AgroLife Scientific Journal - Volume 8, Number 2, 2019
ISSN 2285-5718; ISSN CD-ROM 2285-5726; ISSN ONLINE 2286-0126; ISSN-L 2285-5718

SEED BANKING FOR LONG-TERM CONSERVATION OF GLACIAL RELICT *Ligularia sibirica* (L.) CASS.

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Abstract

The latest assessment of world vascular plant diversity showed that the current rate of their extinction has reached unprecedented high values. Biodiversity loss is driven mainly by anthropogenic causes as habitat destruction and climate changes. Being adapted to cold climate conditions, glacial relict species are particularly vulnerable to present global warming. Ligularia sibirica (L.) Cass. is a typical glacial relict plant species with medicinal value, native in Romania's Flora and protected under Annex II of Habitats Directive. Although at national level the species is protected in situ within Natura 2000 sites, its natural populations are declining. In order to sustain species in situ protection, complementary ex situ conservation measures should be taken. The aim of present study was to develop an efficient protocol to introduce species seed onto a seed bank, for long-term ex situ conservation of plant germplasm. Banked seeds will be a valuable source of plant material for further studies on species active compounds, natural population reinforcement of for habitat rehabilitation.

Key words*: Ligularia sibirica, glacial relict, medicinal plant, seed bank.*

INTRODUCTION

In June 2019 were published the results of the World's largest plant survey which reveals that seed-bearing plants have been disappearing at a rate which is up to 500 times faster than could be estimated as a result of natural events (Humphreys et al., 2019). In addition, more numerous than expected plant species became "functionally extinct" being present only in botanical gardens or in the wild, but in small numbers which cannot ensure long-term population survival, making this category of species candidate to near future extinction (Ledford, 2019).

Addressing the challenges raised by plant diversity loss a broad international initiative was developed under the *Convention on Biological Diversity* (CDB) by adopting the *Global Strategy for Plant Conservation* (GSPC). Under the Objective II of GSPC: *Plant diversity is urgently and effectively conserved*, at Target 8 proposed for period 2011-2020, is stipulated 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). The most developed regional response to the GSPC so far, is from European countries (*Plants 2020* release). Romania, which ratified CDB since 1994, hosted the Fifth *Planta Europa Conference* on the conservation of wild plants *Working Together for Plants.*

Within the conference a new *European Plant Conservation Strategy* was developed under the *Planta Europa Network* and the Council of Europe. The geographical area of this new Strategy comprises 47 European countries including Romania, and follows the objectives and targets of GSPC.

At national level *Vegetal Genetic Resources Bank "Mihai Cristea" of Suceava* (BRGV) is the main *ex situ* conservation unit for seeds and other propagules. The Gene Bank preserves in active collections (conservation for medium term, at 4°C) over 17,800 accessions and in base collections (conservation for long period, at -20°C) a number of 4,560 accessions (BRGV, 2019). The preserved accession refers only to species important for food and agriculture. For *ex situ* conservation of wild endangered plant species, at national level the efforts were concentrated mainly in botanical gardens and researches institutes (Păunescu, 2009).

Ligularia sibirica (L.) Cass. is a glacial relict plant species with a main continuous distribution from east Asia to Southern Siberia and a fragmented range with small and isolated populations in Europe (Meusel and Jager, 1992). The latest assessments reveal that populations with European range, originated in the early postglacial period and thus represent rare remnants of a former continuous distribution (Šmídová et al., 2011). In Europe, species is protected under the Habitats Directive, Annex II of the Council of the European Community (1992). As consequence, *L. sibirica* is protected in Romania *in situ* in some *Natura 2000* sites. A comprehensive assessment of the species` current and potential distribution concluded that the efficiency of *Natura 2000* in Romania for this species is less than optimal (Mânzu et al., 2013).

L. sibirica is important not only as rare glacial relict species but also for its medicinal value. It is widely used in traditional Chinese medicine to reduce expectoration and relieve coughing due to its Eremophilane type sesquiterpenes content (Wu et al., 2016).

Moreover, some pyrrolizidine alkaloids like tussilagine and iso-tussilagine isolated from *L. sibirica* showed antimicrobial and immune system stimulation effect being used for anti-HIV-1, HSV-1 and HSV-2 treatments (Kapas et al., 2009).

In several European countries national seed banks were developed to preserve endangered wild plants. For example, within last decade in Poland, several projects were developed regarding seed banking of indigenous endangered plants, including *L. sibirica* (Puchalski et al., 2014).

Even though at national level there were some efforts to introduce some endangered plant taxa in *ex situ* collections, none of these concern the species *L. sibirica*.

In order to support and complement *in situ* preservation, the aim of our study was to develop an efficient seed banking protocol towards an *ex situ* conservation strategy of *L. sibirica* germplasm.

MATERIALS AND METHODS

Since there are no specific standards for the conservation of seeds from wild plant species (Hay and Probert, 2013) the presented protocol was developed (Figure 1) according to latest release of *Gene bank Standards for Plant Genetic Resources for Food and Agriculture* (FAO, 2014). In addition, when we have tested different storage temperatures and water content, we have considered Roberts's rule which states that seed longevity increases as moisture content and temperature are reduced (Roberts, 1973).

Site identification

A comprehensive screening of available literature allowed us to make a list of national sites where the species was identified.

Seed aquisition

Achenes were collected at maturation stage in late September and early October 2015 from one Natura 2000 site - ROSCI 0055 *Dealul Cetății Lempeș - Mlaștina Hărman* Brașov county and other two non- protected site named *Bahna Mare* from Neamț county and *Stupini Marches* (Brașov county). All material was harvested directly from fructified individuals in order to avoid pathogenic infestation from ground achenes. When establishing a collection, sampling plays a critical role because a maximum genetic diversity that will be preserved must be ensured. We have sampled a number of 5 populations and collected achenes from at least 50 individuals of each. According to Brown and Marshall (1995) recommendation, this sampling formula ensures a capture of 67-83% of the species` alleles and at least one copy of 95% of the alleles that occur in populations at frequencies greater than 0.05. A total number of about 6300 achenes were collected, with an average of 25 achenes per individual. Samples from each individual were collected in a separate sealed envelope. To prevent fresh material hydration, a few grains of silica gel (about ¼ of volume of collected achenes) were disposed in each envelope.

Seed drying and storage

At maximum 2 days after field collection achenes were hand separated from accidentally introduced of alien material and also from pappus. Selected achenes were placed in an incubator with ventilation at 25°C and dried to 9.8% water content. Samples of about 20 achenes from each individual were disposed in cryovials of 1.8 ml, labelled and placed in a freezer at -18°C. A total of about 5000 achenes were stored for preservation.

Viability monitoring - germination test post storage

After one month of cold storage achenes were thawed by immersion in water bath at 37°C, scarified with scalpel and placed onto sterile Petri dishes (6 cm diameter) with sterile cotton fibres wetted with sterile distilled water. The samples were placed in the growth chamber set to a temperature of 20°C and an 8 hours photoperiod.

Seed characterisation

An initial germination test was performed using fresh collected achenes. In order to break seed dormancy a sample of 100 achenes were stratified for 30 days onto wet filter paper at 4°C. After stratification achenes were imbibed in distilled water for 24 hours at 20°C and then transferred in samples of about 30 achenes onto Petri dishes (9 cm diameter) with filter paper wetted with distilled water. For germination the samples were placed in the growth chamber at 20°C and an 8 hours photoperiod.

In order to assess water content samples of 100 fresh achenes were initially weight and then dried in an incubator at 130°C until they reached constant weight between three successive evaluation.

Average weight was assessed on a sample of 200 raw cleaned achenes after pappus removal using a Radwag PS 600/C/2 precision balance.

Achenes dimension was calculated as an average of 50 seeds measured under a Zeiss Stemi 2000C stereo microscope equipped with an AxioERc5S digital camera.

Micromorphological features were observed under scanning electronic microscopy (SEM). For analyses achenes were air dried for 24 hours in an incubator with ventilation at 30°C and then coated with 20 nm thick golden layers and finally examined and photographed under a JEOL JSM-6610LV microscope.

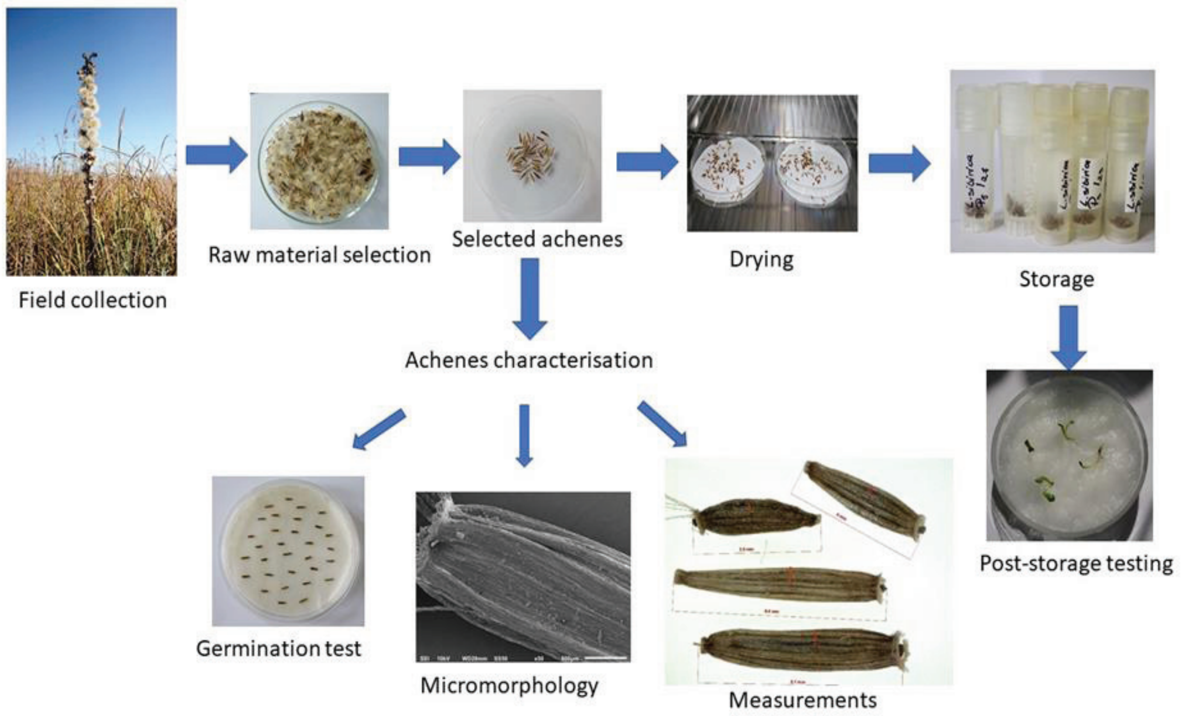

Figure 1. Diagram of seed banking protocol

RESULTS AND DISCUSSION

Species distribution range in Romania

A screening of the available literature showed that species was reported from the following counties: Maramureş (Iezerul Mare, Poiana Săpânţei, Nireşul Săpânţei, Tinovul Vrăticel, Runc, Iezerul Brebului), Cluj (Făgetul Clujului, Valea Morii), Bistriţa-Năsăud (Rodnei Mountains), Harghita (Borsec, Fântâna Brazilor, Bilbor, Dumbrava Harghitei, Beneş and Nadaş Marshes, Volbăşeni), Covasna (Comadău, Răbufnitoarea Marsh), Braşov (Dumbrăviţa, Arinişe, Hărman, Vulcan, Postăvarul), Vâlcea (Olăneşti, Valea Bistriţei), Argeş (Cheile Dâmboviţei, Piatra Craiului), Neamţ (Bahna Mare), Suceava (Lucina, Valea Cârlibabei, Breaza, Ponoare, Negoiasa, Şarul Dornei, Cristişor, Poiana Coşnei, Călimani Mountains) and Bacău (Nemira, Oituz, Valea Jepilor, Cheile Ialomiţei, Căpăţânii Mountains and Parâng Mountains).

Viability post storage

After 47 days, 82% of sown achenes germinated. Surprisingly these figures were better than those obtained from raw stratified seeds. Most of the available reports showed that freezing treatments lower germination percentage. Only a few experiments concluded that freezing could be beneficial for germination, for example those reported by Hosomi and collaborators regarding some *Cattleya* (Orchidaceae) species (Hosomi et al., 2012). This finding could be useful for further experiments in order to enhance germination indices.

Seed morpho-physiological characteristics

Germination

Germination was started 14 days after sowing and last a total of 42 days when a total of 67% of seeds have germinated. The germination process was slow and irregular.

Water content

The average water content of raw achenes was 16%.

Average weight and size

The estimated average weight per 1000 was 2.355 g.

Achenes size varied from 3.9 to 6.9 mm in length, from 0.7 to 1.5 mm in width, and from 0.2 to 0.6 mm in thickness, with averages of 5.335 mm, 1.057 mm, and 0.393 mm, respectively.

Morphology and micromorphology

This species` seeds are enclosed in a dried indehiscent fruit named achene. Seminal coat is thin, membrane-like non-adherent to the sclerified pericarp. The fruit is elongated, narrowed at both endings, brownish in colour and with a rostrum with persistent pappus at apical end (Figure 2).

Figure 2. Achene with pappus as seen in optical microscopy (x 10)

Fruit surface is irregular ribbed and the entire surface is finely grooved as seen in scanning electronic microscopy - SEM (Figures 3 and 4)

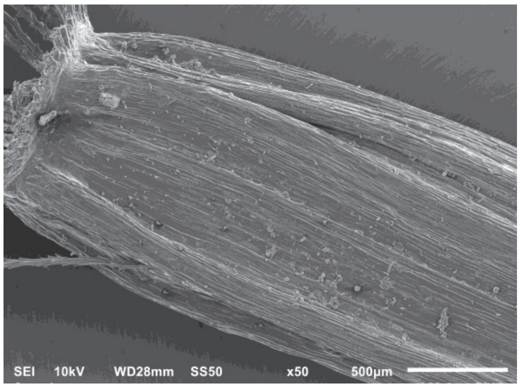

Figure 3. SEM microphotographs of achene surface

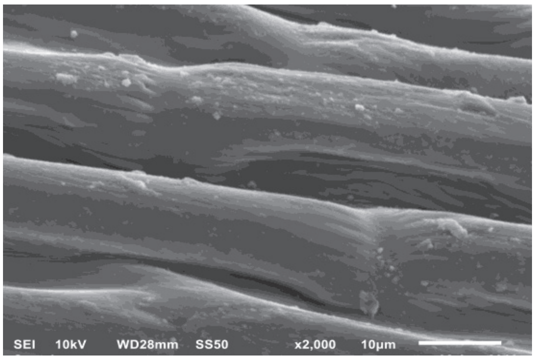

Figure 4. SEM microphotographs showing a finely grooved surface

Onto apical end achenes bears persistent whitish-yellow bristles as long, or longer than the achene (Figures 5 and 6).

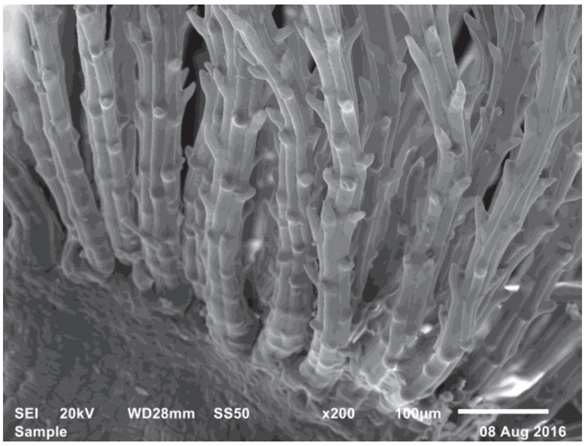

Figure 5. SEM microphotographs of pappus

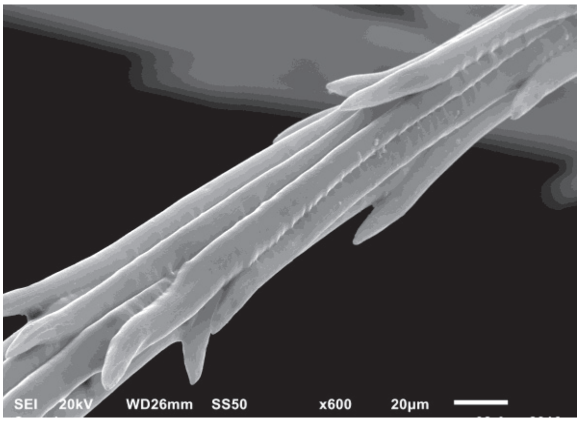

Figure 6. SEM microphotographs of a single bristle

Seed viability could be preserved for very long periods of time. There are many findings arguing that orthodox seeds are excellent plant material that could be preserved for long-term. Examples of exceptional seed longevity are those of ancient seeds of sacred lotus and date palm. One of the first report concerning long-term viability of seed date from 1923 and was released by the Japanese botanist Ichiro Ohga who tested a single-seeded fruit of *Nelumbo nucifera* var. China Antique (sacred lotus) originate from a dry lakebed in Northeast China. The author estimates seed longevity to be 120 years or more (Ohga, 1923). More recently, Miller and collaborators, radiocarbon dated the seeds, from the same provenience, as ~1300 years old, and test it for germination. The centuries-old sacred lotus seeds sprouted, having a germination time of only 3 days (Miller et al., 2013). Another notorious example is those of ancient seed of *Phoenix dactylifera* naturally desiccated in Israel's environment that germinated after 2000 years of storage in Masada archaeological site (Sallon et al., 2008). These achievements are strong arguments that seed banking is a valuable tool for long-term conservation of plant germplasm.

Seed banks are specialized units for long term storage of samples from wild populations and presently are increasingly seen as a central component of *ex situ* plant conservation (Meyer et al., 2014). These banks are sources of characterized and selected good quality plant material to support future researches or conservation efforts like species re-introduction and habitat restoration.

Since 2002 when the Global Strategy for Plant Conservation was adopted, thousands of wild species have been seed banked, mainly due to the endeavour of Millennium Seed Bank (MSB) partnership - (CDB Secretariat, 2009). MSB is recognized as the largest and most diverse wild plant species genetic resource, preserving over 39100 species from 5800 genera (MSB, 2019). Unfortunately, only the species that produces orthodox seeds are seed bankable therefore proper to preserve their germplasm in seed banks. Fortunately, *L. sibirica* is a species whose seeds support desiccation thus with orthodox bankable seeds. Curently, *L. sibirica* is preserved within MSB collection in three accession but from these, only one is originate from wild populations (from Bulgaria) and seeds are not available for distribution from Royal Botanical Gardens Kew. In this respect, developing a local seed bank with wild plant material originating from Romania as a ”back-up” collection of germplasm, could be of particular interest. For instance, *Stupini Marshes* from which a part of preserved seeds originates could be lost in the near future because of the accelerated expansion of Brașov city.

CONCLUSIONS

The developed protocol is a valuable achievement to develop a national seed bank for wild plant species with scientific, medicinal or economic importance. This initiative should be priority within the actual context of biodiversity loss. This study is also an active contribution to achieve Target 8 of Objective II of the *Global Strategy for Plant Conservation* concerning introducing threatened species into available *ex situ* collections.

ACKNOWLEDGEMENTS

The study was funded by project no RO1567-IBB08/2019 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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