Environmental and Experimental Biology (2010) 8: 75–80 *Original Paper*

First results of *ex situ* conservation of endangered wild plants of Latvia in the National Botanic Garden

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Abstract

In 1981, the National Botanic Garden of Latvia began the *ex situ* conservation of rare and endangered native plants. About 70 species of endangered plants of Latvia are now successfully grown *in vitro*. The aim of this study was to determine suitable conditions for *ex situ* cultivation in the territory of the National Botanic Garden, where four artificial habitats (dune and meadow, deciduous tree forest, humid bank and ditch with spring water) were created or used for growth and survival experiments with individuals of 23 endangered wild plant species. Twenty of the species successfully adapted to the implemented growth conditions. *Alyssum gmelini, Dianthus arenarius, Helianthemum nummularium* showed good adaptation in dry and sunny conditions of artificial dune. *Galium schultesii, Pulmonaria angustifolia, Scrophularia umbrosa* showed good adaptation to fertile soil and shady conditions of deciduous forest. The short-lived species as *Spergularia salina* and *Tripolium vulgare* were identified as difficult species for cultivation.

Key words: ecological conditions, *ex situ* conservation, micropropagation, rare wild plants.
Abbreviations: MS, Murashige and Skoog.

Introduction

The threatened and endangered plant species in Latvia are relatively well documented. In total, 319 species of vascular plants are included in the Red Data Book of Latvia (Andrušaitis 2003), and many nature reserves and restricted areas have been established for their protection. Although some species are not very rare on a global scale, they should be protected locally if they are restricted to a smaller geographical region (Broennimann et al. 2005). In addition, conservation of local genetic diversity is also important.

In situ preservation of wild populations of plants is becoming more difficult due to fragmentation of habitats, invasion of alien species, as well as changes in climate. *Ex situ* conservation is an essential part of integrated conservation strategies that are aimed to conserve biodiversity in the wild (Cochrane 2004). According to the Global Strategy for Plant Conservation, 60% of threatened plant species should be accessible in *ex situ* collections by 2010, preferably in the country of origin. Priority should be given to the conservation of critically endangered species in their countries of origin (BGCI 2002). Genetically representative *ex situ* collections provide material for research and minimize impact to wild plant populations, offer potential adaptive management options for *in situ* work and maintain stock to produce material for education, reintroduction and other activities (CPC 1991; Guerrant et al. 2004).

The technical, theoretical and practical aspects of effective *ex situ* conservation are complex. Currently, efficient protocols for planning and implementing *ex situ* plant conservation are urgently needed. These protocols can be built from the developing experience in recovery planning and breeding (Cochrane 2004). The conservation value of cultivated specimens will be affected by the associated provenance data, which identifies both geographic origin and the collection history (Maunder et al. 2001).

Ex situ plant resources can be preserved both as seed banks as well as living plant collections: in pots, in gardens, in field gene banks, in semi-natural environments or as *in vitro* cultures. These collections can be maintained either in special facilities with minimal artificial interference or in large collections with very high level of artificial interference, as traditional botanic gardens usually prefer (Maunder et al. 2004).

Plant micropropagation technology has been developed and redefined continuously during the last 30 years, and it has become a significant tool for plant genetic resource conservation. *In vitro* methods have been successfully used for both propgataion and preservation of many rare and critically endangered wild plants. These methods are especially helpful when a species is difficult to propagate using conventional methods (Fay 1992; Mikulík 1999; Wala, Jasrai 2003; Sugii, Lamourex 2004; Holobiuc et al. 2007; Panayotova et al. 2008).

The need for an integrated approach utilizing both *in situ* and *ex situ* techniques to support wild populations of plants recently has been promoted by botanic gardens (Maunder et al. 2001). Thus, the role of many botanic gardens and arboreta has expanded, shifting from developing traditional plant collections towards active work in plant conservation (Havens et al. 2006).

In the National Botanic Garden of Latvia (NBG) different *ex situ* conservation methods for threatened wild plant species have been successfully performed. Field collections have been established since 1981 and *in vitro* collections since 2003 (Kļaviņa, Šmite 2004). The source material for these collections, seeds and, in some cases, mother plants, was collected in the wild in Latvia. The conservation priorities of the NBG are native threatened species as well as local species listed in the Bern Convention and EU Habitats Directive. The aim of this study was to determine suitable conditions for *ex situ* cultivation of various endangered wild plants of Latvia in the NBG. In the territory of the NBG four artificial habitats were created or used for growth and survival experiments with endangered wild plant species.

Materials and methods

Explants from aseptically germinated seeds were used to establish *in vitro* cultures. Seeds were collected from wild plant populations of Latvia based on the principles described in the ENSCONET seed collection manual (ENSCONET 2009). Seeds were surface sterilized with commercial bleach ACE for 7 to 10 min, rinsed three times in sterile distilled water and germinated in test tubes on agar-solidified (6 g L^{-1}) half-diluted Murashige and Skoog (MS) medium (pH 5.8). The shoots from sterile seedlings were placed on hormone-free half-diluted MS medium or on MS multiplication medium supplemented with growth regulators (0.1 to 0.5 mg L^{-1} 6-benzilaminopurine, 0.1 to 0.5 mg L^{-1} kinetin, 0.1 to 0.5 mg L^{-1} indole-3-acetic acid) according to the needs of particular species and cultivated in a growth room under 16 h photoperiod provided by a fluorescent light with a photon flux density 10 to 15 µmol m^{-2} min^{-1} at 22 to 25 °C. Rooting was carried out on half-diluted MS hormone-free medium with low sucrose level. Plantlets having a well developed root system were transferred to a peat or peat-sand substrate and acclimated in a greenhouse.

In total, 23 species of endangered plants from different natural habitats were included in the growth and survival experiments (Table 1). All of these species showed successful growth and multiplication in tissue culture (excepting *Primula farinosa*, which was not introduced *in vitro*). The survival of plantlets in greenhouse conditions was about 100%. According to putative determinant environmental factors in natural conditions, several artificial habitats were created or semi-natural habitats were chosen for transplantation of *ex vitro* acclimated plants. The planting sites in the chosen habitats were established in spring of 2008: (i) sand dune, (ii) deciduous forest, (iii) pond bank, (iv) ditch with spring water.

A sand and gravel pile approximately 1 m high was created in a dry meadow for cultivation of dune plants. The

Table 1. Species of endangered plants used in the experiments. Meso, mesotrophic; oligo, oligotrophic; eutro, eutrophic

Species	Ecological type	Habitat type	Spreading mode
Alopecurus arundinaceus Poir.	oligo, halophile	coastal meadow	seeds, clonal growth
Alyssum gmelinii Jord.	oligo/meso, calciophyte	dunes	seeds, dwarf shrub
Armeria maritima (Mill.) Willd.	oligo/meso, halophile	coastal meadow	seeds and vegetatively
Dianthus arenarius L.	meso/oligo	dunes	seeds and vegetatively
Galium schultesii Vest.	meso/oligo	forest	seeds and vegetatively
Galium tinctorium (L.) Scop.	meso/oligo	dry forest, meadow	seeds and vegetatively
Glaux maritima L.	oligo, halophile	coastal meadow	vegetatively
Gypsophila paniculata L.	oligo/meso, calciphile	dunes	seeds
Helianthemum nummularium (L.) Mill.	meso/oligo, calciphile	dry grassland	seeds, dwarf shrub
Juncus balticus Willd.	oligo, halophile	dune slacks, coastal lakes	seeds, clonal growth
Linaria loeselii Schweigg.	oligo	dunes	seeds and vegetatively
Lithospermum officinale L.	meso, loam soil, calciphile	meadow	seeds
Prunella grandiflora (L.) Scholler	oligo/meso, calciphile	dry forest, meadow	seeds and vegetatively
Pulmonaria angustifolia L.	oligo/meso	light forest, mainly with pine	seeds
Scrophularia umbrosa Dumort.	meso	river banks	seeds
Serratula tinctoria L.	meso	grassland	seeds
Spergularia salina J. et C. Presl.	oligo, halophile	shore	seeds, annual
Trifolium fragiferum L.	oligo/meso, halophile	coastal lake, river gulf	seeds and vegetatively
Tripolium vulgare Nees (syn. *Aster tripolium* L.)	oligo/meso, halophile	coastal lake, marsh	seeds, short-lived perennial
Veronica montana L.	meso/eutro	forest	vegetatively

soil was dry, with low humus and nitrogen content but rich with mineral elements, especially calcium, magnesium, iron, pH_{KCl} 6.8, EC 0.10 dS m^{-1} (data provided by the Institute of Biology, University of Latvia). The area of the planting site in dune was about 15 m^2. Natural plant cover was formed mainly by *Equisetum arvense* L.

The chosen planting site in deciduous forest had soil rich in humus, with moderate humidity. The deciduous forest was in an area of the arboretum with mature broad-leaved trees *Betula* sp., *Alnus* sp., *Fraxinus* sp. and *Corulus* sp. Mineral element content was characterized as sufficient, with pH_{KCl} 6.6, EC 0.6 dS m^{-1}. Natural plant cover was formed mainly by *Aegopodium podagraria* L. The area of the planting site in the deciduous forest was about 10 m^2.

A humid bank of a pond about 2 m high, with a steep slope, exposed to the north-west, with loamy soil formed a habitat for plants native to relatively moist places. Ponds are a part of NBG drainage system and the water level in the ponds depend on precipitation. The water level at the lower part of bank is variable but the top of bank is associated with a dry meadow. The content of nitrogen in soil was lower than in the forest planting site, pH_{KCl} 7.35, EC 0.52 dS m^{-1}. Good light conditions and variable humidity depending on precipitation and location of the particular place on the slope characterized this planting site. The native plant society consisted mainly of grasses, *Trifolium* sp., *Solidago* sp. and *Heracleum* sp. The area of the planting site was about 25 m^2.

For cultivation of endangered plants a part of the ditch where springs originate was chosen from those in the NBG drainage system. Nitrogen and potassium content in the ditch soil was low but the content of calcium and phosphorus was relatively high in comparison with the other habitats, pH_{KCl} 7.7, EC 0.63 dS m^{-1}.

The competitive flora (*Equisetum arvense, Aegopodium podagraria, Heracleum* sp. etc.) was weeded from the selected planting sites and soil was loosened for planting. Each species occupied about 1 m^2. At each cultivation site, 25 to 30 individuals of a particular species were planted to create a separate group and to exclude any interactions between the species. Several plant species were planted in two or three habitats. Sites were watered only after planting. The survival rate (%), diameter of plant clusters (cm) or number of rosettes, number of inflorescences per plant clusters and seed production were estimated in two vegetation seasons. Mean values of parameters from each plot were calculated.

Results

Dune

The artificial dune was created on a dry meadow by sand loading. In spring of 2008, potted plants grown in the greenhouse were planted on the sand pile, which resembled a dune habitat. Twelve species were planted: *Alopecurus arundinaceus, Alyssum gmelinii, Armeria maritima, Dianthus arenarius, Galium schultesii, Glaux maritima, Gypsophila paniculata, Helianthemum nummularium, Linaria loeselii, Spergularia salina, Trifolium fragiferum, Tripolium vulgare*. Good growth and flowering were observed for *Alyssum gmelinii, D. arenarius, Gypsophila paniculata, Helianthemum. nummularium, Linaria loeselii* and *Trifolium fragiferum* (Table 2). *Linaria loeselii* growth was excellent with high vegetative expansion (by root sprouts). For *Galium schultesii* the applied ecological conditions were not appropriate and the plants showed only 50% survival and depressed growth with no flowering during two vegetation seasons. *Spergularia salina* and *Tripolium vulgare* flowered in the first growing season and suffered mortality in the second year without reestablishment from seeds. *Glaux maritima* showed depressed growth and only a few flowering individuals (Table 2).

Deciduous forest

Six species (*Juncus balticus, Galium schultesii, Galium tinctorium, Prunella grandiflora, Pulmonaria angustifolia* and *Scrophularia umbrosa*) were planted in the deciduous forest. During the first year, the rich soil conditions in the shady forest caused intensive vegetative growth with poor flowering of some species. In the second vegetation season, *Galium schultesii* developed into a continuous plant layer with abundant flowering, but *Galium tinctorium* showed depressed growth and flowering. The height of flowering *Scrophularia umbrosa* plants in the second year reached 1.5 – 1.8 m and surpassed its height in other ecological conditions in the NBG. *Scrophularia umbrosa* and *Galium schultesii* may be assessed as species with high phenotypic plasticity, good adaptation and response to fertile soil conditions.

Pulmonaria angustifolia formed two to three new rosettes without flowers but a few rosettes from the previous year flowered. *Prunella grandiflora* and *Juncus balticus* survived vegetatively, but did not flower (Table 2).

Humid bank of the pond

Nine species were planted on the humid bank: *Juncus balticus, Galium schultesii, Galium tinctorium, Lithospermum officinale, Prunella grandiflora, Scrophularia umbrosa, Serratula tinctoria, Trifolium fragiferum* and *Veronica montana*. All species, except *Scrophularia umbrosa*, showed slow adaptation in this loamy soil. *Galium schultesii, Galium tinctorium, Lithospermum officinale, Trifolium fragiferum* and *Veronica montana* flowered, but vegetative growth of these species, except for *Veronica montana* and *Lithospermum officinale,* was depressed. *Serratula tinctoria* and *Juncus balticus* had poor growth in the first growing season and it died during winter (Table 2).

Ditch with spring water

A ditch with spring water was chosen as an artificial habitat for wetland species. The ditch was suitable for *Dactylorhiza incarnata, Pinguicula vulgaris* and *Primula farinosa*. Good

Table 2. Growth and flowering of transplanted plant species in different artificial habitats during two years of cultivation. *, the flowering of these species was recorded as 'numerous flowers' or 'few flowers' because plants had inflorescences with variable number of extremely small separate flowers difficult to record precisely. **, data not shown because plants were not planted at the same time and area as in others plots

Habitat	Species	First vegetation season			Second vegetation season			
		Survival (%)	Diameter of clusters (cm)	Number of inflorescences per cluster	Survival (%)	Diameter of clusters (cm)	Number of inflorescences per cluster	Seed pro-duction
Dune	*Alopecurus arundinaceus*	100	3.5	5	100	7.8	8.7	+
	Alyssum gmelinii	100	4.7	0	100	10.8	31.6	+
	Armeria vulgaris	95	3.7	0	95	5.2	3.5	+
	Dianthus arenarius	100	9.5	5.7	100	16.2	62.1	+
	Galium schultesii	50	1.5	0	50	5.5	0	–
	*Glaux maritima**	65	2.7	0	50	4.7	numerous	+
	*Gypsophila paniculata**	100	5.3	numerous	100	17.9	numerous	+
	Helianthemum nummularium	100	4.5	1.5	100	15.4	23.3	+
	Linaria loeselii	100	4.5	2.5	100	10.8	12.6	+
	*Spergularia salina**	90	5.5	numerous	0	0	0	–
	Trifolium fragiferum	100	6.8	3.2	100	16.6	17.1	+
	Tripolium vulgare	80	3.5	3	0	0	0	–
Deciduous tree forest	*Galium schultesii**	100	7.5	0	100	continuous layer	numerous	+
	*Galium tinctorum**	100	5.8	0	50	separate plants	few	+
	Juncus balticus	100	2.7	0	100	8.6	0	–
	Prunella grandiflora	100	3.8	0	80	11.1	0	–
	Pulmonaria angustifolia	100	separate plants	0	100	3.1 rosettes	few	+
	Scrophularia umbrosa	100	separate plants	0	100	7.4	3.5	+
Humid bank	*Galium schultesii**	100	3.7	few	100	7.5	numerous	+
	*Galium tinctorium**	75	2.8	few	70	4	numerous	+
	*Lithospermum officinale**	100	separate plants	numerous	100	5.9	numerous	+
	Juncus balticus	100	2.2	0	0	0	0	–
	Prunella grandiflora	90	2.3	0	80	4.1	0	–
	Trifolium fragiferum	100	5.6	few	95	continuous layer	few	+
	Scrophularia umbrosa	100	separate plants	0	100	5.8	3.8	+
	Serratula tinctoria	0	0	0	0	0	0	–
	Veronica montana	100	5.5	0	90	12.4	few	–
Ditch with spring water	*Dactylorhiza incarnata***	–	–	–	–		–	–
	*Pinguicula vulgaris***	–	–	–	–		–	–
	*Primula farinosa***	–	–	–	–		–	–

adaptation and regeneration of these species were observed for many years. Data are not shown for these species as they were not planted at the same time and area as those in other plots.

Discussion

In vitro cultures of all endangered species in the present study were initiated from seeds collected from wild populations of Latvia, without damage to natural resources as suggested by several authors (Fay 1992; Mikulík 1999; Holobiuc et al. 2007; Panayotova et al. 2008). Most of the species chosen for cultivation had a certain risk of extinction.

In scientific publications the possibilities of *ex situ* cultivation of endangered plant species with high potential economic value, suchas medicinal, aromatic plants and ornamental plants, have been widely described (Angelova et al. 1994; Gardner 2002; Wala 2003; Kozuharova 2009). Indigenous species, especially narrow endemic species, have been studied locally (Mikulík 1999; Holobiuc et al. 2007; Panayotova et al. 2008).

It has been well documented that acclimatization often is the most critical part of plant cultivation. For example, survival of *Dianthus superbus* ssp. *superbus* in the greenhouse was observed to be only 26.7%, compared to 61.5% in field conditions (Miculík 1999). Hardened plants of *Curculigo orhioides* showed 96% survival and vigorous growth (Wala, Jasrai 2003). In our experiments, the survival of plantlets in greenhouse conditions was about 100%; survival of hardened plants in planting sites of the NBG varied from 80 to 100% when conditions were suitable and from 50 to 65% in less suitable conditions, such as for *Galium schultesii* and *Glaux maritima* in the dune habitat (Table 2).

The halophile species *Armeria maritima, Alopecurus arundinaceus, Trifolium fragiferum* showed good adaptation to dry and sunny conditions in the artificial sand dune. *Glaux maritima* showed depressed growth but the root system was well developed. Individuals of *G. maritima* successfully established in large containers with peat-sand substrate outdoors in NBG, developing a continuous layer and reproducing with seeds (Kļaviņa et al., unpublished data). Apparently, insufficient moisture was the main limiting factor for *ex situ* growth of *G. maritima* in the present conditions of the artificial dune. The annual halophile *Spergularia salina* and short-lived perennial *Tripolium vulgare* showed good initial adaptation, survival (90 and 80%) and flowered in the first growing season (Table 2). Individuals of these species died during winter and there was not reestablishment from seeds. In natural populations annual and short-lived species need significant regeneration from seeds. It is possible that the lack of germinating seeds was caused by insufficient moisture in the soil or shortage of moderate salinity, as in the natural environment (Ievinsh 2006). In general, the high osmotic potential of the seawater prevents germination of seeds (Ievinsh 2006; Khan et al. 2006) and inhibits growth of mature plants (Shennan et al. 1987; Karlsons et al. 2008).

Dianthus arenarius, Helianthemum nummularium and *Linaria loeselii,* species from dunes and dry grasslands (Table 1), showed good adaptation in the artificially dune. *Linaria loeselli* excelled with high vegetative expansion ability in dry, sandy and sunny conditions of the dune in the second growing season. *Alopecurus arundinaceus* and *Alyssum gmelinii* showed reproduction by seeds on the dune, and *Scrophularia umbrosa* in deciduous forest.

The planting site in the deciduous forest was suitable for species adapted to diminished light conditions in summer, fertile soil and sufficient humidity. *Pulmonaria angustifolia* bloomed early in the spring before the big trees leafed out. *Scrophularia umbrosa* and *Galium schultesii* also showed good adaptation in forest and in humid bank conditions. *Galium schultesii* was unsuitable for dune conditions with poor growth and low survival – only 50% (Table 2). In natural conditions, *Galium schultesii* grows in broad-leaved forests but in *ex situ* conditions it can be designated as a species with high adaptation potential. In deciduous forest *Galium tinctorium* showed depressed growth with only few inflorescences. Apparently, insufficient illumination was the limiting factor for growth of *Galium tinctorium*, since naturally *Galium tinctorium* grows in dry pine forests and calcareous meadows.

Prunella grandiflora showed depressed growth without flowering in both tested conditions. However varieties of *P. grandiflora* are easily cultivated perennial plants and grow successfully in display flowerbeds of NBG. Determining conditions for *ex situ* cultivation of this species is a future task.

The planting site on the humid bank presented harsh conditions for plant adaptation. The content of moisture in the loamy soil was highly variable and delayed plant acclimatization. *Serratula tinctoria* and *Juncus balticus* died, perhaps due to insufficient moisture. In contrast, in exposition boxes of NBG these species grow successfuly (Kļaviņa et al., unpublished data). The planting sites for *Serratula tinctoria* and *Lithospermum officinale* were chosen near the top of the bank; which, perhaps, were not suitable for *Serratula tinctoria* as in the wild it mainly grows in wet flood plain medows. *Lithospermum officinale* is a species adapted to dry and moderately moist meadows, and showed good adaptation to the bank conditions. *Veronica montana* was planted near the water level on the lower part of bank where it adapted and spread well.

In conditions of Latvia, *in situ* conservation is mostly seen as the only appropriate and sufficient strategy for preservation of wild species (National Programme on Biological Diversity 1999). Therefore *ex situ* collections in the natural environment as described by Volis and Blecher (2010) could not be created in Latvia. Instead, various habitats in NBG were used to study plant adaptation in different conditions. A similar approach to determine survival of *ex situ* conserved plants was based on 'ecological similarity' (Wan 2008), i.e., plants from natural ecosystems were allocated in an agro-ecosystem.

In conclusion, the initial observations showed survival and good development of several rare and endangered plant species in the chosen habitats. At this stage of the study, observations of species growth have been made in locations within the botanical garden in semi-natural conditions. Most of the tested species (20 out of 23) showed successful adaptation to the new conditions. In future, when the planted species will spread and fully occupy planting plots, further development of plantings and interactions between the species will be studied.

Acknowledgements

The work was supported by the Foundation for Environment Protection of Latvia. We would like to thank Dr. Ģertrude Gavrilova for support in finding wild populations and species identification. The authors are gratefull to Prof. Ģederts Ieviņš for critical reading of the article.

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Received 30 August 2010; received in revised form 15 October 2010; accepted 1 November 2010