Mycorrhiza (2010) 20:551–557
DOI 10.1007/s00572-010-0302-x

ORIGINAL PAPER

Arbuscular mycorrhiza of *Arnica montana* under field conditions—conventional and molecular studies

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Received: 10 February 2009 / Accepted: 29 January 2010 / Published online: 27 February 2010

Abstract Two distinct populations of *Arnica montana*, an endangered medicinal plant, were studied under field conditions. The material was investigated using microscopic and molecular methods. The analyzed plants were always found to be mycorrhizal. Nineteen arbuscular mycorrhizal fungal DNA sequences were obtained from the roots. They were related to Glomus Group A, but most did not match any known species. Some showed a degree of similarity to fungi colonizing liverworts. Conventional analysis of spores isolated from soil samples allowed to identify different fungal taxa: *Glomus macrocarpum*, *Glomus mosseae*, *Acaulospora lacunosa*, and *Scutellospora dipurpurescens*. Their spores were also isolated from trap cultures.

Keywords *Arnica montana* · Medicinal plants · Endangered plants · Arbuscular mycorrhizal fungi (AMF) · Nuclear ribosomal DNA

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Introduction

Medicinal plants constitute about 10–18% of the world's flora, most of which are being harvested from the wild (Hamilton 2004). An increasing demand for herbal products may endanger many traditionally used and pharmaceutically important plant species and their habitats (Fuchs and Haselwandter 2008). *Arnica montana* L., a member of the Asteraceae family, is a rare plant under strict protection in several European countries (Ellenberger 1998) including Poland (Decree of the Minister of the Environment issued on 09.07.2004, Dz. U. No. 168, entry 1764, published on 28.07.2004). Active protection of this species, aiming at securing its existence and improving its population size, falls under the EU Habitats Directive (The Habitats, Fauna and Flora Directive, Annex V) no. 92/43/EEG and EC Regulation no. 338/97, Annex D. The species is cultured for industrial purposes, but its culture is difficult and non-profitable; therefore, the participation of cultured plants in the overall material pool is minimal (Lange 1998). The programs designed at the reintroduction of arnica to its natural stands, often sponsored by pharmaceutical companies using compounds isolated from this plant, overlook the fact that under field conditions, arnica forms mycorrhizal symbiosis with arbuscular fungi (Heijne et al. 1996; Eriksen et al. 2002). Under some conditions, such as in areas poor in mineral compounds and polluted, the well-developed net of fungal hyphae growing in the soil significantly increases the active root surface, granting the plant access to the otherwise inaccessible water and mineral pools, especially phosphorus. Mycorrhiza is of basic importance for both individual plants and for entire plant communities—it allows to develop a high species diversity on a given site and improves the growth of young seedlings (Van der Heijden et al. 1998). The

mycelium forms a net connecting plants even belonging to different species, where seedlings can plug into and share its water and mineral pools (Turnau and Haselwandter 2002; Jeffries et al. 2003; Renker et al. 2003). Fuchs and Haselwandter (2008) summarized various studies reporting the beneficial influence of arbuscular mycorrhizal fungal (AMF) on endangered medicinal plants belonging to different families. The importance of mycorrhiza for many medicinal plant species and the possibilities of its practical application strengthen the need for identification and cultivation of mycorrhizal fungi present in roots of naturally occurring plants.

The main aims of the present study were to estimate the mycorrhizal status of *A. montana*, to identify arbuscular mycorrhizal fungi forming spores under field conditions, and to select strains that are efficient colonizers of arnica roots. The research presented here is a part of a larger work (Jurkiewicz et al. 2009) and was carried out using a very limited amount of plant material, so it should be regarded as a pilot study.

Material and methods

The study was carried out on material collected in the field on the basis of permits obtained from the Ministry of the Environment and from Karkonosze National Park (DOPog-421-II-36.4/05/msz and 2/2006, respectively). These permits allowed us to collect five specimens from each site. The collection sites were located as follows: Kurpie (Western part of Podlasie, NE Poland) 110 m asl, 53°20′32″ N, 21°40′46″ E; Karkonosze (Western Sudetes, SW Poland) 1,090 m asl, 50°47′11″ N, 15°35′12″ E. The soil and root samples were collected from four stands in Kurpie (growing in the forest close to Charubin village) on bielice-type of soil (pH4–4.5), in a pine forest on lowland (flat area), with *Pinus sylvestris* L., *Picea abies* (L.) H. Karst., *Juniperus communis* L., *Frangula alnus* Mill., and in the herb layer: *Vaccinium myrtillus* L., *V. vitis-idaea* L., *Calluna vulgaris* (L.) Hull, *Hieracium pilosella* L., *A. montana* L., *Melampyrum pratense* L., *Solidago virga-aurea* L., *Hieracium lachenalii* C. C. Gmel., *Scorzonera humilis* L., *Convallaria majalis* L., *Danthonia decumbens* (L.) DC, and *Calamagrostis epigejos* (L.) Roth. In Karkonosze (mountain area), the plant was collected in Jaworowa Łąka (four stands) on acidophilous subalpine grassland soil of pH4.8–5.5. *A. montana* was accompanied by *Picea abies* (L.) H. Karst., *Deschampsia caespitosa* (L.) P. Beauv., *Nardus stricta* L., *Galium* sp., *Gentiana asclepiadea* L., *V. myrtillus* L., *V. vitis-idaea* L., *Potentilla erecta* (L.) Raeusch., *Calamagrostis* sp., and *Dactylis glomerata* (L.) The roots were collected from 0 to10 cm depth in a way to avoid destruction of the plants. The procedure of trap cultures establishment, spore isolation, and identification of AMF spores was precisely described by Błaszkowski (http://www.agro.ar.szczecin.pl/~jblaszkowski/). Spores were isolated by wet sieving and divided into morphotypes.

DNA isolation

DNA was isolated from small samples of fine roots (about 1 to 2 cm per plant). Root samples from the two populations (Karkonosze and Kurpie) were ground in liquid nitrogen, washed twice in 96% ethanol (15 min, shaking at 300 rpm, room temperature). Genomic DNA was isolated with the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.

PCR and sequencing

The primer pair SSU–Glom1 and LSU–Glom1 (Renker et al. 2003) was used to amplify the ribosomal cassette consisting of SSU, ITS1, 5.8S, ITS2, and LSU rDNA. These were the only Glomeromycota-related primers available to the authors during the study. Alternatively, when no amplification products were obtained with SSU–Glom1 and LSU–Glom1, a nested PCR approach with the primer pair ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) was used to amplify 1,000-fold diluted reaction mixtures. PCR were performed in the total volume of 20 µl, containing: 2.5 µl Green GoTaq Buffer, 2.4 µl $MgCl_2$ (25 mM), 0.4 µl dNTPs (10 mM each), 0.8 µl of each primer (10 pM/µl), 9.5 µl nuclease-free water, 0.1 µl GoTaq DNA polymerase (5 u/µl), and 1 µl of DNA template. All PCR reagents except primers were provided by Promega (USA). Amplification cycle conditions were as follows: initial denaturation (94°C, 4 min), 35 cycles of denaturation (94°C, 30 s), primer annealing (55°C, 30 s), product extension (72°C, 30 s), final extension (72°C, 5 min).

PCR products were purified with the Wizard PCR kit (Promega, USA), ligated into pGEM-T vector and cloned into JM109 competent cells (Promega, USA). Due to low cloning efficiency in some samples, about 30 clones in total were analyzed. Clones containing inserts were picked by sterile toothpicks and directly subjected to PCR either with SSU–Glom1 and LSU–Glom1 or ITS1F and ITS4 primer pair, then purified with sodium acetate (3 M) and isopropanol (http://openwetware.org/wiki/Isopropanol_Precipitation_for_PCR_Purification). The PCR products were cycle sequenced with ABI BigDye Terminator ver. 3.1 (Applied Biosystems, USA). The obtained sequences were submitted to GenBank (http://www.ncbi.nlm.nih.gov/) under accession numbers: EU747842–EU747845 and EU747847–EU747861.

DNA data analysis

The obtained sequences were checked using BLAST (Altschul et al. 1990); 5–10 sequences with the highest similarity values and a number other sequences representing most groups of AM fungi from GenBank were selected for further analysis. The dataset was aligned using ClustalW (Thompson et al. 1994). Alignment consisting of total 3,459 characters and enclosing whole regions: internal transcribed spacer 1 (ITS1), 5.8 rRNA, and internal transcribed spacer 2 (ITS2) was used. Phylogenetic relationships between sequences were inferred using the neighbor joining method as implemented in PAUP* ver. 4.0 (Swofford 1998) with the nucleotide substitution model estimated using Modeltest ver. 3.7 (Posada and Crandall 1998), gaps were treated as missing data. Bayesian posterior probabilities of tree topology were calculated using MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck 2003).

Mycorrhizal colonization

The following parameters were assessed in field-collected root samples: frequency of mycorrhiza (F%), mycorrhizal intensity relative (M%) and absolute (m%), and arbuscule richness relative (A%) and absolute (a%). For this, roots were collected, carefully washed in tap water, and cleared in 10% KOH for 24 h at room temperature. Subsequently, after washing in tap water, the roots were acidified for 1 h in 5% lactic acid and stained for 24 h at room temperature in 0.05% aniline blue in pure lactic acid in order to visualize the fungal structures inside the roots. Material obtained this way was cut into 1 cm pieces and mounted on slides in pure lactic acid (approx. 45 root pieces per plant). Mycorrhizal parameters were assessed according to Trouvelot et al. (1986) (http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html) and the abovementioned mycorrhizal parameters calculated.

Data analysis

Data obtained from mycorrhizal evaluation were analyzed with the Kruskal–Wallis one-way analysis of variance using the STATISTICA ver. 7.0 software (Statsoft, USA; $P<0.05$).

Results

Mycorrhizal status of *A. montana* in the field

Analysis of the roots from all stands in two regions showed the presence of arbuscular fungi within all roots. Relatively higher parameters of mycorrhizal colonization were noted in Karkonosze than in Kurpie. Coarse AMF were found at both stands. Slightly lower mycorrhizal colonization parameters were usually observed in the case of plants from Kurpie (Fig. 1). In the same case also, the presence of *Glomus tenue* (a fine endophyte with hyphae of ca. 1 µm in diameter, small vesicles up to 5 µm in diameter and characteristic finger-like branching; see Turnau et al. 2005) was observed.

In Kurpie, *G. tenue* accounted for 0–40% of the total mycorrhizal colonization, and only in one stand were M% and A% higher than the M% and A% values of the coarse endophyte (Fig. 2). *G. tenue* was not found in stands from Karkonosze. In roots collected from the field, dark septated endophytes were also observed.

Identification of mycorrhizal fungi

Soil samples from the field, collected from the rhizosphere of *A. montana* contained spores of *Glomus macrocarpum* Tul. & C. Tul., *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe, *Scutellospora dipurpurascens* Morton & Koske, and *Acaulospora lacunosa* Morton. Except for *G. mosseae*, all the other spore morphotypes were obtained from the trap cultures. In the samples from Kurpie, also *Complexipes moniliformis* Walker, an ectendomycorrhizal associate of pines, was present.

Different results were obtained on the basis of molecular analysis aiming at the identification of efficient AMF colonizers of *A. montana* roots. BLAST results obtained for the investigated sequences revealed that 19 of them belonged to the phylum Glomeromycota. Phylogenetic analysis grouped all the investigated sequence within the Glomus Group A (see Schwarzott et al. 2001) (Fig. 3). Two clades enclosed most of the obtained sequences. The first group (I) contained sequences from Kurpie only, AMF symbionts of a

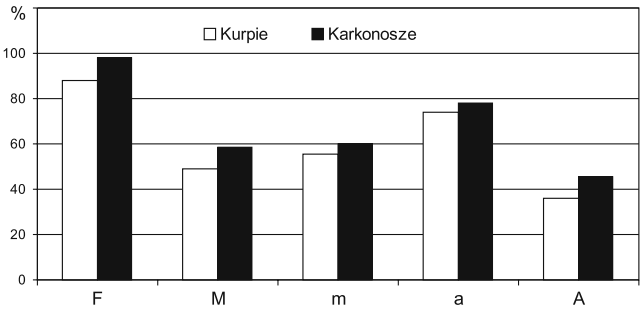

Fig. 1 Mycorrhizal colonization of *A. montana* collected from field stands in Kurpie and Karkonosze; *F* mycorrhizal colonization frequency, *M* relative mycorrhizal root length, *m* intensity of colonization within individual mycorrhizal roots, *A* relative arbuscular richness, *a* arbuscule richness in root fragments where the arbuscules were present; data given in %; no statistically important differences were found

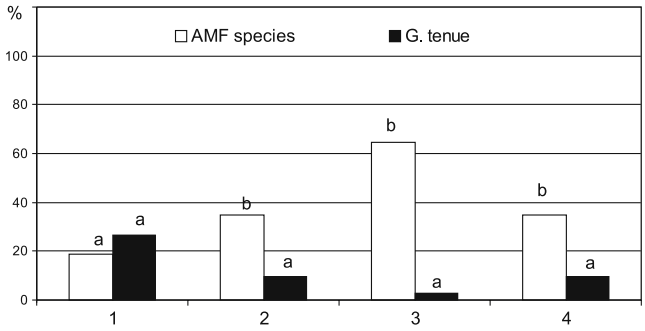

Fig. 2 Total mycorrhizal colonization by coarse fungi (AMF) and by fine endophyte (*G. tenue*) of *A. montana* collected from four field stands in Kurpie; *different letters over bars* indicate statistically significant differences ($p<0.05$)

liverwort, *Marchantia foliacea* and *Glomus sinuosum*. The second clade (II) included only nine sequences obtained from the Karkonosze sample; no other sequences were placed within this group. Two single sequences were placed in two groups showing similarity either to an AM fungus found in *M. foliacea* or two uncultured *Glomus* spp.

Discussion

In the present study, *A. montana* collected from the field sites, both mountain and lowland, was always found to be mycorrhizal. Plants that inhabit poor acidic soils are usually dependent on their symbiotic partner, although researchers mostly pay attention to ericoid or ectomycorrhizal fungi and their plant symbionts. In temperate regions, the symbiotic status is rarely studied in herbs occurring in acid forests or acid meadows. The main reason for this is that such herbs usually have a low economic value, although several have been important sources of medical compounds. Molecular studies carried out presently confirmed that our knowledge of mycorrhizal fungi under such condition is extremely limited. Fungal spores isolated from the rhizosphere, belonging to Glomeromycota, that were isolated from the rhizosphere of *A. montana* are representatives of species known from Europe, also from soils of higher pH values; however, according to molecular analysis, they were not found to be the root colonizers of *A. montana* collected from the field stands. Nineteen sequences of AMF isolated from the roots were found to belong to mostly unknown species, all belonging to Glomus Group A. Most of the obtained sequences were divided into two groups according to the origin of the plant population. The sequences from Kurpie were grouped together with sequences of fungi colonizing liverworts (Russell and Bulman 2005) and *G. sinuosum*. The second group showed no close similarity to any other sequences available in GenBank and consisted only of sequences from Karkonosze.

We are aware that primer pairs used for this study can lead to an amplification bias towards *Glomus* species and other non-AM fungi. This can be omitted using recently published primers (Lee et al. 2008; Krüger et al. 2009) showing both high specificity to Glomeromycota versus other fungi and possibility to amplify DNA of all AMF lineages. As we were only allowed to collect a very limited number of specimens, and generally, the root system of arnica makes DNA isolation difficult (few fine roots from which DNA could be isolated), these studies need further work.

Classical studies on AMF diversity in soils were mainly done using morphological features of spores formed in the rhizosphere or in roots. However, such approach resolves only fungal species that form spores and not necessarily are present in roots as functional symbionts, while the morphology of other AMF structures (hyphae, arbuscules, vesicles) gives only little taxonomical information (Brundrett et al. 1996). On the contrary, fungi-colonizing roots might not sporulate (Clapp et al. 1995; Sanders 2004). Since late 1990s, molecular approaches were designed to evaluate the AMF diversity within roots of plants. Most of them are based on the variability of the ribosomal gene domains (i.e., van Tuinen et al. 1998; Turnau et al 2001; Renker et al. 2003; Lee et al. 2008). Due to these improvements, an unexpected diversity and specificity of AMF was found (Vandenkoornhuyse et al. 2002). Thus, only a small number of mycorrhizal fungal taxons present in the roots might be matched to those described using spore morphology. It seems possible that the efficient root colonizers of *A. montana* do not sporulate or rarely sporulate under the field conditions and that they are also not easy to isolate under trap culture conditions, where they are often substituted by other strains. This might be caused by the composition of the substratum in trap culture, and also by the plant used to bait the fungal strains or other methodological reasons. A typical example is given by *G. tenue* that was demonstrated in the present paper as a fine root colonizer on the basis of presence of well-stained mycelium of ca. 0.8–1.3 μm, often forming typical fan-shaped structures and vesicles of 3–7 μm diameter (Turnau et al. 2005). It is a common fungus in a wide range of soils (including agricultural and forest soils) and at a wide range of altitudes, often recorded in lowlands and in high mountain soils. Due to the small size of spores (10–15 μm), the species is difficult to isolate using conventional methods. As strains of this fungus are tolerant to pasteurization, they might be separated from other AMF by heat treatment. Still, this species is not easy to maintain under greenhouse conditions; to our knowledge, no DNA sequences of this fungus are available that could be compared to our data. Moreover, according to microscopic observations (unpublished data), the strains from different plants and sites seem to be relatively diverse, what would suggest that in fact we

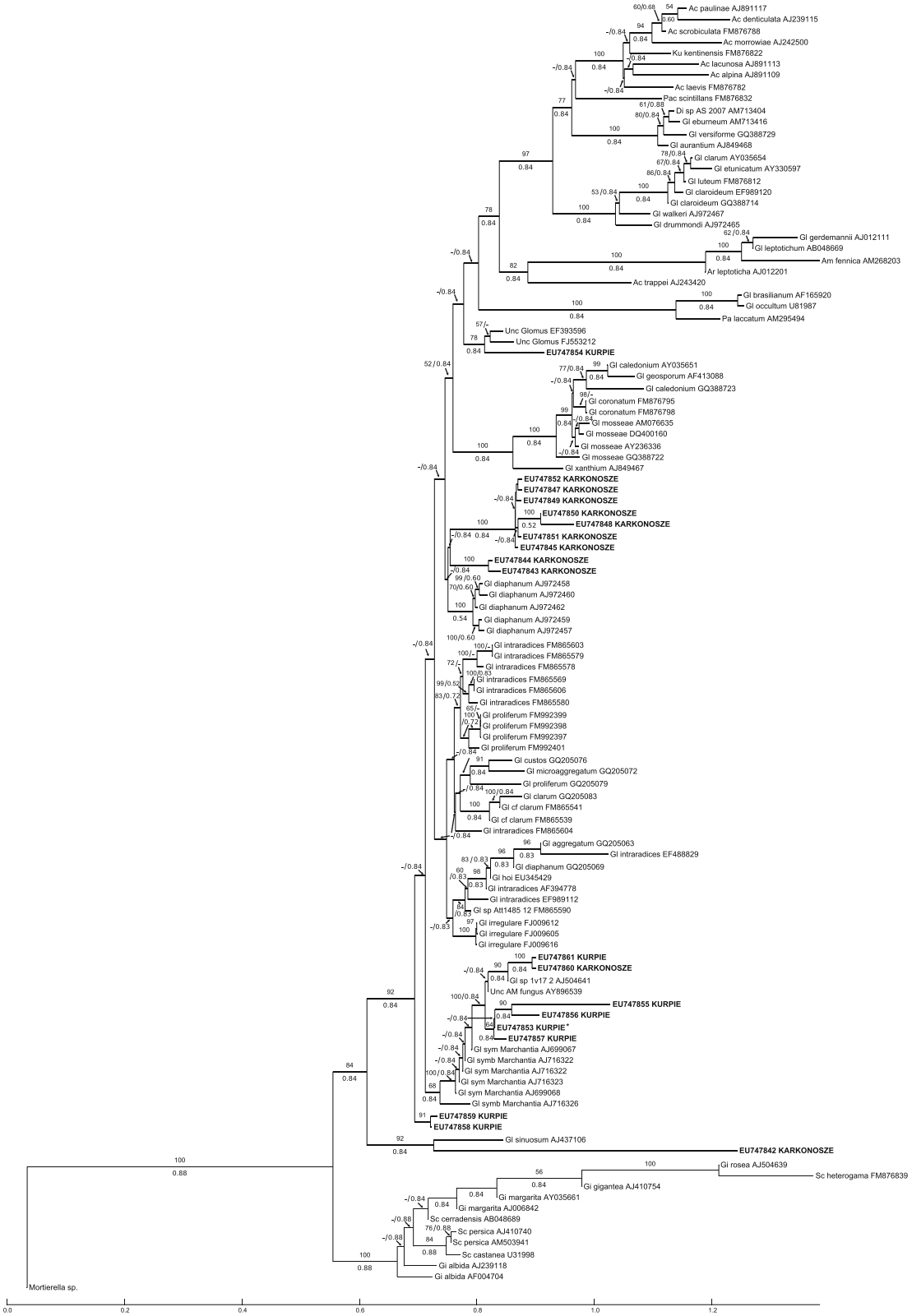

Fig. 3 Neighbor-joining tree showing phylogenetic relations among rDNA sequences of AMF colonizing roots of *A. montana*. *Mortierella* sp. was used as an outgroup. The tree was calculated using likelihood settings from best-fit model (GTR+G) selected by hLRT in Modeltest. The percentages of 10,000 bootstrap replications are indicated by *numbers above branches*, while the posterior probabilities are shown *below the branches*. Only values over 50% and 0.50, respectively, are shown. Sequences obtained during this study are given in *bold*; *asterisks* indicate sequences obtained with ITS1F/ITS4 primer pair; Karkonosze, Kurpie—*A. montana* sampling sites

have several different taxa that could be related to this fungus.

While the field-efficient colonizers are crucial under the natural conditions, it is still important to have strains that are easily multiplied and sporulate under laboratory conditions in order to produce well-defined inoculum for practical applications. AMF strains, different from those efficient under field conditions, may be equally or even more efficient as symbiotic partners while multiplying plant material prior for reintroduction into the natural stands or if the plant is envisaged to cultivate in the nurseries. Such a case was described by Zubek et al. (2008) for *Plantago atrata* and *Senecio umbrosus*. The native AMF isolates were equally effective as strains available in laboratory. Moreover, AMF related to the rhizosphere of *Pulsatilla slavica* in the natural habitat proved to be less effective than lab strains. The comparison between different strains in terms of efficiency of growth stimulation and secondary metabolite production will be the next step of the work aiming at the optimization of *A. montana* cultivation.

Acknowledgements The authors are grateful to MSc Katarzyna Piszczek (student of Jagiellonian University) for the help in culturing arnica and doing pilot studies. This work was supported by the Polish Ministry of Science and Higher Education (Grant No. 2 P04G 109 28) in years 2005–2007.

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