ABA	Abscisic acid
PVS2	Plant vitrification solution 2
DMSO	Dimethyl sulfoxide
LN	Liquid nitrogen

Introduction

The trends observed in the loss of plant biodiversity over the last 100 years have been a matter of great concern (Heywood and Dulloo 2005; Hooper et al. 2012; Vellend et al. 2017). Conversion of primary vegetation to agriculture, climate change combined with habitat loss and fragmentation are the main key factors affecting biodiversity and to cause species to become extinct (Thomas et al. 2004; Mantyka-Pringle et al. 2012; Vellend et al. 2017). The situation today is still very alarming, despite the efforts made to conserve plant diversity. It has been shown that increase in global temperature will increase the species turnover rate and decrease the mean stable area of species in all biomes (Alkemade et al. 2011). Changes in mean climate variables and greater risks of extreme weather, including prolonged drought and storms, are events that biomes will have to adapt to (Lindner et al. 2010). Climate change has also known to be related to the observed northward and uphill distribution shifts of many European plant species (Feehan et al. 2009). Changing climate will cause a myriad of changes, and therefore, different kinds of conservation strategies related to in situ or *ex situ* maintenance of plant populations need to be applied. From these, in situ conservation, preservation of threatened plant population in the original environment is no doubt of highest priority (Hannah et al. 2002). However, in situ conservation is suitable mainly for the species and populations that can be preserved in the original environment of the species, e.g. in protected areas in natural reserves and conservation corridors. In a situation, where in situ conservation is not possible, *ex situ* conservation i.e. conservation outside the original environment—is the method of choice. Furthermore, *ex situ* conservation is applicable as an additional conservation method to in situ method working as a backup collection for the most vulnerable material (Li and Pritchard 2009; Hawkes et al. 2012). For example, clonal field repositories, botanical gardens, seed banks and in vitro collections are widely applied for both economically important and endangered plant species. Thus, *ex situ* conservation is applied as an additional measure to supplement in situ conservation. The Target 8 of the Global Strategy for Plant Conservation (agreed at the CBD meeting in Nagoya in 2010) is the *ex situ* conservation of at least 75% of threatened plant species, with at least 20% available for recovery and restoration programs (https ://www.cbd.int/gspc/targets.shtml).

Cryopreservation process and methods allow long-term survival of organisms at liquid-nitrogen (LN) temperatures (− 196 °C). In LN-storage, one of the benefits is the long-term deferment of regeneration costs. Although no biological sample is immortal, the specimens in LN storage will have indefinite lifespans (Li and Pritchard 2009). Cryopreservation is the only *ex situ* conservation method for long-term preservation of species that cannot be stored in seed banks, e.g. clonal crops or species with a low number or recalcitrant seeds. Moreover, requiring only a minimum space and maintenance efforts, cryopreservation has turned out to be a very important tool for the long-term storage of plant genetic material (Engelmann 2004; Matsumoto 2017).

The most commonly used cryopreservation techniques are vitrification, encapsulation dehydration, controlled rate cooling, and dormant bud preservation (Benson 2008; Benelli et al. 2013; Jenderek and Reed 2017). Vitrification is a physical process where solution solidifies into a metastable glass at very low temperatures and avoids crystallisation (Sakai et al. 2008). Many plant vitrification protocols use two main techniques: the addition of cryoprotective additives at very high concentrations and the removal of water by evaporative desiccation and osmotic dehydration. A cryoprotectant acts as an antifreeze substance and must be non-toxic to the cell at the concentration that is required for it to be effective (Benson 2008).

More than 200 plant species, also including a few species from genus *Rubus*, Rosaceae, have been successfully cryopreserved using the PVS-based vitrification protocol (Reed 2008; Matsumoto and Niino 2014). For example, Gupta and Reed (2006) cryopreserved shoot tips of four genotypes representing three *Rubus* species (*R. ursinus*, *R. crataegifolius* and *Rubus* hybrid cv. Chehalem) successfully with a PVS2-based vitrification method. The four genotypes showed 71% average regrowth after cryopreservation. In particular, the vitrification and encapsulation-dehydration methods have been continuously developed and are thus the most frequently employed methods for cryopreservation of clonally propagated plant species. Genus *Rubus*, with around 500 species, is one of the most diverse plant genera (Skirvin et al. 2005). *R. humulifolius* is a rhizome-forming perennial plant, found in northern areas of Eurasia from the Lake Onega to the coast of the Pacific Ocean. A separate population of the species was found native in Finland in the beginning of the twentieth century in the Jyväskylä area (Mikkola 1927). Because of human activity, the natural population disappeared in 1957 (Kypärä 2012). Reintroductions with the same plant material were carried out but with no long-term success. In 1986, a micropropagation project was initiated and a successful reintroduction into the wild in Jyväskylä in a nature conservation area was carried out (Kypärä 2012). Despite the presence of this reintroduced population, *R. humulifolius* has been classified as regionally extinct (RE) (Rassi et al. 2010) and more recently as critically endangered (CR) (Hyvärinen et al. 2019) in Finland.

Rubus humulifolius plants from the Jyväskylä population have been maintained in vitro in the Botanical Garden of the University of Oulu since 2006 (20 plantlets all presenting one *R. humulifolius* clone). More recently, *R. humulifolius* was included as a target species into the EU Life+ project Ex Situ Conservation of Finnish Native Plant Species (ESCAPE) project (LIFE+ 2011 BIO/FI/917 ESCAPE; https://luomus.fi/en/exsitu-conservation-finnish-native-plant-species). During this project, we developed the cryopreservation protocol for *R. humulifolius* to enable a long-term conservation of the germplasm of the separate populations of the species.

Materials and methods

Plant material

In vitro cultures of micropropagated *R. humulifolius* C. A. Mey plants maintained at the Biotechnology Laboratory of Botanical Gardens at the University of Oulu were used as explant material for the present study. The plant material was originally received from Natural Resource Institute, Finland where the micropropagation protocol

was developed in order to restore the species representing the most western habitat of *R. humulifolius* (Kypärämäki, Jyväskylä, Finland ETRS-TM35FIN: N 6901644, E 432210).

In vitro cultivation

The in vitro cultivation/maintenance of *R. humulifolius* plants was carried out on ½ MS (Murashige and Skoog 1962) medium with 0.1 mg/l BAP, 0.05 mg/l NAA, 100 mg/l myo-inositol, 30 g/l sucrose and 7.6 g/l agar at 22 °C under 16/8 photoperiod. The illumination was provided by fluorescent tubes (Osram L 30 W/830) with intensity of 107–128 μmol/m^2/s. The plants were transferred to the fresh medium at 3-month interval.

Cryopreservation protocol

For cryopreservation of *R. humulifolius,* a droplet vitrification method was used. In the present study, cryopreservation was done like in encapsulation vitrification protocol originally developed for raspberry (*Rubus idaeus*) but without encapsulation step (Wang et al. 2005). We studied the effect of a 10-day pretreatment with or without abscisic acid (0, 2, or 4 mg/l ABA) performed on 1-month-old buds dissected from multiple shoots of in vitro plants. For control, the cryopreservation protocol (see below) was applied immediately after dissection (Treatment 1 in Table 2). We studied also the effect of propagation interval on the cryopreservation success of *R. humulifolius*. The buds were collected from 1-, 2- or 4-months-old donor plants (from last subculture), later referred as in vitro age. All experiments/treatments included non-cryopreserved control buds (treated like cryopreserved ones but without freezing in LN) and all the work steps during cryopreservation procedure were done aseptically. The buds in each treatment had three replicates (three Petri plates/treatment) and the experiment was done once.

For cryopreservation, the buds (2–4 mm size) either with or without pre-treatment were first precultured on ½ MS medium with 0.1 mg/l BAP, 0.05 mg/l NAA, 100 mg/l myo-inositol including 0.25, 0.5 and 0.75 M sucrose for one day per concentration (Fig. 1a). After that, the buds were transferred into the loading solution (0.75 M sucrose, 2 M glycerol, ½ MS) for 90 min. Thereafter, the buds were vitrified in plant vitrification solution 2 (PVS2: 30% glycerol, 15% ethylene glycol, 15% DMSO, 0.4 M sucrose in ½ MS) for 3 h. Incubations in loading solution and PVS2 were carried out by placing the buds on small Petri plates moistened with 2 ml of each solution at a time. Preculture, loading and vitrification steps were performed at room temperature (RT). After cryoprotection in PVS2, aluminum foil pieces (0.5×1.5 cm) were prepared with 2–3 μl droplets of PVS2 (Fig. 1b) and the buds were transferred to the droplets (5 per foil) and cryopreserved by transferring the foils quickly into cryotubes filled with liquid nitrogen (LN) for at least 1 h.

Recovery after cryopreservation

The first step in recovery of cryopreserved *R. humulifolius* buds was thawing the cryotubes at 40 °C water bath for 3 min. Thereafter, the cryotubes were opened and foils with buds transferred to 30 ml unloading solution (1 M sucrose in ½ MS) at RT. After that, the excess moisture was removed by transfer to filter paper and the buds were cultivated on ½ MS medium with 0.1 mg/l BAP, 0.05 mg/l NAA, 100 mg/l myo-inositol and 30 g/l

Fig. 1 **a**. *Rubus humulifolius* buds after sucrose treatment. **b**. Foil strips with PVS2 droplets. **c**. Regenerating bud 2 weeks after cryopreservation. **d**. Cryopreserved material 2 months after cryopreservation. **e**. Cryopreserved material 2 weeks after transfer to ex vitro conditions. **f**. Cryopreserved overwintered *R. humulifolius* plants

sucrose in Petri plates for 4 days in dark before transferring to light in the culture room. Survival (S) and regeneration (R) of buds were estimated at several time points (between 1 and 5 weeks) under stereomicroscope. Buds classified as survived ones (S) included both regenerating buds (R) i.e. buds forming new shoots (Fig. 1c) and buds which were alive but not regenerating.

Transfer of cryopreserved material into ex vitro conditions

Approximately 4 weeks after thawing the cryopreserved buds started to produce shoots which were transferred into larger tissue-culture jars containing ½ MS medium with 0.1 mg/l BAP, 0.05 mg/l NAA and 100 mg/l myo-inositol (Fig. 1d). After a few months, the shoots started to produce roots either with or without 0.1 mg/l indole-3-butyric acid (IBA) treatment. Altogether 19 rooted shoots were transferred, from in vitro to in vivo conditions (Fig. 1e). During the first weeks in vivo, high moisture atmosphere was maintained. The plants were let to overwinter in an unheated greenhouse (temperature maintained above 0 °C at the Botanical Gardens of the University of Oulu).

Statistical analysis

A Chi square test of independence was performed for both survival and regeneration results for cryopreserved and control buds. If the p value was < 0.05, the result was not independent of the treatment and pairwise comparisons by Chi square test of equal proportions with Holm correction were carried out. The statistical program R (R Core Team 2012) was used to perform the statistical analyses.

Results

The in vitro material of *R. humulifolius* was successfully cryopreserved by droplet vitrification method. The highest survival (62.0) and regeneration (52.0) percentages of cryopreserved buds were obtained from 1-month-old buds without any pretreatment (Tables 1, 2). Longer period from the last in vitro subculture had negative effect on post-cryopreservation survival as only 29.4%, representing the buds derived from 2-months-old donor plants survived, but no survival was observed when the buds were dissected from 4-months-old donor plants (Table 1).

For control buds (no cryopreservation, no pretreatment) the survival percentages were 73.3% for buds originating from 1-month-old donor plants whereas the corresponding survival percentages for 2- and 4-months-old donor plants were 60.0% and 50.0%, respectively (Table 1). Accordingly, survival percentage of control buds (no cryopreservation, no pretreatment) derived from 1-month-old donor plants was 61.2%.

Table 1 The effect of in vitro age on *R. humulifolius* post-cryopreservation survival (S) and regeneration (R) percentage (%)

In vitro age	−LN			+LN		
	n	S (%)	R (%)	n	S (%)	R (%)
1	30	73.3	66.6	105	62.8	48.6
2	5	60.0	na	17	29.4	na
4	4	50.0	na	8	0.0	na

+LN, cryopreserved, −LN, non-cryopreserved control. In vitro age, time from last subculture (months)

n the number of buds in each treatment, *na* not available

Table 2 The effect of a 10-day ABA pre-treatment on post-cryopreservation survival (S) and regeneration (R) percentages (%) of *R. humulifolius*

Treatment no.	Pre-treatment	−LN			+LN		
		n	S (%)	R (%)	n	S (%)	R (%)
1	–	49	61.2 a	51.0 a	50	62.0 a	52.0 a
2	0 mg/l ABA	33	84.8 ac	75.8 ac	34	47.1 ac*	35.3 ac*
3	2 mg/l ABA	31	93.5 bc*	90.3 bc**	34	17.6 b**	5.9 b**
4	4 mg/l ABA	31	96.8 bc**	87.1 bc**	35	17.1 bc**	8.6 bc**
	X^2, df = 3		21.16	19.94		20.24	25.21
	p-value		**	**		**	**

Before cryopreservation, the dissected buds (1–3 mm) were incubated 10 days on MS media with 0, 2 or 4 mg/l ABA (Treatments 2–4). For comparison, freshly disseiscted buds without 10-day pre-treatment were included (Treatment 1)

+LN, cryopreserved, −LN, non-cryopreserved control, ABA, the concentration of abscisic acid, "–" no pre-treatment n, the number of buds in each treatment

χ^2-value represents the result of Chi squared test of independence (df = 3) at 0.95 (*) and 0.99 (**) level within each column for −LN and +LN separately. Significant differences are marked with asterisks at 0.95 (*) and 0.99 (**) level for −LN and +LN treatments. The letters followed by asterisks denote significant differences according to pairwise comparisons (Chi squared test of equal proportions with Holm correction for p-value)

The effect of pretreatment

Pretreatment with or without ABA had a significant effect in both control and cryopreserved *R. humulifolius* buds. However, the effect of pretreatment differed between non-cryopreserved control buds and cryopreserved ones.

For non-cryopreserved controls, all three pre-treatments (0, 2 or 4 mg/l ABA) had positive effect on both survival and regeneration. The survival percentage of freshly dissected buds was 61.2% while the 10-day pre-treatment without ABA (0 mg/l) increased survival to 84.8%. In the presence of 2 or 4 mg/l ABA the survival percentages reached to 93.5% and 96.8% (Table 2), respectively. The same phenomenon was observed for regeneration percentages—the regeneration of control buds without pretreatment was 51.0% while after the 10-day pre-treatment without ABA the regeneration increased to 75.8% whereas with 2 or 4 mg/l ABA the regeneration reached 90.3 and 87.1%, respectively (Table 2).

In the case of cryopreserved buds, the effect of the 10-day pretreatment with 0, 2 or 4 mg/l ABA had a negative effect on recovery after cryopreservation. The survival percentage of freshly dissected buds was 62% while the 10-day pretreatment without ABA (0 mg/l) decreased the survival percentage to 47.1%. The effect was more severe when ABA was included—only 17.6 and 17.1% of buds survived from 2 and 4 mg/l ABA pretreatments (Table 2), respectively.

The regeneration percentages of ABA-treated buds were even lower. For freshly dissected buds, the regeneration percentage was 52.0 while after 10-day post-dissection incubation on ABA-free medium the regeneration percentage was 35%. In the presence of 2 or 4 mg/l ABA, only 5.9 and 8.6% of buds regenerated after thawing (Table 2), respectively.

Transfer from in vitro to ex vitro

Eight of the 19 rooted shoots transferred from in vitro to in vivo soil conditions survived throughout the first summer. These eight plants overwintered successfully in unheated greenhouse and were healthy and growing after 1 year of transfer (Fig. 1f).

Discussion

In the present study, *R. humulifolius*, critically endangered native plant in the wild in Finland, has been successfully cryopreserved to enable the long-term conservation of the species.

Cryopreservation protocol for germplasm conservation of *R. humulifolius* has not been reported before. However, genus *Rubus* includes several economically important berry species, and therefore several cryopreservation protocols including controlled rate cooling, encapsulation–dehydration, encapsulation-vitrification, PVS2-vitrification and droplet vitrification have been applied for several species in this genus (Reed 1993; Chang and Reed 1999; Vysotskaya et al. 1999; Wang et al. 2005; Gupta and Reed 2006; Ukhatova et al. 2017). For Finnish raspberry (*Rubus idaeus*) cultivars, an encapsulation–vitrification protocol has been developed (Wang et al. 2005). Therefore, for the native endangered *R. humulifolius* genotype, the encapsulation–vitrification protocol was used as a reference for development of the droplet vitrification protocol successfully applied in the present study resulting high survival and regeneration percentages (62% and 52%, respectively, Table 2, Treatment 1).

The addition of ABA to growth media before cryopreservation has previously been proposed to further increase the survival percentage of cryopreserved buds and therefore it has been applied in many cryopreservation protocols of different plant species. For example, for adventitious shoots of *Begonia x erythrophylla,* a 7-day pre-growth period on 1.9 or 3.8 μM ABA before dissecting the shoots for cryopreservation, significantly increased the shoot regrowth from 24 to 35% or 43%, respectively (Burritt 2008) while 7.6 μM ABA resulted in 20% regrowth. Similarly, when in vitro shoots of pear (*Pyrus cordata*) were pre-cultured on culture media with 50, 75 and 150 μM ABA for 3 weeks before bud dissection and cryopreservation, a significant increase in regrowth from 0 to 7%, 10% or 18%, respectively, was observed (Chang and Reed 2001). Survival of *Vanda pumila* was also significantly increased when shoot primordia were cultivated for 3–6 days on media including ABA (1.0 mg/l) (Na and Kondo 1996). Therefore, in the present study prior to cryopreservation, dissected buds were pretreated for 10 days on media with 0, 2 or 4 mg/l ABA.

According to our results, pre-treatment with ABA has a divergent effect on control and cryopreserved buds on *R. humulifolius* (Table 2). The same divergent effect of ABA pretreatment has been observed for in vitro sugar beet (*Beta vulgaris*) shoot tips in which the survival of control buds increased in the presence of ABA from 57 to 70% after 1 week of ABA pre-treatment. Contradictory, 37% of cryopreserved shoot tips survived cryopreservation without ABA treatment and with 1-week ABA treatment survival decreased from 37 to 23% (Vandenbussche and Proft 1998).

For several species, cold acclimation is needed to support ABA treatment. For example, five *Rubus* genotypes (three blackberry and two raspberry cultivars), ABA had no effect on recovery at room temperature whereas cold acclimation significantly increased the recovery percentages. Moreover, for some genotypes, cold together with ABA synergistically

increased the recovery (Reed 1993). For example, for pear (*P. cordata*), ABA treatment alone produced 18% regrowth whereas the highest post-cryopreservation regeneration (> 70%) was obtained when a 2-week cold treatment was combined with increment of 50 μM ABA in the pre-treatment medium. Moreover, the presence of ABA significantly decreased the time of cold pretreatment needed for successful recovery after cryopreservation (Chang and Reed 2001). For *Beta vulgaris*, synergistic effect of cold and ABA treatment was also observed—ABA alone increased survival from 23 to 45% whereas under cold and ABA combination survival increased to 70% after freezing (Vandenbussche and Proft 1998). Thus, for future studies, a cold pretreatment could also be included in the protocol to see whether it could improve post-cryopreservation recovery of *R. humulifolius*.

In vitro cultivation interval as well as time from original initiation of in vitro cultures are known to affect cryopreservation outcome. For example, the survival of young (22 months from initiation) in vitro cultures of silver birch (*Betula pendula*) was significantly higher (37.5%) than that from 55 months old cultures (14.8%) (Ryynänen and Häggman 2001). In the case of *Dianthus caryophyllus*, the survival of shoot tips increased with the time from last subculture reaching 21% after 3 days, 94% after 14 days and 98% after 3 weeks and 2 months of the last subculture (Dereuddre et al. 1988).

In the present study, the best recovery after cryopreservation of *R. humulifolius* in vitro buds was achieved when 1-month-old cultures (from the last subculture) without any pretreatment were used. In detail, the optimal cryopreservation procedure in the present study was following. (1) Preculture on ½ MS medium with 0.1 mg/l BAP, 0.05 mg/l NAA, 100 mg/l myo-inositol including 0.25, 0.5 and 0.75 M sucrose for 1 day per concentration. (2) Osmoprotection (loading) in 0.75 M sucrose, 2 M glycerol in ½ MS for 90 min. (3) Vitrification in plant vitrification solution 2 (PVS2: 30% glycerol, 15% ethylene glycol, 15% DMSO, 0.4 M sucrose in ½ MS) for 3 h followed by transfer of the buds to aluminum foil pieces (0.5 × 1.5 cm) with 2–3 μl droplets of PVS2. (4) Cooling: The quick transfer of foils with buds into cryotubes filled with liquid nitrogen (LN) and storage in LN container − 196 °C for at least 1 h. (5) Rewarming: Immersion of the cryotubes at 40 °C water bath for 3 min. (6) Dilution: Transfer of foils with buds to 30 ml unloading solution (1 M sucrose in ½ MS) at RT. (7) Regeneration: The removal of excess moisture by filter paper and cultivation on ½ MS medium with 0.1 mg/l BAP, 0.05 mg/l NAA, 100 mg/l myo-inositol and 30 g/l sucrose on Petri plates for 4 days in dark before transfer to light in the culture room.

There are no previous cryopreservation experiments for *R. humulifolius*. However, the results can be compared to the ones obtained for other species of the same genus. Gupta and Reed (2006) reported 96–100% survival and 45–78% regrowth of four *Rubus* (blackberry and raspberry) genotypes after cryopreservation by PVS2 vitrification. Correspondingly Wang et al. (2005) reported 46–90% survival and 50–85% regrowth after cryopreservation of 7 raspberry (*Rubus idaeus*) genotypes. Recently, Ukhatova et al. (2017) reported 85–100% survival and 24–89% regeneration of 12 red raspberry cultivars. Thus, the present results, 61% survival and 51% regeneration after LN exposure, are in line with the ones presented so far for the species of genus *Rubus*. Actually, the survival and regeneration of freshly dissected control buds (−LN, treated with all the solutions except freezing in LN) were the same as cryopreserved ones (62% survival and 52% regeneration) (Treatment 1 in Table 2) showing that all the shoot tips that survived cryopreservation treatments could withstand freezing in LN. Therefore, in future, it could be tested whether different incubation times in loading solution and PVS2 could affect the survival and regeneration of both control and cryopreserved buds of *R. humulifolius*.

At present, cryopreserved *R. humulifolius* in vitro buds are safely conserved in the cryo-facility and the in vitro plants are maintained in the tissue culture facility of Botanical Gardens at the University of Oulu, Finland. Moreover, the success of the cryopreservation has allowed the transfer of the cryopreserved plants from in vitro to in vivo conditions. Thus the existing material in cryo-facility, in the greenhouse and in outdoor garden allow additional reintroductions of the species in the future. In order to protect threatened plant species, including *R. humulifolius*, it is important to combine both *ex situ* and in situ conservation tools. To address the unpredictable effects of climate change, *ex situ* plant storages, including storage in LN, play an important role in the conservation of biodiversity (Liu et al. 2018). Cryopreservation is a beneficial conservation method for several reasons, the main ones arguably being the reduced risk of microbial contamination and reduced costs (Li and Pritchard 2009). In comparison to in vitro culture, cryopreservation reduces the risk of genetic instability related to prolonged in vitro culture (Dulloo et al. 2010). The minimum space and maintenance requirements make cryopreservation an essential tool for the long-term storage of plant material. The fact that *R. humulifolius* has become extinct in its native growing areas in Finland is yet another example of the irreversible effects of human activity. Reintroduction of *R. humulifolius* has also been difficult because of increased human activity land use for construction in the area and in the suitable nearby areas (Kypärä 2012). Reintroduction of threatened species into their native environments is challenging (Sarrazin and Barbault 1996). Increased land use activity, as in the case of *R. humulifolius*, is often combined with other habitat deterioration under changing climate. Finnish *R. humulifolius* is an example of rather unique case where the whole population would have gone extinct without *ex situ* conservation tools. Developing cryogenic protocol, which was carried out in this study, is additional tool to *ex situ* conservation toolbox and helps to maintain the plant both in *ex situ* and in situ repositories in the future. To conclude, the *ex situ* conservation of plant species e.g. in botanical gardens and in tissue culture facilities as well as in cryogenic tanks continues to be an important tool in maintaining biodiversity also in the future (Reed 2017; Liu et al. 2018).

Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interest.

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