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Tissue culture technology in conservation of threatened plant species of Latvia

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Abstract

To meet the requirements of the Convention on Conservation of Biological Diversity and internationally accepted botanical garden targets, the National Botanic Garden of Latvia started the elaboration of *in vitro* conservation methods for threatened plant species of Latvia. The objective of the present work was to develop a reliable protocol for *in vitro* conservation of threatened plants. Seeds and other materials of 40 threatened plant species from natural habitats were collected. In total 37 species were introduced in sterile conditions. For 20 species culture was initiated by seed germination and for six species – from shoot apex explants. Seed germination in sterile culture was not observed for 11 of experimental species. In five cases seed germination failure might be due to incomplete development of seeds. Normal organogenesis was observed in medium without hormones. In culture, the introduced species had different growth potentials *in vitro*. Development of germplasm conservation technique by minimal growth storage was started for species with a high multiplication rate.

Key words: conservation of biological diversity, endangered wild plants, establishment *in vitro*, multiplication, rooting.

Introduction

As a result of the degradation and destruction of natural habitats, many species have become threatened *in situ* and need conservation. One of the main tasks of Botanical Gardens is local flora conservation, including the elaboration of *ex situ* conservation measures (Wyse Jackson 2000). In accordance to the *ex situ* conservation strategy, it is not desirable to propagate plants from small and genetically restricted samples, because depletion of genetic variability may reduce the ability to adapt in habitats. Therefore, the material obtained from natural populations must represent a wide genetic base (Malda et al. 1999). Tissue culture is used for conservation of biological diversity for multiplying of endangered species that have extremely small populations, for species with reproductive problems and for recovery and reintroduction (Bromwell 1990).

The development of a reliable *in vitro* protocol are of great importance for producing plant material and for conservation of rare plant species, and to offset the pressure on the natural populations as well as plants for medical and ornamental purposes. The selection

of plant material and media for *in vitro* propagation and conservation requires considerable empirical research. The present investigations are focused on the development of *in vitro* techniques for collecting and preserving germplasm of threatened species.

Materials and methods

Material from 40 species from susceptible natural habitats of Latvia including a narrow endemics or species on their border of distribution was collected (Table 1). Explants from aseptically germinated seedlings were used to establish *in vitro* cultures. In several cases when seeds were not available actively growing shoots were excised from plants grown in the field. Explants were surface sterilised with commercial bleach ACE, in case of seeds – without dilution, in cases of shoot apexes – half diluted, for 7 to 20 min, then rinsed in sterile distilled water for three times. The sterile explants were placed in culture vessels on hormone free Murashige and Skoog (1962; MS) basal medium or on MS medium supplemented with growth regulators [0.1 to 0.5 mg l^{-1} 6-benzylaminopurine (BAP), 0.1 to 0.5 mg l^{-1} kinetine, 0.1 to 0.5 mg l^{-1} indole-3-acetic acid (IAA)]. They were stored in a growth chamber at 24 ºC with a 16-h photoperiod.

Results and discussion

In total, 37 species were introduced in aseptic conditions. From these, 22 species were germinated in sterile conditions but in six cases culture was initiated from aseptic apexes. Seeds of 11 species failed to germinate due to incomplete development of seeds in five cases and perhaps due to the rest period and dense seed coats. For such species the scarification of the seed coats is essential to obtain good germination or by pre-treatment by immersion in water at 90 ºC and soaked for 24 h (Iriondo et al. 1995).

Table 2 provides information on seed germination of various species in aseptic conditions. The percent germination and time for germination differed considerably for each species. In most cases, mature seeds were used and it was found that for germination of some species it is very important to use freshly harvested seeds (*Scrophularia umbrosa*, *Onobrychis arenaria*). Water supply and moisture conditions were very important in the germination process (Oboucheva 1999). Low germination was noted for some species (D'Antuono et al. 2003).

Growth characteristics of species *in vitro* are presented in Table 3. The growth character of species in aseptic conditions was similar to growth in wild conditions: growth in height or formation of rosettes and clumps of multiple shoots. According to multiplication potential, the cultivated species could be divided in three groups: species with a high multiplication rate, species with a medium or low multiplication and species which failed to multiplicate so far. The growth features on medium, mostly without hormones, are summarized in Table 3. The highest proliferation rate (10 to 30) was obtained for *Dianthus arenarius* (both subspecies), *Galium tinctorium* and *Dracocephalum ruyschiana* on MS medium without growth regulators. For propagation of these species with high multiplication rate, growth regulators were not necessary. In contrast, for the Czech Republic endemic plant *Dianthus arenarius* ssp. *bohemicus*, a relatively high BAP concentration (1 mg l^{-1}) was used to obtain a comparably high multiplication (Kovac 1995). For species with a medium or low multiplication potential, considerably increased proliferation was observed only

Table 1. Threatened species of Latvia for conservation *in vitro*. RDB, species listed in the *Red Data Book of Latvia* (category). ED, species protected by *The Europe Union Directive on Conservation of Species and Habitat*s. NM, species included in the *National Biological Diversity Monitoring Programme*. (+) only for limited time

Species	Status of protection			Initiation *in vitro*	
	RDB	ED	NM	Seed germination	By apex culture
Agrimonia pilosa	+	+		–	
Ajuga pyramidalis	1			+	
Alisma gramineum	1		+	(+)	
Allium ursinum	3		+	–	
Angelica palustris	1	+	+	+	
Arenaria procera	2			+	
Armeria maritima	1			+	
Carex davalliana	3			+	
Carex ornithopoda	3			+	
Carex pilosa	1		+	–	
Carex supina	1		+	–	
Cinna latifolia	3	+		+	
Corydalis cava	1				
Cypripedium calceolus	2	+	+	–	
Delphinium elatum	2		+	–	
Dianthus arenarius ssp. *arenarius*	–	+		+	
Dianthus arenarius ssp. *borussicus*	+			+	
Dracocephalum ruyschiana	2		+	–	+
Equisetum telmateia	1				
Euphorbia palustris	2		+		(+)
Galium schultesii	2		+	+	
Galium tinctorium	1		+	–	+
Gladiolus imbricatus	3			+	
Helianthemum nummularium	3			+	
Ligularia sibirica	1	+	+	+	
Linaria loeselii	3	+		+	
Liparis loeselii	3	+	+	–	
Lunaria rediviva	4		+	–	
Onobrychis arenaria	3			+	+
Ophrys insectifera	1			–	
Pentaphylloides fruticosa	1		+		+
Peucedanum oreoselinum	3			+	
Polygonatum verticillatum	3			+/–	
Prunella grandiflora	1			+	
Pulmonaria angustifolia	2		+		
Pulsatilla patens	4	+	+	+	
Saussurea esthonica	1	+	+	–	
Scrophularia umbrosa	1		+	+	(+)
Trifolium fragiferum	2			+	
Veronica montana	1		+		+

Table 2. Seed germination *in vitro* of some threathened species. *, results from a separate experiment; **, results from the same experiment

Species	Germination (%)	Germination time (days)
Ajuga pyramidalis	25	78
Angelica palustris	40	28
	55*	112*
Arenaria procera	50	7
	92**	66**
Carex davalliana	28	132
	44*	266*
Carex ornithopoda	60	73
	73*	115*
Cinna latifolia	43	48
Dianthus arenarius ssp. *arenarius*	100	44
Dianthus arenarius ssp. *borussicus*	83	24
Galium schultesii	50	51
Gladiolus imbricatus	17	51
Helianthemum nummularium	100	48
Ligularia sibirica	67	30
Linaria loeselii	13	98
Onobrychis arenaria	88	38
Peucedanum oreoselinum	20	75
Pulsatilla patens	62	96
Scrophularia umbrosa	85	28
Trifolium fragiferum	11	26
	17	228*

after addition of cytokinins to the growth medium (*Arenaria procera*, *Carex ornithopoda*, *Pulsatilla patens*). For species without regeneration (*Ajuga pyramidalis*, *Angelica palustris*, *Gladiolus imbricatus*, *Peucedanum oreoselinum*, *Trifolium fragiferum*) various combinations of growth regulators will be tested for induction of propagation. In contrast to commercial propagation, for endangered species we must avoid elevated cytokinin levels in proliferation optimisation, as it is necessary to produce relatively few plantlets of each genotype for reintroduction or tissue culture collection. Minimal cytokinins and auxins in culture media would avoid somaclonal variation and efficiently produce true-to-type plantlets (Edson et al. 1996). Some authors have suggested the possibility of widening the genetic base of a species by somaclonal variation *in vitro* as a method of generating new vigour into natural populations of endangered species (Bromwell 1990). However, this conservation strategy is disputable.

In all cases rooting occurred on MS medium without growth regulators and no special rooting medium was used. Nearly all species had good rooting potential, except

Table 3. Growth *in vitro* of some threatened species. *, with citokinin BAP. Proliferation rate: ax., the number of axillary shoots obtained from one explant; nod., the number of nodes obtained from one explant. Rooting: + poor; +++ exelent

Species	Growth type	Proliferation rate	Rooting
Ajuga pyramidalis	rosette	1	++
Angelica palustris	rosette	1	+
Arenaria procera	in height	1.3 / 6.0*	+++
	clusters		(81 %)
Carex davalliana	clusters	7.0*	+++
Carex ornithopoda	clusters	1.5 / 4.2*	+++
			(100 %)
Dianthus arenarius ssp. *arenarius*	in height	4.8 ax.	+++
	axillary shoots	10.4 nod.	(100 %)
Dianthus arenarius ssp. *borussicus*	in height	5.0 ax.	+++
	axillary shoots	14.5 nod.	(100 %)
Dracocephalum ruyschiana	in height	4.2 ax.	++
	axillary shoots	14.5 nod.	(61 %)
Galium schultesii	in height	4	++
	axillary shoots		
Galium tinstorium	in height	7 ax.	++
	axillary shoots	30 nod.	
Gladiolus imbricatus	rosette	1	+
Helianthemum nummularium	in height	2 - 4 nod.	++
	axillary shoots		
Ligularia sibirica	clusters	1.3	++
Linaria loeselii	in height	2.7 ax. + nod.	++
	axillary shoots		
Onobrychis arenaria	clusters	1 - 3	
Peucedanum oreoselinum	rosette	1	+ (from seeds)
			+/– (from apex)
Pentaphyloides fruticosa	in height	4 ax. + nod.	+
	axillary shoots		
Pulsatilla patens	rosette	2 / 7*	+/–
Scrophularia umbrosa	in height	2 ax.	+++
	axillary shoots		
Trifolium fragiferum	rosette	1	+
Veronica montana	in height	1.4 ax.	++
	axillary shoots	3.4 nod.	

Onobrychis arenaria introduced by shoot apex and *Pulsatilla patens* after the seedling stage. Similarly, some authors have reported a high rooting percetage of *Dianthus* sp. with different auxines or without them (Kovac 1995; Prolic et al. 2002).

Several species have been introduced *ex vitro*. After transferring the plantlets to the greenhouse, a 28 to 100 % survival rate was achieved (*Dianthus arenarius* 69 %, *Galium tinctorium* 84 %, *Dracocephalum ruyschiana* 28 %, *Carex ornithopoda*, *Prunella grandiflora* 95 %, *Ligularia sibirica*, *Helianthemum nummularium* 90 %).

A total of 37 threatened plant species of Latvia have been introduced in sterile conditions. Seed germination time and percent *in vitro* differed considerably among the species. Seedlings for shoot culture initiation were obtained for 20 species. Shoot culture was initiated from shoot type for five species. Cultivated species can be conditionally divided in three groups: species with a high proliferation rate, with a medium or low proliferation rate and species which failed to multiplicate so far. Multiplication potential differed considerably on medium without growth regulators. Introduction of seven species *ex vitro* in greenhouse conditions was successful. Elaboration of slow growth conditions for species with a high multiplication potential is in progress.

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