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# *In vitro* propagation of cryptic species of *Aneura pinguis* (Hepaticae, Metzgeriales)

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**Abstract** – Axenic cultures of three cryptic species of *A. pinguis* were established from the apical parts of gametophytes. Concentrations: 1, 2, 5, 25, 35 and 50% of commercial ACE bleach as well as 50% and 35% alcohol were tested in order to find optimal method of sterilization. The best outcomes resulted from the application of a lower concentration – 2% ACE solution and a longer time of exposure – 8 min. In order to find the optimal conditions of growth for *A. pinguis*, three different pH values (5.6, 6.0, 7.0) of MS 1× medium and different media were tested too. Regeneration of plants of *A. pinguis* were successfully achieved on two hormone-free media: special liverwort medium (Lukavsky, 1991) and MS medium (Murashige & Skoog, 1962).

**Liverworts / *Aneura pinguis* / cryptic species / axenic cultures**

## INTRODUCTION

*Aneura pinguis* (L.) Dumort. is a subcosmopolitan and locally common liverwort species showing wide ecological amplitude. It occurs on acid to strongly base-rich, moist or wet clay, loam, sand or peaty soil, but it is the most common on Ca-rich sites (Schuster, 1992; Paton, 1999). In Poland, *A. pinguis* spreads from the northern lowlands (Western Pomerania, Masurian Lakeland) through the central plains to the higher elevations in the mountains. It grows in various habitats such as on lime rocks, basic humus, peat bogs, fallen decorticated logs or on the wet sand on the bank of oligotrophic lakes (Szweykowski, 1958; Szweykowski, 2004). Recently,

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isozyme studies have shown that *A. pinguis* is a complex of three genetically different cryptic species tentatively called as A, B and C, correlated with different type of habitat (Szweykowski & Odrzykoski, 1990; Andrzejewska, 2000). To continue the biosystematic studies through molecular biology and cytological techniques we have established *in vitro* cultures of all cryptic *A. pinguis* species.

*A. pinguis* is a thallose, dioecious species with a simple morphological structure. Its thalli are fleshy with smooth surface and thin-walled epidermal cells. The species show a wide range of variation in thalli size, from very small plants (10-20 mm long × 2-3 mm wide) to large (30-50 (– 60) mm long × 4-6 (– 10) mm wide) (Furuki, 1991; Schuster, 1992). *In vitro* cultures give a possibility to bulk up small plants to obtain sufficient material for DNA isolation and chromosome analyses from one isozymatically identified plant. *A. pinguis* thalli usually contain endophytic fungi (Pocock & Duckett, 1984; Read *et al.*, 2000), thus *in vitro* culture gives also an opportunity to obtain the material free of fungal contamination, which is especially important for molecular studies. Cytological studies are also difficult to conduct among liverworts because of the low mitotic index in a meristematic region. *In vitro* cultures with well-dividing tissues are one of the method of accumulation of mitotic cells that enables good chromosomes preparation (Fujisawa *et al.*, 2001; Nakayama *et al.*, 2001).

Since sterilization of sporangium surface is easy and most effective, axenic cultures in bryophytes are most easily obtained from spores. Moreover spores enclosed in capsule are usually not infected with fungi or bacteria (Kowalczyk *et al.*, 1997; Duckett *et al.*, 2004). Axenic cultures of *A. pinguis* were also successfully established from spores (Read *et al.*, 2000). However in this species similarly to other hepatics, sporophytes display a very seasonal nature, which constitutes in this case a real problem to use spores as inocula. Yet, while sporophytes absence, asexual propagules (gemme) or gametophyte fragments can be used to obtain axenic cultures (Kowalczyk *et al.*, 1997; Duckett *et al.*, 2004). *In vitro* culture of *A. pinguis* from gametophyte pieces were also established in two cases (Tazaki *et al.*, 1995; Fiedorow & Szweykowska-Kulińska, 1998), however sterilization method and medium requirements were not discussed in detail. This investigation focuses on the development of methods for *in vitro* multiplication of all cryptic species of *A. pinguis* in order to obtain material for molecular and cytological studies. *In vitro* propagation can ensure the availability of plant material throughout the year.

## MATERIAL AND METHODS

A total of 53 isozymatically identified samples of all cryptic *A. pinguis* species collected mainly from different regions of Poland (Tab. 1) were used in this study. After identification, each sample was divided into two parts, one of which was deposited as a voucher at POZW Herbarium, whereas the second one was used for greenhouse culture. Plants for *in vitro* cultures were taken from a greenhouse after 3-6 months of growth. The experiment was carried out at three stages. At the first stage, the sterilization method for gametophyte pieces was tested. We tested 50 and 35% alcohol and various concentrations: 1, 2, 5, 25, 35 and 50% of ACE (commercial bleach solution, 4.9% w/w active chlorine) (Tab. 2). In the next stages of the study, three values of pH: 5.6, 6.0, 7.0 and different concentrations of nutrients in medium were subjected also to tests.

Table. 1. Collection sites of all studied populations of *Aneura pinguis* cryptic species .

| *N°. of population* | *Location* | *Herb. N°.* | *Cryptic species* | *Substratum* | *Collector* | *Date of collection* |
|---|---|---|---|---|---|---|
| 1 | Wielkopolska region, Wełna stream near Oborniki city | 40239 | C | humus | IO&SO | 02.05.2003 |
| 2 | Wielkopolska region, Poznań city | 40231 | B | humus | KB | 11.09.2003 |
| 3 | Wielkopolska region, Diabli Skok Reserve, near Wałcz city | 40143 | C | humus | KB | 11.09.2003 |
| | | 40142 | C | humus | KB | 11.09.2003 |
| | | 40141 | C | humus | KB | 11.09.2003 |
| | | 40140 | C | decaying wood | KB | 11.09.2003 |
| | | 40139 | C | humus | KB | 11.09.2003 |
| | | 40138 | C | humus | KB | 11.09.2003 |
| | | 40133 | B | decaying wood | KB | 11.09.2003 |
| 4 | Białowieski National Park, Aurochs Reserve | 39864 | B | humus | MA&AA | 26.09.2002 |
| | | 39865 | B | humus | MA&AA | 26.09.2002 |
| | | 39867 | C | humus | MA&AA | 26.09.2002 |
| | | 39868 | A | humus | MA&AA | 26.09.2002 |
| | | 39871 | B | humus | MA&AA | 26.09.2002 |
| | | 39873 | B | humus | MA&AA | 26.09.2002 |
| | | 39874 | B | humus | MA&AA | 26.09.2002 |
| 5 | Białowieski National Park, section 254 Dc | 39875 | C | decaying wood | MA&AA | 27.09.2002 |
| | | 39881 | C | humus | MA&AA | 27.09.2002 |
| | | 39877 | C | humus | MA&AA | 27.09.2002 |
| | | 39879 | B | humus | MA&AA | 27.09.2002 |
| 6 | Białowieski National Park, Wysokie Bagno Reserve | 39883 | C | humus | MA&AA | 24.092002 |
| | | 39885 | C | humus | MA&AA | 24.092002 |
| | | 39887 | C | humus | MA&AA | 24.092002 |
| 7 | Bieszczaday Mts, Moczarne in valley of Górna Solinka stream | 40181 | B | humus soil | HB&IO | 05.07.2002 |
| | | 40214 | B | sandy soil | HB&IO | 05.07.2002 |
| | | 40215 | B | sandy soil | IO&HB | 05.07.2002 |
| | | 40146 | B | sandy soil | KB | 14.07.2003 |
| | | 40150 | B | sandy soil | KB | 14.07.2003 |
| 8 | Bieszczaday Mts, Sianki, Niedzwiedzi stream | 40217 | B | clay soil | IO&EB | 06.07.2002 |

Table. 1. Collection sites of all studied populations of *Aneura pinguis* cryptic species *(suite)*.

| *N°. of population* | *Location* | *Herb. N°.* | *Cryptic species* | *Substratum* | *Collector* | *Date of collection* |
|---|---|---|---|---|---|---|
| 9 | Bieszczaday Mts, old quarry at the road from Brzegi Górne to Nasiczne | 40222 | B | clay soil | HB&IO | 07.07.2002 |
| 10 | Bieszczaday Mts, S slope of Ryczywół Mt. | 40224 | B | clay soil | IO&HB | 07.07.2002 |
| 11 | Małe Pieniny Mts, N slope of Repowa Mt. | 40199 | A | humus soil | KB&BCH | 28.08.2002 |
| | | 40200 | A | soil | KB&BCH | 28.08.2002 |
| | | 40190 | A | rock detritus | KB&BCH | 28.08.2002 |
| | | 40197 | A | rock detritus | KB&BCH | 30.08.2002 |
| 12 | Małe Pieniny Mts, Skalskie stream | 40192 | A | clay soil | KB&BCH | 29.08.2002 |
| | | 40194 | A | humus | KB&BCH | 29.08.2002 |
| | | 40196 | A | clay soil | KB&BCH | 29.08.2002 |
| | | 40195 | A | soil | KB&BCH | 29.08.2002 |
| | | 40207 | A | rock detritus | KB&BCH | 31.08.2002 |
| | | 40209 | A | moss | KB&BCH | 31.08.2002 |
| 13 | Tatry Mts, Jaworzynka Valley | 40164 | A | soil | AB&KB | 06.08.2002 |
| 14 | Tatry Mts, ski road on NE slope of Skupniów Upłaz Mt. | 40093 | A | humus | KB&AB | 08.08.2002 |
| | | 40094 | A | humus | KB&AB | 08.08.2002 |
| | | 40097 | A | soil | KB&AB | 08.08.2002 |
| 15 | Tatry Mts, Chochołowka Valley, Wielka Sucha Woda stream | 40103 | A | soil | AB&KB | 09.08.2002 |
| | | 40104 | A | rock detritus | AB&KB | 09.08.2002 |
| | | 40105 | A | rock detritus | KB&AB | 09.08.2002 |
| | | 40119 | A | rock detritus | AB&KB | 09.08.2002 |
| 16 | Tatry Mts, Pañszczyca Valley | 40110 | A | soil | KB&AB | 12.08.2002 |
| 17 | Scotland, North Ebudes, Rum, (D.G. Long N° 33262) | 40163 | B | humus | DL | 28.06.2004 |
| 18 | Ireland, West Galway, near Ooeyuna, (D.G. Long N° 33343) | 40237 | B | humus | DL | 13.07.2004 |
| 19 | Ireland, West Galway, Broaboy, Glencorbet, Twelve Bens, (D.G. Long N° 33363) | 40238 | B | humus | DL | 15.07.2204 |

Collectors: AA – Artur adamczak, AB – Alina Bączkiewicz, BCH – Błażej Chmielewski, DL – David Long, HB – Hanna Barczak, IO – Ireneusz Odrzykoski, KB – Katarzyna Buczkowska, MA – Małgorzata Adamczak, SO – Samuel Odrzykoski.

Table. 2. Sterilization conditions.

| *Time of sterilization* / *Solution* | *30 s* | *60 s* | *3 min* | *5 min* | *6 min* | *8 min* | *10 min* | *15 min* |
|---|---|---|---|---|---|---|---|---|
| 50% ethanol | + | | | | | | | |
| 35% ethanol | + | + | | | | | | |
| 50% ACE | + | + | | | | | | |
| 35% ACE | + | + | + | | | | | |
| 25% ACE | + | + | + | | | | | |
| 5% ACE | | + | + | + | + | | | |
| 2% ACE | | + | + | + | + | + | + | |
| 1% ACE | | | + | + | + | + | + | + |

*Sterilization of plant material.* Thalli taken from the greenhouse were cleaned with a fine paintbrush and rinsed several times in distillated water. Cleaned thalli were left for 24-48 h in Petri dishes on the blotting paper soaked with distillated water. After that the thalli were sterilized and rinsed 3 × in sterile distillated water. Only the top parts of the thalli (about 5-6 mm) were taken for sterilization.

*Media.* The *in vitro* cultures were grown on MS (Murashige & Skoog, 1962) basal salt mixture medium (Sigma) and modified special liverworts medium (Lukavsky *et al.*, 1991). Both media were supplemented with sucrose (2.0 g/l) which is an important source of carbon (Bopp & Knoop, 1984). The media were solidified with phytagel in concentration of 2.0 g/l. The medium pH was adjusted with 0.1N KOH before adding the phytagel and autoclaving at 121°C and 118 kPa for 25 min. All media were mixed up with myoinositol 100 mg/l and vitamins: nicotinic acid 0.5 mg/l, pyridoxine HCl 0.5 mg/l, thiamine HCl 0.1 mg/l, glycine 2 mg/l. Nine variants of media differing in pH value and in concentration of nutrients were tested (Tab. 3). Because *A. pinguis* grows mainly at base-rich sites, media n° 2, 4 and 6-9 were supplemented with $CaCO_3$ as an additional source of

Table. 3. Types of tested media.

| *N°* | *medium* | *pH* | *Additional* $Ca^{2+}$ *(mg/l)* |
|---|---|---|---|
| 1 | MS1 × | 5.6 | – |
| 2 | MS1 × | 5.6 | + 120 |
| 3 | MS1 × | 6.0 | – |
| 4 | MS1 × | 6.0 | + 120 |
| 5 | MS1 × | 7.0 | – |
| 6 | MS1 × | 7.0 | + 120 |
| 7 | MS1 × | 6.0 | + 120 |
| 8 | MS1/2 × | 6.0 | + 120 |
| 9 | liverwort medium 1 × | 6.0 | + 120 |

$Ca^{2+}$. After sterilization, pieces of thalli were put on 1× MS medium, pH 5.6, without sucrose, vitamins and $CaCO_3$, next sterile explants were transferred to different media. The explants were regarded as sterile, if they remained clean (without visible fungal or bacterial contamination) during 2 weeks after sterilization. The number of thalli sterilized per sample depends on the sample size in a greenhouse culture. On average 20-30 thalli were taken from each sample, except for small samples, where only 10-15 thalli could be taken from. The cultures were grown under stable conditions: 21°C, dark-light rhythm 16:8 hour, light at 60 μM $m^{-2}$ $s^{-1}$ was supplied by cool white fluorescent lamps.

## RESULTS AND DISCUSSION

In the first stage of the study the sterilization method of gametophyte pieces was tested. Sterilization with 50 and 35% alcohol as well as by 50, 35 and 25% of ACE solution for *A. pinguis* gametophytes was apparently too strong. After 1 min. of sterilization the material was aseptic but completely colorless and damaged. However, when the sterilization time was shortened to 30 s. almost all cultures became infected. Similarly a low concentration of ACE (1%) applied even for 15 min was not effective. The application of 2 and 5% ACE solutions seemed to be the most effective sterilization method for *A. pinguis* gametophyte, the results of which are shown in Tab. 5. A sterile explant was achieved for 566 out of 1659 pieces of thalli subjected to sterilization. The best results were obtained from the application of a lower concentration – 2% solution and a longer time of exposure (479 sterile explants). Contrary at a shorter time of exposure and a higher – 5% solution concentration the obtained results were lower (87 sterile explants) (Tab. 5). The pieces of thalli just after sterilization remained pale green, but during several hours they become colorless, except from apical cells that usually retain their green color. Some differences in the easiness of obtaining sterile explants were observed between cryptic species. Sterile explants were more easily obtained from the plants of the C species (40.6%) than from plants of the A species and C and (23.5% and 26.6%, respectively).

Obtaining sterile explants is the main difficulty in the case of establishing *in vitro* cultures from fragments of gametophytes in bryophytes (Kowalczyk *et al.*, 1997; Sabovlejevic *et al.*, 2003; Duckett *et al.*, 2004). There is a necessity to develop the technique of gametophyte tissue sterilization individually for each bryophyte species. For some liverworts species, such as *Cephalozia bicuspidata* (L.) Dumort., *Conocephalum conicum* (L.) Dumort., *Pellia epiphylla* (L.) Corda and *Ptilidium ciliare* (L.) Hampe, the application of 50% ACE solution for 0.5-1 min. appeared to be the most effective method of sterilization (Kowalczyk *et al.*, 1997). However for *A. pinguis*, a lower concentration and a longer time of sterilization are apparently more suitable, than in the case of *Pellia* species (Fiedorow & Szweykowska-Kulińska, 1998), or moss *Eurhynchium praelongum* (Hedw.) B., S. & G. (Sabovlejevic *et al.*, 2003). We have found that in the case of *A. pinguis* it was much easier to obtain sterile explants, while leaving the cleaned thalli before sterilization for 2 days in Petri dishes on blotting paper soaked with distillated water, than if they were sterilized just after taking from a greenhouse culture.

In order to choose the optimal pH for *A. pinguis*, three different pH values of 1× MS medium supplemented or not supplemented with $CaCO_3$ were

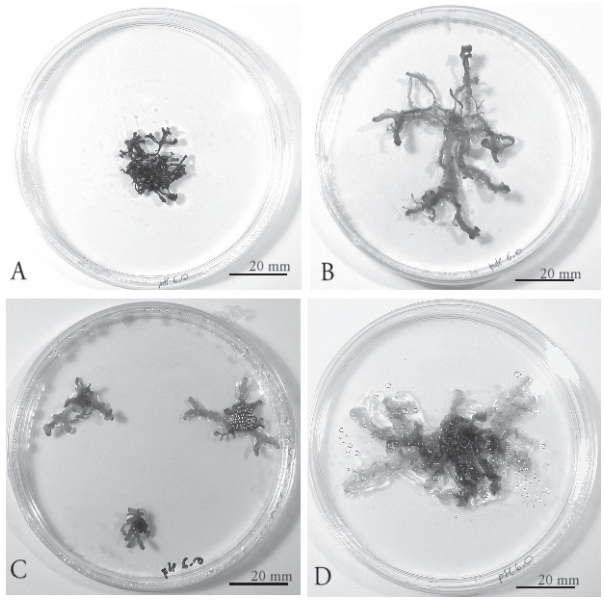


Fig. 1. Gametophyte of *A. pinguis*: **A** and **B** – species C POZW 40141 (A – after 6 months of culture on MS medium, B – after 3 months of culture on special liverwort medium), **C** – species B POZW 40238 after 1 month of culture on special liverwort medium, **D** – species B POZW 39865 –after 3 months of culture on special liverwort medium.

tested, variants n° 1-6 (Tab. 3). In this part of the study one sample from each cryptic species (big enough to take large material for sterilization) was used. Ten sterile thalli fragments from each sample were placed on each of 1-6 medium variant. For establishing *in vitro* culture of the cryptic *A. pinguis* species a medium with pH 6.0 supplemented with $CaCO_3$ (medium n° 4) was more suitable than other variants of tested media. *A. pinguis* is a calcicole species. In nature it can occur in different habitats, however, it is the most frequent on the base-rich sites (Schuster 1992). It has been observed that calcicole species, e.g. *Chiloscyphus polyanthos* (L.) Corda, *Cratoneuron filicinum* (Hedw.) Spruce, are relatively indifferent to calcium status, but they are strongly intolerant of low pH (Bates, 2000). Our *in vitro* cultures proved the importance of both, pH value and the concentration of calcium are important for *A. pinguis*. Only pH increase even above 6.0 without supplementing the medium with Ca have caused that regeneration and the growth rate were very slow.

Different concentrations of basal nutrients (media n° 7-9) were tested in the next stage of the experiments (Tab. 3). From eight to ten sterile fragments of thalli from 17 samples of three cryptic species from different populations were placed on each medium variant (Tab. 6). Samples with only few sterile explants were excluded from this part of the study. The special liverwort medium with pH 6.0, supplemented with $CaCO_3$ was the best for establishing *in vitro* culture of *A. pinguis*, 36.0% of explants placed on this medium regenerated and grew fast (Tab. 6). Regeneration of thalli from gametophyte fragments with green apical cells on this medium took place after ca. 1-2 weeks of establishing the culture, after that the growth rate was relatively fast. After ca. 1 months, plants 2-3 cm in diameter and consisting of 3-5 thalli developed from some explants (Fig. 1c).

Table. 4. Differences in composition of macro- and microelements between MS and special liverwort medium.

| *Macro- and microelements* | *MS medium (mg/l)* | *Modified special liverwort medium (mg/l)* |
|---|---|---|
| $NH_4NO_3$ | 1650 | 120 |
| $KNO_3$ | 1900 | – |
| $MgSO_4 \times 7\ H_2O$ | 370 | 246 |
| $KH_2PO_4$ | 170 | 700 |
| $CaCl_2 \times 2$ H2O | 340 | 3. 40 |
| KJ | 0.83 | – |
| $H_3BO_3$ | 6.2 | – |
| $MnSO_4 \times 4\ H_2O$ | 16.9 | – |
| $ZnSO_4 \times 7\ H_2O$ | 8.6 | – |
| $Na_2MoO_4 \times 2\ H_2O$ | 0.25 | – |
| $CuSO_4 \times 5\ H_2O$ | 0.025 | – |
| $CoCl_2 \times 6\ H_2O$ | 0.025 | – |
| $Na_2EDTA$ | 37.3 | – |
| $FeSO_4 \times 4\ H_2O$ | 27.8 | – |
| $FeCl_3$ | – | 30 |

Table 5. Results of sterilization.

| *Cryptic species* | *N° of samples* | *N° of sterilized thalli* | *N° of explants sterilized and sterile in brackets* | | *N° of sterile explants* | *% of sterile explants* |
|---|---|---|---|---|---|---|
| | | | *2% ACE solution, 8-10 mm* | *5% ACE solution, 5-6 min* | | |
| A | 20 | 548 | 275 (117) | 270 (12) | 129 | 23.5 |
| B | 17 | 526 | 263 (125) | 259 (15) | 140 | 26.6 |
| C | 14 | 731 | 368 (237) | 364 (60) | 297 | 40.6 |
| Total | 53 | 1659 | 906 (479) | 893 (87) | 566 | 34.1 |
| % of sterile explants | | | 52.8 | 9.7 | | |

After 3 months plants formed colony up to 5-7 cm in diameter, they had normal morphology (Fig. 1b, d), and in some cases formed a male sex organs. The MS 1× medium with pH 6.0, supplemented with $CaCO_3$ was also suitable for those species. However on this medium the growth rate was much slower. Regeneration of thalli took place after ca. 3-4 weeks, plants 2-3 cm in diameter and consisting of several small thalli developed from some explants after 4-5 month. Yet, the plants were darker-green than those on the special liverwort medium (Fig. 1a). Both media differed considerable in concentration of basal nutrients (Tab. 4). In the special liverwort medium concentration of phosphorus ($KH_2PO_4$) was 4× higher,

Table 6. Number of obtained cultures on different types of media. The plants with male sex organs were marked with an asterisk.

| *N° of Population* | *N° of samples* | *Cryptic species* | *N° of explants put on each medium* | *N° of obtained cultures* | | |
|---|---|---|---|---|---|---|
| | | | | *medium n° 7* | *medium n° 8* | *medium n° 9* |
| 1 | 40239 | C | 10 | 1 | – | 2 |
| 2 | 40231 | B | 10 | 1 | – | 2* |
| 3 | 40141 | C | 10 | 3 | 1 | 6 |
| | 40139 | C | 10 | 7 | 4 | 9 |
| 4 | 39865 | B | 8 | 1 | – | 3 |
| | 39867 | C | 10 | 1 | – | 8 |
| | 39874 | B | 9 | 1 | 1 | 2 |
| 5 | 39875 | C | 10 | 4 | 1 | 2 |
| 6 | 39883 | C | 9 | – | – | 1 |
| 7 | 40150 | B | 10 | 2 | 1 | 1 |
| 9 | 40222 | B | 9 | – | – | 4* |
| 10 | 40190 | A | 10 | – | – | – |
| 11 | 40194 | A | 10 | – | 1 | - |
| 15 | 40119 | A | 10 | 2 | – | 12 |
| 16 | 40110 | A | 10 | – | – | 2 |
| 17 | 40163 | B | 9 | – | – | 2 |
| 19 | 40238 | B | 10 | – | – | 3 |
| Total | 17 | | 492 | 23 | 9 | 59 |
| % | | | | 14.0 | 5.5 | 36.0 |

however concentrations of nitrogen ($NH_4NO_3$) was 14× lower than in MS medium. Concentration of Fe, Mg and Ca was similar in both kinds of media, but in the special liverwort medium microelements (except from iron) were absent. The use of half-strength MS medium did not provide satisfying results (Tab. 6) because plant regeneration was very slow. Many thalli fragments did not regenerate in spite of having green apical cells and died after several weeks.

Our studies show that regeneration of *A. pinguis* plants can be achieved on hormone-free media. In all cases plant regeneration took place exclusively from apical, merystematic cells and thalli were built directly without secondary protonema and callus stage. It seems that hormones are not indispensable for successfully *in vitro* cultures establishment, at least for some liverwort species. Establishing of *in vitro* culture on medium without hormones was also achieved for other liverworts (Fiedorow & Szweykowska-Kulińska, 1998) and for some mosses (Sabovlejevic *et al.*, 2003). The special liverwort medium can be recommended when a large amount of material have to be obtain in relatively short time. Plants on this medium grown fast, but they required fresh media every 2-3 months. Plants cultured on the MS medium grown slower but they are able to grow on the

same medium about 4-5 months. Although MS medium and media based on MS are often used for *in vitro* culture of bryophytes (Kowalczyk *et al.*, 1997; Fiedorow & Szweykowska-Kulińska, 1998; Sabovlejevic *et al.*, 2003), the special liverwort medium was used too (Fiedorow & Szweykowska-Kulińska, 1998).

We have succeeded in establishing *in vitro* culture of 91 plants from 17 different samples (Tab. 6) representing three cryptic species of *A. pinguis* (Szweykowski & Odrzykoski, 1990; Andrzejewska 2000). Differences in the easiness of sterilization and in the growth rate between plants belonging to different cryptic species were observed. Plants of cryptic species C regenerated the more easily (50 plants) in comparison to plants of cryptic species A and B (17 and 24 plants respectively). Establishment of axenic cultures from gametophytes of *A. pinguis* is complicated by the presence of fungal endophytes (Pocock & Duckett, 1984; Read *et al.*, 2000; Kottke *et al.*, 2003). However not all *A. pinguis* samples are infected by the fungi and green, actively growing apical region of thalli are usually free from fungal infections (Pocock & Duckett, 1984). It makes possible to led *in vitro* cultures from the apical parts of gametophytes. *A. pinguis* material collected in the natural habitats in Poland contains some samples not infected by fungi. Micrscopic analysis in cross-section of the thalli from *in vitro* cultures have shown that they are free from fungal hyphae.

The material from *in vitro* cultures free from contamination was used for molecular studies of the complex species (Wachowiak *et al.* in preparation). On account of many dividing cells material from *in vitro* cultures can also be used in the cytological studies. In Bryophytes this method was used on *Marchantia polymorpha* (Nees) Burgeff (Fujisawa *et al.*, 2001; Nakayama *et al.*, 2001; Ishizaki *et al.*, 2002). In our study highest mitotic index and no poliploid sets of chromosomes was observed in meristems fixed from 2 weeks growing thalli. It could be usefull for chromosome analyses only when the number of chromosomes is well known. After longer time (over 4 weeks) *in vitro* cultures can show some changes of the karyotype (due to ploidy level) (Chudzińska *et al.* in preparation). Establishment of *in vitro* cultures of *A. pinguis* cryptic species makes it possible to access to the same defined plants in the further studies.

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