Scientific Papers. Series B, Horticulture. Vol. LXV, No. 2, 2021
Print ISSN 2285-5653, CD-ROM ISSN 2285-5661, Online ISSN 2286-1580, ISSN-L 2285-5653

BIOTECHNOLOGICAL APPROACHES FOR *EX SITU* CONSERVATION OF MEDICINAL SPECIES *LIGULARIA SIBIRICA* (L.) CASS.

Mihnea VLADIMIRESCU, Cristian BANCIU, Ioana Cătălina PAICA, Gabriel Mihai MARIA, Anca MANOLE

Plant and Animal Cytobiology Department, Institute of Biology Bucharest, Romanian Academy, 296 Splaiul Independentei, 060031, Bucharest, Romania

Corresponding author email: cristi.banciu@ibiol.ro

Abstract

Currently, habitat destruction, exploitation, and climate change are driving biodiversity loss. Glacial relict species - remnants from the last glaciation - most of them cold-adapted, are particularly vulnerable to climate change. Ligularia sibirica (L.) Cass. is a typical glacial relict plant species with medicinal uses, protected in situ within Natura 2000 sites. Despite in situ conservation, in Romania the species is declining, therefore complementary conservation measures should be taken. In this respect, we have tested and selected some efficient biotechnological tools such as in vitro culture and cryopreservation, for medium and long-term ex situ conservation of L. sibirica germplasm.

Key words*: conservation biotechnology, Ligularia sibirica.*

INTRODUCTION

According to Millennium Ecosystem Assessment substantially and largely irreversible biodiversity loss has been occurring over the past 50 years. The most recent report of Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES, 2018) estimates that 33 per cent of the assessed vascular plants living exclusively in Europe and Central Asia are threatened. Moreover, the world's largest and most recent plant survey reveals that since 1900, nearly 3 species of seed-bearing plants have disappeared per year, showing that the extinction rate is 500 times faster than would be expected as a result of natural forces (Humphreys et al., 2019).

The main causes of plant endangerment include habitat alteration, spread of invasive species overexploiting, urbanization, unsuitable agriculture, pollution, global warming and loss of genetic diversity (Pitman and Jorgensen, 2002). Particularly vulnerable are species from wetlands mainly because wetland spread has declined by 50 per cent since 1970 (IPBES, 2018). Moreover, if adapted to cold and wet climate conditions, as glacial relicts are, species are under the pressure of both habitat loss and global warming.

Addressing the conservation and sustainable use of biological diversity a broad international strategy was established- Convention on Biological Diversity (CDB) - ratified by Romania since 1994. Under CDB an agreement was developed for integrated plant conservation, the Global Strategy for Plant Conservation, where target 8 established for period 2011-2020 stipulates that at least 75 per cent of threatened plant species in ex situ collections, preferably in the country of origin, and at least 20 per cent available for recovery and restoration programmes (GSPC, 2011). Following the targets of GSPC all over the world strategies were developed for endangered plant conservation in terms of in situ and ex situ conservation.

Ligularia genus (Asteraceae) originates from Central China in mid-Cretaceous and includes 129 species (Liu et al., 1994; Liu, 2004) with a large distribution range from Asia to Europe. Species *Ligularia sibirica* (L.) Cass. has a main continuous distribution from east Asia to Southern Siberia and a fragmented range with disjunct populations in Europe - Estonia, Latvia, Poland, Hungary, Romania, Croatia, Bulgaria, the Slovak Republic, the Czech Republic, Austria and France - (Meusel and Jager, 1992). The populations with European distribution originated in the early postglacial period and thus represent rare remnants of a former continuous distribution (Šmídová et al., 2011) being protected at European level under the Habitats Directive, Annex II of the Council of the European Community (1992). In

Romania, species is protected exclusively in situ under Natura 2000 network but latest assessment suggests this standalone measure is less than optimal (Mânzu et al., 2013).
Conservation measures should be taken for *L. sibirica* not only because of its scientific importance but also for species medicinal value (Manole et al., 2019). Studies on chemical constituents showed that *L. sibirica* is an important source of bioactive secondary metabolites like sesquiterpenes and pyrrolizidine alkaloids with cytotoxic, antibacterial, antitumor and anti-HIV effect (Kapas et al., 2009; Wu et al., 2016; Şuţan et al., 2020).
In order to complement *in situ* conservation of this vulnerable species and in response to the main targets of Global Strategy for Plant Conservation, the aim of our study was to add new conservation strategy to previously developed seed banking protocol (Manole et al., 2019) by the means of available biotechnological tools.

MATERIALS AND METHODS

Field collection
Mature achenes were collected in October 2015 from Natura 2000 site - ROSCI 0055 *Dealul Cetății Lempeș - Mlaștina Hărman*. In order to ensure coverage of maximum variability, achenes were collected from at least 50 individuals, sampled randomly from the entire population. From each individual the achenes were collected in separate sealed envelopes. To prevent fresh material hydration, in each envelope a few grains of silica gel were disposed (about ¼ of volume of collected achenes).
Dormancy breaking
Achenes were stratified for 30 days at 4°C
In vitro culture
For medium-term *ex situ* conservation of *L. sibirica* germplasm an *in vitro* culture protocol was established, following multiple stages as presented in Figure 1 and Table 1.
Ex vitro acclimatization
Rooted plantlets were transferred onto glass tubes (3 cm diameter, 14 cm high) filled with MS liquid media with low sugar content (2mg/l sucrose), without hormones and maintained at 20°C and 70% humidity. After for two weeks plantlets were transferred onto culture pots filled with aseptic substrate mixture (peat: sand: perlite/2: 1: 1) and maintained at 20°C and 70% humidity. After four weeks in solid substrate plantlets were transferred onto culture pots with acidic substrate mixture (soil: peat/ 1: 1).
Seed cryopreservation
For long-term *ex situ* conservation was developed a protocol for seed preservation under extremely low temperature at -196°C. The main stages for both seed cryo-storage and recovery are shown in Table 2.

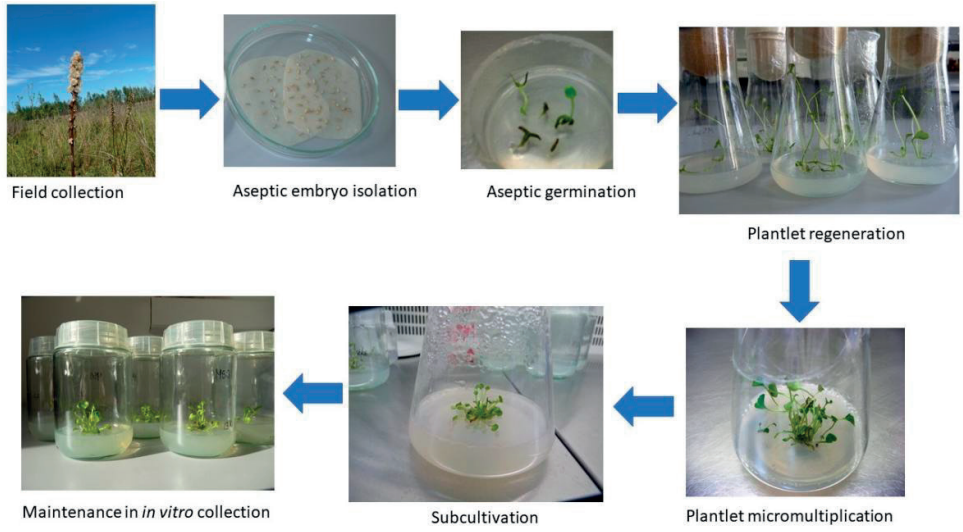


Figure 1. Diagram for medium term *ex situ* conservation protocol

Table 1. Stages for an *in vitro* collection establishment

Stages/sub-stages	A	b	c	d	E
Sterilization	pre-sterilisation pre-imbibition in $KMnO_4$ 0.5% (w/v), 1 hour	imbibition at 20°C for 24 hours in double distilled sterile water	pre-sterilization post-imbibition in ethylic alcohol 70° for 30 sec	sterilization for 2 min in $HgCl_2$ 0.1% (w/v)	3 washes with double distilled sterile water, each for 10 min
Plant regeneration	aseptic embryo isolation	embryo culture on Petri dishes onto MS media without hormone	culture maintenance at 20°C and 16 hours photoperiod	-	-
Micropropagation	single plantlet culture on Petri dishes onto MS[1] media supplemented with 0.1 mg/l IBA[3] and 2.5 mg/l BAP[4]	culture maintenance at 20°C and 16 hours photoperiod	-	-	-
Subcultivation	single shoot culture on Petri dishes onto MS basal media supplemented with 15 g/l manitol and low sugar content (15 g/l sucrose)	culture maintenance at 20°C and 8 hours photoperiod	subcultivation on same condition at 60 days interval	-	-
Rooting	shoot separation	single shoot culture on Petri dishes onto ½ MS[2] basal media supplemented with 0.3 mg/l NAA[5]	culture maintenance at 20°C and 16 hours photoperiod		

[1]MS basal media - Murashige Skoog media formulation (1962), [2]½ MS - Murashige Skoog media with half-strenght salt content; [3]IBA- Indole-3-butic acid; [4]BAP - 6- benzylaminopurine; [5]NAA - 1-naphthylacetic acid

Viability test

Fluorescence-based live assay was used to evaluate the seed viability post-cryotreatment. Seeds recovered after 3 days of storage in liquid nitrogen were immersed in a 0.1% solution (w/v) of fluorescein diacetate (FDA) which stains viable cells. FDA is taken up by cells which convert the non-fluorescent FDA into the green fluorescent metabolite fluorescein. Treated seeds were hand sectioned and analysed by fluorescent microscopy using a Zeiss Axio Imager M2 microscope equipped with fluorescent lamp and multiple fluorescence channels. Taking into account that fluorescein has a maximum absorption at 495 nm and maximum emission at 517 nm we have used a filter with wavelength range between 457 and 538 nm.

Germination test

In order to asses germination potential after cryogenic storage, samples of seeds were placed onto sterile Petri dishes (6 cm diameter) with humid cotton substrate. The samples were placed in the growth chamber set to a temperature of 20°C and an 8 hours photoperiod.

Table 2. Stages for seed cryo-storage

Stages/ sub-stages	A	b	c	d	e
Sample preparation	fresh seed colected from field were placed in an air-tight desiccator jar over calcium sulphate desiccant at 12% relative humidity for 1week	samples of 5 seeds were disposed in cryogenic vials of 2 ml	-	-	-
Cryoprotective treatment	in each cryogenic vial was added 1ml of 0.5M sucrose for 24 hours	solution removal	in each cryogenic vial was added 1ml of 1M sucrose for 24 hours	solution removal	in each cryogenic vial was added 1ml of PVS2[6] for 30 min
Controlled freezing onto *Freeze Control CL-3300* (Cryologic) system	CryoGenesis Programme version 5.0	cooling samples to 0°C cooling rate at 2°C/min	freezing samples to -4°C freezing rate at 1°C/min	2 min pause	freezing samples to -45°C freezing rate at 1°C/min
Freezing in liquid nitrogen and storage	immersion in liquid nitrogen for 1 hour	storage at -196°C in MVE Cryosystem 4000 containers	-	-	-
Seed recovery	vials with samples were thawed by direct immersion in water bath at 37°C	-	-	-	-

[6]PVS2 - Plant Vitrification Solution 2 (Sakai and Engelmann, 2007)

RESULTS AND DISCUSSIONS

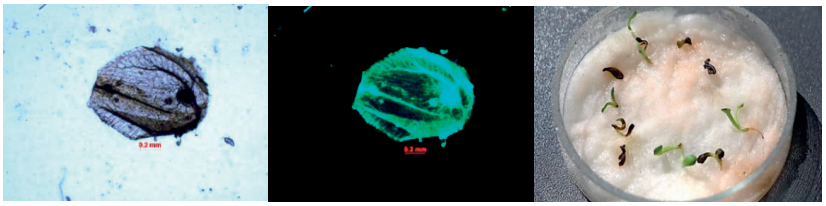
In vitro culture

One of the critical stages when initiating an *in vitro* culture is explant sterilization which has to be efficient to ensure an aseptic culture but also mild in order to ensure explant viability (Sarasan et al., 2006). The proposed sterilization protocol proved to be efficient resulting in 89% embryo survival and also, no contamination (Table 1, *Sterilization* stage). After 30 days of culture the embryos developed plantlets which served as source of plant material for micro multiplication (Table 1, *Plant regeneration* stage). When cultivated on multiplication media plantlets developed multiple shoots, after 4 weeks of culture multiplication efficiency being an average of 5 shoots/single plantlet (Table 1, *Micropropagation* stage). Under slow growth conditions provided by cultivation on maintenance media regenerants showed reduced growth and could be maintained without any alteration by periodical subcultivation at minimum 60 days period (Table 1, *subcultivation* stage). When necessary, shoots could be rooted in about 4 weeks of culture on rooting media (formulated as shown in Table 1), with an efficiency of 97% (Table 1, *Rooting* stage).

Another critical phase when developing an *in vitro* protocol is acclimatization (Hazarika, 2003). Our findings showed that rooted plants could be successfully acclimatised ex *vitro* within 8 weeks with a survival rate of 87%. As a result, following the proposed protocol plant material maintained in *in vitro* collection could be ready for field cultivation in approximately 4 months.

Seed cryopreservation

When treated with FDA, cryostored seed displayed fluorescence meaning that seed tissues are viable (Figure 2 a and b). Seed viability was validated also by germination test which showed that after 4 weeks on humid media 79% of tested seeds were able to germinate (Figure 2 c)



a b c

Figure 2. Viability test - Seed tissues after cryo-treatment a - in direct light, b - in fluorescence, c - germination test

Ex situ collections are valuable sources to increase populations that are declining or to restore the lost populations from rehabilitated habitats (Puchalski et al., 2014). *L. sibirica* populations are declining most probably because of different herbivores that can have a dramatic effect on the fitness of plant populartions (Heinken-Šmídová and Münzbergová, 2012). Consequently, with habitat degradation the moss layerproved to be the most suitable microhabitat for *L. sibirica* seed germination (Heinken-Šmídová and Münzbergová, 2012), is disturbed and appropriate germination and seedling recruitment is hampered. In this context developing *ex situ* collection of good quality material of *L. sibirica* as reserve for future actions for population reinforcement are necessary. Moreover, establishing an *in vitro* collection of a threatened plant species responds to target 8 of *Global Strategy for Plant Conservation* being an active contribution towards the aim "*20 per cent available for recovery and restoration programs*".

Acclimatization is considered a major bottleneck in the micropropagation of many plants that imply complex adaptive processes (Hazarika, 2003; Pospíšilová et al., 2007). When transferred from an atmosphere with high level of humidity and a medium rich in nutrients, including sugars, that allow heterotrophy, to external conditions, plantlets survival becomes critical. Although the proposed *ex situ* conservation protocol comprises the stages shown in Figure 1, we have also provided an efficient method to achieve roots and to acclimatize the micro-multiplicated plantlets in order to make plant material maintained in *in vitro* collections proper for transfer in natural habitats. In addition, the protocol developed within the present study could provide high quality plant material as an important source of bioactive compounds without any collection pressure from natural populations.

In order to back-up *in vitro* collections of *L. sibirica* germplasm the present study provides an efficient protocol for cryogenic storage of this species seeds (Table 2). Cryopreservation provides a safe and cost-effective method for long term-storage of plant tissues (Kaczmarczyk et al., 2011; Panis and Lambardi, 2006). Some estimations showed that until 2010 a few reports were published regarding only 52 wild endangered plant species for which cryoconservation protocols were established (Berjak et al., 2011). Enriching the knowledge in the field is also very important since currently there are still unavailable effective cryoconservation protocols for diverse plant tissues and genotypes (Popova et al., 2015).

CONCLUSIONS

Present paper provides for the first time, two simple and efficient methods, using biotechnological tools, to establish *ex situ* collections for medium and long-term conservation of *L. sibirica* germplasm. Live material preserved in collection may constitute a valuable source of plants for further restoration programs or for valuable bioactive metabolites extraction. These methods also provide insight into the development of protocols for the establishment of *ex situ* collections of other endangered species from *Ligularia* genus.

ACKNOWLEDGEMENTS

The study was funded by project no RO1567-IBB08/2020 supported by Romanian Academy and project PeatRo- RO02-65469. The authors would like to thank the Commission for the

Protection of Natural Monuments from Romanian Academy for permission to collect the plant material (No. 3896)

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