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Micropropagation of the narrow endemic *Hladnikia pastinacifolia* (Apiaceae)

Jana Ambrožič-Dolinšek[1, 2, 3*], Terezija Ciringer[1], Mitja Kaligarič[1, 3]

[1] University of Maribor, Faculty of Natural Sciences and Mathematics, Koroška 160, Maribor, Slovenia
[2] University of Maribor, Faculty of Education, Koroška 160 Maribor, Slovenia
[3] University of Maribor, Faculty of Agriculture and Life Sciences, Pivola 10, 2311 Hoče, Slovenia

Abstract – The monotypic *Hladnikia pastinacifolia* Rchb. is a narrow endemic species, with an extremely small distribution area in Slovenia, prone to any kind of threat that could lead to species extinction. Tissue culture techniques are proposed as a conservation measure for rapid propagation and *ex-situ* conservation. Tissue culture was initiated from seeds and juvenile plants obtained from natural sites on a solid Murashige and Skoog (MS) medium, with and without growth regulators. We tested various combinations and concentrations of growth regulators, and the best proliferation of axillary shoots, on average 14, was obtained on MS medium with 5 μM BAP and 3 μM IBA and 3% sucrose. Rooting was achieved after transferral of the shoots to an MS medium with 2 μM IBA and 3% sucrose. The rooted plants were acclimatized on a mixture of limestone sand, potting soil and vermiculite in a ratio of 10:2:2, with pH in the range of 7.5–8.0. In vitro propagation methods provide an important opportunity for the propagation and preservation of *H. pastinacifolia* by rapidly increasing the number of plants, without disturbing the wild population.

Keywords: Apiaceae, biotechnology, conservation, endemic, *Hladnikia pastinacifolia*, micropropagation, tissue culture

Abbreviation: BAP – 6-benzylaminopurine, IBA – indol-3-butyric acid, 2iP – 2-isopentinyl adenine, K – kinetin, MS – Murashige and Skoog medium, NAA – naphthalene acetic acid, TDZ – thidiazuron, Z – zeatin

Introduction

In the distant past and during more recent geological periods, complex biogeographical and evolutionary processes resulted in an extraordinarily high number of endemic alpine taxa (Berthouzoz et al. 2013). Five hundred and one endemic taxa have been described in the Alps, comprising ca. 9% of the 4,485 taxa inhabiting this mountain range (Aeschimann et al. 2011). The most emblematic species are the narrow endemics. However, only a few of them have been objects of intensive ecological and/or genetic studies (Berthouzoz et al. 2013). *Hladnikia pastinacifolia* Rchb. (Apiaceae) is one of the four endemic monotypic genera of the Alps – in addition to *Berardia, Physoplexis* and *Rhizobotrya* (Wraber 2009). It has an extremely narrow distribution area of 4 km^2 within the high karst plateau of Trnovski gozd in Slovenia (Aeschimann et al. 2004), which represents a bridge between the Alps and the North-Western Dinaric Alps. The species is also famous for its uniquely isolated phylogenetic position within the Apiaceae family (Šajna et al. 2012). It is not restricted to a specific niche; on the contrary, it thrives in a variety of the habitats (Šajna et al. 2012, 2014) that are available within the entire area of the High plateaus of the Dinaric Alps (Mayer 1960). It is still unknown why the distribution range is so narrow. Thus, the species' inability to populate favorable habitats in an area wider than a few isolated populations within a few square kilometers is the reason for concern regarding its persistence and has placed the plant at risk. Rare narrow endemic species occurring in a few small populations have to cope with random genetic drift, inbreeding, a stronger founder effect, and a greater potential for demographic bottlenecks that result in low genetic variability (Gaston and Kunin 1997). Small habitat populations decrease the chances of the plant outcrossing. The low potential for range expansion and recolonization was additionally explained by poor seed dispersal (Šajna et al. 2012). Thus, along with all mentioned restrictions for its dispersal across appropriate habitats and potential for further adaptation, its unique taxonomic position too makes *H. pastinacifolia* vulnerable and a potential candidate for extinction, especially on account

* Corresponding author, e-mail: jana.ambrozic@um.si

of the forecast global climatic changes (Dawson et al. 2011). Species that persist in small, isolated populations with low genetic diversity will have limited ability to adapt to new climate conditions (Lavergne et al. 2010).

Hladnikia pastinacifolia has another peculiarity: the small distribution range is divided into two populations by a large dense forest, which is unsuitable as habitat, in between. It has been presumed that such isolated distribution would have caused genetic divergence after the last glaciation. For that reason, its genetic variability has been tested, but divergence was not confirmed. A general loss of genetic diversity has been proved with molecular techniques using RAPD markers (Šajna et al. 2012). The authors concluded that the genetically homogeneous populations of *Hladnikia pastinacifolia* are the result of severe bottlenecks, which dramatically reduced or eliminated some populations, regardless of the time of colonization (before or after glaciation); the extant populations were founded by a single lineage, starting from a founding population.

The conservation status of *Hladinika pastinacifolia* is satisfactory. It is protected by the Decree on rare and threatened wild plant species (Official Gazette of the Republic of Slovenia 2002, 2010), with a status 4, meaning that measures for favorable conservation status on its habitat should be implemented. Furthermore, this species is listed as a "Natura 2000 species" in Annex II of the Habitat Directive (Council Directive 1992), which also envisages the designation of a Natura 2000 site on the basis of its occurrence. There have already been some attempts at ex situ conservation in the Botanical Garden of the University of Ljubljana, where a seed collection and living exemplars are stored. Both methods have disadvantages. One is connected with the short life cycle, since monocarpic *H. pastincifolia* (Šajna et al. 2009, 2011, 2014) flowers once in its lifetime, and dies after flowering. The other is connected with problematic seed germination established in the Apiaceae family (Hendrawati et al. 2012, Baskin and Baskin 2014). For such exceptional botanical rarities, it is nowadays usual to apply, besides the conventional propagation methods, in vitro propagation methods, which provide an important opportunity for the propagation and preservation of endemic, rare and/or endangered plant species in general (Fay 1992, Rao 2004, Pence 2010, 2011, Cruz-Cruz et al. 2013).

Only in cases where a previously optimized and tested propagation protocol exists would this kind of plant escape extinction from sudden changes in natural conditions or other factors that place the plant at risk of local extinction (which is here the definitive level). Over-collecting and competition with other species, along with reduction and fragmentation or degradation of natural habitats could diminish the population substantially. Micropropagation is a convenient measure suggested for preserving endangered species by rapidly increasing the number of plants in the case of natural population loss in a relatively short period of time and introducing them to their natural or to new environments (Sarasan et al. 2006, Kapai et al. 2010, Cruz-Cruz et al. 2013, González-Benito and Martín 2011). Beside this, tissue culture allows genotype conservation (Tavares et al. 2010b), constitutes a kind of living ex situ collection and is a basic method for other biotechnological conservation measures like cryopreservation of shoots (Cruz-Cruz et al. 2013). Several institutions, like the Kew and Missouri Botanical Gardens (Sarasan et al. 2006), and the Botanic Garden of the University of Coimbra for endemic Apiaceae, are now using this strategy to propagate and maintain endangered and endemic species (Tavares et al. 2009–2010, 2010b). The problems with such propagation include the potential for somaclonal variation, as a consequence of either genetic or epigenetic changes in the tissue culture-propagated material (Bairu et al. 2011). To obtain plant material that is as genetically stable and uniform as possible, with a low probability of genetic or epigenetic variation, micropropagation must be carried out through shoot tip or axillary bud proliferation (Pierik 1991).

The aim of the present work was to establish and evaluate the in vitro propagation protocol for *Hladnikia pastinacifolia* through axillary shoots in order to obtain enough plants that can be used for several purposes allowed because of legislative restrictions connected with sample quantities and for establishment of ex situ collections in botanical gardens, production of seeds for seed banking, reintroduction to the natural environment, for basic research on the ecology and biology of threatened plant species, and for long-term maintenance in a tissue culture collection – all, without disturbing the wild population.

Material and methods

Plant material, explant source and culture condition

All plant material, seeds and juvenile plants were collected at natural secondary sites near the route to Predmeja, Slovenia (Figs. 1a, b), with the permission of the responsible authority in 2004 and 2013. Seeds and juvenile plants of *Hladnikia pastinacifolia* were used as initial explants (Figs. 1c, d). Since the species is protected by law, we performed only one culture initiation by seed germination, and one initiation of the culture from the juvenile plants collected at the natural sites in 2004. Tissue culture was initiated once more in 2013, with sterilized juvenile plants collected at the natural sites. Each plant was the source of one tissue culture line for the propagation of plant material.

Seeds and all plant material were surface sterilized with 70% ethanol (Sigma Aldrich) for 30 seconds and then soaked in 1% or 2% commercial bleach 6.7% NaOCl (Šampionka), with a drop of the detergent Tween 80 (Merck) for 15–20 min and rinsed three times with sterile deionized water.

We tested several factors to increase the germination rate: cold treatment, scarification and sterilization of seeds. Sterilized and non-sterilized seeds germinated in sterile petri-dishes closed with parafilm (Bemis), on moistened paper bridges at 20 °C in the growth chamber or in the fridge at 4 °C in the dark. The cultures in the growth chamber were kept at 23 ± 2 °C, with a photoperiod of 16 h at 38–50 μmol m^{-2} s^{-1} (Osram L 58W/77 – Fluora) and 50% relative humidity.

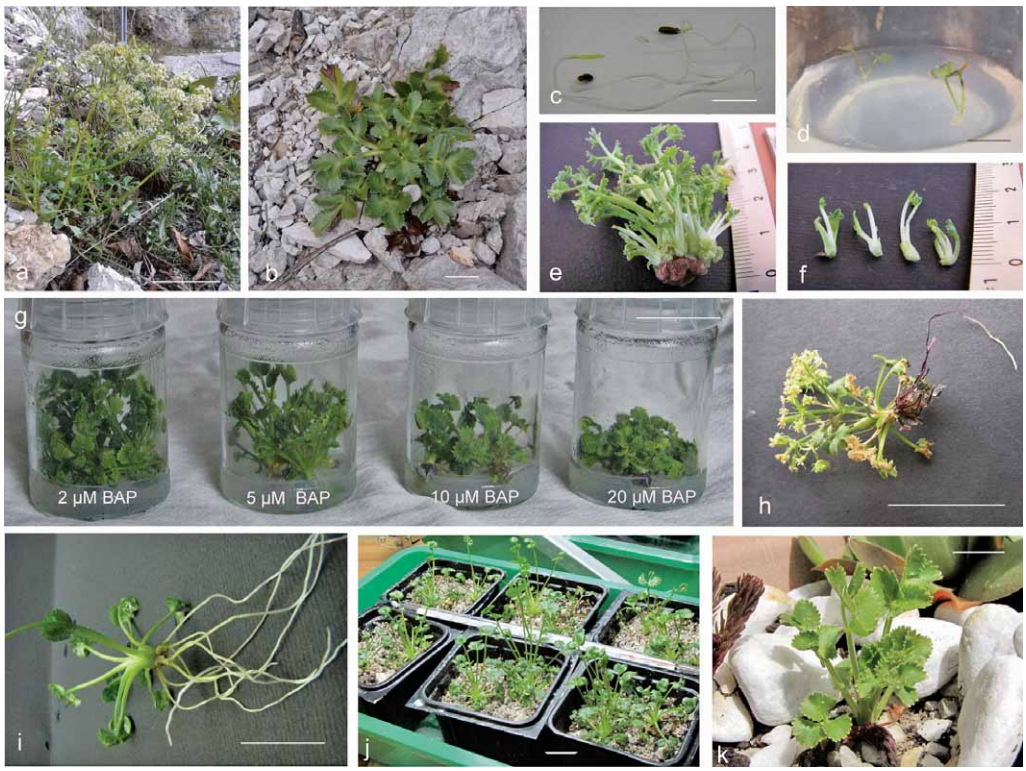

Fig. 1. Micropropagation of *Hladnikia pastinacifolia*: a, b) plants from the natural habitat in Predmeja (bar = 5 cm); c) sterilized seeds germinated into sprouts (bar = 1 cm); d) initiation of the culture with sprouts from natural sites (bar = 1 cm); e) the shoots developed on MS medium with 5 µM 6-benzylaminopurine (BAP) and 3 µM – indol-3-butyric acid (IBA); f) detached juvenile shoots are the most suitable for multiplication of *H. pastinacifolia* shoots; g) the effect of BAP on the length of shoots (bar = 2.5 cm); h) flower shoots developed sporadically from vegetative shoots (bar = 2.5 cm); i) rooted *H. pastinacifolia* shoots after 4 weeks on MS medium with 2 µM BAP (bar = 2.5 cm); j) rooted plants transferred to plastic pots with substrate for acclimatization (bar = 2.5 cm); k) acclimated plants after 6 months at outdoor conditions (bar = 1 cm).

Culture medium for shoot proliferation and multiplication

In 2004 and the next two years, tissue culture was initiated from 12 sterilized juvenile plants from the natural sites and 10 sprouts from seeds (Figs. 1c, d), and the best concentrations were additionally tested on juvenile plants from the natural site in 2013. The sterilized juvenile plants and sprouts were placed on solid MS medium (Murashige and Skoog 1962). supplemented with 0.8% Difco-bacto-agar (Medias International), with 3% sucrose (Duchefa Biochemie), with and without growth regulators: 6-benzylaminopurine (0–20 µM BAP, Sigma Aldrich), 2-isopentinyl adenine (0–20 µM 2iP, Sigma Aldrich), thidiazuron (0–10 µM TDZ, Sigma Aldrich), kinetin (0–20 µM K, Sigma Aldrih), zeatin (0–20 µM Z, Sigma Aldrich), naphthalene acetic acid (0–1 µM NAA, Sigma Aldrich), indol-3-butyric acid (0–3µM IBA, Sigma Aldrich) (Tab. 1) adjusted to pH 5.7–5.8, and later on autoclaved.

Root induction and acclimatization

The regenerated shoots were rooted on MS media with different combinations and concentrations of the growth regulators BAP (0–1 µM) and IBA (2–10 µM). In the rooting experiment, the following combinations and concentrations of the growth regulators were tested: 2 µM IBA and 1 µM BAP; 5 µM IBA and 1 µM BAP; 10 µM IBA and 1 µM BAP; 5µM IBA and 2 µM BAP; 10 µM IBA and 2 µM BAP; 2 µM IBA and 0 µM BAP; 5 µM IBA and 0 µM BAP; 10 µM IBA and 0 µM BAP.

Rooted plants were transferred to the prepared substrate containing a mixture of limestone sand : potting soil : vermiculite (10**:**1**:**2) with a pH of 7.0–7.5, or in a mixture of limestone sand : potting soil : vermiculite (Vermit Group) (10**:**2**:**2) with a pH of 7.5–8.5, and transferred to in vivo greenhouse conditions. The plants were protected against excessive water loss by regular spraying with water, and by plastic covers with adjustable aeration openings. They were acclimatized with a progressive increase in aeration through the adjustable aeration openings for the first two weeks, and with progressive opening of the whole cover over the next three weeks.

Statistical analysis

The statistical package SPSS® 21.0 was used for data analysis. Student's t-test and the 2 x 2 Chi-squared test (χ^2) were used for evaluating levels of statistical significance (P) between treatments. Statistical significance was shown between control on MS without and with growth regulators, unless otherwise denoted. The symbols used in the figures are as follows: NS denotes not significant, symbol (*) denotes $P < 0.05$, symbol (**) denotes $P < 0.01$, symbol (***) denotes $P < 0.001$. All experiments were repeated twice with similar results.

Tab. 1. Effect of different cytokinins and auxins on shoot development of detached shoots of *Hladnikia pastinacifolia* after two months of culture. BAP – 6-benzylaminopurine, 2iP – 2-isopentinyl adenine, TDZ – thidiazuron, K – kinetin, Z – zeatin, IBA – indol-3-butyric acid, 2,4-D – 2,4-dichlorophenoxyacetic acid, NAA – naphthalene acetic acid, SD – standard deviation. * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$. Significance was shown between shoots on MS without and with hormones, except if it is denoted different: [a]with 2 μM BAP; [b]with 5 μM BAP; [c]with 10 μM BAP; [d]with 0 μM TDZ.

Hormones [μM]								Explants with new shoots [%]	Average number of shoots ± SD
BAP	2iP	TDZ	KIN	Z	IBA	2,4-D	NAA		
0	0	0	0	0	0	0	0	9.3	3.7±0.4
2	0	0	0	0	0	0	0	27.3	6.4±1.5 * [a]
2	0	0	0	0	2	0	0	69.5	10.0±1.9 * [a]
2	0	0	0	0	3	0	0	71.0	9.1±2.5 * [a]
2	0	0	0	0	0	3	0	–	callus
2	0	0	0	0	0	5	0	–	callus
2	0	0	0	0	0	10	0	–	callus
5	0	0	0	0	0	0	0.5	25.0	6.8±1.1 **
5	0	0	0	0	0	0	1	10.3	6.1±0.9 *
5	0	0	0	0	0	0	0	38.5	6.6±0.7*** [b]
5	0	0	0	0	0.5	0	0	37.0	5.8±0.7 **
5	0	0	0	0	1	0	0	39.7	5.2±0.2 **
5	0	0	0	0	2	0	0	68.0	10.5±1. 6 ** [b]
5	0	0	0	0	3	0	0	90.2	14.0±2.7 ** [b]
5	0	0	0	0	0	10	0	–	callus
10	0	0	0	0	0	0	0	23.3	7.7±1.1 ** [c]
10	0	0	0	0	0	0	0.5	57.0	6.5±1.0 *
10	0	0	0	0	0	0	1	43.5	6.3±0.4 ***
10	0	0	0	0	0.5	0	0	35.0	6.0±0.6 **
10	0	0	0	0	1	0	0	55.3	7.3±1.4 ***
10	0	0	0	0	2	0	0	67.3	12.0±2.2 ** [c]
10	0	0	0	0	3	0	0	72.7	10.1±2.3 * [c]
20	0	0	0	0	0	0	0	17.0	5.6±0.3 ***
20	0	0	0	0	3	0	0	53.3	8.5±2.1
0	2	0	0	0	0	0	0	10.3	4.5±0.7
0	5	0	0	0	0	0	0	20.0	6.3±1.4 *
0	10	0	0	0	0	0	0	39.0	8.1±1.7 **
0	10	0	0	0	0	0	0.5	40.0	6.8±1.6 *
0	10	0	0	0	0.5	0	0	27.3	5.3±1.0
0	20	0	0	0	0	0	0	50.3	8.6±2.0 **
0	20	0	0	0	0	0	0.5	34.7	4.7±0.5
0	20	0	0	0	0.5	0	0	38.0	5.4±1.8 *
0	0	0.25	0	0	0	0	0	46.3	7.3±1.7 *
0	0	0.5	0	0	0	0	0	74.3	8.50±2.12 *
0	0	0.5	0	0	2	0	0	73.7	9.81±1.5 ***
0	0	1	0	0	0	0	0	34.7	5.1±1.1
0	0	2	0	0	0	0	0	39.0	6.2±1.0 *
0	0	5	0	0	0	0	0	26.3	5.2±0.8
0	0	10	0	0	0	0	0	27.0	4.3±0.7
0	0	0	1	0	0	0	0	6.3	3.6±0.3
0	0	0	2	0	0	0	0	14.0	4.1±0.6
0	0	0	5	0	0	0	0	14.0	4.0±0.3
0	0	0	10	0	0	0	0	11.3	3.8±0.4
0	0	0	20	0	0	0	0	13.3	4.2±0.7
0	0	0	0	0.5	0	0	0	8.5	3.6±0.3
0	0	0	0	2	0	0	0	21.0	5.5±1.2
0	0	0	0	3	0	0	0	21.0	5.2±1.4
0	0	0	0	5	0	0	0	28.0	4.5±0.6
0	0	0	0	10	0	0	0	52.5	4.6±2.2 *
0	0	0	0	20	0	0	0	34.0	4.1±0.1
0	0	0	0	0	0	1	0		callus
0	0	0	0	0	0	3	0		callus
0	0	0	0	0	0	5	0		callus
0	0	0	0	0	0	10	0		callus

Results

In vitro seed germination and initiation of the culture

The seeds of *H. pastinacifolia* started to germinate after 4 months in the culture (Fig. 1c) and achieved their highest germination rate after one year. We tried several factors which could influence the germination rate: room temperature and cold treatment, scarification and sterilization of seeds. Only those seeds under permanent cold treatment germinated but failed to germinate at all if they were kept in the growth chamber. Scarification did not promote germination. Between 7% of the sterilized and 30% of the unsterilized seeds germinated after 4 months and between 45% of the sterilized and 60% of the unsterilized seeds germinated after one year of cold treatment. Sterilization decreased and slowed the rate of early germination. This was significantly evident ($\chi^2 = 10.960$; df = 1; P = 0.001) after four months of germination but no longer significantly evident after one year of germination. Tissue culture was initiated from shoots excised from juvenile sprouts (Fig. 1c) germinated from sterilized seeds, from sterilized juvenile sprouts germinated from the unsterilized seeds and from juvenile plants from the natural sites (Fig. 1d).

Shoot proliferation and multiplication

Permission for the application, which allowed only a small number of explants, limited our research. That is why we began our experiments with a small number of initial explants. In these early experiments we first cultivated a small number of explants on MS medium with and without 2–20 μM BAP (data not shown); this was later repeated several times on a higher numbers of explants (Fig. 2a). Shoot multiplication was observed on all MS media, with or without growth regulators. Shoot proliferation was optimal when 5 and 10 μM BAP were used, and the best growth medium produced on average, 7.3 shoots per explant (Fig. 2a). This plant material was used for further experiments, where several concentrations and combinations of cytokinins and auxins were tested (Tab. 1).

In further experiments, the development of new shoots was observed on almost all media tested (Tab. 1). There was a significant difference within the various treatments using different combinations and concentrations of growth regulators. The addition of auxins to cytokinins additionally improved shoot development (Tab. 1). New shoots developed on almost all the explants. The best proliferation of shoots, on average 14 (Fig. 1e, f, g), was obtained on MS medium with 5 μM BAP and 3 μM IBA (Fig. 2b, Tab. 1). Shoots developed on 90% of explants. Slightly fewer shoots, on average 12, were obtained on MS medium with 10 μM BAP and 2 μM IBA (Tab. 1). Shoots developed on 67% of these explants. The new shoots began to proliferate after 10 to 14 days. Over the next 15 to 20 days, the shoots elongated to a length appropriate for detachment and rooting.

These detached shoots exhibited both organized and unorganized growth (Tab. 1). Unorganized growth was observed only on the MS medium with growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) (Tab. 1). Flowers developed sporadically from the new shoots. Well-developed flower shoots formed several inflorescences at the shoot tip, which developed into a considerable number of flowers (Fig. 1h).

Growth regulators did influence the shoot length. With an increased concentration of BAP, smaller shoots developed, if BAP alone was added to the media. This was significantly evident when concentrations exceeded 5 μM BAP (Fig. 1g).

Root development and acclimatization

Roots did not develop on those media without growth regulators. To obtain roots, we tested several combinations and concentrations of growth regulators. We started by decreasing the amounts of cytokinin BAP and increasing the auxin IBA and finished by increasing only the auxin IBA.

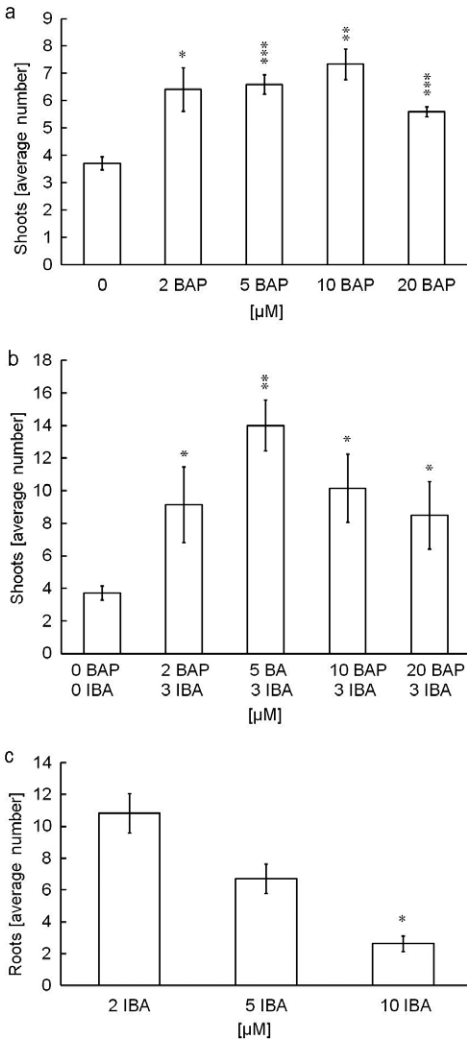

Fig. 2. Effect of plant growth regulators on the shoot and root development in the micropropagation of *Hladnikia pastinacifolia*: a) the effect of 6-benzylaminopurine (BAP) on the number of shoots (n = 28) after 35 days of culture; b) the effect of BAP and indol-3-butyric acid (IBA) on the number of shoots (n = 28) after 35 days of culture; c) root development (n = 25) on MS media supplemented by different concentrations of IBA after 4 weeks of culture. Statistically significant differences (t-test) are shown between the control without hormones (a, b) or MS medium with 2 μM IBA (c) and different treatments. SD -standard deviation, * denotes P < 0.05, ** denotes P < 0.01, *** denotes P < 0.001.

The best rooting was achieved on the MS medium with IBA alone (Fig.. 1i, 2c). Roots started to develop after 2 weeks. Optimal rooting was obtained on the MS medium with 2 μM or 5 μM IBA after 4 weeks of culture. The greatest number, 44% of shoots with roots, was obtained on the MS medium with 2 μM BAP and the smallest, 28% of shoots with roots, was obtained on the MS medium with 5μM IBA. Shoots with roots developed an average 10.8 of roots on medium with 2 μM IBA. A lower average of 6.9 roots developed on the medium with 5 μM IBA, and the significantly lower average of 2.6 roots appeared on the MS medium with 10 μM IBA. The higher concentration, 10 μM IBA, induced both roots and callus to develop. Roots developed on the detached side of the shoots.

Two growth substrates with different pH were assessed for efficiency in supporting ex vitro growth. Both mixtures are attempts to imitate conditions in the natural habitat. They were prepared with limestone sand, which increases the pH of the substrate, potting soil and vermiculite. After 5 months, 66% of the plants survived in the substrate with a higher pH between 7.5 and 8.5 (Fig. 1j) and only 22% in substrate with a lower pH between 7 and 7.5. Plants of *H. pastiancifolia* obtained by this procedure are transferred to outside conditions (Fig. 1k).

Discussion

Like many other alpine plant endemics (Aeschimann et al. 2011), *Hladnikia pastinacifolia,* has an extremely restricted distribution. Due to its inability to colonize appropriate habitats in a wider range due to several reasons, due to genetic depauperation, which make its capacity of adaptation to climate change even weaker, *Hladnikia pastinacifolia* is a strictly protected species (Šajna et al. 2012). This is the reason for the restrictions on the collecting of seeds for seed banks and research activities. Micropropagation of *H. pastinacifolia* provides the possibility for conservation in tissue culture collection, without disturbing the wild population and allows rapid propagation of *H. pastinacifolia* for research efforts.

Seed-banking is the primary method for ex-situ conservation. We had shown that *H. pastinacifolia* had problematic seed germination. Seeds from cold wet environments have been shown to be relatively short lived in storage, and successful long-term seed conservation for alpine plants may be difficult (Mondoni et al. 2011). Therefore *H. pastinacifolia* has to be conserved either in living collections or through micropropagation and/or cryogenic storage, and this article presents the latter method.

In vitro seed germination and initiation of the culture

Micropropagation was started with the introduction of plant material in aseptic conditions. We started with limited sample quantities and with different sources of plant material. Seeds, juvenile sprouts from seeds, and juvenile plants of *H. pastinacifolia* were used as initial explants for initiation of the culture. The starting material had a range of disadvantages connected with germination and/or the establishment of aseptic culture conditions.

H. pastinacifolia has problematic seed germination. These seeds did not germinate in conditions that usually promote germination. When they did germinate, they did so neither in high percentages nor immediately and simultaneously. For that reason, the initiation of culture from seeds was time consuming, uncertain in outcome and in need of further consideration. The seeds began to germinate between four and twelve months after initiation of the culture. The seeds in our experimental system germinated only after cold treatment and did not germinate at all in growth chamber conditions. The reason for this late and low germination could be dormancy, which is not unusual for seeds of the Apiaceae family (Hendrawati et al. 2012, Baskin and Baskin 2014), or possible loss of viability (Makunga et al. 2003, Mondoni et al. 2011). Sterilization only slowed the rate of early germination and slightly influences the final germination of cold treated seeds. That is why the initiation of the culture through seeds or juvenile sprouts from seeds could be problematic, since this lengthens the initial stage of micropropagation by several months. The introduction of unsterilized plant material in aseptic conditions was also problematic, because of damage during sterilization. When we started the culture with sterilized seeds, they germinated into viable, juvenile sprouts. When we started the culture with unsterilized seeds that germinated into unsterilized juvenile sprouts, there were losses due to contamination and/or mechanical damage during their transfer to aseptic conditions by sterilization. Problems with the establishment of an aseptic culture make seeds more appropriate than juvenile sprouts from seeds.

When tissue culture was initiated from sterilized juvenile plants from the natural sites, the initiation of the culture was problematic because sterilization did not always remove contamination. These sources of contamination include surface sterilization resistant plant endophytic microorganisms and common environmental microorganisms, both of which may become pathogenic in culture (Cassells 2012). Contamination can resist sterilization and sooner or later damage plant material. Cover contamination makes juvenile plants taken from natural sites the least appropriate for tissue culture initiation.

Shoot multiplication

The development of new shoots was observed on almost all media tested, even on the MS medium free of growth regulators. Growth regulators on optimal media more than tripled the number of shoots. The best proliferation of shoots was obtained on the MS medium with 5 μM BAP and 3 μM, and slightly worse on the MS medium with 10 μM BAP and 2 μM IBA. A combination of BAP and IBA was used for shoot proliferation of Apiaceae *Anethum graveolens* (Sharma et al. 2004) and *Centella asiatica* (Banerjee et al. 1999), first in a combination of 2.2 μM BAP and 0.5 μM IBA, and second in a combination of 8.9 μM BAP and 0.5 μM IBA (Banerjee et al. 1999, Sharma et al. 2004). Other researchers (Hosain et al. 2000, Nath and Buragohain 2003, Sharma and Wakhlu 2003, Karuppusamy et al. 2007, Das et al. 2008, Jaheduzzaman et al. 2012) used a combination of BAP and NAA or a combination of BAP

and IAA (Gaddaguti et al. 2013) for proliferation of shoots in Apiaceaes and an optimal concentration ranging from 2 to 17.5 μM of BAP and from 0.5 to 2.7 μM of NAA. The combination of BAP with NAA in our case was not as effective in shoot regeneration.

Growth regulators influence the shoot length of *H. pastinacifolia*. With an increased concentration of BAP, when BAP alone was added to the media, smaller shoots developed. However, the trend toward shoot lowering was not evident when BAP was combined with different concentrations of auxin IBA (data not shown). The opposite trend was observed when 2iP alone was added to the media, where with the increased concentration of 2iP, taller shoots developed (data not shown).

Sporadically observed flower development, instead of vegetative shoots, indicates the end of the life cycle for these plants. Since it occurred infrequently, it did not disturb the micropropagation procedure. Flower shoots with inflorescences were previously observed in Apiaceae tissue culture (Tavares et al. 2010a).

Root initiation

Rooting of shoots during propagation is genotype-dependent, and shoots of some species which root naturally without a rooting stage need a special rooting medium, with or without growth regulators. *Hladnikia pastinatifolia* shoots developed roots only on the MS medium with growth regulators. Optimal rooting was obtained on MS medium with 2 μM IBA. Concentrations higher than 5 μM IBA induced both roots and the development of callus. Root development with an intermediary callus indicated that at that roots and shoots were not well connected with vascular tissue and that plants are insufficiently supplied with nutrients (George 1993). Successful rooting in other members of the Apiaceae family was also achieved with 1–5 μM IBA (Tavares et al. 2010a, Das et al. 2008, Jana and Shekhawat 2011, Coste et al. 2012, Jaheduzzaman et al. 2012), and with higher concentrations of IBA (Hossain et al. 2000, Sharma and Wakhlu 2001, Banerjee et al. 1999, Tavares et al. 2009–2010), with NAA or IAA (Karuppusamy et al. 2007, Jaheduzzaman et al. 2012) and only rarely without growth regulators (Makunga et al. 2003).

Acclimatization and transfer to in vivo conditions

Acclimatization of *H. pastinacifolia* required a specially prepared substrate. The substrates usually used for acclimatization with other members of that family (Sharma and Wakhlu 2001, Nath and Buragohain 2003, Tavares et al. 2010a, Coste et al. 2012, Hendrawati et al. 2012, Jaheduzzaman et al. 2012) were far different from the substrate which promoted acclimatization of our plant species. *H. pastinacifolia* did not acclimatize at all if the substrates were rich in organic matter (data not shown). That is why we tested two artificial substrates, which imitated the crumbling granulated texture and the alkaline nature of the substrate from the natural sites (Čušin 2004). We used two mixtures composed of limestone sand, potting soil and vermiculite in different ratios. Our plants survived better in the substrate with a more alkaline reaction, with a pH between 7.5 and 8.5. Nevertheless, less than half the shoots were successfully acclimatized. Plants of *H. pastiancifolia* obtained by this procedure are now growing in outside conditions, showing the potential of this method to increase the number of plants available for conservation purposes.

Do we need alternative ex situ conservation programs for alpine plants, especially endemic species like *H. pastinacifolia*? Alpine plants and their habitats are extremely fragile, easily disturbed and prone to any kind of climate change effects. During the past few decades, human activity has increased in the alpine environment, and disturbance is probably the greatest threat to alpine plants. Protected areas with specific conservation measures are an efficient way to protect Alpine biodiversity, but uncertainty still persists, owing to a range of different climate-change driven effects. More than half the species growing within the European Natura 2000 network might lose suitable habitats in these areas. Hence, it will be necessary to prepare alternative ex situ measures (Vittoz et al. 2013), of which the micropropagation presented in this paper is claimed to be the most efficient.

Conclusion

As an ancient paleoendemic and one of the most remarkable among the Pleistocene survivors, *H. pastinacifolia* is of great importance for understanding the effects of changing environment and deserves further research efforts in the direction of biodiversity studies and conservation programs (Šajna 2012). In our study we established a protocol for in vitro propagation of *Hladnikia pastinacifolia,* from preparation of plant material, establishment of aseptic culture, multiplication, in vitro rooting, transplanting and acclimatization of plants. Furthermore, tissue culture collection has been successfully propagated continuously since 2006. We have maintained it for 10 years without loss of either juvenile character or shoot and root proliferation capacity, the only exception being the sporadically observed flower development, which indicates the end of the life cycle for these plants. Since the rest of the material retained its juvenility, we can confirm that we did achieve the goal of having a long-term collection of *H. pastinacifolia* in tissue culture. In 2013, we additionally started a tissue culture of *H. pastinacifolia* and obtained several new lines. With these lines, we additionally tested the pre-designed micropropagation protocol, with repeated results.

The tissue culture of *H. pastinacifolia* represents a living collection or back-up gene pool of this species. New plants can be used for several purposes: reintroduction to nature, assisted migration, strengthening the natural population, for ex situ collections in botanical or rock gardens, for studies on the ecology and biology of species, for its nutritional value, or simply for educational and ornamental purposes. Further studies on this species will focus on other specific biotechnological tools successfully used in several programs of plant conservation, like assessment of the degree of genetic variability of plants in culture and introduction of techniques for long-term storage at low temperatures.

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References

Aeschimann, D., Lauber, K., Moser, D. M., Theurillat, J. P., 2004: Flora alpina: ein atlas sämtlicher 4500 gefäßpflanzen der Alpen, 1108–1109. Haupt, cop. Bern, Stuttgart, Wien.

Aeschimann, D., Rasolofo, N., Theurillat, J. P., 2011: Analyse de la flore des Alpes. 1: historique et biodiversité. Candollea 66, 27–55.

Bairu, M. W., Aremu, A. O., Van Staden, J., 2011: Somaclonal variation in plants: causes and detection methods. Plant Growth Regulation 63, 147–173.

Banerjee, S., Zehra, M., Kuma, S., 1999: In vitro multiplication of *Centrella asiatica*, a medical herb from leaf explants. Current Science 76, 147–148.

Baskin, C. C., Baskin, J. M., 2014: Seeds: ecology, biogeography, and evolution of dormancy and germination 2nd edn. Academic Press, Elsevier, Oxford.

Berthouzoz, M., Maendly, S., Bétrisey, S., Mangili, S., Prunier, P., Lexer, C., Kozlowski, G., 2013: Some like it cold: distribution, ecology and phylogeny of *Arenaria bernensis* Favarger (Caryophyllaceae) from the western Prealps in Switzerland. Alpine Botany 123, 65–75.

Cassells, A. C., 2012: Pathogen and biological contamination management in plant tissue culture: phytopathogens, vitro pathogens, and vitro pests. In: Loyola-Vargas, V. M., Ochoa-Alejo, N. (ed.) Plant cell culture protocols, 57–80. Springer Science+Business Media. New York.

Coste, A., Halmagy, A., Buticu-Keul, A. L., Deliu, C., Coldea, G., Hurdu, B. 2012: In vitro propagation and cryopreservation of Romanian endemic and rare *Hypericum* species. Plant Cell Tissue and Organ Culture 110, 213–226.

Council Directive, 1992: Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. Retrieved August 14, 2014 from http://ec.europa.eu/environment/nature/legislation/habitatsdirective/index_en.htm

Cruz-Cruz, C. A., González-Arnao, M. T., Engelmann, F., 2013: Biotechnology and conservation of plant biodiversity. Resources 2, 73–95.

Čušin, B., 2004: *Hladnikia pastinacifolia* Rchb. In: Čušin, B., (ed.) Natura 2000 in Slovenia – Plants, 107–113. ZRC SAZU, Ljubljana (In Slovenian).

Das, R., Hasan, M. F., Hossain, M. S., Rahman, M., 2008: Micropropagation of *Centrella asiatica* L. an important medicinal herb. Progressive Agriculture 19, 51–56.

Dawson, T. P., Jackson, S. T., House, J. I., Prentice, I. C., Mace, G. M., 2011: Beyond predictions: biodiversity conservation in a changing climate. Science 332, 53–58.

Fay, M. F., 1992: Conservation of rare and endangered plants using in vitro methods. In Vitro Cellular & Developmental Biology – Plant 28, 1–4.

Gaddaguti, V., Reddy, K. S., Deepthi, R. S., Lavanya, K., Rama, C., Talluri, V. R., Allu, P. R., 2013: In vitro flower induction and multiple shoot regeneration studies in *Centella asiatica* from nodal and leaf explants. Annals of Plant Sciences 2, 55–58.

Gaston, K. J., Kunin, W. E., 1997: What is rarity? In: Kunin, W. E., Gaston, K. J., (ed.) The biology of rarity, causes and consequences of rare-common differences, 30–47. Chapman and Hall, London.

George, E. F., 1993: Plant propagation by tissue culture; Part 2: in practice, 2nd edn. Exegetics, Edington.

González-Benito, M, E., Martín, C., 2011: In vitro preservation of spanish biodiversity. In Vitro Cellular and Developmental Biology – Plant 47, 46–54.

Hendrawati, O., Hille, J., Woerdenbag, H. J., Quax, W J, Kayser, O., 2012: In vitro regeneration of wild chervil (*Anthriscus sylvestris* L.). In Vitro Cellular and Developmental Biology – Plant 48, 355–361.

Hossain, S. N., Rahman, S., Joydhar, A., Islam, S., Hossain, M., 2000: In vitro propagation of thankuni (*Centella asiatica* L.). Plant Tissue Culture and Biotechnology 10, 17–23.

Jaheduzzaman, M. D., Habib, M. A., Akter, S., Banu, N. A., Rahman, R. B., Banu, T. A., Khan, S., Islam, S., 2012: In vitro regeneration of an important medicinal plant *Centella asiatica* L. Urban. Bangladesh Journal of Scientific and Industrial Research 47, 269–272.

Jana, S., Shekhawat, G. S., 2011: Plant growth regulators, adenine sulfate and carbohydrates regulate organogenesis and in vitro flowering of *Anethum graveolens*. Acta Physiologiae Plantarum 33, 305–311.

Kapai, V. Y., Kapoor, P., Rao, I. U., 2010: In vitro propagation for conservation of rare and threatened plants of India–a review. International Journal of Biological Technology 1, 1–14.

Karuppusamy, S., Aruna, V., Kiranmai, C., Pullaiah, T., 2007: In vitro propagation of an endemic umbellifer, *Hydrocotyle conferta*. Indian Journal of Biotechnology 6, 541–544.

Lavergne, S., Mouquet, N., Thuiller, W., Ronce, O., 2010: Biodiversity and climate change: Integrating evolutionary and ecological responses of species and communities. Annual Review of Ecology, Evolution, and Systematics 41, 321–350.

Makunga, N. P., Jäger, K. G., Van Staden, J., 2003: Micropropagation of *Thapsia garganica*-a medicinal plant. Plant Cell Reports 21, 967–973.

Mayer, E., 1960: Endemic flowering plants in the southeastern limestone Alps, their foothills and transitional Illyrian territory. In: Lazar, J. (ed.) Proceedings on the 150th anniversary of the Ljubljana Botanical Garden. Univerza v Ljubljani, Ljubljana (in Slovenian).

Mondoni, A., Probert, R. J., Rossi, G., Vegini, E., Hay, F. R., 2011: Seeds of alpine plants are short lived: implications for long-term conservation. Annals of Botany 107, 171–179.

Murashige, T., Skoog, F., 1962: A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15, 473–497.

Nath, S., Buragohain, A. K., 2003. In vitro method for propagation of *Centrella asiatica* (L) Urban by shoot tip culture. Journal of Plant Biochemistry and Biotechnology 12, 167–169.

Official Gazette of the Republic of Slovenia, 2002: Official Gazette of the Republic of Slovenia, 82/02. Retrieved September 24, 2013 from http://www.uradni-list.si/1/objava.jsp?urlurid=20024055

Official Gazette of the Republic of Slovenia, 2010: Official Gazette of the Republic of Slovenia 42/10. Retrieved May 28, 2014 from http://www.uradnili-st.si/1/objava.jsp?urlurid=20102122

Pence, V. C., 2010: The possibilities and challenges of *in vitro* methods for plant conservation. Kew Bulletin 65, 593–547.

Pence, V. C., 2011: Evaluating costs for the *in vitro* propagation and preservation of endangered plants. In Vitro Cellular and Developmental Biology – Plant 47, 176–187.

Pierik, R. L. M., 1991: Commercial aspects of micropropagation. In: Prakash, J., Pierik, R. L. M., (ed.) Horticulture – new technologies and applications, 141–153. Kluwer Academic Publishers, Dordrecht.

Rao, N. K., 2004: Plant genetic resources: Advancing conservation and use through biotechnology. African Journal of Biotechnology 3, 136–145.

Sarasan, V., Cripps, R., Ramsay, M. M., Atherton, C., Mcmichen, M., Prendergast, G., Rowntree, J. K., 2006: Conservation in vitro of threatened plants – Progress in the past decade. In Vitro Cellular and Developmental Biology – Plant 42, 206–214.

Sharma, R. K., Wakhlu, A. K., 2001: Adventitious shoot regeneration from petiole explants of *Heracleum candicans* Wall. In Vitro Cellular and Developmental Biology – Plant 37, 794–797.

Sharma, R. K., Wakhlu, A. K., 2003: Regeneration of *Heracleum candicans* Wall plants from callus cultures through organogenesis. Journal of Plant Biochemistry and Biotechnology 12, 71–72.

Sharma, R. K., Wakhlu, A. K., Bolaria, M., 2004: Micropropagation of *Anethum graveolens* L. through axillary shoot proliferation. Journal of Plant Biochemistry and Biotechnology 13, 157–159.

Šajna, N., Kušar, P., Slana-Novak, L., Novak, T., 2009: Notes on thermo- and hygropreference in *Leiobunum roseum* C. L. Koch, 1839 (Opiliones: Sclerosomatidae) in a habitat of *Hladnikia pastinacifolia* Reichenbach, 1831 (Spermatophyta: Apiaceae). Contributions to Natural History 12, 1111–1123.

Šajna, N., Kušar, P., Slana-Novak, L., Novak, T., 2011: Benefits of low-intensive grazing: co-occurrence of umbelliferous plant (*Hladnikia pastinacifolia* Rchb.) and opilionid species (*Phalangium opilio* L) in dry, calcareous grassland. Polish Journal of Ecology 59, 777–786.

Šajna, N., Kavar, T., Šuštar-Vozlič, J., Kaligarič, M., 2012: Population genetics of the narrow endemic *Hladnikia pastinacifolia* Rchb. (Apiaceae) indicates survival in situ during the Pleistocene. Acta Biologica Cracoviensia Series Botanica 54, 84–96.

Šajna, N., Šuštar-Vozlič, J., Kaligarič, M., 2014: New insight into the anatomy of an endemic *Hladnikia pastinacifoia* Rchb. Acta Biologica Cracoviensia Series Botanica 73, 19–28.

Tavares, A. C. P., Salgueiro, L., Canhoto, J., 2009–2010: In vitro conservation of Angelica pachycarpa, an Iberian endemic Apiaceae of the Portuguese Berlanga Island. Revista del Jardin Botanico Nacional 30–31, 109–111.

Tavares, A. C., Salgueiro, A. L., Canhoto, J., 2010a: In vitro propagation of the wild carrot *Daucus carota* L. subsp. *halophilus* (Brot.) A. Pujadas for conservation purposes. In Vitro Cellular and Developmental Biology – Plant 46, 47–56.

Tavares, A. C., Salgueiro, A. L., Canhoto, J., Paiva, J., 2010b: Iberian endemic Apiaceae: A reassessment for conservation purposes in Portugal. Studia Botanica 29, 13–37.

Vittoz, P., Cherix, D., Gonseth, Y., Lubini, V., Maggini, R., Zbinden, N., Zumbach, S., 2013: Climate change impacts on biodiversity in Switzerland: A review. Journal for Nature Conservation 21, 154–162.

Wraber, T., 2009: The alpine flora. In: Bizjak, J. [texts], Lenarčič, M. [photos]: The Alps: a bird's-eye view, 479–481. PanAlp, Nazarje.