Propagation in vitro and ex situ cultivation of Woodsia alpina (Bolton) S.F. Gray

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ABSTRACT: Woodsia alpina is a rare fern with circumpolar distribution. The species is protected or endangered in many countries. In Poland, it is known from the Sudetes Mts. (1 location) and the Tatra Mts. (3 locations). The reproduction of this plant by spores is difficult and thus there is no method of cultivation available. An in vitro tissue culture technique is promising in propagation of many recalcitrant and demanding species. This technique was used to determine the propagation method on purpose to maintain the Sudeten population of Woodsia alpina in botanical collections and in the supplementary habitat in the Karkonoski National Park. Spores collected in the glacial circus Mały Śnieżny Kocioł were sown in vitro and germinated during 3–9 months. Prothalli cultured in vitro spread vegetatively in various manners. Gametangia were formed on prothalli in different developmental stages. On heart-shaped prothalli archegonia and occasionally antheridia were formed, whereas on filamentous ones only antheridia occurred. Sporophytes were hardly ever produced with single sporophytes present per thousands of gametophytes. At apical meristems of the primarily formed sporophytes, green globular bodies (GGB) proliferated. These bodies could further regenerate forming secondary sporophytes, which developed nearby the initial ones. Sporophytes at this stage of development often became brown and lost fronds, however many of them renewed their growth after the dormancy period. Plants, which were transferred in spring to a greenhouse and planted in a commercial mix for ferns or neutralized peat substratum, acclimated in 70%. The growth cycle of W. alpina cultivated at the altitude of 120 m a.s.l. in the Botanical Garden of the University of Wroclaw was short and fronds were lost.
in the middle of August. Next year not all rhizomes initiated the growth; only the biggest ones displayed this ability. Plants cultivated in the supplementary habitat in the Jagniśtków nursery of the Karkonoski National Park at the altitude of 650 m a.s.l. performed perfectly well.

ABSTRAKT: Woodsia alpina jest rzadką paprocią o rozmieszczeniu wokółbiegunowym. Gatunek ten w wielu krajach uznawany jest za zagrożony i ginący, w Polsce zaś występuje w Sudetach (1 stanowisko) i w Tatrach (3 stanowiska). Reprodukcja tej rośliny przez zarodniki jest trudna, a żadna metoda uprawy nie jest obecnie dostępna. Kultura in vitro jest obiecująca techniką w rozmnażaniu wielu opornych i trudnych gatunków roślin. Została ona zastosowana w opracowaniu metody rozmnażania i zabezpieczenia sudeckiej populacji Woodsia alpina w kolekcji ogrodu botanicznego i na stanowisku zastępczym w Karkonoskim Parku Narodowym. Zarodniki zebrane z Małego Śnieżnego Kotła wysiane in vitro kiełkowały podczas 3–9 miesięcy. Uprawiane przedroślą rozrastały się wegetatywnie kilkoma sposobami. Gametangia powstawały na przedroślach znajdujących się w różnych stadiach rozwoju. Na gametofitach sercowatych różnicowały się rodnie i plemnie, a na nitkowatych jedynie plemnie. Sporofity powstawały bardzo rzadko; na tysiące gametofitów rozwijały się zaledwie pojedyncze sporofity. Na inicjalnie utworzonych sporofitach z merystem wierzchołkowego proliferowały zielone globularne ciała (GGB). Ciała te mogły wytwarzać następne sporofity, które rozwijały się w pobliżu inicjalnego. Powstały sporofity w tym stadium rozwoju często brązowiły i traciły liście, chociaż wiele z nich odnawiało wzrost po okresie spoczynku. Rośliny wysadzone wiosną do szklarni w handlową mieszankę ziemi dla paproci lub zneutralizowany substrat torfowy aklimatyzowały się w 70%. Okres wzrostu W. alpina we Wrocławiu, na wysokości 120 m n.p.m. był krótki, a rośliny zrzucały liście w połowie sierpnia. Następnego roku nie wszystkie klązba inicjowały wzrost; zdolność tę posiadały tylko te największe. Rośliny uprawiane na stanowisku zastępczym w Gospodarstwie Szkółkarskim Karkonoskiego Parku Narodowego w Jagniśtkowie, na wysokości 650 m n.p.m., rosły i rozwijały się bardzo dobrze.

KEY WORDS: Woodsia alpina, in vitro cultures, endangered ferns
threatened are endemic plants with a limited number of specimens in their populations. From the botanical point of view, particularly interesting are uncommon and rare species which significantly contribute to the local (Nilsson et al. 1988) and regional (Gentry 1986) species biodiversity. Such species are extremely endangered due to the small population size and restricted distribution. Detailed information on the biology of species and a knowledge of the factors essential for their growth make it possible to define conservation priorities for plant taxa.

Pteridophytes are an ancient group of plants with a large number of relict and endemic species. As evolutionary old plants they represent a specific biology, which determines on their weakness and strength and their ability to colonize new habitats. The limitations are identified as the handicap of an independent gametophyte stage, a single initial cell in the growing-points of sporophytes, the slow plant growth rate, intolerance to fluctuating environmental conditions, poorly controlled evaporative potential, uncontrolled high reproductive commitment and dependence on water to breed (Page 2002).

Woodsia alpina is a rare or endangered plant in several countries, including Canada, the USA, Great Britain, and Spain. This species is listed by the U.S. federal government and the state governments of Main and Michigan as threatened, and of New York and Vermont as endangered (Goff et al. 1982; USDA Natural Resources Conservation Service).

Woodsia alpina – an alpine cliff fern, alpine woodsia or northern woodsia is a species with a circumpolar distribution, which includes Europe, Asia, Greenland and North America, and the Arctic Islands. In Europe it occurs in the Pyrenees, the Apennines, the Alps, the Sudetes Mts. and the Carpathians. The alpine cliff fern is an arctic-alpine glacial relict. It grows in Poland in single location in the Sudetes Mts. and the Carpathians. In the Sudetes Mts. it is known only from one location in a glacial circus Małý Śnieżny Kocioł (Karkonosze Mts.). The other reported locations in this mountain range, at which woodsia however became extinct, were in the southern part of the Karkonosze Mts. and Pradziad Massive in the Czech Republic. In the Tatra Mts. the plant is known from 3 locations (Fabiszewski, Piękoś-Mirkowa 2001; Piękoś-Mirkowa, Delimat 2002).

The plant is a small, rock loving fern typically growing in dense, upright clumps. Its leaves are linear to narrowly oblong, reaching 10–15 cm in length, singly pinnate; pinnae are ovate and obtuse, pinnately lobed, smooth above, hairy but not scaly. W. alpina grows in clumps that usually have several crowns (Brown 1964; http://hardyfernlibrary.com). It has a short erect rhizome, compact and stiff; fronds usually are shorter than 10 cm. It sporulates in summer and in early autumn.
The species is included in the Polish Red Data List (Zarzycki, Szeląg 2006) and according to the Polish Red Book is classified as critically endangered (Fabiszewski, Piękóś-Mirkowa 2001).

Isozyme studies confirm the longstanding hypothesis that *W. alpina* is an allotetraploid derived from hybridization between *W. glabella* and *W. ilvensis*. A considerable disagreement exists concerning the chromosome number of *W. alpina* but 2n=160 seems to be the most likely, given the numbers reported for the two parental species. Additionally, hybrids between *W. alpina* and *W. ilvensis* have been reported from both Europe and North America. These morphologically intermediate triploids with malformed spores have been named *W. × gracillis* (Lawson) Butters (Wagner 1987; Windham 1987).

*Woodsia alpina* typically occurs in crevices, steep slopes and cliffs, ridges containing slate and calcareous rocks, especially limestone of pH 5.1 to 7.8. Occasionally it grows on slopes covered mostly by grained rocks. The plant colonizes substrates with a low organic content. The only location in the Mały Śnieżny Kocioł glacial circus is unusual habitat for this plant, because there it grows on a basaltic body of sub-volcanic intrusion (Zagożdżon, Zagożdżon 2006). Basalts differ from other crystalloid rocks in respect of their acidity and belong to rocks richer in base, which explains why *W. alpina* can grow there.

The reproduction of this plant by spores is difficult and there is no method of cultivation available. An *in vitro* tissue culture technique is promising in propagation of many demanding and resistant species. It was used to determine the propagation method on purpose to preserve the Sudeten population of *Woodsia alpina* in botanical collections and in the supplementary habitat in the Karkonoski National Park.

The best method of the species protection is *in situ* conservation based on preserving of natural habitats. Particular species, such as *Woodsia alpina*, require *ex situ* conservation either by multiplication by conventional methods or by *in vitro* tissue culture or spore culture technique. Such multiplied plants may be protected and maintained in the botanical gardens or in chosen supplementary habitats.

1. Material and methods

Several leaf blades of *Woodsia alpina* with sori were collected at the beginning of July (10.07.2003) and August (6.08.2004) from the only natural locality in Lower Silesia in the Mały Śnieżny Kocioł glacial circus. Collected fragments of sporophylls were dried out between sheets of paper at room temperature. The obtained spores were packed in small packages of blotting-paper, and after superficial sterilization sown on culture media. Spores were sown at different period of time according to the collection date (13, 42, 76, 88, 119 days).
In sterilization procedure packages were immersed in 70% alcohol for 3 minutes and followed by shaking for 5 or 10 min in a solution of 3% sodium hypochlorite. In next year (2005), spores from four plants were sterilized by immersion in 70% alcohol for 30 s followed by treatment in 5–8% sodium hypochlorite for 5–12 min.

Spores were sown on 1/2 or 1/4 of MS (Murashige, Skoog 1962) medium supplemented with vitamins, glycine (2.0 mg•l⁻¹), sucrose (30 g•l⁻¹) and agar (8 g•l⁻¹). The pH of media was adjusted to 6.8 with 1M KOH or HCl prior to autoclaving for 15 min at 121°C. Flasks with spores were incubated in different temperatures and light regimes: 25°C and 16-h light photoperiod at the irradiance of 14.2 μmol•m²•s⁻¹; 10°C at the irradiance of 0.15 μmol•m²•s⁻¹. The influence of sucrose concentration on the growth of gametophytes and sporophytes was also analysed.

Prothalli were subcultured in a three-month interval; newly formed sporophytes were separated and cultured on 1/2 MS medium.

Older sporophytes of various sizes were planted in a greenhouse in a neutralized peat soil or a commercial mix for ferns (Krone, Floro-hum). Pots were covered with polyethylene foil, which was gradually removed. Five weeks later, the potted plants were transferred to a hotbed and the growth of ferns was observed in the following years. Two supplementary habitats were created, one in the Botanical Garden of the University of Wrocław and the second, bigger one, in the Jagniątków nursery of the Karkonoski National Park.

2. Results and discussion

Sterilized spores of W. alpina coming from the first trial in 2003 and sown on 1/2 MS medium were infected in a low percent (13.6%).

In the sowing experiment in 2004, 29% of contaminations were noted. To prevent infections it is necessary to separate carefully spores from debris before sterilization.

The germination rate was very low, and only single prothalli emerged after 7 months in culture. In the second experiment, spores of four individual plants collected in the natural location were sown separately on medium. The best-germinated spores came from the plant signed with the number 4, weaker from the plant numbered as the first, and spores of a specimen with the number 3 did not germinate at all. The differences in the ability to form prothalli are probably connected with the degree of the spore maturity. Spores of the specimen No. 4 were much darker brown than the others, which means that they were fully ripe, however only 6% of them germinated.
Fern spores usually persist in a viable, yet metabolically inactive state for a long period of time. The sequence of sowing 13, 42, 76, 88, 119 days after collecting did not answer our question of the effective time of germination, because of a generally very low level of spore germination.

The ability to germinate persisted from 1.5 to 9 months for *W. alpina* spores cultured *in vitro*. The developmental block, resulting in the failure of perfectly viable spores to germinate, even when they are in conditions that promote germination, points to their dormancy. Spores of several ferns including those of Hymenophyllaceae (Atkinson 1960) and Grammitidaceae (Stokey, Atkinson 1958) hardly ever exhibit any dormancy, whereas others like the members of Schizaceae and Adiantaceae are definitely dormant. Apart from the primary requirement to hydrate the spores prior to germination, in many cases the mechanisms triggering their germination are not understood (Raghavan 1989).

Performed experiments showed a very low germination rate of *W. alpina* spores in comparison to *W. ilvensis* sterilized by the same method (unpublished data). Also in natural conditions, the germination of these two species is different and much more difficult in case of *W. alpina*, what can significantly limit the spreading of this species populations.

Germinating spores form two cells – rhizoid and protonema initials. The spore germination in *W. alpina* is according to the Vittaria-type pattern (Brown 1964), and prothallial development is similar to Drynaria-type (Nayar, Kaur 1971). This means that germinating spores develop the primary rhizoid and protallus initials based on the fixed sequence of cell divisions and the direction of growth. According to literature mentioned above, germination starts from the unequal cell division, perpendicular to the spore polar axis. A primary rhizoid is formed from a smaller cell, whereas a prothallus initial from a bigger one at the opposite side. The second division of protonema initials is perpendicular to the first one.

*W. alpina* spores cultured *in vitro* formed filaments. The meristematic activity of the apical cell of a filament gives rise to adult prothalli. The characteristic feature of prothallus formation in *Woodsia alpina* is the variability in the process of development. In the next step, a broad plate is formed, which consists of the meristem located at the bottom of the notch, and a thick, meridian midrib surrounded by broad, one-cell-thick wings on its either side. The adult prothallus, after 3–4 months of culture, has a cordate-thalloid shape with doubled curved wings and hairs on the surface (1–5 mm). The morphology of prothalli depends on the density of their growth and vary from cordate to more elongated structures in loose and compact groups on the medium, respectively (Fig. 1).

Gametophytes spontaneously propagated in culture at least in two ways: from filaments and in the adult stage of growth. The filament has an unlimited ability to branch out. The cordate-like prothalli also regenerate the progeny
usually as outgrowths of one of the wings. In this way, gametophytes prolong their growth. In the older regions of adult prothalli, progressive degeneration takes place and this part forms additional numerous filamentous protonema and extra branches of the independent prothallus.

The adult prothallus forms in the middle an elongated cushion, which bears the sexual organs, i.e. archegonia, and rhizoids on the ventral surface (Fig. 2). Antheridia in *W. alpina* are formed in the posterior half of the thallus (Fig. 3), while archegonia occupy marginal regions in the anterior region. The archegonia are visible only in the adult heart-shaped gametophytes, mainly on the midrib, whereas antheridia arise rather occasionally in adult thalli, mostly in filaments (Fig. 4). During 3 months of culture, the flasks were filled with gametophytes, which only rarely formed sporophytes (Fig. 5). In one flask, a limited number of sporophytes occurred. In spite of a big number of prothalli, the amount of sporophytes born was extremely low. Usually only 1–3 sporophytes were noticed in one flask with thousands of gametophytes. In some cases, from one clump of prothalli several sporophytes arose.

Prothalli after 2 months of culture comprised of elongated heart-shaped structures and filaments, which entwined and covered them. This type of expansion, where heart and filamentous prothalli were close together, created favorable conditions for fertilization. The examination of such clumps of *W. alpina* revealed that initially formed sporophytes decayed but from an apical meristem, green globular bodies (GGB) proliferated similarly to those described by Fernández and Revilla (2003). These bodies could further regenerate into new sporophytes in a vegetative way of propagation. The phenomenon of regeneration may explain why in one clump of prothalli the new sporophytes arise more numerous close to the initial, first formed sporophyte (Fig. 5).

Sporophytes left on the same medium become brown and lost fronds after next 3 months of culture. They could renew their growth after separation from gametophytes. Rhizomes transferred to a fresh medium may renew gametophyte-like growth as a result of proliferation of growing centers (Fig. 6). The reason of this phenomenon is unknown. It can be a natural rest time of the plant or it can be forced by leaf necroses induced by unfavourable growth conditions and insufficient nutrition. Generally, it is almost impossible to separate two *W. alpina* generations, gametophytes and sporophytes. Probably, quick growing gametophytes cover the sporophytes and impede the uptake of nutrition. Fernández and Revilla (2003) stated that gametophytes of *Dryopteris affinis* sp. *affinis* cultured on MS media and supplemented with growth regulators for one month, produced sporophytes from the apical notch strictly in an apogamic way, since sexual reproduction was not possible due to a lack of archegonia. However, facultative apogamy is possible but not obligatory in this species. The developing sporophytes were of the same size as those found in natural
environments, what may indicate their diploid character. During cultivation in vitro the growth regulators were excluded. It should in some way prevent the unwanted proliferation of asexual sporophytes. Apogamy is triggered in the callus and gametophytes growing on the medium containing 2,4-D, high doses of sucrose and ethylene (Raghavan 1989). To determine the sexual or asexual origin of sporophytes, the content of DNA in the prothalli and in sporophytes should be estimated.

The life cycle of ferns comprising of two generations, haploid gametophytes and diploid sporophytes, requires creation of specific conditions in culture in vitro for the proper development of gametophytes and formation of an increased number of embryos.

In spring, specimens of W. alpina propagated in vitro were planted in a greenhouse (Fig. 7) in a commercial mix for ferns or in a neutralized peat substratum. Transplanted micropropagules were covered with polyethylene foil or with inverted glass beakers inside the growth chamber. After developing new fronds, ferns were transferred to a hotbed initially covered with a shaded glass and later with a green plastic net. Propagules survived in about 70% after the weaning process (Fig. 8). The species had fronds, which did not persist throughout the winter. The growth cycle of W. alpina was rather short in our altitude (the Botanical Garden of the University of Wroclaw) and fronds became brown and lost in the middle of August. Next year not all the plants renewed the growth. Probably, specimens that did not form the rhizomes big enough during the previous season hardly ever renewed their growth in the following season (Fig. 9). According to literature, the species endures temperatures up to -40°C in winter but requires a cold summer. At our altitude it may be too warm in summer, because the comparison of plants in the Botanical Garden (120 m a.s.l.) and at the supplementary locality at the Jagniątków nursery (650 m a.s.l.) showed the better state of Woodsia alpina plants in the second location.
Fig. 5. *In vitro* culture of *W. alpina* gametophytes with formed sporophytes on 1/2 MS medium (phot. D. Poturała)

Ryc. 5. *Kultura in vitro* gametofitów *W. alpina* z powstającymi sporofitami na pożywce 1/2 MS (fot. D. Poturała)

Fig. 6. Sporophytes developing from resting rhizomes of *W. alpina* after 11 months in culture (phot. D. Poturała)

Ryc. 6. Sporofity rozwijające się ze spoczynkowych kłęby *W. alpina* po 11 miesiącach uprawy (fot. D. Poturała)
3. Conclusion

To our knowledge, this is the first paper describing the possibility of propagation of *Woodsia alpina* in tissue culture. Since the species can be reproduced only by spores and this propagation is difficult, the presented here method is the only available way of preservation and preventing the extinction of the small populations of this species.
Fig. 8. Plants of *W. alpina* in the first year of cultivation (phot. D. Poturala)
Ryc. 8. Rośliny *W. alpina* w pierwszym roku uprawy (fot. D. Poturala)

Fig. 9. Alpine cliff fern in a hotbed in the second year of cultivation (phot. D. Poturala)
Ryc. 9. Rozrzutka alpejska w inspekcji w drugim roku uprawy (fot. D. Poturala)
References


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