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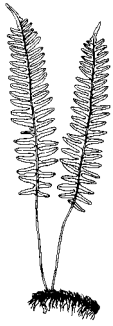
# *IN VITRO* PROPAGATION AND *EX SITU* PRESERVATION OF ENDANGERED FERNS FROM LOWER SILESIA

KRYSTYNA KROMER, JOWITA MARSZAŁ-JAGACKA, KATARZYNA KEMPIŃSKA, TOMASZ NOWAK, LUDWIK ŻOŁNIERZ, DOROTA POTURAŁA, KRZYSZTOF ŚWIERKOSZ



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**Abstract.** *In vitro* propagation and *ex situ* preservation was studied in two serpentine ferns, *Asplenium adulterinum and A. cuneifolium,* as well as species not characteristic of serpentine rock but occurring on it: *Asplenium septentrionale, Polypodium vulgare,* and two species from *Woodsia* genus, *Woodsia ilvensis* and *W. alpina.*

The growth and development of six fern species on modified 1/2 MS gave the best results in terms of fresh weight of gametophytes, which had typical shape and formed many primary and secondary gametophytes. When the sucrose concentration in the medium was between 20 and 30 g $l^{-1}$ the fresh weight of *Asplenium adulterinum* and *Polypodium vulgare* gametophytes was higher. Decreasing the sucrose concentration (0.5 and 10 g $l^{-1}$) reduced the fresh weight and size of gametophytes. A high sucrose dose (40 g $l^{-1}$) diminished the growth of gametophytes without causing discoloration or decay. The influence of glucose, fructose, mannose, sucrose, maltose and lactose at 0.087 M concentration on the growth and development of the gametophytes was studied. Every fern species had the highest fresh mass increase and the largest gametophytes on medium with the addition of sucrose. Fructose turned out to be the most toxic.

It was shown that *in vitro* cultures are an effective method for maintaining a prothallia gene bank of serpentine fern species. The best medium for long-term storage of *Asplenium adulterinum* and *A. cuneifolium* prothallia was 1/2 MS medium without phytohormones, and low temperature of +8°C, under cool white fluorescent light at intensity of 0.15 umol $m^{-2}$ $s^{-1}$.

Not many sporophytes were produced in cultures of gametophytes of the six studied species. Fertilization was rare; 3-10 sporophytes per flask formed after 2 months of culture. The spo-

rophytes were separated from the gametophytes and propagated on 1/2 MS medium supplemented with KIN and NAA. The developing clumps with fronds were rooted and hardened on liquid medium with perlite. All ferns were successfully acclimated in a greenhouse and planted outside. They formed sporangia after 2-3 years.

**Key words:** *Asplenium adulterinum, A. cuneifolium, A. septentrionale, Polypodium vulgare, Woodsia ilvensis, W. alpina,* gametophytes, *in vitro* culture, long-term storage, sporophyte propagation.

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## INTRODUCTION

Ferns make up a heterogeneous group; the majority represent high polyploids as well as hybrid forms, created as a result of interspecific and intergeneric crosses (Zenkteler 2000). In Poland *ca* 44% of fern species are threatened (Zarzycki, Szeląg 2006). Among the endangered species are two serpentine ferns, *Asplenium adulterinum* and *A. cuneifolium* (Swierkosz 1992; Fabiszewski 1993; Żołnierz 1993; Kaźmierczakowa, Zarzycki 2001; Swierkosz, Szczęśniak 2003) as well as *Woodsia ilvensis* and *W alpina*. They occur in isolated populations containing few individuals, which sooner or later may also disappear (Holderegger, Schneller 1994; Schneller, Holderegger 1996). Many localities of other fern species such as *Asplenium septentrionale* and *Polypodium vulgare* are also disappearing.

*Asplenium adulterinum* Milde and *A. cuneifolium* Viv. occur in scattered and isolated localities in Lower Silesia. They grow in clusters in crevices of serpentine rock. The substrate is characterized by an exceptionally low calcium/magnesium ratio, low content of N, P and K, high content of Ni, Co and Cr, and high pH of 7.2 (Gams 1938; Sarosiek, Sadowska 1961; Proctor, Woodell 1975; Roberts, Proctor 1992).

Two other studied species of the *Woodsiaceae* family - *Woodsia ilvensis* (L.) R. Br. and *Woodsia alpina* (Bolton) S. F. Gray - are light-demanding plants with a circumpolar range, growing in rock crevice vegetation of the order *Androsacetalia vandellii*. *Woodsia ilvensis* grows in Poland in one locality in the Gorce Mts on andésites devoid of calcium carbonate. The alpine cliff fern *Woodsia alpina* grows in the Sudety Mts on basalts and in the Tatras on Triassic dolomites and

granites. *Asplenium septentrionale* is a mountain species occurring mainly in the Carpathians and the Sudety Mts, and rarely in lowland. In Poland, *Polypodium vulgare* is a fairly frequent, legally protected species.

One *ex situ* method of preserving threatened plant species is *in vitro* culture of isolated tissues or cells. *In vitro* culture is a very good way to propagate many recalcitrant species. Tissue culture can help in understanding the plant's nutritional requirements and life cycle.

The aim of the study was to elaborate an *in vitro* culture method for six fern species *{Asplenium adulterinum, A. cuneifolium, A. septentrionale, Polypodium vulgare, Woodsia alpina, W. ilvensis)* using spores gathered mostly from natural habitats.

## MATERIAL AND METHODS

### CULTURE OF GAMETOPHYTES

Spores of the studied fern species were collected mainly from sporophytes growing in natural habitats: *Asplenium cuneifolium* from Książnica and Przemiłów, *A. adulterinum* from Książnica, *A. septentrionale* and *Polypodium vulgare* from Osola, and *Woodsia alpina* from Śnieżne Kotły, all localities in Poland. Spores of *Woodsia ilvensis* were collected from plants preserved in the Botanical Garden of University of Wrocław.

A two-step method of disinfection was used. After trials of disinfection first in 70% ethanol for 3, 5, 7 and 10 min, and then the second step of disinfection in 0.1% NaCIO for 3, 5 and 10 min, the method of disinfection in 70% ethanol for 3 min and then 5 min in 0.1% NaCIO was chosen.

Single leaves of ferns with developed sporangia were collected in paper envelopes and left to dry at room temperature for 7-10 days. Next the spores pouring out from the sporangia were packed in paper filter bags, soaked in disinfecting solutions, and washed three times with sterile distilled water. Aseptic spores were sown in 100 ml flasks containing 35 ml solid medium. The medium for spore germination was 1/4 MS (Murashige, Skoog 1962) supplemented with 30 g $l^{-1}$ sucrose and solidified with 8 g $l^{-1}$ agar. Media pH was adjusted to 6.2 before sterilization (1 atm, 121°C, 18 min). With the sown spores 2 ml sterile *distilled* water was added to the flask to increase germination. All cultures were incubated at 18-20°C under a 16 h photoperiod (cool white fluorescent light, 14.2 jumol $m^{-2}$ $s^{-1}$).

The growth and development of the gametophytes of four fern species were compared in the experiments. The prothallia populations were cultured in full-strength, 1/2, 1/4 or 1/8 MS with 6.8 g $l^{-1}$ agar, 20 g $l^{-1}$ sucrose, pH 6.8-7.0, and on own modified medium with the proportions of macroelements altered ($NH_4NO_3$ - 825 mg $l^{-1}$; $KNO_3$ - 950 mg $l^{-1}$; $CaCl_2 \times 2\,H_2O$ - 110 mg $l^{-1}$; $MgSO_4 \times 2\,H_2O$ - 370 mg $l^{-1}$; $KH_2PO_4$ - 42.5 mg $l^{-1}$) and half a portion of MS glycine.

The influence of sucrose dose (0, 5, 10, 20, 30 or 40 g $l^{-1}$) on the growth and development of the gametophytes was analyzed for *Asplenium adulterinum, A. septentrionale* and *Polypodium vulgare.* The percentage of decaying gametophytes was determined after five weeks of culture. After another five weeks the fresh weight and number of prothallia were measured.

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The influence of several sugars (glucose, fructose, mannose, sucrose, maltose, lactose) at 0.087 M concentration on gametophyte growth and development was studied. The osmotic pressure of all media was kept constant.

Further experiments with gametophytes employed 1/2 MS medium supplemented with 30 g $l^{-1}$ sucrose, solidified with 8 g $l^{-1}$ agar (pH 6.2). Ten single heart-shaped gametophytes of *Asplenium adulterinum, A. cuneifolium, A. septentrionale* and *Polypodium vulgare* were transferred to 100 ml flasks containing 35 ml medium. Each experimental treatment was done in 40 replicates. Cultures were kept at 18-20°C under a 16 h photoperiod (cool white fluorescent light, 14.2 umol $m^{-2}s^{-1}$).

To create a gene bank the prothallia of *Asplenium adulterinum* and *Asplenium cuneifolium* were stored for one year at +2°C. Single heart-shaped gametophytes were transferred to 50 ml flasks containing 20 ml medium. Five gametophytes per flask (twenty flasks per treatment in all experiments) were cultured on 1/2 MS medium supplemented with KIN (0.8 mg $l^{-1}$), IAA (0.1 mg $l^{-1}$) and $GA_3$ (1.0 mg $l^{-1}$). The cultures were stored for a year at +2°C under continuous darkness, and at +8°C under a 16 h photoperiod (cool white fluorescent light, 0.15 umol $m^{-2}s^{-1}$). After twelve months the prothallia were subcultured on fresh 1/2 MS medium, and the survival rates and sizes of gametophytes of the *Asplenium adulterinum* and A *cuneifolium* were evaluated.

### CULTURE OF SPOROPHYTES

Sporophytes 2-3 cm high were separated from gametophytes and subcultured on 1/2 MS medium. Propagated sporophytes were divided and cultured on 1/2 MS medium enriched with various doses of KIN and 0.1 mg $l^{-1}$ NAA.

### ACCLIMATIZATION OF PLANTS BEFORE TRANSFER TO GREENHOUSE

Before planting, ferns were transferred from agar medium to liquid medium with perlite, where the sporophytes developed roots, and next for the last 7 days of culture placed under light irradiance of 34.6 umol $m^{-2}$ $s^{-1}$. Then the flasks were placed in a greenhouse and after 5 days the aluminum foil covers were taken off and 3 days later the plants were potted into soil.

### PLANTING OF FERNS IN GREENHOUSE

Survival of sporophytes of *Asplenium adulterinum, A. cuneifolium, A. septentrionale, Polypodium vulgare, Woodsia alpina* and *W. ilvensis* was tested on various gardening soils: serpentine soil, pH 7.2 (from natural localities); Floro-hum commercial mix for ferns, pH 5.0; mixture of peat, leaf mould and sand (3:2:1); and Kronen commercial mix for ferns, at pH 6.5 and at pH 7.0. The pots were covered with polyethylene foil, gradually removed. After 4 weeks the number of growing plants was counted. The plants were transferred to a hotbed 5 weeks later. The significance of differences between treatments was assessed by the method of standard error.

## RESULTS AND DISCUSSION

Spores of *Polypodium vulgare* (20 days) and *Asplenium adulterinum* (30 days) germinated first, followed by *A. septentrionale* (40 days) and *A. cuneifolium*

(40 days). Heart-shaped gametophytes with sex organs formed after 3 months of culture in *Polypodium vulgare* and *Aspleniwn adulterinum,* and after 4 months in *A. septentrionale* and *A. cuneifolium.* Six months later, sporophytes could be observed in *Polypodium vulgare* and *Asplenium adulterinum,* and eight months later in *A. septentrionale* and *A. cuneifolium.*

Spore germination and protonema formation occurred between 70 and 96 days of culture in *Woodsia ilvensis,* and after 56 days of culture in *W. alpina.* The percentage of spore germination was high in *W. ilvensis,* 80-90% after 19 weeks. Germination of *W. alpina* was the lowest of all studied species, barely 6% after one year.

## CULTURE OF GAMETOPHYTES

The influence of full-strength, 1/2, 1/4 and 1/8 MS medium and our own modification of MS medium was tested in experiments on serpentine ferns.

Assessed in terms of fresh weight increase, the growth and development of gametophytes of all serpentine fern species was best on modified 1/2 MS and our modification of the medium (Fig. 1). The gametophytes were properly shaped, and formed many primary and secondary gametophytes (Figs 2, 3).

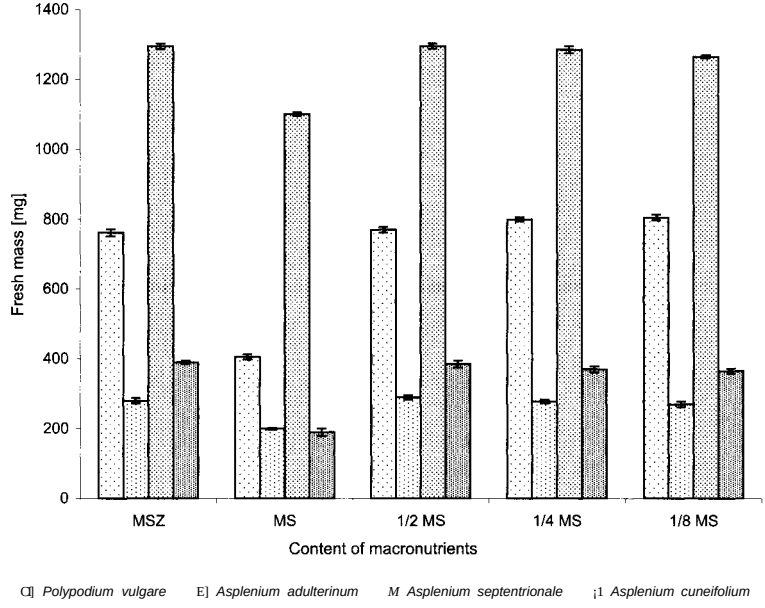


**Fig. 1.** Influence of MS medium and our modification of it (MSZ) on the growth and development of gametophytes of *Polypodium vulgare, Asplenium adulterinum, Asplenium septentrionale* and *Asplenium cuneifolium* after 2 months of culture.

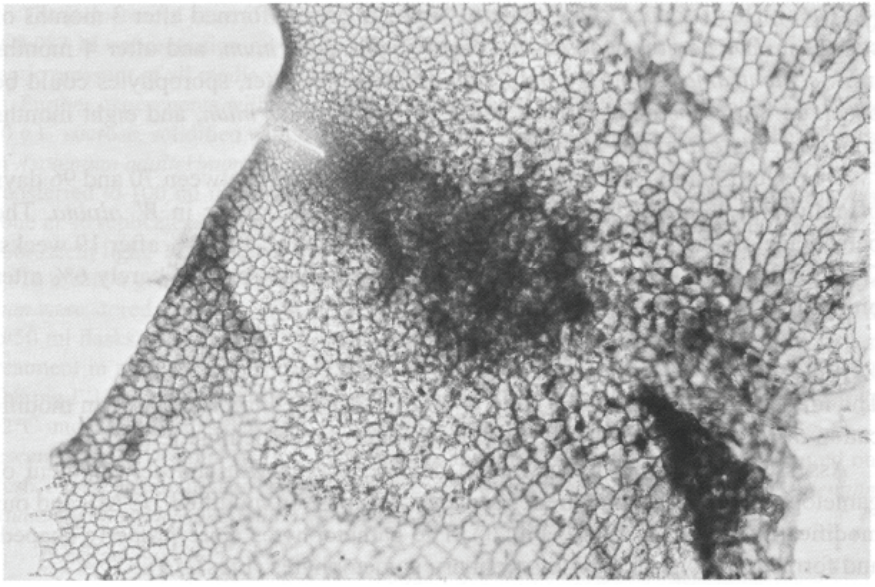

**Fig.** 2. Gametophyte of *Woodsia ilvensis* with pluricellular meristem in apical notch and archegonia below.

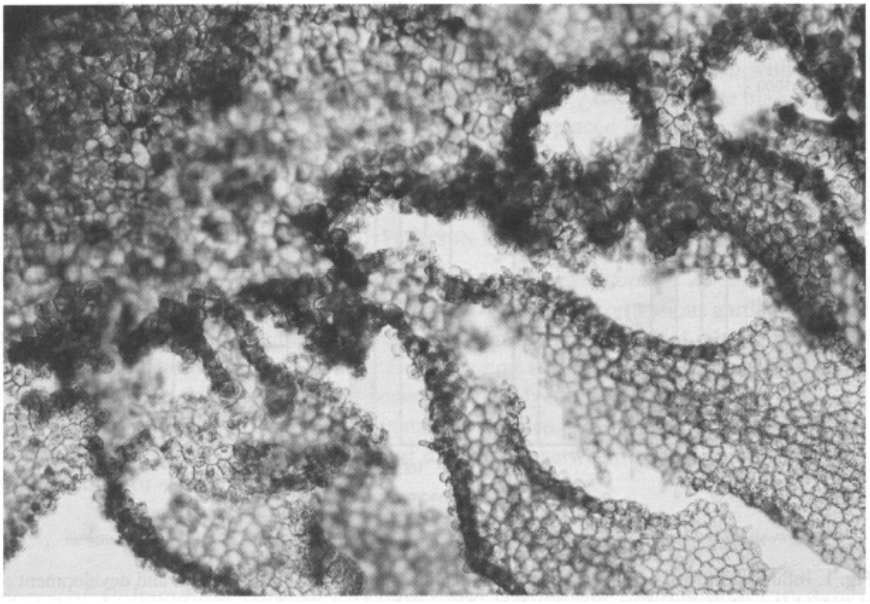

**Fig. 3.** Proliferation of young gametophyte arising from the margins of prothallus of *Woodsia ilvensis.*

**Table 1.** Influence of MS medium and our modification (MSZ) on necrosis of isolated gametophytes of *Polypodium vulgare, Asplenium adulterinum, Asplenium septentrionale* and *Asplenium cuneifolium* after one month of culture.

| Species | Decaying gametophytes [%] | | | | |
|---|---|---|---|---|---|
| | Media | | | | |
| | MSZ | MS | 1/2 MS | 1/4 MS | 1/8 MS |
| *Polypodium vulgare* | 36.7 | 66.7 | 36.7 | 33.4 | 33.4 |
| *Asplenium adulterinum* | 3.0 | 23.4 | 0 | 3.0 | 3.4 |
| *Asplenium septentrionale* | 0 | 10.0 | 0 | 0 | 3.4 |
| *Asplenium cuneifolium* | 0 | 50.0 | 0 | 0 | 0 |

Gametophytes grew worst on modified full-strength MS, with low fresh weight (Fig. 1). Some gametophytes became necrotic. One of the species whose gametophytes were most sensitive to medium nutrients was *Asplenium cuneifolium* (decaying of 50% gametophytes); the least sensitive were gametophytes of *A. septentrionale* (decay of 10% gametophytes; Table 1). The number of gametophytes of *Asplenium septentrionale, A. adulterinum* and *A. cuneifolium* diminished and fresh weight, was less on 1/8 MS of the medium, but in the case of *Polypodium vulgare* the number of pincers increased.

Measurements of the fresh weight of *Asplenium adulterinum* and *Polypodium vulgare* gametophytes revealed that growth was enhanced at 20 and 30 g $l^{-1}$ sucrose concentration in the culture medium (Fig. 4). At lower concentrations (0.5, 10 g $l^{-1}$) the gametophytes were smaller and weighed less. At 40 g $l^{-1}$ sucrose concentration the growth of gametophytes was slowed, but they showed no discoloration or decay. *Asplenium septentrionale* tolerated all the concentrations of sucrose; in the absence of sucrose the fresh weight and number of its gametophytes were lower. On media with sucrose the fresh weight of the gametophytes of all fern species increased (Table 2). Increased fresh weight (to 70 mg) was noted in *Asplenium adulterinum* on maltose, and in *Polypodium vulgare* and *Asplenium septentrionale* on glucose in the medium. *Asplenium cuneifolium* gametophytes had high fresh weight on medium supplemented with glucose and mannose. In all fern species, fructose caused necrosis of gametophytes, and lactose reduced their size. The gametophytes were largest in *Asplenium adulterinum* and *Asplenium cuneifolium* on medium with sucrose. Each of the tested fern species had the highest fresh weight and largest gametophytes on medium with sucrose. Glucose in the medium gave similar results, except in *Asplenium adulterinum,* which responded better to maltose. The other sugars reduced the fresh weight of prothallia or caused necrosis.

The best medium for storing prothallia of *Asplenium adulterinum* and *A. cuneifolium* was 1/2 MS. *Asplenium adulterinum* gametophytes performed well at

**Table** 2. Influence of sugars applied in 1/2MS medium on the growth and development of gametophytes of *Asplenium adulterinum, Asplenium cuneifolium, Asplenium septentrionale* and *Polypodium vulgare* after 2 months of culture.

| Species | Fresh mass of gametophytes [mg] | | | | | |
|---|---|---|---|---|---|---|
| | Sugars 0.087 M | | | | | |
| | fructose | maltose | glucose | mannose | sucrose | lactose |
| *Asplenium adulterinum* | necrosis of gametophytes | 70 | 26.25 | 38.75 | 66.25 | 21.25 |
| *Asplenium cuneifolium* | necrosis of gametophytes | 41.25 | 161.25 | 161.25 | 177.5 | 7.5 |
| *Asplenium septentrionale* | necrosis of gametophytes | 193.75 | 1351.25 | 453.75 | 1705.0 | 166.25 |
| *Polypodium vulgare* | necrosis of gametophytes | 123.75 | 293.75 | 81.25 | 253.75 | necrosis of gametophytes |

+8°C under cool white fluorescent light at 0.15 umol m~$^{2}$s$^{-1}$ irradiance, regardless of the phytohormones in the medium. Only high sucrose content together with phytohormones influenced gametophyte size. *Asplenium cuneifolium* gametophytes on MS medium containing KIN developed badly; they were smaller and

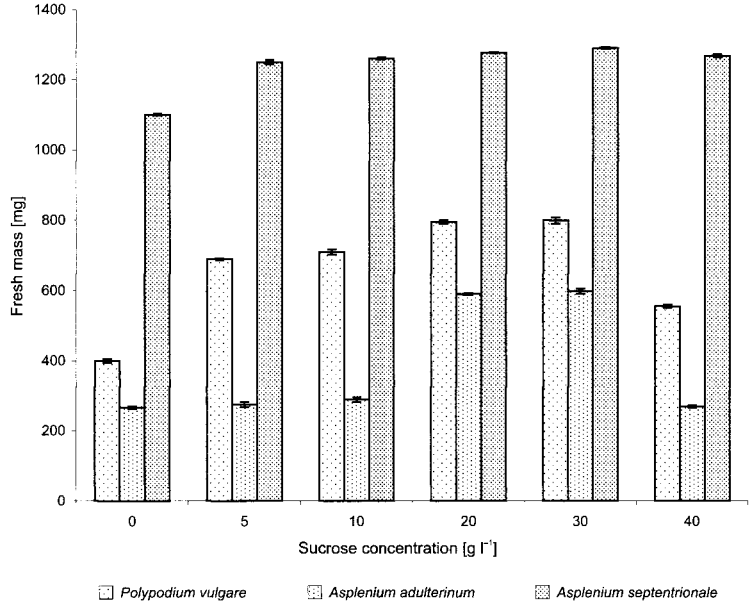


**Fig.** 4. Influence of sucrose concentration in medium on the growth and development of gametophytes of *Polypodium vulgare, Asplenium adulterinum* and *Asplenium septentrionale* after 2 months of culture.

**Table 3.** Condition of gametophytes *oî Asplenium adulterinum* and *Asplenium cuneifolium* stored for 12 months at +2°C in darkness on 1/2 MS medium with phytohormones added.

| Species | Phytohormone [mg 1$^{-1}$] | | | |
|---|---|---|---|---|
| | 1/2 MS | 0.1 IAA | 0.8 KIN | 1.0 $GA_3$ |
| *Asplenium adulterinum* | gametophytes small, light-green, pincers present, many young gametophytes | gametophytes small, light-green, pincers present, many young gametophytes | gametophytes small, yellow, pincers absent, no young gametophytes | gametophytes small, light-green, pincers present, many young gametophytes |
| *Asplenium cuneifolium* | Gametophytes small, light-green, with very well developed pincers, absence of young gametophytes | | | |

yellowed. MS medium containing 1.0 mg l$^{-1}$ $GA_3$ in combination with a high dose of sucrose strongly reduced the size of *Asplenium cuneifolium* gametophytes. MS medium containing IAA affected only the state of *Asplenium cuneifolium* gametophytes which was average.

Prothallia of the *Asplenium adulterinum* and *A. cuneifolium* stored at +2°C in continuous darkness were strongly decreased in size regardless of the phytohormones, agar and sucrose in the medium. KIN slowed the development of new gametophytes of *Asplenium adulterinum* (Table 3). *In vitro* culture proved to be an effective method for creating a prothallia gene bank of serpentine fern species. The best medium for long-term storage of *Asplenium adulterinum* and *A. cuneifolium* was 1/2 MS medium without phytohormones at +8°C under cool white fluorescent light at 0.15 umol m$^{-2}$s$^{-1}$ intensity. Different doses of agar and sucrose had no influence on the condition of stored gametophytes.

## CULTURE OF SPOROPHYTES

The number of sporophytes obtained in gametophyte cultures of all studied species was low. From the thousands of gametophytes growing in flasks, 3 to 10 sporophytes per flask were developed after 2 months of culture. Analyses of the sexual phenotypes revealed that the number of female gametophytes was high, and male ones were rare. The low number and even absence of gametophytes with antheridia is very likely a limiting factor for fertilization and development of sporophytes.

Ferns such as *Ceratopteris richardii* are attractive genetic model systems because their isolated spores develop into hermaphrodite gametophytes. The gametophyte becomes a male if exposed to antheridiogen pheromone during early development. After this initial period, the gametophyte is insensitive to antheridiogen and develops into a hermaphrodite, producing and secreting an antheridiogen. The differences between male, female and hermaphrodite gametophyte development allows screening for C. *richardii* mutants with altered sex de-

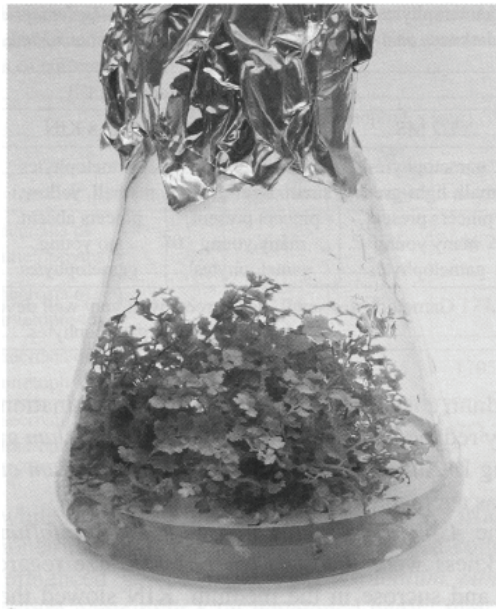

**Fig. 5.** Sporophytes of *Asplenium adulterinum* on 1/2 MS medium with kinetin (0.5 mg $l^{-1}$) and NAA (0.1 mg $l^{-1}$) after 2 months of culture.

termination. Studies of such mutants have led to a model for sexual determination in fern gametophytes, which is often independent of the genotype (Banks 1997).

Since the work of Naf *et al.* (1975) and Dopp (1962), several substances with antheridiogenic activity have been identified. The gibberellins have been identified as the major antheridiogens in *Anemia mexicana* gametophytes, and $GA_{103}$ and $GA_{107}$ have been shown to be the probable biosynthetic precursor of the antheridic acid of *Anemia phyllitidis* (Pour *et al.* 1998). Kazmierczak (2003) found that treatment of *Anemia phyllitidis* gametophytes with gibberellic acid induced precocious antheridia formation. Methyl ester of $GA_{73}$ from *Lygodium japonicum* prothallia has been shown to be a potent antheridiogen, inducing antheridia in prothallia of *L. circinnatum* (Wynne *et al.* 1998). Antheridiogen from *Pteridium aquilinum* induces antheridia in 38 fern species from 25 genera and 8 families (Raghavan 1989), but not in *Ceratopteris richardii, Lygodium japonicum* and *Anemia phyllitidis.* Identification of the antheridiogen or antheridiogens of the genus *Asplenium* could help increase generation of sporophytes in prothallia culture.

Propagation of sporophytes was tested on media containing KIN and NAA. KIN at doses not higher than 1.0 mg $l^{-1}$ was found to stimulate development of fronds (Fig. 5). Further increases of the KIN concentration caused inhibition

of sporophyte growth. High doses of KIN decreased their size but increased the number of initiated buds. Higher numbers of formed buds were noted for 0.5 mg $l^{-1}$ KIN in A. septentrionale and for 1.0 mg $l^{-1}$ KIN in A. cuneifolium. Further increases of the KIN concentration inhibited sporophyte growth, and 6.0 mg $l^{-1}$ KIN was toxic.

Perlite was used for rooting ferns in vitro because transfer from agar on liquid medium with perlite increased rooting and improved hardening, making acclimation easier. This substrate yielded similar effects in culture of apple (Kromer 1987) and fern Cyathea australis (Goller, Rybczyński 1995). This method used for hardening sporophytes propagated in vitro in the present experiments provided a useful stage between in vitro culture and cultivation in the greenhouse.

In vitro plants are characterized by considerable hydration, week lignification, incomplete formation of epidermal tissue, and nonfunctioning stomata and roots (Pierik 1989). That is why in the first period of acclimation it was so important for the plants to be under polyethylene foil, protected against drying and unsuitable temperature and humidity. This foil was gradually removed and after two weeks the plants were left completely uncovered.

The influence of various soils on the survival of sporophytes transferred from in vitro culture to the greenhouse was evaluated. The best soil for ex vitro cultivation of all investigated species of ferns was Kronen commercial mix for ferns at

Fig. 6. Sporophytes of Woodsia alpina after acclimation in greenhouse.

pH 6.5; for serpentine ferns, soil taken from the natural environment at pH 7.2 was equally good. For *A. septentrionale* and *P. vulgare,* a 3:2:1 mixture of peat, leaf mould and sand was suitable. We can conclude that serpentine ferns grow equally well in serpentine soils as in gardening soli with neutral pH. Sarosiek and Sadowska (1961) suggested that serpentine ferns probably are physiologically adapted to the special conditions of serpentine soil. *Alyssum bertolonii* transplanted from serpentine rock to horticultural soil grew badly (Pancaro *et al.* 1978); this may mean that an elevated level of nickel is indispensable to its life process, but in the case of serpentine ferns heavy metals were not indenspensible for their growth.

Cultivation of the investigated fern species proceeded satisfactorily when it was carried out according to standard procedures. All plants acclimated well outside in a garden frame where they were initially shaded (Fig. 6). *A. adulterinum* and *P. vulgare* sporophytes reached maturity and produced spores in the second year of cultivation. *A. septentrionale* formed sporangia in the third year after transfer to soil. *A. cuneifolium,* whose roots are susceptible to insects *(Sciarids),* showed the worst growth. This species did not grow outside the frame.

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