

Ex situ conservation of endangered plant species of Latvia by slow growth storage

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Abstract

Ex situ conservation of endangered plant species is the main objective of work of the Department of Ecophysiology of the National Botanic Garden of Latvia. In order to create a gene bank in tissue culture, use of appropriate methods for long-term preservation of living plant material is important. The objective of this study was to find ways to limit rate of shoot growth and to maximize the vitality period of shoot cultures. Several endangered plant species with high multiplication rate and sufficient number of explants were chosen for slow growth experiments. Murashige and Skoog medium supplemented with 1 to 6% carbohydrates (sucrose, sorbitol, mannitol), activated charcoal, and, for some species, cytokinin or other ingredients was used. After planting explants in experimental media, cultures were kept for four to five weeks at 25 °C, later at 5 °C. To evaluate explant growth in relation to various media conditions and genotype, root structure was analyzed using the program Win RHIZO 2002 C. Reduced shoot and root growth was observed for 23 species with sorbitol (for example, *Armeria maritima*, *Galium tinctorium*, *Spergularia salina*) and for eight species with mannitol (for example, *Dianthus arenarius*, *Hydrocotyle vulgaris*, *Juncus gerardii*). Few species grew only on sucrose-containing medium. At 5 °C, most species survived for 24 and more months of storage without subcultivation. Some influence of storage medium composition on shoot first re-growth after storage was observed, but all cultures retained growth capacity after storage.

Key words: storage, endangered plants, *in vitro* conservation, polyols.

Abbreviations: BA, benzyladenine; MS, Murashige and Skoog.

Introduction

There are various approaches for achieving the current targets of the Global Strategy for Plant Conservation for species conservation. Besides species conservation *in situ*, also *ex situ* preservation including *in vitro* banks of taxa is being widely promoted. International Botanic Gardens programmes emphasize that botanic gardens should be the centres of *ex situ* conservation of plant species and elaborate *ex situ* conservation protocols for all groups of vascular plants. Alongside with seed banks, tissue culture technology is an additional way for saving biological diversity *ex situ* (Bramwell 1990).

The National Botanic Garden of Latvia started to elaborate *ex situ* conservation methods for threatened species of Latvia in 2003 (Kļaviņa et al. 2004). There are several methods for long-term and slow-growth conservation: cultivation of shoot cultures at low temperature and variation of media components. Successful combination of these factors ensures reduced growth rate of particular species together with extended subculture period. Temperature for *in vitro* storage depends on plant species; differences in appropriate storage temperatures among species might be related to the cold hardiness of a particular plant (Lambardi et al. 2006; Joshi, Jadhav 2013).

Cultivation at low temperature can be used for plant species of the temperate zone origin, because of their natural cold tolerance. Increased sucrose concentration in cultivation medium usually stimulates *in vitro* growth (Voračkova et al. 1998) and allows for long-term preservation success of woody plants (Kļaviņa, Ievinsh 2008). For various perennials, a standard sucrose concentration of 2 to 3% in media is usually used.

Variation of mineral element concentration in media and use of abscisic acid and osmotic growth retardants can allow to find the most appropriate medium composition. Polyols or sugar alcohols, mainly mannitol and sorbitol, are essential components that can significantly reduce growth in certain plant cultures (Flecher 1994; Lipavska, Vreugdenhil 1996; Negash et al. 2001; Preira et al. 2003; Charoensub, Phansiri 2004; Ankita, Animesh 2013; Joshi, Jadhav 2013; Ghahery et al. 2015). Polyol media amendments are generally considered to be metabolically inert, and *in vitro* polyol-dependent phenomena are defined predominantly as mere osmotic effects (Steinitz 1999). Polyols may serve as reserve carbohydrates, but also as sinks for photosynthetically produced reducing compounds. The physiological role of polyols as agents of translocation and endogenous osmoregulation has been considered, together with more biochemical aspects of

their utility, and for co-enzyme regulation (Lewis, Smith 1967; Mioti et al. 2009). Mannitol is readily taken up by plants and transported to shoots rather quickly. As there are probably great differences in the ability of various plants to accumulate mannitol and use it as a source of carbon and energy, it is necessary to test the response of particular plant (Lipavska, Vreugdenhil 1996).

The aim of the current study was to test effect of media with polyols on cold storage of *in vitro* cultures of threatened plant species.

Materials and methods

For *in vitro* storage, microcuttings were transferred to modified Murashige-Skoog medium (MS) to test efficiency of different cultures for inducing slow growth. For experiments, *in vitro* cultures of endangered plant species of Latvian wild origin were used (totally 47 species). All of the used species showed good regeneration and propagation potential of earlier established cultures (Kļaviņa et al. 2004; 2006). Storage results were obtained for 32 species (Table 1). Shoot type or rosette explants of species without growth regulators in previous subculture were cultivated on MS medium (with half reduced nitrate content) with plant agar 6 g L⁻¹, pH 5.8, sucrose 1 to 6% and metabolically inactive carbohydrates sorbitol and mannitol in concentration 1 to 4% (w/v) as the sole carbon source or with sucrose (0.1, 0.5, 1, 2 or 3%) together with sorbitol or mannitol in cases when explants did not grow on polyol alone (Table 1). Various other additives were tried depending on requirement of species: activated charcoal 0.075 to 0.1 %, sodium chloride (100 to 200 mM), cytokinin (0.1 to 0.3 mg L⁻¹ kinetin, 0.1 to 0.5 mg L⁻¹ 6-benzyladenine, 0.1 and 0.2 mg L⁻¹ topolin). For each species 5 to 20 media variations were tested. For

each treatment 15 to 50 explants were used. The cultures were adapted on the media at 25 °C (photoperiod 16 h, photon flux density of photosynthetically active radiation 40 μmol m⁻² s⁻¹) for four to five weeks. After that, plant growth (rooting, additional shoot number, necrosis, vitrification) was evaluated and cultures were transferred for cold storage at 5 °C (photoperiod 16 h, photon flux density of photosynthetically active radiation 30 μmol m⁻² s⁻¹). Cultures were maintained at low temperature for several months up to a few or more years without transfer onto fresh medium. Plant survival was controlled by re-growth of plantlets on fresh MS medium with half reduced nitrate content, with plant agar 6 g L⁻¹, pH 5.8, sucrose 2% in room conditions at 25 °C (photoperiod 16 h, photon flux density of photosynthetically active radiation 40 μmol m⁻² s⁻¹). After four weeks re-grown plantlets were counted and survival evaluated.

Root structure was analysed to characterize explant growth reaction in relation with various cultivation conditions and species. Root analysis using calibrated scanner STD-1600+ and computer program Win RHIZO 2002 C was conducted for 15 species (shown eight). Total root length of each plant in storage vessel, root surface area and volume, and number of root tips were measured for 5 to 12 plants. Root scanning measured parameters were compared among variants with ANOVA and Tamhane post-hoc test using the SPSS 16 programme (Morgan et al. 2004).

Results

In standard conditions, plant culture growth was ensured on medium containing 20 g L⁻¹ sucrose 20 g L⁻¹; in these conditions plant tissues grew rapidly and filled tubes and jars within a few months. To prevent rapid growth, various carbohydrate concentrations and their combinations in media were tested for each species (Table 1). Effect of

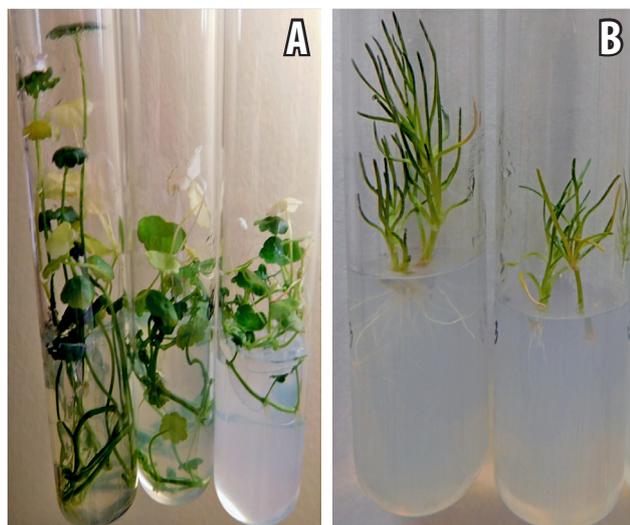


Fig. 1. Cultures of *Hydrocotyle vulgaris* (A) and *Spergularia salina* (B) after month precondition period at 25 °C before cold storage on media supplemented with 20 mg L⁻¹ sucrose, sorbitol and mannitol (only for *H. vulgaris*) from the left, respectively.



Fig. 2. Cultures of *Scrophularia umbrosa* after month precondition period at 25 °C before cold storage on media supplemented with 20 mg L⁻¹ sucrose and sorbitol from the left, respectively.



Fig. 3. Cultures of *Spargularia salina* (A) and *Trifolium fragiferum* (B) after a year of cold storage on media supplemented with 20 mg L⁻¹ sucrose and sorbitol from the left, respectively.



Fig. 4. Cultures of *Tripodium vulgare* during cold storage (from left) on media supplemented with 20 mg L⁻¹ sucrose, 40 mg L⁻¹ sorbitol and 20 mg L⁻¹ mannitol.

media components on shoot cultures appeared during pre-storage culturing under normal temperature conditions (Fig. 1, 2) and in many cases a growth retarding effect of polyols was evident (Fig. 3, 4, 5). This allowed to choose more appropriate storage media.

Initial growth-reducing effect of sorbitol and mannitol was tested for long-term storage as well. During prolonged

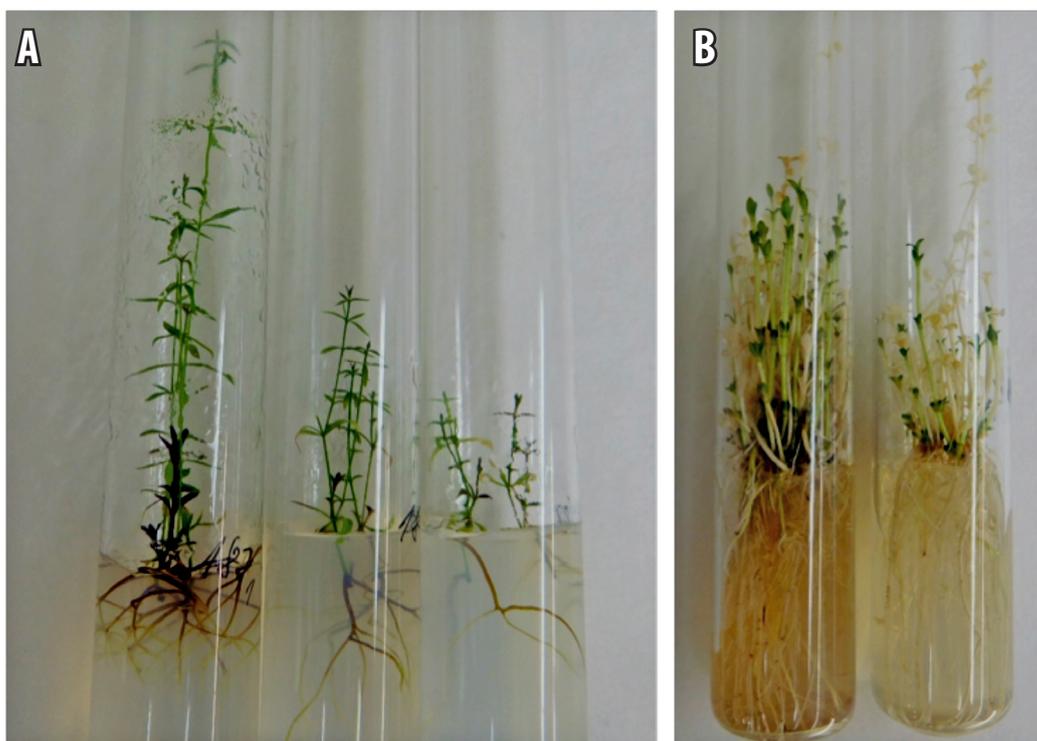


Fig. 5. Cultures of *Galium tinctorium* (A) after 4 month cold storage (from left) on media supplemented with 30 mg L⁻¹ sucrose, 30 mg L⁻¹ and 40 mg L⁻¹ sorbitol and *Glaux maritima* (B) after three years cold storage supplemented with 40 mg L⁻¹ sucrose and 40 mg L⁻¹ sorbitol.

cultivation some additional effects of polyols on plant growth appeared. Mannitol caused necrosis, vitrification and completely arrested root growth for some species (*Galium schultesii*, *Glaux maritima*, *Prunella grandiflora*, *Pulmonaria angustifolia*, *Saussurea esthonica*, *Spergularia salina* etc., Table 2). The majority of species tested showed successful growth on media with sorbitol (Table 2);

Table 2. Survival time and response of rare plant species tested on polyols in media. +, living explants; r, reduced growth; -, unsuitable for growth. *EU protected species

Species	Storage time (years)	Response on sorbitol	Response on mannitol
<i>Alyssum gmelinii</i>	3	+r	+r
<i>Angelica palustris</i>	1	+	+/-
<i>Arenaria procera</i>	5	+	+r
<i>Armeria maritima</i>	3	+r	+r/-
<i>Carex davalliana</i>	3	-	-
<i>Carex otrubae</i>	> 2	+r	+r/-
<i>Cinna latifolia</i> *	1	+	+r
<i>Circea lutetiana</i>	1	+r	+r/-
<i>Dianthus arenarius</i>	3	+r	+r
<i>Dianthus arenarius</i> ssp. <i>arenarius</i> *			
<i>Eryngium maritima</i>	2	+r	+r
<i>Galium schultesii</i>	3	+r	-
<i>Galium tinctorium</i>	4	+r	+r/-
<i>Geum hispidum</i>	> 1	+r	+r/-
<i>Glaux maritima</i>	3	+r	-
<i>Helianthemum nummularium</i>	4	+r	
<i>Hydrocotyle vulgaris</i>	8	+r	+r
<i>Juncus gerardii</i>	4	+	+r
<i>Lithospermum officinale</i>	1	+r/-	-
<i>Ligularia sibirica</i> *	3	+r	+r
<i>Linaria loeselii</i> *	3 – 5	+r	+r
<i>Plantago maritima</i>	3	+r	+r/-
<i>Prunella grandiflora</i>	3	+r	+r/-
<i>Pulmonaria angustifolia</i>	2	+r	+r/-
<i>Saussurea esthonica</i> *	1	-	-
<i>Schoenus ferrugineus</i>	2	+r	+r/-
<i>Scrophularia umbrosa</i>	5	+r	+r
<i>Scutellaria hastifolia</i>	> 2	+r	+/-
<i>Spergularia salina</i>	3	+r	-
<i>Tragopogon heterospermus</i>	> 1	+	-
<i>Trifolium fragiferum</i>	5	+r	+r/?
<i>Tripolium vulgare</i>	5	+r	+r
<i>Veronica montana</i>	3	+r	+

nevertheless, there were some exceptions, as *Lithospermum officinale* and *Carex davalliana*. The precondition period of *Carex davalliana* was successful on six media (Table 1, Fig. 6) and there was limited growth of shoot and especially root number with sorbitol and mannitol (in comparison with sucrose). After a nine-month storage period plants on media with both polyols and their combinations with sucrose became necrotic and seemed to be unsuitable for growth of this species during a longer storage period. This might be explained by weak root development on media with polyols: plants on sucrose media had well branched roots, but those on media with polyols were without branching.

During four years of storage *Carex davalliana* remained vital only on media with sucrose. Similar results in respect to polyols were obtained with *Saussurea esthonica*, but storage time of its cultures was shorter, about one year. For storage of *Saussurea esthonica* and *Eryngium maritimum*, new shoot formation was essential and therefore cytokinins were required. Topoline 0.1 mg L⁻¹ and benzyladenine (BA) 0.1 and 0.3 mg L⁻¹ were used for *Eryngium maritimum*. These additives promoted new shoot production; the best media were with sucrose 30 g L⁻¹ + topline or BA 0.1 mg L⁻¹.

Differences observed among species corresponded to their sensitivity to osmolytes in media. Retarded growth and good vitality were observed for *Armeria maritima*, *Pulmonaria angustifolia*, *Scrophularia umbrosa*, *Spergularia salina* (Fig 3, 2), *Tripolium vulgare* with sorbitol 20 g L⁻¹, *Galium tinctorium* with sorbitol 20 g L⁻¹ and 40 g L⁻¹, and *Dianthus arenarius* (Table 3) with mannitol 20 g L⁻¹. Results with *Veronica montana* and *Scutellaria hastifolia* showed large species plasticity: their growth was similar on seven media with sucrose, sorbitol, mannitol or combinations of these substances. Shoot growth of *Veronica montana* was slightly retarded only on media containing sorbitol while rooting did not differ significantly (Fig 7). *Gallium schultesii*, in contrary, was very sensitive to media composition: it accumulated anthocyanins (maximum on sucrose 40 mg

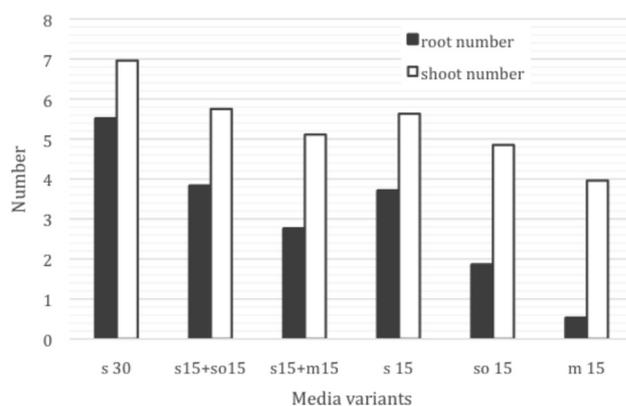


Fig. 6. Effect of different media on *Carex davalliana* growth after preconditioning period one month. s, sucrose; so, sorbitol; m, mannitol. Numbers indicate concentration (mg L⁻¹).

Table 3. Effect of different media on root morphology (mean \pm SE) of rare plants during slow growth storage *in vitro*. s, sucrose; so, sorbitol; BA, benzyladenine; kin, kinetin; C, activated charcoal. Different letters indicate significant differences between treatments for a particular species ($p < 0.05$)

Species	Media	Root length (cm)	Surface area (cm ²)	Root tips (number)
<i>Dianthus arenarius</i>	s 20	54.8 \pm 3.2 a	3.9 \pm 0.4 b	240 \pm 32 a
	s 20C	114.2 \pm 13.3 a	7.2 \pm 1.0 b	771 \pm 104 c
	s 20 + so 20	49.6 \pm 3.9 a	3.3 \pm 0.3 b	138 \pm 21 ac
	s 40	59.0 \pm 5.5 a	4.1 \pm 0.4 b	123 \pm 12 ab
	so 20	26.2 \pm 3.5 b	1.8 \pm 0.3 ab	110 \pm 20 b
	so 40	21.7 \pm 1.8 b	1.7 \pm 0.2 ab	103 \pm 13 b
	m 20	22.7 \pm 5.0 b	1.6 \pm 0.3 a	82 \pm 22 b
<i>Glaux maritima</i>	s 30	9.2 \pm 0.8 a	0.6 \pm 0.1 a	17 \pm 1 a
	s 30 C	9.1 \pm 0.8 a	0.6 \pm 0.1 a	19 \pm 3 a
	s 30 NaCl 100	8.1 \pm 1.1 a	0.5 \pm 0.1 a	17 \pm 3 a
	so 30	7.3 \pm 0.8 a	0.5 \pm 0.1 a	17 \pm 3 a
<i>Helianthemum nummularium</i>	s 20	69.9 \pm 8.4 a	6.6 \pm 0.8 a	135 \pm 18 a
	so 20	19.3 \pm 2.3 b	2.0 \pm 0.3 b	40 \pm 6 b
	so 20 + s 5	118.7 \pm 11.4 c	9.6 \pm 0.8 c	161 \pm 14 a
<i>Pulmonaria angustifolia</i>	s 20 + so 20	111.1 \pm 22.4 c	11.1 \pm 2.2 c	153 \pm 30 a
	s 20	112.4 \pm 14.7 cd	33.3 \pm 4.8 ac	902 \pm 125 a
	s 20 BA	81.0 \pm 13.0 c	26.3 \pm 4.9 a	754 \pm 107 a
	s 40	124.0 \pm 9.6 d	38.9 \pm 2.6c	927 \pm 83 a
	s 20 + so 20	117.0 \pm 18.9 cd	33.6 \pm 5.0 ac	727 \pm 125 a
	s 20 + so 20 C	130.0 \pm 25.2 cd	27.7 \pm 5.4 ac	443 \pm 134 b
	so 20	13.3 \pm 3.3 b	3.3 \pm 0.8 b	43 \pm 13 c
<i>Saussurea esthonica</i>	so 40	2.8 \pm 0.5 a	0.9 \pm 0.1 b	11 \pm 3 c
	s 30	22.4 \pm 3.5 b	4.3 \pm 0.6 b	15 \pm 2 b
	s 30 BA	8.4 \pm 3.8 bc	1.5 \pm 0.6 b	7 \pm 3 b
	s 50	43.1 \pm 6.4 a	8.2 \pm 1.2 a	26 \pm 4 a
	s 50 BA	17.4 \pm 4.2 bc	3.2 \pm 0.8 b	10 \pm 2 b
	s 30 + so 20	33.7 \pm 7.5 a	6.0 \pm 1.2 ab	19 \pm 3 b
	s30 + so 30 BA	30.7 \pm 8.2 a	6.2 \pm 1.7 ab	18 \pm 5 b
	so 30	7.1 \pm 1.0b	1.7 \pm 0.3 b	6 \pm 2 b
	so 30 BA	12.0 \pm 6.4 bc	2.1 \pm 1.1 b	13 \pm 5.9 b
	so 50	9.4 \pm 1.6 c	2.3 \pm 0.4 b	9 \pm 1 b
<i>Trifolium fragiferum</i>	so 50 BA	19.3 \pm 11.0 bc	4.2 \pm 2.3 b	23 \pm 11 ab
	s 30	82.8 \pm 10.7 a	7.9 \pm 1.1 a	158 \pm 54 a
	s 30 C	52.3 \pm 9.3 a	5.3 \pm 1.1 ab	137 \pm 23 a
	s 30 C BA	47.4 \pm 4.4 a	4.7 \pm 0.6 ab	123 \pm 25 a
	s 30 NaCl 100	34.7 \pm 12.9 a	4.2 \pm 1.6 ab	62 \pm 20 a
<i>Tripolium vulgare</i>	so 30	31.1 \pm 7.6	2.3 \pm 0.6 b	73 \pm 20 a
	s 20	204.4 \pm 37.9 a	52.6 \pm 9.0 a	1154 \pm 295 a
	so 20	34.0 \pm 7.1 b	6.5 \pm 1.6 b	119.6 \pm 34 b
	so 30	26.0 \pm 3.9 b	5.0 \pm 1.0 b	27 \pm 6 c
<i>Veronica montana</i>	so 40	17.7 \pm 6.1 b	3.0 \pm 1.2 b	15 \pm 5 cb
	s 30	14.7 \pm 2.1 a	1.1 \pm 0.1 ab	34 \pm 4 a
	s 30 kin	20.6 \pm 3.4 ab	1.4 \pm 0.2 ab	44 \pm 9 ab
	s 30 C kin	14.5 \pm 2.9 a	1.0 \pm 0.2 a	36 \pm 5 a
	s 30 + so 15	21.3 \pm 1.8 ab	1.7 \pm 0.1 b	37 \pm 3 a
	s 30 + so 30	33.1 \pm 3.1 b	2.8 \pm 0.3 b	54 \pm 6 b
	s 60	35.2 \pm 4.6 b	2.6 \pm 0.4 ab	56 \pm 10 b
so 30	19.2 \pm 2.4 ab	1.6 \pm 0.2 ab	30 \pm 4 a	

L⁻¹) and had necrotic apices and leaves. After few months of cold storage greener shoots occurred on medium with sucrose 20 g L⁻¹ + BA 0.2 mg L⁻¹.

Root growth on media with various ingredients is shown in Table 3. Among media tested, root growth on medium with sucrose and sorbitol differed significantly in most of cases (except for *Glaux maritima* and *Veronica montana*). The root surface area of *Trifolium fragiferum* was significantly larger in treatments with sucrose in comparison with sorbitol (activated charcoal and NaCl additives gave intermediate results, Table 3).

It was observed that after 10-month cold storage, for example, root length of *Pulmonaria angustifolia* with 2% sorbitol was tenfold less in comparison to 2% sucrose in medium (13 ± 3 and 112 ± 15 cm, respectively). Root structure analysis revealed two more suitable indices: root length and number of root tips; surface area and root volume showed the same tendencies. Root length and surface area of *Tripolium vulgare* on media with sorbitol were significantly smaller than on media with sucrose (Table 3). In the case of *Dianthus arenarius*, activated charcoal stimulated root growth (root length, number of root tips, root volume); 2/3 of roots were very small. Sorbitol and mannitol decreased these root parameters and also number of additional shoots.

Basic reactions of rare plant species tested on media with osmolytes under germplasm bank conditions are shown in Table 2. Plantlets maintained vitality at low growth rate without subcultures one year for species *Cinna latifolia*, *Lithospermum officinale*, and *Saussurea esthonica*, two years for *Pulmonaria angustifolia* (Fig. 8), three years for *Dianthus arenarius*, *Glaux maritima*, *Gallium schultesii*, *Plantago maritima*, and *Spergularia salina* and more years for *Arenaria procera*, *Galium tinctorium*, *Hydrocotyle vulgaris*, *Scrophularia umbrosa*, and *Trifolium fragiferum*. *Eryngium maritimum* could be stored at 5 °C about two years due to additional shoot formation during storage.

Survival and regrowth capacity were estimated after storage (Table 2). Survival differed considerably between

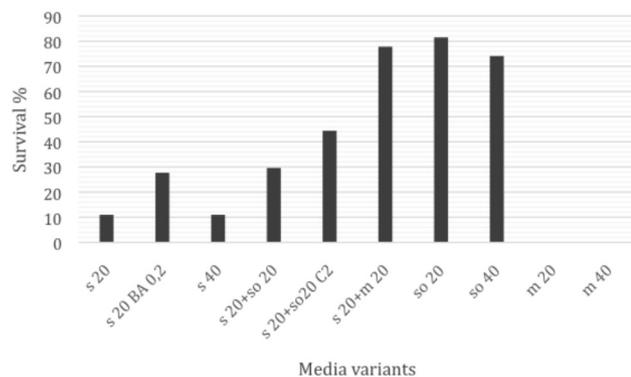


Fig. 8. Survival (%) of *Pulmonaria angustifolia* after two-year storage at 5 °C. s, sucrose; so, sorbitol; m, mannitol. Numbers indicate concentration (mg L⁻¹).

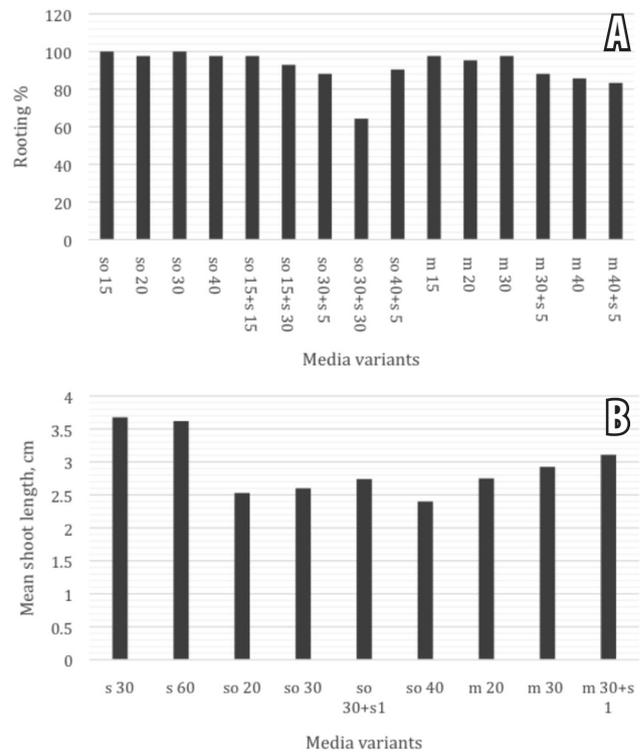


Fig. 7. Effect of different media on *Veronica montana* rooting (A) and shoot length in the other experiment with 100% rooting (B) after adaptation period. s, sucrose; so, sorbitol; m, mannitol. Numbers indicate concentration (mg L⁻¹).

media used, for example *Plantago maritima in vitro* cultures could be stored better on media with sorbitol than on sucrose or mannitol (Fig. 9). Successful regrowth was obtained for all species and growth parameters were estimated for five of these species. Recovery of species from slow growth storage depended on effect of storage medium on subsequent growth in normal conditions. Shoot multiplication was reduced in cases when the previous medium retarded growth. For instance, media with activated charcoal, BA, sorbitol or proline reduced growth and shoot production during cold storage of *Glaux maritima*. The same tendency remained after storage during the first subculture in normal conditions (Fig. 10). Effect of storage medium on shoot

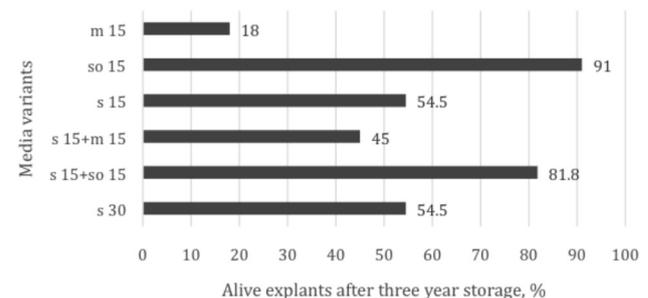


Fig. 9. *Plantago maritima* living explants after three-year storage at 5 °C temperature on media with sucrose (s), sorbitol (so), mannitol (m) and their combinations.

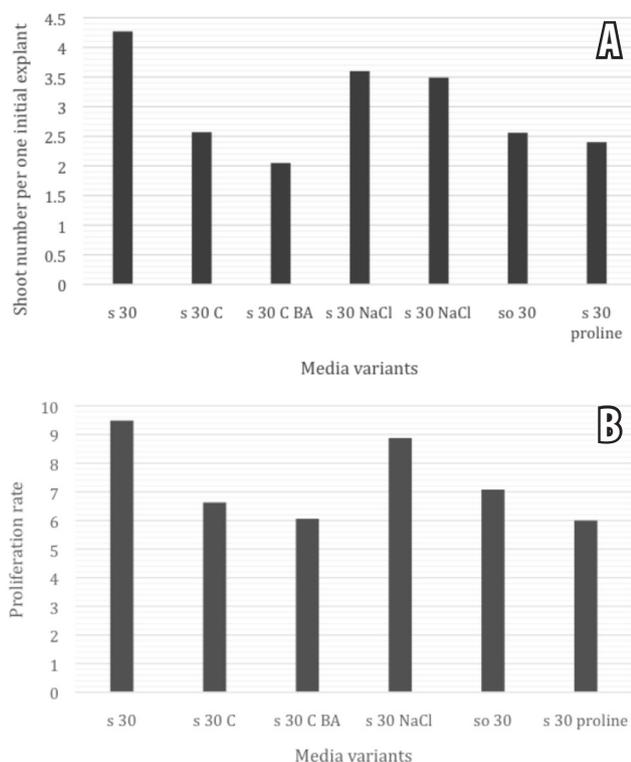


Fig. 10. Influence of cold storage medium on shoot growth of *Glaux maritima* during storage (A) and after storage on MS medium with sucrose 20 g L⁻¹ (B). s, sucrose; so, sorbitol; m, mannitol. Numbers indicate concentration (mg L⁻¹).

growth after storage was observed. All cultures retained re-growth capacity after storage.

Discussion

Possible tools for conserving *in vitro* cultures of rare plants and reducing supplementary handling of the *in vitro* preserved material were developed. Media modifications by using sorbitol and mannitol are useful for medium and long-term cold storage of threatened species. Development of process for rare species storage at low temperature at slow growth conditions has started quite recently (Holobiuc et al. 2008; Mitoi et al. 2009). These methods have been previously widely used commercially for plant cold storage in *in vitro* (Bertrand-Desbrunais et al. 1992; Fletcher 1994; Negash et al. 2001; Pereira et al. 2003; Lambardi et al. 2006; Capuana, Ponti 2008; Joshi, Jadhav 2013). It was found that species with easy cultivation in tissue culture in experiments at growth-limiting conditions were able to adapt for growth on media with osmolytes (sorbitol, mannitol). For instance, Lipavska and Vreugdenhil (1996) found a dramatic decrease in dry matter at higher concentrations of mannitol. Growth retarding effect of these osmolytes in appropriate concentrations coincided with results of our experiments (Fig. 1 to 5, Table 3). Plant cell division is inhibited by osmolytes introduced into cultivation media

and, consequently, plant growth is reduced (Xiong, Zhu 2002). Therefore, in many cases with polyols, we obtained smaller plants during the precondition period. Estimated reaction of 47 species was quite different on cultivation in media with polyols as the only carbon sources, as cold storage under the studied experimental conditions was suitable for 32 species for medium or longer storage period (Table 2).

Carbohydrates have two main roles: (i) a carbon and energy source and (ii) an osmotic agent. Those sugars that are not metabolized can be used to alter the osmotic conditions in the tissue, which also seems to influence adventitious shoot and root formation (Welandar, Pawlicki 1994). In addition to essential roles as substrates in carbon and energy metabolism and in polymer biosynthesis, sugars have important hormone-like functions as primary messengers in signal transduction (Rolland et al. 2002). Other studies indicate positive effect by radical scavenging of sorbitol and mannitol in plant storage and life processes (Cassels, Curry 2001; Molassiotis et al. 2006, Mitoi et al. 2009) and of carbon transport compounds in the phloem (Gaspar et al. 2002). A physiological role of polyols as agents of translocation and endogenous osmoregulation has been considered, together with more biochemical aspects of their utility as reserve carbohydrates, as sinks for photosynthetically produced reducing compounds and for co-enzyme regulation (Lewis, Smith 1967; Miotti et al. 2009). For germplasm preservation, media with sorbitol or mannitol have been widely used and shown to maintain cultures viable at low growth rate without subcultures for four or six months or more (Negash et al. 2001; Hao, Deng 2003, Pereira et al. 2003; Mitoi et al. 2009). Our study implemented a longer storage time, and showed that at least cultures of 27 species can be stored for more than year (Table 2).

Slow growth during *in vitro* cultivation generally is explained by low photosynthetic ability, as photosynthetic rate is restricted by low CO₂ concentration during the light period. High amounts of reserve saccharides are more important for plant survival than full autotrophy (Voračkova et al. 1998). Therefore, elevated concentration of sucrose in cultivation medium is needed for plant survival in unsuitable conditions like cold growth storage (Kļaviņa, Ievinsh 2008) and saccharide supplement in cultivation media is important. Heterotrophic cultures can adjust their metabolism from consumption of saccharides to consumption of polyols as an alternative carbon-energy source when the medium is sucrose-deficient (Steinitz 1999). However, ability to change metabolism or grow on media with polyols differs between species. In the present study, *Carex davaliana*, *Eryngium maritima*, *Saussurea esthonica* could be preserved only on medium with sucrose. On the other hand, some polyols are characteristic for particular families, e.g., sorbitol in Rosaceae (Lewis, Smith 1967) or higher taxonomic units, as mannitol is the most common

form of polyols in Angiosperms. In our experiments, more species were successfully preserved on media with sorbitol (24) than with mannitol (11).

When explants are exposed to low temperature, the level of reactive oxygen species increases (El-Sheekh, Rady 1995). Oxygen toxicity during cold storage can be reduced by plant endogenous mechanisms, such as peroxide metabolism enzymes as well as media components with antioxidant features. Both osmotic and saline media significantly increased non-enzymatic antioxidant activity (Molassiotis et al. 2006). Mannitol and sorbitol act also as osmoprotectants and quenchers of reactive oxygen species (Jennings et al. 1998). Perhaps the successful long term preservation success of some species in our experiments was due to this additional function of used polyols. Inclusion osmolytes in culture media causes morphological changes, e.g. inhibition of shoot growth (Xiong, Zhu 2002). This was confirmed for numerous wild plants, for example, *Pulmonaria angustifolia* (Fig. 9); maintaining on medium with sorbitol was more successful than for that with sucrose or mannitol, due to slower shoot growth and ability to form roots.

Storage of cultures at 5 °C on appropriate medium appears to be a promising technique for maintenance and medium-term conservation of rare species with economy of labour and materials for transfer. Species can be cold-stored from six months up to a few years or more. Survival rate during storage was influenced by media and genotype. Sorbitol and mannitol were appropriate growth retarding substances for cold storage of rare species, only their concentration and combinations with other compounds must be specified for each species to achieve successful regeneration capacity. *In vitro* culturing for an extended period under slow growth conditions (1 to 8 years) may allow storage of rare species germplasm and utilization of stored material when needed for National Botanic garden expositions, experimental purposes or reintroduction.

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