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ORIGINAL PAPER

Optimization of culture conditions of *Arnica montana* L.: effects of mycorrhizal fungi and competing plants

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Abstract *Arnica montana* is a rare plant that needs special protection because of its intensive harvesting for medicinal purposes. The present work was aimed at finding optimal culture conditions for *Arnica* plants in order to enable their successful reintroduction into their natural stands. Plants were cultivated under controlled greenhouse conditions on substrata with different nitrogen (N) concentration. As *Arnica* is always colonized by arbuscular mycorrhizal fungi (AMF) in nature, a fact that has been overlooked in other similar projects, we, here, applied and tested different inocula. We found that they differed in their effectiveness, both in establishing symbiosis, assessed by the colonization parameters, and in improving the performance of *Arnica*, evaluated by the photosynthetic parameters derived from the fluorescence transients (JIP-test), with the inocula containing *G. intraradices* or composed of several *Glomus* strains being the most effective. The comparison was possible only on substrata with medium N, since high N did not permit the formation of mycorrhiza, while at low N, few nonmycorrhizal plants survived until the measurements and mycorrhizal plants, which were well growing, exhibited a high heterogeneity. Analysis of secondary metabolites showed clearly that mycorrhization was associated with increased concentrations of phenolic acids in roots. For some of the inocula used, a tendency for increase of the level of phenolic acids in shoots and of sesquiterpene lactones, both in roots and in shoots, was also observed. We also studied the interactions between *A. montana* and *Dactylis glomerata*, known to compete with *Arnica* under field conditions. When specimens from both species were cultured together, there was no effect on *D. glomerata*, but *Arnica* could retain a photosynthetic performance that permitted survivability only in the presence of AMF; without AMF, the photosynthetic performance was lower, and the plants were eventually totally outcompeted.

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Introduction

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 July 9, 2004, Journal of Laws number 168, item 1,764, published on July 28, 2004). Active protection of this species, aimed at securing its existence and improving its population size, falls under the EU Habitats Directive (The Habitats, Fauna and Flora Directive, Annex V) number 92/43/EEG and EC Regulation number 338/97, Annex D. *Arnica* extracts stimulate blood flow, promote healing, and soothe arthritic pains. The species was used for medicinal purposes already since the Middle Ages. It became more widely

used since the 18th century against rheumatism and externally, as a cure for swellings and subcutaneous bleedings. Nowadays, the anti-inflammatory and antiseptic extracts of *Arnica* are used mostly external for treating bruises, swellings, and diseases of the mouth. Extracts of *Arnica* are also used in homeopathy (Kohlmünzer 2000; Strzelecka and Kowalski 2000).

A. montana contains several groups of active secondary compounds (Kohlmünzer 2000): sesquiterpenoids in the form of lactones, mainly helenalin and its derivatives (0.2–0.8%); flavonoids (0.4–0.6%), mainly in the form of glycosides (apigenin, hispidulin, eupafolin, kaempherol, and quercetin); and essential oils (0.2–0.5%), composed mainly of fatty acids. Among other components of *A. montana* extracts, some coumarins (umbeliferon and scopoletin) and phenolic acids (caffeic acid and cynarin) were found.

A. montana is cultured for industrial purposes, but its culture is difficult and nonprofitable; therefore, the participation of cultured plants in the overall material pool is minimal (Lange 1998). The programs designed for the reintroduction of *Arnica* to its natural stands, often sponsored by pharmaceutical companies that use compounds isolated from this plant (i.e., the project sponsored by Weleda concerning the Vosges mountains in France and the area of Grison in Switzerland—Ellenberger 1998), overlook the fact that under field conditions, *Arnica* forms mycorrhizal symbiosis with arbuscular fungi (Heijne et al. 1996; Eriksen et al. 2002). This aspect was recently studied using molecular and conventional methods (Ryszka, Błaszkowski, Jurkiewicz, and Turnau; manuscript submitted to Mycorrhiza—now under revision), all analyzed *Arnica* plants in natural sites were found to be colonized by a wide range of arbuscular mycorrhizal fungi. However, since any nonmycorrhizal *Arnica* plants can hardly be found to permit the comparison with mycorrhizal plants, the impact of symbiosis in the field cannot be evaluated.

It is known that plants, depending on the species and on particular conditions in the soil, differ in their dependence on symbiosis. Under some conditions, such as in areas poor in mineral compounds and are 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 both for individual plants and for the 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. 1998a, b). The mycelium forms a net, connecting plants even belonging to different species, where seedlings can plug into and use its water and mineral pools (Turnau and Haselwandter 2002; Jeffries et al. 2003; Renker et al. 2003). The interaction of arbuscular mycorrhizal fungi (AMF) and plants is beneficial for both partners (Hayman 1983); fungi use the easily accessible carbon compounds produced by plants during photosynthesis and the plant benefits by increased uptake of nutrients such as P and N and increased interface between roots and soil by means of increased penetration of soil by the mycelium (Smith and Read 1997). AMF positively increase the succession rate (Turnau and Haselwandter 2002) and the tolerance to heavy metals (Turnau et al. 2006), water stress, extreme salinity (Ruiz-Lozano and Azcon 2000), pathogenic fungi, and nematodes (Azcon-Aguilar and Barea 1997; Pozo et al. 2002). Mycorrhizal plants, sometimes, show larger biomass and faster growth rate than nonmycorrhizal ones (Gupta and Janardhanan 1991). Mycorrhiza may also improve seedling survival both in natural stands and laboratory conditions, especially during the critical stage of weaning (Grotkass et al. 2000). Mycorrhiza was also found to influence the level of plant secondary metabolites (Abu-Zeyad et al. 1999; Fester et al. 1999; Strack et al. 2003; Copetta et al. 2006; Khaosaad et al. 2006).

It was, therefore, of high interest to investigate whether mycorrhization plays such a beneficial role in the case of *A. montana*. The main aims of the present study were: (i) optimization of culture methods of *A. montana*, supported by mycorrhizal fungi; (ii) determination of quantitative and qualitative differences in secondary metabolites; and (iii) recognition and evaluation of interactions between *A. montana* and other plant species. Since preliminary experiments performed in garden conditions proved to be unsuccessful, we focused on the cultivation of the plants under chosen controlled conditions in greenhouse. Applying different combinations of substrata and mycorrhizal inocula, we investigated the extent of mycorrhizal colonization and the impact of mycorrhization on plant performance and on the production of secondary metabolites with special focus on sesquiterpene lactones and phenolic acids, which are on the list of mountain *Arnica*-derived substances (Kohlmünzer 2000) and according to pilot studies conducted previously, could be identified in roots and leaves of plants inoculated with mycorrhizal fungi and noninoculated. Plant performance was assessed by shoot biomass, as well as by biophysical methods, the JIP-test, which translates the polyphasic chlorophyll (Chl) *a* fluorescence transient OJIP exhibited by plants upon illumination (Strasser et al. 1995) to biophysical parameters evaluating photosynthetic performance. The JIP-test, which has been proven to be a very useful noninvasive tool for the investigation of stress effects on plants (for reviews, see Strasser et al. 2000, 2004), has been also successfully used for the evaluation of the beneficial role of mycorrhization (Calantzis et al. 2000; Tsimilli-Michael et al. 2000, 2008; Pinior et al. 2005; Corrêa et al. 2006; Strasser et al. 2007; Tsimilli-Michael and Strasser 2008; Zubek et al. 2009). We

also investigated, for the optimal combination of substratum/inoculum, the effect of the presence of *Dactylis glomerata*, known to compete with *A. montana* under field conditions.

Materials and methods

Inocula preparation and testing

The inocula used were: (1) *Glomus geosporum* UNIJAG PL 12-2 (G. geos.); (2) *G. constrictum* 265-5 Walker (G. cons.); (3) *G. intraradices* BEG 140 (G. intr. 140); (4). *G. intraradices* UNIJAG PL24-1 (G. intr. 24); (5) a mixture composed of the above and of *G. mosseae* BEG 12 (MIX); (6) crude inoculum obtained from samples collected in Kurpie (Kurpie); and (7) crude inoculum obtained from samples collected in Karkonosze (Karko) (Abbreviations, written in brackets after each name, are employed for the sake of figures' simplicity). The fungi for the inocula one to five were multiplied in pots (3 l) on sterile substratum (sand:expanded clay 3:1, *v*:*v*, supplied with rock phosphate, 50 g/l) with *Zea mays* and *Plantago lanceolata* as host plants. After 4 months of cultivation, the shoots were harvested, and the inocula were allowed to air dry for 30 days.

The quality of the prepared inocula was assessed as follows: *P. lanceolata* seedlings were inoculated with 50 g of dried inoculum per 500 ml pot, and after 6 weeks of cultivation, the roots were harvested, stained, and examined for AM colonization according to Trouvelot's method (Trouvelot et al. 1986). The same procedure was followed for assessing the quality of the crude inocula (6–7), but mixing 100 g of soil containing plant roots, spores, and mycelium from the original soil with the substratum in a 500 ml pot. It was found that inoculation resulted in colonization by AMF of over 90% of root length.

Plant cultivation

The study was carried out on material cultured in the laboratory from seeds obtained from a commercial supplier (Planta-Naturalis, Markvartice, Czech Republic; http://www.plantanaturalis.com). Seeds of *A. montana* were germinated on wet filter paper in Petri dishes. The germination started after 7 days, and the fraction of germinated seeds reached the 50–70% after 14 days. One week-old seedlings were transferred into 400 ml pots with different substrata in the absence or presence of the above-described inocula. The substrata composed of a mixture of garden soil (containing peat, sand, pine bark, and compost enriched with organic fertilizers and dolomite), sand, and expanded clay at different ratios; their nitrogen concentration was determined with Kjeldahl method and the phosphorus concentration with the Vanadian method as described by Lityński et al. (1968). Ten weeks old individual plants were transferred into 200 ml pots (7–12 plants for each type of inoculum depending on the survival of the plants) and cultivated for 3 months more. The pots were kept in sealed sun bags (Sigma–Aldrich, Poland) under greenhouse conditions at 20°C under the following light regime: $100 - 110\mu$ mol PAR photons $\times$ m^{-2} $\times$ s^{-1}, 12 per 12 h (similar to conditions for understory plants). Sun bags are transparent for light and equipped with a ventilation system. The pots were watered in excess and allowed to stabilize for 1 day. Excess water was removed from the bag, and the saturated culture weight was recorded. Water was added to a pot when its weight (checked weekly) was below the saturated culture weight (in grams) multiplied by 0.85 plus 30 g (protocol by Christopher Walker, personal communication). This system allowed for keeping the culture under constant moisture regime. Experiments were also conducted by planting *D. glomerata* seedlings together with *Arnica* seedlings in the same pots or alone with or without mycorrhizal fungi.

Mycorrhizal colonization

The following parameters were assessed in 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, and cleared in 10% KOH for 24 h at room temperature. Subsequently, after careful 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 lactic acid, in order to visualize the fungal structures inside the roots; material obtained in this way was cut into 1 cm pieces and mounted on slides in lactic acid (approximately 45 root pieces per plant). Mycorrhizal parameters were assessed and calculated according to Trouvelot's method (Trouvelot et al. 1986; the method can be also found at http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

Chlorophyll *a* fluorescence measurements and the JIP-test

The Chl *a* fluorescence measurements were conducted on dark-adapted leaves (five rosette leaves) still attached to the plants. Data presented in this paper were obtained at the end of the cultivation period and for each of 7–12 plants not inoculated or inoculated with a particular fungal strain.

The fluorescence transients OJIP (Strasser et al. 1995), measured with a Handy PEA fluorimeter (Plant Efficiency Analyser, Hansatech Instruments, GB) were

induced by red light (peak at 650 nm) of 600W × m^{-2}(3,000μ mol photons × m^{-2} × s^{-1}) provided by an array of three light-emitting diodes and recorded for 1 s, with the first reliable measurement at 20 μs after the onset of illumination. The data acquisition was every 10 μs (in the interval from 10 μs to 0.3 ms), every 0.1 ms (0.3–3 ms), every 1 ms (3–30 ms), every 10 ms (30–300 ms), and every 100 ms (300 ms to 1 s).

Each transient was analyzed according to the JIP-test (Strasser et al. 2004; Tsimilli-Michael and Strasser 2008), with the *Biolyzer* software (Laboratory of Bioenergetics, University of Geneva, Switzerland). The analysis utilizes the following original data: the maximal measured fluorescence intensity, F_P, equal here to F_M, since the excitation intensity is high enough to ensure the closure of all photosystem (PS) II reaction centers (RCs); the fluorescence intensity at 20 μs, considered as the intensity F_0 when all RCs are open; the fluorescence intensities at 2 ms (J-step; F_J) and at 30 ms (I-step; F_I); the complementary area (Area) above the fluorescence curve, i.e., the area between the curve, the horizontal line $F=F_M$ and the vertical lines at $t=20$ μs and at $t=t_{FM}$ (the time at which F_M is reached); and the fluorescence intensities at 50 and 300 μs ($F_{50\mu s}$ and $F_{300\mu s}$) required for the calculation of the initial slope M_0 of the V-kinetics $\left(M_0 = (\mathrm{d}V/\mathrm{d}t)_0 \cong V_{300\mu s}/250\mu s\right)$, where V is the relative variable fluorescence between F_0 and F_M:$V=(F-F_0)/(F_M-F_0)$. For the evaluation of the photosynthetic performance, the following biophysical parameters were chosen to be calculated in the present study, all referring to the condition of the sample at time zero (onset of fluorescence induction): (a) the quantum yields of primary photochemistry, $TR_0/ABS = 1 - F_0/F_M$, of electron transport from Q_A^- (the primary electron quinone acceptor of PSI) to plastoquinone (PQ), $ET_0/ABS = 1 - F_J/F_M$, and of the reduction of PSI end electron acceptors, $RE_0/ABS = 1 - F_I/F_M$; (b) the efficiencies/probabilities with which a trapped exciton can move an electron from Q_A^- to PQ, $ET_0/TR_0 = 1 - V_J$, and from the reduced PQ to the PSI end electron acceptors, $RE_0/ET_0 = (1 - V_I)/(1 - V_J)$; (c) the amount (arbitrary units) of active reaction centers per absorption, $RC/ABS = (TR_0/ABS)/(TR_0/RC) = (1 - F_0/F_M)/(M_0/V_J)$; (d) the total electron carriers per reaction center, $EC_0/RC = \mathrm{Area}/(F_M - F_0)$; and (e) the performance index PI_{ABS}, which combines the parameters RC/ABS, TR_0/ABS, and ET_0/TR_0 and the performance index PI_{total}, which combines PI_{ABS} and the parameter RE_0/ET_0. The samples were also compared in respect to the energetic connectivity (grouping) among the PSII units, by comparing the initial part (50–300 μs) of the transients after normalization between $F_{50\mu s}$ and $F_{300\mu s}$, as previously described (see e.g., Strasser et al. 2004; and Zubek et al. 2009).

Analyses of secondary metabolites of *A. montana*

Extraction for the sesquiterpene lactone analysis was performed using the modified method described by Douglas et al. (2004). Santonine was used as the external standard. Dried tissues were ground in a mortar, and 20 ml of methyl chloride was added. Extraction was performed in ultrasonic bath for 10 min and was repeated. Both extracts were pooled and evaporated to dryness on a rotatory evaporator (temperature 40°C for 30 min). The dry residue was dissolved in methanol, filtrated, and analyzed on HPLC system. All analyses were performed on Agilent Technologies 1100 HPLC System equipped with photodiode array detector (DAD) and MS API ESI with quadrupole mass analyzer.

Mobile phase: acetonitrile/water, gradient 50–60%, 0–5 min; 60–80%, 5–9 min; and 80–50%, 9–13 min and flow rate 1 ml/min; chromatographic column Eclipse XDB-C18 75×4.6 mm pore size 3.5 μm (Agilent Technologies, USA), thermostated at 25°C; DAD settings: 225 nm; MS settings: API APCI mode, drying gas flow 13 l/min, nebulizer pressure 40, drying gas temperature 275°C, capillary voltage 4,000 V, and corona current 15 μA; and negative single ion mode: 245, 261, 263, 303, 305, 329, 331, 343, 345, and 347 m/z, fragmentor 70, and gain 3.

The residual plant material (after the sesquiterpene lactones extraction) was air dried for 1 day to eliminate methyl chloride, then 10 ml of the extraction mix (methanol:water:acetone; (3:1:1; *v*:*v*:*v*)) was added. Extraction lasted 20 min and was repeated twice. Both extracts were pooled and evaporated to dryness on a rotatory evaporator (temperature 40°C for 120 min), and the dry residue was dissolved in methanol and analyzed on HPLC after filtration.

Mobile phase: acetonitrile/formic acid 0.1%, gradient 15–25%, ACN 0–15 min; 25–40%, 15–30 min; 4–15%, 30–33 min and flow rate 0.8 ml/min; chromatographic column HyPurity Aquastar 100×3 mm pore size 3 μm (Thermo, USA), thermostated at 25°C; DAD settings: 200 nm and 360 nm; and MS settings: API ESI mode, drying gas flow 10 l/min, nebulizer pressure 60, drying gas temperature 270°C, capillary voltage 4,000 V, positive scan mode 220–800 m/z, fragmentor 70, and gain 3.

The phenolic acids analyses were carried out according to the procedure described by Zidorn et al. (2005), without the use of standards; therefore, the results we obtained are in relative units. However, they can still be used to compare concentrations of the analyzed phenolic acids among tested plants. Specific compounds were identified by their specific molecular masses and UV spectra, and results were referred to those of Spitaler et al. (2006).

Statistical analysis

Statistical analysis of the photosynthetic parameters calculated from the measurements of Chl *a* fluorescence transients was conducted with the nonparametric Kruskal–Wallis test (STATISTICA version 7.0 software (Statsoft, USA)) and of the secondary metabolites with the *t* test (for both, $P<0.05$).

Results

Preliminary experiments

Preliminary experiments done in garden conditions were unsuccessful, as *Arnica* plants were strongly attacked by snails and soil insects, probably attracted by the secondary metabolites. Hence, we focused on the cultivation of the plants in greenhouse. We tried different conditions and found that *Arnica* cultivation becomes possible, though still with problems, but only under the controlled conditions described in the "Materials and methods" section; e.g., the nonmycorrhizal plants grown without the sun bags were very susceptible to heat and drought stress. Different combinations of substrata and inocula were tried, and each combination was examined in respect to the mycorrhizal colonization parameters and the photosynthetic parameters (JIP-test), searching for conditions under which the plants could survive and give reproducible results and also representative of substrata with high, medium, and low N and P concentrations.

After these preliminary experiments, our investigation was carried out on plants cultivated in mixtures of garden soil, sand, and expanded clay at 5:1:1 ratio (*v*:*v*:*v*)—characterized by high N (0.7%) and P (0.2%) concentrations; 5:4:1 ratio (*v*:*v*:*v*)—characterized by medium N (0.4%) and P (0.1%); and at 5:8:1 ratio (*v*:*v*:*v*)—characterized by low N (0.3%) and P (0.06%); the pH for all substrata was around 6.

Plant growth and mycorrhizal colonization

Culturing plants in pots filled with garden soil with high N and P (mixture 5:1:1) resulted in almost no development of mycorrhizal fungi, and no differences between plants were visible (data not shown). At the end of the cultivation period, the survival rate was about 90%.

At the other extreme, the low N and P (mixture 5:8:1), the mycorrhizal plants were developing well, and their survival rate at the end of the cultivation period was about 90%. On the contrary, nonmycorrhizal plants were several times smaller and dark green, and their survival rate strongly decreased with time, going down to about 16% at the end of the cultivation period. No statistically important differences in dry weight were found among plants inoculated with different AMF strains; however, there were differences concerning mycorrhizal colonization parameters, as shown in Fig. 1. Chemical analysis was not possible because the biomass of nonmycorrhizal plants was not sufficient.

At medium N and P (mixture 5:4:1), there were no statistically significant differences among the differently inoculated plants or between inoculated and noninoculated, in respect to shoot and root biomass. Their survival rate was also the same, being about 90% at the end of the cultivation period. However, statistically significant differences were found concerning the mycorrhizal colonization parameters, as shown in Fig. 2. The frequency of mycorrhizal colonization was about 100% for all, but mycorrhizal intensity—relative (M%) and absolute (m%)—were the highest when the laboratory-made mixture of various species of genus *Glomus* (MIX) was used, while arbuscule richness—relative (A%) and absolute (a%)—were the lowest when the crude inoculum from Kurpie was used.

Photosynthetic parameters of plants grown at different soil conditions, with or without inoculation

The photosynthetic parameters of plants grown under greenhouse conditions in pots filled with substratum rich

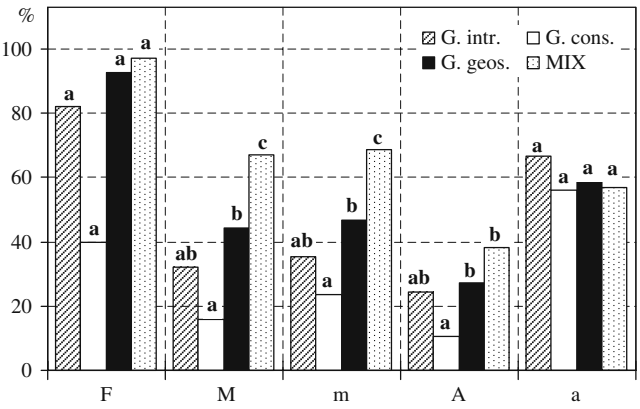

Fig. 1 Mycorrhizal colonization parameters determined in *A. montana* plants cultivated under greenhouse condition in substratum composed of a mixture of garden soil, sand, and expanded clay at 5:8:1 (*v*:*v*:*v*), characterized by low N and P. The substrata were inoculated, as indicated in the figure, with *G. intraradices* BEG 140 or UNIJAG PL24-1 (G. intr.; same for both strains), *G. constrictum* 265-5 Walker (G. cons.), *G. geosporum* UNIJAG PL 12-2 (G. geos.), or a laboratory-made mixture of *Glomus* fungi (MIX). The depicted parameters are: *F*—mycorrhizal colonization frequency, *M*—relative mycorrhizal root length, *m*—intensity of colonization within individual mycorrhizal roots, *A*—relative arbuscular richness, and *a*—arbuscule richness in root fragments where arbuscules were present. The numbers are given in percent, according to the parameter definitions by Trouvelot et al. (1986). Different *letters above columns* referring to the same parameter indicate statistically significant differences ($P<0.05$). For other details, see "Materials and methods" section

in N and P (5:1:1) were very similar, and no statistically significant differences were found between samples concerning any of the parameters (data not shown). This is in accordance with the visually recognized similarities and the finding that there was no development of mycorrhizal fungi.

Significant differences between treatments were neither observed in the case of plants cultivated at low N and P (substratum 5:8:1; data not shown), though mycorrhiza was well developed in inoculated plants (Fig. 2) and non-mycorrhizal plants were a few times smaller and their survival rate was much lower (see above in "Plant growth and mycorrhizal colonization" section).

When the plants were cultivated at medium N and P (substratum 5:4:1), the situation was different. The differences among inoculated plants and between them and the nonmycorrhizal are clearly revealed by the comparison of the fluorescence transients (Fig. 3), even before their analysis with the JIP-test. As shown in Fig. 3, which depicts the average fluorescence transients (30 replicates per treatment), normalized on F_0 to exclude differences of chlorophyll density, the transients exhibit different values of the F_M/F_0 ratio, which is a parameter related with the maximum quantum yield of primary photochemistry, $TR_0/ABS(= 1 - F_0/F_M)$. Still, this parameter is the least sensitive of all photosynthetic parameters (Strasser et al. 2004). The full JIP-test analysis showed differences concerning all other parameters, i.e., yields/efficiencies, RC/ABS, and EC/RC (see definitions in "Materials and methods" section; for their analytical description see Tsimilli-Michael and Strasser 2008). Here, we chose to present (Fig. 4) only the performance indexes PI_{ABS} and PI_{total}, which are products of terms expressing *potentials* for photosynthetic performance (partial performances) at the sequential energy bifurcations from exciton to PQ reduction and to the reduction of PSI end acceptors, respectively, and therefore, evaluate the overall photosynthetic performance. As shown in Fig. 4, the plants inoculated with laboratory prepared strains exhibited higher PI_{ABS} and PI_{total} than nonmycorrhizal plants and plants where crude inocula originating from Kurpie and Karkonosze were used. It should be mentioned that each of the parameters composing PI_{ABS} and PI_{total}, showed the same trend as their products.

The standard deviation (SD) of the photosynthetic parameters in plants cultivated at medium N and P was found to be higher in the case of nonmycorrhizal than in the cases of inoculated plants; for example, the SD for PI_{ABS}, expressed as percentage of the average value, was about 50% and in the range of 23–32%, respectively. On the contrary, in plants cultivated at low N and P, the SD was lower in the case of nonmycorrhizal than in the case of inoculated plants; for PI_{ABS}, it was 19% and 28–37%, respectively.

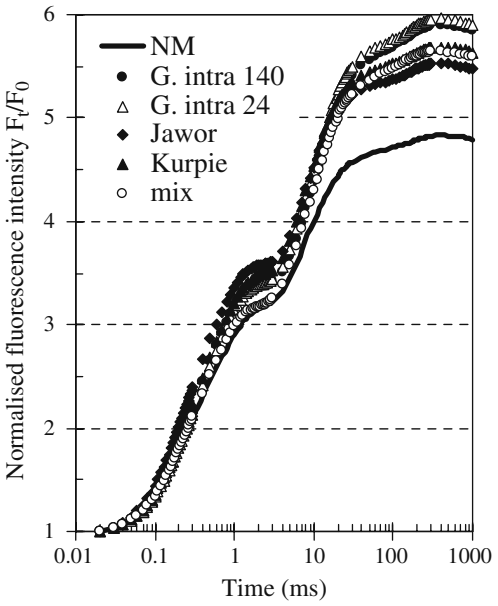

Fig. 3 Chl *a* fluorescence transients (OJIP) of dark-adapted leaves of the *A. montana* plants whose colonization parameters are presented in Fig. 2, as well as of noninoculated plants (NM). The inocula used (and NM) are indicated in the figure. Each *transient*, plotted on logarithmic time scale from 20 μs to 1 s, represents the average of raw fluorescence transients from 30 replicates, expressed as F_t/F_0 (F_0: the fluorescence intensity at 20 μs). For other details, see "Materials and methods" section

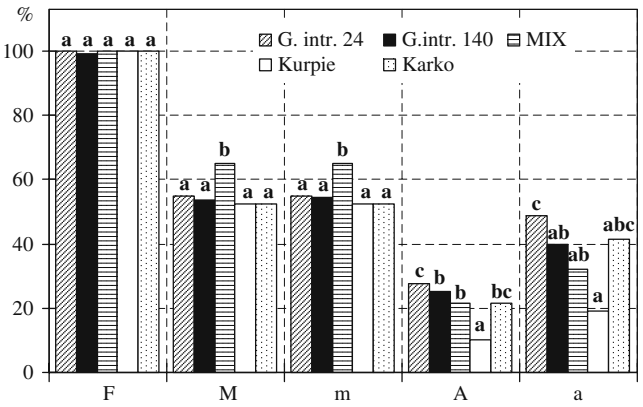

Fig. 2 Mycorrhizal colonization parameters (as in Fig. 1) determined in *A. montana* plants cultivated under greenhouse conditions in substratum composed of a mixture of garden soil, sand, and expanded clay at 5:4:1 (*v*:*v*:*v*), characterized by medium N and P. The substrata were inoculated, as indicated in the figure, with *G. intraradices* BEG 140 (G. intr. 140), *G. intraradices* UNIJAG PL24-1 (G. intr. 24), a laboratory-made mixture of *Glomus* fungi (MIX), or crude inocula obtained from samples collected in Kurpie or in Karkonosze (Karko). Different *letters above columns* referring to the same parameter indicate statistically significant differences ($P<0.05$)

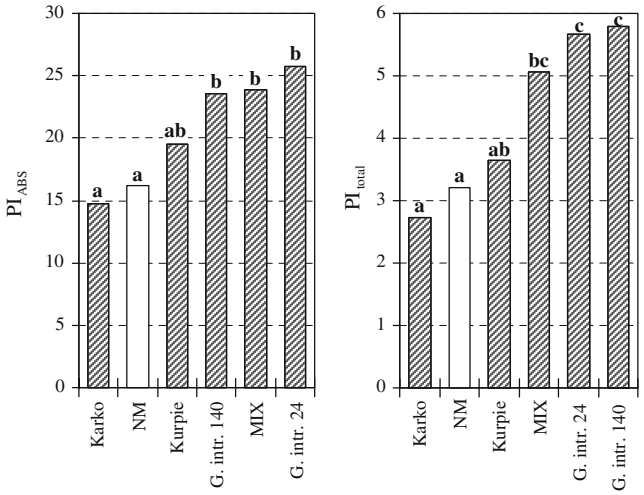

Fig. 4 The performance indexes (average values) PI_{ABS} (*left panel*) and PI_{total} (*right panel*) for the treatments presented in Fig. 3. The indexes were calculated, as described in the "Materials and methods" section, from the analysis of the same transients (30 replicates per treatment) whose averages are depicted in Fig. 3. In each panel, the *columns* are presented in *ascending order*; a different color (*white*) was used for the NM column, to facilitate visual comparison. Different *letters above* the *columns* in the same panel indicate statistically significant differences ($P<0.05$)

Further than the photosynthetic parameters related with regulations of activity, we also compared the different treatments concerning the extent of energetic connectivity (grouping) among the PSII photosynthetic units; a higher grouping results in bigger utilization of excitation energy and moreover, a higher stability of the photosynthetic system (Strasser et al. 2004). Our investigation (data not shown) revealed that there were no differences among treatments in the case of 5:8:1 substratum. On the other hand, in the case of 5:4:1 substratum, the plants for which the laboratory prepared inocula and the crude inoculum from Kurpie were used had higher grouping than the nonmycorrhizal plants and those for which the crude inoculum from Karkonosze was used.

Sesquiterpene lactones and phenolic acids in mycorrhizal and nonmycorrhizal plant roots and shoots

The results from the analysis of roots and shoots concerning sesquiterpene lactones and phenolic acids are presented in Tables 1 and 2, respectively. Peaks assignable to sesquiterpene lactones (s1–s7) and to phenolic acids (p1–p7) were numbered consecutively with increasing retention time. For each group of secondary metabolites and each inoculation condition, the concentration of the

Table 1 Sesquiterpene lactone mean concentrations (three replicates per case) in nanogram per gram dry weight of roots (R) and shoots (S) of *Arnica montana* plants

		s1	s2	s3	s4	s5	s6	s7	total
NM	R	1,185	0	2,086	1,837	0	0	0	5,108
Karko	R	790	0	1,563	335	0	0	0	2,687
Kurpie	R	1,633	0	3,608	1,296	0	0	0	6,537
G. intr. 24	R	831	0	1,034	882	0	0	0	2,746
G. intr. 140	R	1,425	0	3,513	2,079	0	0	0	7,017
MIX	R	548	0	1,144	0	0	0	0	1,693
NM	S	128,575	35,946	239,061	111,082	86,488	36,830	32,863	670,844
Karko	S	114,877	16,676	292,028	92,552	100,627	27,523	25,255	669,538
Kurpie	S	75,461	78,175	225,623	199,993	106,562	60,259	32,481	778,554
G. intr. 24	S	48,796	28,933	169,211	158,504	64,820	41,063	26,287	537,613
G. intr. 140	S	66,874	59,931	203,058	173,051	84,082	47,070	29,796	663,862
MIX	S	88,799	14,369	215,810	56,057	84,320	20,753	37,732	517,839

Sesquiterpene lactone mean concentrations (three replicates per case) in nanogram per gram dry weight of roots (R) and shoots (S) of *Arnica montana* plants cultivated under greenhouse conditions in substratum composed of a mixture of garden soil, sand, and expanded clay at 5:4:1 (*v*:*v*:*v*), characterized by medium N and P (like in Figs. 2-4), noninoculated (NM) or inoculated with *G. intraradices* BEG 140 (G. intr. 140), *G. intraradices* UNIJAG PL24-1 (G. intr. 24), a laboratory-made mixture of *Glomus* fungi (MIX), or crude inocula obtained from samples collected in Kurpie or in Karkonosze (Karko). The substances found in the extracts, characterized by molar weight (m) and retention time (rt) were identified according to the literature as: acetyl-dihydrohelenalin (s1; m=306; rt=1.61), acetyl-helenalin (s2; m=304; rt=1.74), isobutyryl-helenalin (s3; m=332; rt=2.61), methacryloyl-helenalin/isobutyryl-dihydrohelenalin (s4; m=330; rt=2.84), isovaleryl-helenalin (s5; m=346; rt=3.14), tigloyl-helenalin (s6; m=344; rt=3.43), and 2-methylbutyryl-dihydrohelenalin/isovaleryl-dihydrohelenalin (s7; m=348; rt=3.85)

For each inoculation condition, the total concentration is also depicted. Concentrations are written in bold and underlined when they exhibited a statistically significant difference (at $P<0.05$) from the corresponding concentration in the nonmycorrhizal plants

Table 2 Phenolic acid mean concentrations (three replicates per case) in nanogram per gram dry weight of roots (R) and shoots (S) of *Arnica montana* plants for which the sesquiterpene lactones' concentrations are depicted in Table 1

		p1	p2	p3	p4	p5	p6	p7	total
NM	R	4,002	3,509	652	654	586	1,322	93	10,819
Karko	R	15,063	12,769	2,034	**1,921**	**2,779**	4,398	130	**39,094**
Kurpie	R	**7,691**	**17,633**	**3,235**	**2,201**	**3,088**	**5,605**	**538**	**39,991**
G. intr. 24	R	4,754	**11,488**	**1,904**	**1,503**	**2,451**	**3,327**	**281**	**25,708**
G. intr. 140	R	5,573	6,920	1,010	1,151	2,859	2,053	151	**19,717**
MIX	R	6,933	**13,641**	2,171	1,185	1,760	**3,549**	295	**29,535**
NM	S	18,318	14,930	4,425	1,191	206	11,334	646	51,051
Karko	S	47,579	62,790	12,020	3,454	286	20,170	1,014	**147,312**
Kurpie	S	14,690	31,408	6,584	2,223	194	17,581	973	73,652
G. intr. 24	S	12,843	29,954	6,012	1,479	175	12,247	812	63,522
G. intr. 140	S	9,882	16,314	3,398	**372**	172	7,487	465	38,091
MIX	S	11,070	10,923	2,847	**260**	134	9,211	622	35,067

The substances found in the extracts, characterized by molar weight (m) and retention time (rt), were identified according to the literature as: isomeric dicaeoylquinic acids (p1; m=516; rt=6.6), 1-methoxyoxaloyl-3,5-dicaeoylquinicacid or its isomer (p2; m=602; rt=7.1), 1-methoxyoxaloyl-3,5-dicaeoylquinicacid or its isomer (p3; m=602; rt=8.7), hitherto unknown methoxyoxaloyl-tricaeoylquinic acid derivative (p6; m=764; rt=16.22), and unidentified substances p4 (m=246; rt=9.83), p5 (m=764; rt=13.41), and p7 (m=764; rt=17.97)

For each inoculation condition, the total concentration is also depicted. Concentrations are written in bold and underlined when they exhibited a statistically significant difference (at $P<0.05$) from the corresponding concentration in the nonmycorrhizal plants

individual substances (three extracts' replicates per case), as well as their total concentration, are depicted in nanogram per gram dry weight of roots (R) and shoots (S).

The analysis revealed that helenalin and its derivatives were the major sesquiterpenes in the studied plant, with their total concentration in shoots being much bigger than in roots (about 80–300 times). Due to a wide heterogeneity among replicates, only in very few cases, the differences between inoculated and noninoculated plants are statistically significant (Table 1); the comparison cannot be clear. Still, we observe higher total concentration in roots upon inoculation with *G. intraradices* BEG 140 or with fungi from Kurpie, with the latter resulting also in an increase in shoots; all other inocula result in lower total concentrations. In roots, only the s1, s2, and s4 were detected. The same substances were, in general, more abundant in shoots, with the concentration of s3 being the highest, both in roots and in shoots.

Clear results were obtained concerning the phenolic acids in roots (Table 2). The total concentration of these substances was in all cases statistically higher in inoculated than in noninoculated plants; the differences were the biggest in the cases of inoculation with fungi collected in Kurpie or in Karkonosze. Concerning the total phenolic acid concentration in leaves, it was statistically higher (much higher) only in plants inoculated with fungi from Karkonosze. Still, we can observe that inoculation with *G. intraradices* BEG 24 and fungi from Kurpie resulted in some increase, while the other two inocula resulted in a decrease. We also observe that the total concentration in shoots is bigger than in roots, but only by up to about 4.7 times. Both in roots and in shoots, the p2 is the most abundant, followed by p1 and p6.

Plant interactions

D. glomerata is one of the many species known to compete with *A. montana* under field conditions. We were, therefore, interested to investigate this competition, with or without inoculation, under the selected controlled greenhouse conditions. We used the substratum with medium N and P concentrations (5:4:1), which, as shown above, is the only to bring significant differences among treatments and, for inoculation, the two strains of *Glomus intraradices,* which were found to bring the widest differences from the nonmycorrhizal plants (Fig. 4).

In this experiment, we studied all combinations: *A. montana* and *D. glomerata* were grown both individually or together, either with or without inoculation. Inoculated *D. glomerata* was colonized up to 10% but arbuscules were rare. No differences were found in mycorrhizal colonization rate and in plant growth of *D. glomerata* in the presence or absence of *A. montana*. On the contrary, the differences in these parameters were clear in the case of *A. montana*. When *A. montana* was grown with *D. glomerata*, the mycorrhizal colonization parameters were significantly lower (data not shown). At the beginning, the survival of *A. montana* seedlings was 100% in all groups. However,

1 month old seedlings growing alone (mycorrhizal and nonmycorrhizal) already showed better growth than those cultivated in the presence of *D. glomerata*, and this difference became more pronounced with time. Also, the differences between mycorrhizal and nonmycorrhizal plants started to be more visible; the presence of *D. glomerata* strongly inhibited the growth, especially of nonmycorrhizal *A. montana*, and plants older than 3 months started to die.

It was very interesting to find that the fluorescence measurements revealed the big effect of *D. glomerata* on *A. montana* almost from the beginning of their coexistence, as well as that the effect of *A. montana* on *D. glomerata* is less extended. This is even recognized without the JIP-test analysis, from the differences among the average fluorescence transients (normalized on F_0) obtained from the different combinations (Fig. 5). Applying the JIP-test, we found (data not shown) that the rate of electron transport from PQ to the PSI end electron acceptors was the same in all combinations, except in the case of nonmycorrhizal *Arnica* in the presence of *D. glomerata*, where it was smaller; this was also associated with a higher RE_0/ET_0 and a higher EC_0/RC, mainly related with a wide decrease of RC/ABS, which indicates that the main effect was a down regulation of the PSII function while the PSI electron acceptor side was not affected. In all other efficiencies of energy conservation (and the RC/ABS ratio), the following trend was observed: in *Arnica*, inoculation resulted in an increase and the presence of *D. glomerata* in a decrease, which was eliminated when *Arnica* was inoculated. For *D. glomerata*, inoculation brought an increase, though less extended than in the case of *Arnica*, but the presence of *Arnica* did not appear to play any significant role. This trend is shown, indicatively, by the PI_{ABS} in Fig. 6. The grouping extent (data not shown) was also found to follow the same trend, being the smallest (highest instability) in the nonmycorrhizal *Arnica* grown in *D. glomerata* presence.

For both species, the SD of the photosynthetic parameters was found to be higher in nonmycorrhizal than in mycorrhizal plants, being the highest in the case of nonmycorrhizal *Arnica* plants in *D. glomerata* presence. For example, the SD of PI_{ABS}, expressed as percentage of the average value was as follows (using the same abbreviations as in Fig. 6): A−, 53%; A+, 25%; A−, D, 78%; A+, D, 12%; D−, 28%; D+, 24%; D−, A, 38%, and D+, A, 11%.

Discussion

Plants that inhabit poor acidic soils, as it is in the case of *Arnica*, are usually dependent on their symbiotic partner. Researchers mostly pay attention to ericoid or ectomycorrhizal fungi and their plant symbionts and, so far, there is little knowledge on the AMF influencing plants, especially those growing in forest or forest clearings.

In nature, *Arnica* is always colonized by arbuscular mycorrhizal fungi. However, as revealed by conventional and molecular research previously carried out on *Arnica*

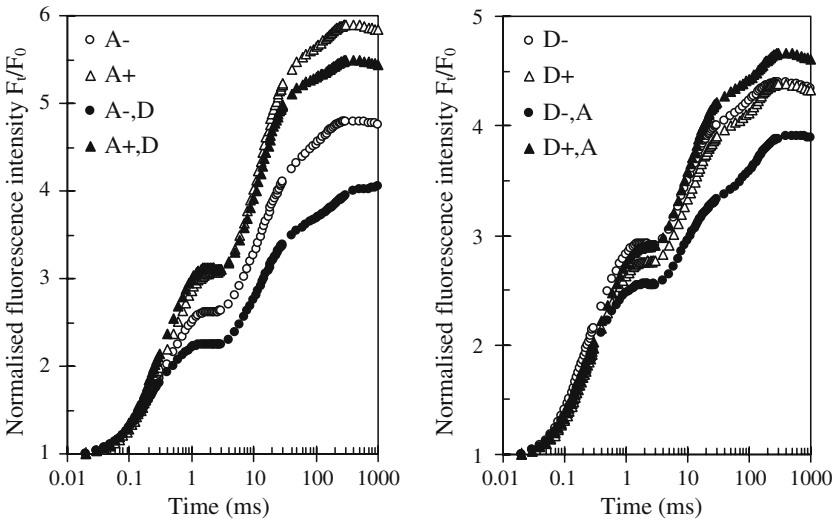

Fig. 5 Chl *a* fluorescence transients (OJIP) of dark-adapted leaves of *A. montana* (A) and *D. glomerata* (D) plants cultivated under greenhouse condition in the substratum characterized by medium N and P (substratum 5:4:1; as in Figs. 2-4), inoculated (*positive sign*) or not (*negative sign*) with the two strains of *G. intraradices*. The plants were cultivated individually (A+, A−, D+, and D−) or in the same container; in the second case the abbreviated descriptions indicated in the figure are *A*− and *D* and *A*+ and *D* for *A. montana* in *D. glomerata* presence and *D*− and *A* and *D*+ and *A* for *D. glomerata* in *A. montana* presence. Each *transient* represents the average of raw fluorescence transients from 10 replicates, expressed as F_t/F_0. For other details see legend of Fig. 3

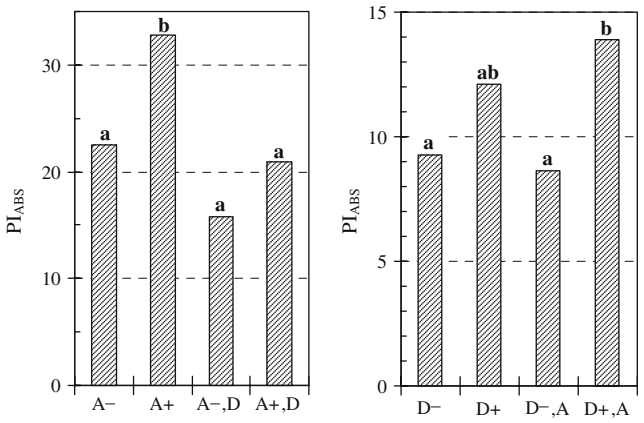

Fig. 6 The performance index PI_{ABS} (average values) calculated, as described in the "Materials and methods" section, from the same *transients* (10 replicates per treatment) whose averages are depicted in Fig. 5. The results for *A. montana* are depicted in the *left panel* and those for *D. glomerata* in the *right panel*. Different *letters above* the *columns* in the same panel indicate statistically significant differences ($P<0.05$)

collected from natural stands (Ryszka, Błaszkowski, Jurkiewicz, and Turnau; manuscript submitted to Mycorrhiza—now under revision), the AMF strains that effectively colonize *Arnica* roots at natural sites are not isolated in the trap cultures, but only fungi that are forming spores around the roots without colonizing them. Still, as shown in the present paper, the strains originating from such spores obtained from samples collected in Kurpie and in Karkonosze, are effective as root colonizers of *Arnica* in pot cultures. The same was found to be true for other fungi, available at the laboratory, namely *G. geosporum* UNIJAG PL12-2, *G. constrictum* 265-5 Walker, *G. intraradices* BEG 140 and *G. intraradices* UNIJAG PL24-1, as well as for a mixture of the above with *G. mosseae* BEG 12. Though it is known that arbuscular mycorrhiza is usually a little specific with regard to the fungus and plant species, there are cases where significant differences in the intensity of colonization and the effectiveness of symbiosis were observed (Orlowska et al. 2005a, b; Cesaro et al. 2008). Therefore, our finding is not trivial and, moreover, opens important perspectives since *Arnica* is a rare and endangered plant. Strains obtained from trap cultures, though not being colonizers of the plants in natural site, as well as commonly available fungi, proved to be useful in precultivation of *Arnica* plants, which can then be reintroduced into the original sites; the fungi present in their roots will help the establishment of the plant, although they will be, eventually, substituted by other fungi.

In our study, parallel to the investigation of the effectiveness of mycorrhizal development, assessed by the mycorrhizal colonization parameters, we also studied the survival rate of the plants and investigated the impact on plant photosynthesis, evaluated by the photosynthetic parameters derived from the fluorescence transients (JIP-test). At low N and P, the survival rate of noninoculated plants was only about 16% and of mycorrhizal plants about 90%, which is in good agreement with studies on *Baptisia tinctoria* where an increase in survival of up to 95% when seedlings were inoculated with mycorrhizal fungi was demonstrated (Keller et al. 1997; Hutter et al. 2001). It is worth noting that a survival rate of about 90% was also found at high N and P, where inoculation was not successful, but also at medium N and P both for mycorrhizal and noninoculated *Arnica* plants.

The JIP-test, which has been shown in many previous reports (cited in the "Introduction" section) to be a very useful tool for recognizing and evaluating the role of symbiosis in plants' performance, proved also to be very useful in our present study. The JIP-test has the added advantage of being a noninvasive method, which is especially recommended in research concerning valuable plant material like endangered species, which should not be destroyed as, e.g., by biomass measurements. It can, therefore, be also applied in our future work for monitoring the establishment of reintroduced species in natural sites, even more, because it provides an early diagnosis of vitality differences (Strasser et al. 2004).

Preliminary experiments showed that the cultivation of *Arnica* plants, even of those that were mycorrhizal, can be successful only under controlled conditions in a greenhouse. Different combinations of substrata and inocula were tried, and each combination was examined in respect to the mycorrhizal colonization parameters and the photosynthetic parameters. Even under the optimal external conditions chosen on the basis of the preliminary experiments, mycorrhiza development and efficient stimulation of plant photosynthesis were found to depend strongly on the N and P concentration in the substratum.

It was found that a high concentration of N (substratum with soil:sand:clay; ratio 5:1:1; *v*:*v*:*v*) did not permit the formation of mycorrhiza; concomitantly, no differences concerning the photosynthetic parameters were detected between inoculated and noninoculated plants neither among plants inoculated with different strains. However, it should be emphasized that these nonmycorrhizal plants managed not only to grow and survive but also to have high photosynthetic parameters because of the chosen controlled conditions for their cultivation. This would not happen in nature if N concentration would be similarly high and hence, forbid mycorrhization. The nonmycorrhizal *Arnica* plants are very sensitive, and they would be outcompeted by plants that have better developed root system.

On the other hand, at too low N and P levels (substratum with soil:sand:clay; ratio 5:8:1; *v*:*v*:*v*) mycorrhization was

rather successful but the plants exhibited a high heterogeneity in respect to the mycorrhizal colonization parameters, which masked possible differences among plants inoculated with different strains, though the average values of the parameters appear different, most of their differences are statistically insignificant. In accordance, the same was also found concerning the photosynthetic parameters. Neither the differences of the photosynthetic parameters between mycorrhizal and nonmycorrhizal plants were statistically significant, though the nonmycorrhizal ones had very low survival rate (16% versus 90% for the mycorrhizal plants).

This finding should not be considered as a paradox, since the survival rate is a statistical description of the whole group of planted seedlings, while the photosynthetic parameters refer, inevitably, only to the few plants that survived until the measurements. Moreover, this finding shows that the nonmycorrhizal plants that survived, though they were smaller and their growth was arrested, did not have a lower photosynthetic capability than the mycorrhizal plants; it is worth to remind here that all the calculated photosynthetic parameters are on absorption basis (and not on plant basis). It can be reasonably assumed that these few plants, which appeared healthy concerning the photosynthetic performance, were those that had managed, even in the absence of mycorrhizal mycelium and even from a poor in nutrients substratum, to uptake an amount of nutrients, though still not enough to permit a normal growth. On the other hand, under poor soil conditions, the mycorrhizal benefit was not the same for all replicates of inoculated plants and therefore, the evaluated photosynthetic performance represents the average of *more* healthy and *less* healthy (but still surviving) inoculated plants. This explanation is supported by the finding that the standard deviation of the photosynthetic parameters was lower in the case of nonmycorrhizal than in the case of inoculated plants; in other words, there was lower heterogeneity among the (survived) nonmycorrhizal plants than among the heterogeneously benefited inoculated plants.

At medium N and P concentrations (substratum with soil:sand:clay; ratio 5:4:1; *v*:*v*:*v*), which was found to support mycorrhizal and nonmycorrhizal plants, with both producing similar biomass, the photosynthetic parameters were found to be lower in nonmycorrhizal than in mycorrhizal plants. However, it is clear that the level of plant benefit depends on the fungal strain and species, which fits well with previous findings (Orłowska et al. 2005b). The most effective under the greenhouse conditions of the present work in stimulating plant activity were inocula containing *G. intraradices* and the inoculum composed of several AMF strains. The inocula originating from Kurpie and Karkonosze Mountains (Karko) were not effective, probably because forest fungi require much lower pH than pH=6 used in our experiments.

The finding that the standard deviation of the photosynthetic parameters was higher in the case of nonmycorrhizal than in the cases of inoculated plants is in accordance with the explanation we gave above for the standard deviations in the case of the 5:8:1 substratum at medium N and P substratum, more nonmycorrhizal plants managed to survive (90%; the same as for mycorrhizal plants); hence, to be measured, but with bigger heterogeneity among them concerning their vitality (from *less* to *more* healthy) than among the inoculated replicates that benefited both from the soil conditions and mycorrhization.

It is worth commenting that higher photosynthetic performance was followed, in all studied cases (including the case of *A. montana* and *D. glomerata* interaction), by higher energetic connectivity (grouping) among the PSII units, which is related not only with a bigger utilization of excitation energy, but also with a higher stability of the photosynthetic system (Strasser et al. 2004; Tsimilli-Michael and Strasser 2008).

As known from the literature, mycorrhiza induces many changes in plant physiology (Morandi 1996; Strack et al. 2003). Dehne and Schönbeck (1979) showed that *G. mosseae* increases the total soluble phenol concentration of the roots. Phenolic compounds were shown to be involved in many plant functions and many reports underline their protective role against oxidative stress that originates from various environmental factors (Rice-Evans et al. 1997; Santiago et al. 2000; Jung et al. 2003). This might be in line with the effect of mycorrhiza on increased survival in mycorrhizal forming plants. Increased phenolic metabolism in plant roots has been suggested as part of the mechanism involved in biocontrol, although, still, some controversy exists. Cell wall-bound phenolics are known to strengthen the cell wall and thus, prevent invasion by pathogens. The colonization of roots by mycorrhizal mycelium causes the induction of specific genes and the production of specific proteins (Bestel-Corre et al. 2002). This results in increased resistance of the plant towards harmful environmental conditions and pathogen attack. Interesting results have been also obtained concerning secondary metabolite concentration in *Arnica* flowers (see e.g., Spitaler et al. 2006; and Ganzera et al. 2008).

The analysis of secondary metabolites carried out in the present study was done on younger plants and only on roots and shoots. The analysis was only performed on plants grown at medium N and P, since the biomass of the plants grown on the poorer substratum was not sufficient and the plants grown at high N and P were not mycorrhizal. Concerning the total sesquiterpenes content, though there are wide differences among reported data for flowers, we can roughly conclude from our present study that this content is lower in shoots than in flowers and much lower in roots.

Our analysis showed that the concentration of sesquiterpene lactones is much higher in shoots than in roots. We would reasonably expect that the concentration in roots of these potentially antimicrobial and antiherbivorial substances would be higher in inoculated than in noninoculated plants, resulting in an increase of resistance against soil pathogens. Though we observe higher total concentration in roots upon inoculation with *G. intraradices* BEG 140 or with fungi from Kurpie (with the latter resulting also in an increase in shoots), the comparison between mycorrhizal and nonmycorrhizal plants is not clear due to a wide heterogeneity among replicates.

In leaves, the total phenolic acids concentration was much higher (and statistically significant) in plants inoculated with fungi from Karkonosze, but a tendency for increase was also observed upon inoculation with *G. intraradices* BEG 24 and fungi from Kurpie. A really clear picture is revealed concerning the phenolic acid concentrations in roots. For all inocula used, mycorrhizal formation is shown (Table 2) to be associated with an increase of the total phenolic acid concentration, thus, providing good evidence that mycorrhization is enhancing the formation of substances that play a protective role.

Overall, our results suggest that mycorrhizal impact on the formation of secondary metabolites, which is different for sesquiterpene lactones and for phenolic acids, is realized by complex mechanisms that are not controlled only by the extent of mycorrhization or the type of fungus and thus, needing a further investigation.

A. montana population has declined greatly in Europe due to loss of habitat, overharvesting, and fertilizer application causing encroachment of shrubs and trees in pastures. According to Ellenberger (1998), this plant can grow only in unimproved meadows not subjected to any nitrogen fertilizers for at least 60 years. The tendency to outcompete *Arnica* under field conditions was also observed in Poland. The changes of plant community composition of low mountain swards (*Nardetalia*) caused by cessation of pasture and mowing result in retreat of many rare plants like *Arnica*. As pointed out by Fabiszewski and Wojtuń (2001), the main cause of the decreasing population size in the Karkonosze Mountains is the change in soil environment connected with anthropogenic nitrogen fertilization. The large inflow of mineral nitrogen from the atmosphere is the reason of accelerated rate of decomposition of organic matter and intensified nitrification. The high concentration of nitrates in the soil cause expansion of graminoids, while under such conditions *Arnica* is able to survive only if it is cultivated alone, as also confirmed by our results which are as follows: (a) increased concentration of nitrogen in the substratum used in one of the experiments caused the total loss of mycorrhizal symbiosis; and (b) in the experiment with *D. glomerata* the induced loss of mycorrhizal symbiosis was the reason for the low survival of *A. montana*, similarly to the experiment in which both plants were devoid of mycorrhizal fungi. Decreasing the N concentration of the substratum was the only way to allow the development of mycorrhiza and an increased tolerance to stress.

A. montana studied under greenhouse conditions was far more responsive to the presence of mycorrhizal fungi than *D. glomerata* that can compete with *A. montana* under field conditions. In the presence of AMF, *A. montana* could survive easily between *D. glomerata* specimens; while without AMF, it was eventually totally outcompeted. Both the mycorrhizal parameters and the photosynthetic parameters were strongly decreased if both plants were cultivated in the same containers and the differences became more pronounced with time. After 3 months of cocultivation, *Arnica* plants started to die; while *D. glomerata* was not affected in terms of mycorrhizal and photosynthetic performance. These results might explain the challenges that *A. montana* faces in natural stands and also the problems encountered when trying to grow this species outside natural sites.

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