Features of *in vitro* culture initiation of *Ligularia sibirica* (L.) Cass.

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Abstract. *Ligularia sibirica* (L.) Cass., a glacial relict, asociated with wetlands and also forested habitats, needs both *in situ* and *ex situ* conservation strategies. This paper presents a preliminary study conducted on *Ligularia sibirica in vitro* culture initiation, as a first stage in conservation strategy, before repopulation of habitats with laboratory-grown transplants. Depending on nutritive medium composition, the morphogenetic response was different, the variant V2 including 2 mg L^{-1} naphtyl acetic acid (NAA) and 1 mg L^{-1} of 2.4 D (dichlorophenoxyacetic acid) triggered a more pronounced sprouting.
Key Words: *in vitro*, *Ligularia sibirica*, regeneration potential, wild habitat.

Introduction. Only in Romania, about 107 bibliographic studies are focused on *Ligularia sibirica* (L.) Cass. (Matei 2018). Included in IUCN Red List, described by botanical enciclopedies like a glacial relict (Hendrych 2003; Smidova et al 2011), extremly rare in intra and intercarpatine swamp, *L. sibirica* was identified in our country in 1950 by Emil Pop.

L. sibirica is a perennial plant of the Asteraceae (Compositae) family, with 130-170 cm in height, vigorous, growing on sunny swampy lands (Slavik 2004), claying soils (or sandy) with a 6.5-7.5 pH. The species has a Eurosiberian distribution. It grows in groups of 2-5 frames. The leaves are dark green, simple. The flowers are yellow, star-grouped shaped, grouped in racem influorescences. In *L. sibirica* prevails sexual reproduction. Flowering period starts at the middle of July and lasts until the middle of August. The seeds are achenes with pappus (adaptation for anemophilous pollination); matures in late August or September. The seed are spread by the wind. Vegetative reproduction is realised by fragmenting the rhizome, but it is ineficient due to this slow growth, about 6 mm per year (Sammul 2001). The numerous sprouting is an solution to this inconvenience. Germination capacity is very low in open field, but in greenhouses conditions it reaches a 50% percentage.

L. sibirica is exposed to abiotic stress factors as water stress and salt stress (Matei et al 2016). Concerning the plant distribution, Mânzu & Cîșlariu (2019) corelated the recent distribution data of *L. sibirica* with previous data and personal observations collected from the field. Survival of the *L. sibirica* species is influenced by the biogeographical conditions, the main reason that determines the decrease of the number of subpopulations and affects their viability. Optimal conditions for *L. sibirica* populations development depend on the nutrient content of the substrate and biotic interactions within phytocoenoses (Cîșlariu et al 2018, 2021). Althought global warming is a challenge for relict glacial plants as *L. sibirica*, studies revealed that the niches have been conserved, being influenced by cold and wet climate conditions (Mânzu et al 2013). *L. sibirica* is found in [B] [DH. 2] Habitats directive - Annex II [B] and Bern Convention - Annex I. Genus *Ligularia* comprises 140 species (Chen et al 2018) and in the last two decades, knowledge about *L. sibirica* has reached a remarkable extent. The natural reservations of Harghita County are the most important conservative formations of relicts. *Caricetum nigrae* (*fusca*) (Braun - Blanquet 1915) is the association with the largest spatial spread, both in the Ciucului basin and in Giurgeului, which assimilates

glacial relict species *L. sibirica* (Albert 2009). Among other associantions in which this species is found, there are: As*. Carici flavae - Eriophoretum* Soo (1944) 1947, *Caricetum diandrae* (Jonas 1932) Oberdorfer 1957, from Nadas marsh (Tusnad Sat). Although in Romania is a rare species, benefiting of an advanced protection, in wetlands of the upper basin of Olt and Mures is very widespread. Populations with a large number of individuals, in which the flowering stage is not affected, grow in sunny pastures, located near running waters. Asociated with wetlands and also forested habitats, *L. sibirica* receive shelter and protection from neighbouring plants (Lanno & Sammul 2014). Without careful management of *L. sibirica* populations, it could become extinct in most of its habitats in the upcoming decades (Kukk 2003). Essential measures are being taken in order to avoid inadequate harvesting and to improve demographic situation by managerial activities of carefully monitoring. A list of national sites where the species was identified is available; for repopulation and multiplication of the individuals in controlled conditions of medium, cryopreservation of seeds is a reliable method to storage for long term (Manole et al 2019).

The aim of this paper is to presents the particularities of introducing in *in vitro* culture the species *L. sibirica*.

Material and Method. Optimal conditions of multiplication for *in vitro* conservation involve the vegetal material analysis in order to highlight the extent to which the conditions of the experiment influence the normal pattern of development and also the possible genetic variations (Banciu 2009). An important stage before inoculation on culture medium *in vitro* is the seeds preparation by sterilizing agents beside fortifying substances (Shelifist et al 2015).

Period of shoot cultures is prolonged by growth retarding substances as mannitol or sorbitol (Kļaviņa & Kļaviņa 2018).

Before the seeds sterilisation procedure, the dandelion formation called papus, around the seed, must to be removed. Then, seeds must be washed under tap water and maintained over night (12 hours) in a bowl of water, in order to soak seminal coat. Scarcing seeds previous to asepsis before inoculation procedure stimulated the germination process (Figure 1).

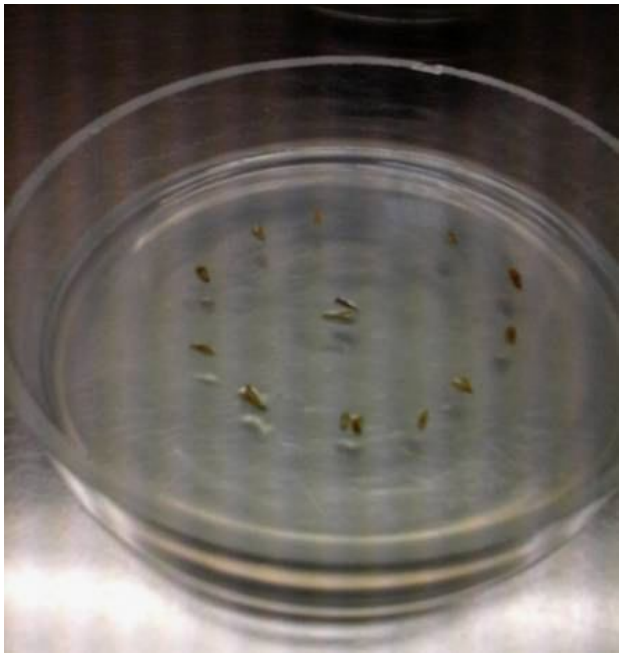

Figure 1. Seeds inoculi on Murashige-Skoog medium.

For sterilizing the seeds, in addition to 70% ethanol, amenable for surface contaminants removing, we used two kinds of sterilizing agents, namely dichloroisocyanuric acid in concentration of 0.5 mg L^{-1} and $HgCl_2$ in concentration of 0.1% associated with a few

drops of Tween 80. Between these steps of sterilizing process, the explants were washed with distilled water for 20 minutes for removing sterilizing solutions and to avoid the negative effects of these on vegetal tissue. The plantlets obtained by seeds germination, cut into small pieces, are the source of inoculi.

The cultures were maintained in growth chamber specially arranged, with an ambiental temperature of 25^{o}C and a photoperiod of 16h/day light and an light intensity of 2000 lucsi.

For medium conservation cultures we used solidified Murashige-Skoog (MS) medium with 8 g L^{-1} plant agar and fortified with the folllowing components: sucrose, mannitol, naphtyl acetic acid, benzylaminopurine, 2.4-D (2,4-dichlorophenoxyacetic acid), kinetin, in different proportions as in variants:

- V1: MS and 20 mg L^{-1} sucrose;
- V2: MS with 2 mg L^{-1} ANA and and 1 mg L^{-1} of 2.4 D;
- V3: MS with 1 BAP, 1 kinetin and 0.5 mg L^{-1} ANA;
- V4: MS withouth sucrose;
- V5: MS with 20 mg L^{-1} mannitol;
- V5: MS ½ .

Result and Discussion. Time of sterilizing and agent sterilizing type determine the success of sterilization process, a favorable results being 1 minute for ethanol 70% and 3 minutes for dichloroisocianuric acid sodium salt 0.5%.

Isocianuric acid sodium salt in concentration of 0.5 mg L^{-1} determines a efficiciency of sterilization protocol of 85.37% and 0.1% $HgCl_2$ with Tween 80 associated, a percentage of 95% sterilization.

Moisturizing the seeds endosperm stimulate the faster germination, beside removing the seed coat. In 14 days from the experiment initiation, the seeds germinate in percentage of 24.39% at 25^{o}C alternating a 16 hours light and 8 hours dark. The rate of plantlets growth reach 2 cm week^{-1}; in three weeks, the height reached 14 cm.

Moist filter paper enhanced the process of germination in order to obtain plantlets as source of inocullum (Figures 2 and 3).

The success of germination process depends on numerous factors as biological characteristics of seeds and storage conditions (Belokurova & Kuchuk 2014). Long storage of seeds before inoculation *in vitro* determines the loss of germination capacity due to the deterioration in time of its quality. Germinative response is evident after five days. Within two weeks, the germinated inoculli developed into transferable plantlets, exceeding the height of Petri dishes. Culture vessels amenable for *L. sibirica* stem are Erlenmeyer dishes of high capacity.

Figure 2. *Ligularia sibirica* seeds coated on paper filter.

Figure 3. Uncoated seeds on wet filter paper discs.

Depending on nutritive medium composition, the morphogenetic response was different. V2 variant of the culture medium triggered a more pronounced sprouting than the first variant. V3 variant allowed maintaining regeneration capacity but it requires repeated cultures on the same variant at a period of 30 days because of the phenolic substances delivered in the dishes. Culture media variants with a halved mineral salts concentration allow explants conservation more that 12 months. Vitrification process was not obvious.

Osmolite substances as mannitol, sucrose, sorbitol, ancymidol have growth delaying associated also with decreasing of the temperature to 10^{o}C (Blîndu 2009).

Lower temperatures triggered morphogenetic process delay beside mannitol as in V5 variant. The inoculli developed shoots in groups of 5-6 that exceeded also the length of the glass in about six weeks. Subcultures must be performed at two-weeks interval, when the *in vitro* plants looked withered and water steam was accumulating. Stem fragments can be used to further micropropagate the culture.

Acclimatization of regenerants assumed several stages:

- their extraction from culture vessels under sterile conditions;
- removing and washing the agarised medium from root level with sterile distilled water, to avoid bacterial contamination;
- transfer into glass tubes with the diameter of 3 cm and the height of 14 cm, containing liquid medium, with the aim of sustain high stems;
- coating (covering) them with sterile plastic wrap (preacclimatisation), in order to ensure proper humidity;
- planting in plastic pots with different combinations of soil, sand and perlit, sterilised and moistened with sterile distilled water.

The development period *in vitro* for *L. sibirica* plantlets last between 3-5 months. To detach gradually from anoxic *in vitro* conditions the regenerants, the sterile plastic wrap have been daily removed for 5-10 minutes.

A number of anatomical features contribute to the installation of the shock of acclimatization - the leaves of micropropagated plants are thinner, the palisade tissue is poorly developed and has large intercellular spaces, the vascular tissues of the veins and petiole are less developed, and chloroplasts have a modified structure and contain little chlorophyll. *In vitro*, the sustaining tissues - colenchim and sclerenchim - are poorly represented. That is why in the future experiments we must try to obtain regenerants with a smaller phenotype and with many shoots (rameti), before acclimatization stage. Usually, the roots obtained *in vitro* are devoid of absorbent hairs, are thick and brittle (Roșu 2006), but the phenotype obtained by us is characterized by long stems, vigorously roots and large leaves (Figure 4). Regenerants acclimatization encounter some inconvenients as in the findingd of Kumar & Rao (2012), as a disproportion between stems and wide leaves surface for the acclimatization process, the leaves mass being more heavy (Figure 5). The regenerants obtained *in vitro* can be source for callus cultures initiation and secondary metabolites production.

Figure 4. Large leaf regenerants of *Ligularia sibirica*.

Figure 5. Regenerants prepared to the stage of acclimatization.

Conclusions. There are two ways of initiation the *Ligularia sibirica* (L.) Cass cultures *in vitro*: starting from seeds as inocullum or starting from plantlets developed from seeds, this second procedure avoiding supplementary contamination.

Ligularia sibirica has an *in vitro* developmental rythm very appreciable, the height of the regenerants exceeding the Erlenmeyer vessels.

The *L. sibirica* differentiate *in vitro* different phenotypes depending on medium composition. Further studies will involve initiating callus cultures in large quantities as source for secondary metabolites extraction from the *in vitro* obtained regenerants, an importan objective for the research field starting from seeds or regenerants obtained *in vitro*.

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Conflict of interests. Author declares that there is no conflict of interest.

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