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Vesicular-arbuscular mycorrhiza of *Calamagrostis villosa* supplied with organic and inorganic phosphorus

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Abstract

Plants of *Calamagrostis villosa* were cultivated in nutrient solution alone or in association with a vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus etunicatum*. They were supplied with two levels of inorganic phosphate (P_i, 0.1 or 1 mM) and with or without organic phosphate (dinatriumphenylphosphate, P_o; 1 mM). Depression of growth and enhancement of root respiration of mycorrhizal plants in comparison with non-mycorrhizal plants were observed after 12 weeks of cultivation in a growth cabinet. Root colonization was not influenced by the higher phosphorus availability in contrast to the extraradical mycelium (ERM). The lengths of ERM hyphae both attached to the root surface and in the substrate were decreased substantially by higher phosphorus supply, irrespectively of its form.

Additional key words: dehydrogenase activity, extraradical mycelium, *Glomus etunicatum*, growth depression, root colonization.

Introduction

Pollution-induced decline of Norway spruce forest is a very serious ecological problem in central European mountains. Among other ecosystem changes, the rapid expansion of perennial grasses through damaged forest and clearcuts occurs (Fiala 1989, Vosátka *et al.* 1991) and has significant consequences, *e.g.* on the soil properties and further ecosystem development. *Calamagrostis villosa* is one of such invasive grass species. Although it can be regarded as a sun species, it shows significant adaptability to low irradiance, similarly as it was found for co-occurring species *Calamagrostis arundinacea* (Gloser and Gloser 1996).

Like the most grass species with C_3 photosynthetic pathway, *C. villosa* may be regarded as a facultative mycotroph, *i.e.* its responses to VAM symbiosis depend on

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the environmental (climatic, edaphic *etc.*) conditions. Among these, irradiance is of a great importance: the roots of *C. villosa* grown under tree canopy with low irradiance were found less colonized by VAM fungi compared to the roots of plants grown under high irradiance on declined forest stands or clearcuts (Kasowska 1994). The abundance of mycorrhizas formed often well correlate with the main potential positive effect, the improved plant growth by enhanced uptake of immobile nutrients from soil, especially of phosphorus (Bolan 1991, George *et al.* 1995).

In the forest surface soils, a great portion of the total P (often more than 50 %) is in the organic forms (P_o; Attiwill and Adams 1993). They can be utilised by plants after their mineralization into inorganic (P_i) forms by phosphatases or by abiotic hydrolysis. The phosphatases are widely distributed and in the soils they are produced by bacteria, fungi, protozoa as well as by plant roots. The role of VAM fungi in phosphatases production is not fully clear, but some reports suggest that hyphae of VAM fungi do not produce these enzymes and that the role of VAM fungi consist in stimulation of exudation of root phosphatases, both acid and alkaline (Dodd *et al.* 1987) and in better uptake of P_i released after mineralization (Joner and Jakobsen 1995).

It has been reported many times that the P supply influences mycorrhizal development (for review see Koide and Schreiner 1992). The largest extent of mycorrhizal colonization occurs when soil P concentration is suboptimal for plant growth and by contrast the restriction of the symbiosis formation is often detected under high P availability. Stimulatory effect on the development of extraradical mycelium was found when organic matter was added into soil (Joner and Jakobsen 1995). At present there are no reports about the decrease of ERM development due to the high P_o supply.

The aim of this study was to evaluate the effects of mycorrhizal inoculation and different forms and concentrations of phosphorus on the growth, root respiration and mycorrhiza of *Calamagrostis villosa*.

Materials and methods

We used three-factorial experimental design: *1)* inoculation by VAM fungus, *2)* two concentrations of inorganic phosphorus (P_i) and *3)* the addition of organic phosphorus (P_o) into nutrient solution. Seeds of *Calamagrostis villosa* (Chaix) J.F. Gmelin collected on an acid rain polluted site in the Krkonoše Mts. were sown in sterilized sand/perlite mixture (1:1, v/v, sand particle size > 0.2 mm). Three-week-old seedlings were transplanted into 1000 cm^3 pots containing the same mixture. Half of the plants was left non-mycorrhizal (-M) and half was inoculated (+M) by spores of *Glomus etunicatum* Becker & Gerd. (isolate S 328, University of Florida, Gainesville) from four-month maize pot culture, surface sterilized in 2 % aqueous solution of *Chloramin T* for 3 min. Plants were cultivated in growth cabinet with fluorescent tubes (photoperiod 16 h, irradiance 150 μmol(PAR) m^{-2} s^{-1}). Light/dark air temperatures were 26/24 °C. Nutrients were supplied in the solution containing $N\text{-}NO_3$ (3.8 mM), $N\text{-}NH_4$ (1.2 mM), K (5 mM), Mg (2 mM), Ca (1.3 mM),

Na (2 mM) and the tracers of microelements. Following treatments of P supply were designed: *1)* inorganic phosphorus in the forms KH_2PO_4 (40 % of P_i) and K_2HPO_4 (60 % of P_i) was added up to the final concentrations of 0.1 or 1 mM; *2)* organic phosphorus as dinatriumphenylphosphate was added at the concentration of 1 mM P_o or not supplied. Each pot received 200 cm^3 of the solution, amount sufficient to full field capacity, in intervals which were continually shortened from once a week at the beginning to three days intervals in the last two weeks of cultivation.

Plants were harvested after 12 weeks of cultivation and dry mass of roots and shoots was recorded. Roots were cleared in 10 % KOH and stained by 0.05 % trypan blue in lactophenol (Phillips and Hayman 1970) and root colonization was evaluated by grid line intersect method (Giovannetti and Mosse 1980). The total length of extraradical mycelium (ERM) and its portion showing dehydrogenase (DH) activity was evaluated according to Sylvia (1988) using *INT* vital staining. Two parts of ERM were evaluated in this way, the ERM extracted from the substrate by membrane filter technique (Jakobsen *et al.* 1992) and the hyphae attached to the roots 1 cm segments. Root respiration rate was measured by Clark's oxygen electrode (PP 15 μm membrane, *Labio,* Praha, Czech Republic) combined with *MEM 102* oxymeter (*Laboratorní přístroje,* Praha, Czech Republic) as a decrease of O_2 concentration in full air saturated nutrient solution. The acid phosphatase (APase) activity in roots was used to evaluate the phosphorus deficiency in the plant tissues of -M plants. APase was extracted from root tissue by extraction to the solution consisted of 0.1 M Na acetate and 0.25 M saccharose, pH 4.5. After two centrifugations (1500 *g* for 15 min followed by 18000 *g* also for 15 min) was the enzyme incubated with *p*-nitrophenyl phosphate (*p*-NPP). P_i released from the *p*-NPP was detected spectrophotometrically at 660 nm (Kummerová 1986).

The biomasses, number of tillers and root colonization were evaluated in 7 replicates, the lengths of ERM in five, and APase activity in three replicates. Data were analysed by the series of *ANOVA*s using statistical package *Statgraphics v. 6.0.*

Results

Both above- and underground plant biomasses and numbers of tillers were substantially and consistently reduced by mycorrhizal inoculation (Table 1). The negative correlations between root colonization by VAM fungus and biomass formation have been found (r = -0.433 for shoot dry mass and r = -0.529 for number of tillers, $P < 0.05$ for both coefficients). The higher P_i level in nutrient solution as well as P_o addition had a positive effect on shoot growth and tiller formation. The addition of P_o into nutrient solution promoted shoot growth of non-inoculated plants whereas no effects of P_o were found when plant were mycorrhizal (Table 1).

VA mycorrhizal plants had approximately twice higher root respiration rate compared to non-inoculated plants (Table 1). The addition of P_o enhanced the respiration rate of mycorrhizal but not of non-inoculated plants.

Extraradical mycelium (ERM) of *Glomus etunicatum* was found to be more sensitive to higher phosphorus availability than the intraradical mycorrhizal

structures (Table 2). At the end of the experiment, all types of intraradical structures were well developed and no differences in root colonization at different phosphorus supply were recorded. In contrast, the development of ERM was highly depressed by higher concentration of P_i or by the addition of P_o. This finding has been consistent for both parts of ERM - hyphae attached to roots surface and extracted from substrate. Interaction of P_i level with P_o addition was detected for the length of ERM attached to the root surface. Although the general effect of higher P_i as well as P_o nutrition was detected, there was no further decrease in the ERM length when nutrient solution contained both P_i and P_o in the 1 mM concentration.

Table 1. Dry biomass, number of tillers and root respiration rate of *C. villosa* plants grown at two levels of P_i, with or without the addition of P_o and inoculated with VAM fungus *G. etunicatum* (+M) or left non-inoculated (-M) (means ± standard errors). The significances for main effects, each with one degree of freedom, and their interactions (n.s. - not significant differences; * - $P < 0.05$; ** - $P < 0.01$; *** - $P < 0.001$) are presented in the lower part of the Table.

Treatment	Biomass [g] aboveground	underground	Tillers [plant^{-1}]	Root respiration rate [mg O_2 g^{-1} (d.m.) s^{-1}]
Mycorrhizal plants	0.272 ± 0.115	0.036 ± 0.047	10.0 ± 2.28	16.9 ± 1.24
Non-inoculated plants	3.332 ± 0.102	0.936 ± 0.042	52.5 ± 2.03	8.9 ± 1.09
0.1 mM P_i	1.697 ± 0.107	0.483 ± 0.044	26.4 ± 2.13	12.5 ± 1.17
1.0 mM P_i	1.907 ± 0.110	0.489 ± 0.045	36.0 ± 2.19	13.4 ± 1.17
0.0 mM P_o	1.617 ± 0.110	0.472 ± 0.045	28.0 ± 2.18	11.2 ± 1.12
1.0 mM P_o	1.987 ± 0.108	0.500 ± 0.044	34.4 ± 2.14	14.7 ± 1.22
0.0 mM P_o+M	0.286 ± 0.172	0.042 ± 0.070	10.2 ± 3.42	13.1 ± 1.68
1.0 mM P_o+M	0.257 ± 0.152	0.030 ± 0.062	9.7 ± 3.03	20.8 ± 1.83
0.0 mM P_i–M	2.947 ± 0.136	0.902 ± 0.056	45.9 ± 2.70	9.2 ± 1.48
1.0 mM P_i–M	3.717 ± 0.152	0.969 ± 0.062	59.1 ± 3.03	8.6 ± 1.61
mycorrhizal inoculation	***	***	***	***
P_i level	n.s.	n.s.	**	n.s.
P_o addition	*	n.s.	*	*
myc. × P_i	n.s.	n.s.	n.s.	n.s.
myc. × P_o	*	n.s.	*	*
P_i × P_o	n.s.	n.s.	n.s.	n.s.
myc. × P_i × P_o	**	n.s.	n.s.	n.s.

As regards dehydrogenase (DH) activity of ERM, negative effects of higher phosphorus nutrition were detected (Table 2). The percentage of DH active length of ERM attached to the root surface decreased substantially when plants were supplied with 1 mM P_i. The same negative effect was found for P_o addition. The DH active length of ERM extracted from substrate was negatively affected by the addition of P_o into nutrient solution.

Because the lack of root biomass caused by the growth depression of plants in +M treatments, there were not enough material to conduct root APase measurement on

Table 2. VA mycorrhizal colonization of *C. villosa* roots with *G. etunicatum*, total and DH active lengths of ERM attached to roots or extracted from the substrate and the root APase activity of non-mycorrhizal plants (means ± standard errors). The significances for main effects, each with one degree of freedom, and their interactions (n.s. - not significant differences; * - $P < 0.05$; ** - $P < 0.01$; *** - $P < 0.001$) are presented in the lower part of the Table.

Treatment	Root colonization	ERM attached to roots [m m^{-1}]			ERM in substrate [m g^{-1}(d.m.)]			APase activity
	[%]	total length	active length	% active	total length	active length	% active	[nmol P_i g^{-1}(f.m.) s^{-1}]
0.1 mM P_i	59.6 ± 7.3	0.916 ± 0.142	0.822 ± 0.145	73.2 ± 5.51	33.52 ± 3.96	16.59 ± 2.13	47.92 ± 5.51	5.33 ± 1.24
1.0 mM P_i	45.2 ± 7.9	0.112 ± 0.142	0.038 ± 0.145	32.7 ± 5.51	13.39 ± 3.96	7.02 ± 2.13	44.03 ± 5.51	5.02 ± 1.24
0.0 mM P_o	53.5 ± 8.1	0.931 ± 0.142	0.812 ± 0.145	59.6 ± 5.51	29.95 ± 3.96	16.69 ± 2.13	56.40 ± 5.51	6.36 ± 1.24
1.0 mM P_o	51.2 ± 7.1	0.098 ± 0.142	0.048 ± 0.145	46.3 ± 5.51	16.96 ± 3.96	6.92 ± 2.13	35.55 ± 5.51	4.00 ± 1.24
P_i level	n.s.	**	**	***	**	**	n.s.	n.s.
P_o addition	n.s.	***	**	n.s.	*	**	*	n.s.
P_i x P_o	n.s.	**	**	*	n.s.	n.s.	n.s.	*

these plants. APase activity of -M plants was used as a marker of plant phosphorus supply (Table 2). The highest value of root APase was found in the treatment with basal amount of P_i.

Discussion

In our experiment we found that *C. villosa* plants did not benefit from the association with *G. etunicatum*. Several possible mechanisms are suggested to explain the plant growth depression after mycorrhizal inoculation. Firstly, the different responsiveness of plant families to the presence of VAM fungi must be considered. Secondly, the environmental conditions can also influence the outcome of the VAM symbiosis. The growth inhibition occasionally occurs even in the case of truly VA mycorrhizal plants. The explanation for this phenomenon may be found in host-fungus competition for photosynthates under conditions of low irradiance, especially when P supply is adequate for non-mycorrhizal plants growth (Kiernan *et al.* 1983). Buwalda and Goh (1982) reported such growth depression of ryegrass grown under irradiance of approximately 400 μmol m^{-2} s^{-1}. They concluded that transfer of organic compounds from the host to the fungus may deplete the host non-structural carbon pool thereby restricting amino acid and protein synthesis. Even growth of leek was found inhibited under irradiance of 250 μmol m^{-2} s^{-1} by mycorrhizal inoculation when the plants were P fertilized (Pearson *et al.* 1991). In our experiment, the plants were grown under irradiance of 150 μmol m^{-2} s^{-1}. The purpose of this low irradiance used was to simulate the conditions when the grass grows in the shade under trees canopy. But under such conditions the plants were probably not able to cover the photosynthate requirements both of the fungus and themselves. And finally, the growth depression observed may consist in the host-fungus combination used. Functioning of different fungal species can differ significantly (Pearson and Jakobsen 1993). In our study, we used *G. etunicatum* as available mycorrhizal inoculum, but the use of VAM fungi isolated from the natural habitats of *C. villosa* with very acid soils would be more appropriate.

The root respiration rate of mycorrhizal *C. villosa* was found twice greater compared to non-mycorrhizal plants. This result is in quite good agreement with findings of other authors. Baas *et al.* (1989) estimated the increase of ATP production in root respiration process of mycorrhizal *Plantago lanceolata* up to 80 % compare to non-mycorrhizal plants. Mycorrhizal plants tend to have a higher rate of photosynthesis per plant, partly due to their greater leaf area. Therefore, as far as P supply is limiting, mycorrhizal plants usually grow faster, despite the large carbon sink of the VAM symbiosis (Lambers *et al.* 1996). In our experiment, the plants grew under very low irradiance and VAM fungi were probably not able to outweigh the cost of photosynthates required from the plants. This was probably the primary mechanism of plant growth depression suggested also by other authors (Buwalda and Goh 1982).

P_i starvation of plant tissues induces *de novo* synthesis of intra- and extracellular APases in whole plants (Barrett Lennard 1982). The highest APase activity found in

the roots of plants grown at the lowest P supply thus indicate the P-deficiency for *C. villosa* plants at this treatment.

Many researchers have focused on the role of plant phosphorus status in the host regulation of VAM colonization. There are many reports about the negative correlation between plant P status and degree of mycorrhizal colonization (for review see Koide and Schreiner 1992). High amendments with P_i have been shown to reduce both root colonization and the growth of ERM (Schwab *et al.* 1983, Abbott *et al.* 1984). We have found differential response of intraradical and extraradical mycorrhizal structures to higher P supply. Root colonization was not decreased significantly by high P availability but very apparent changes were detected on ERM. These effects have been found for both P_i and P_o. The length of extraradical mycelium (about 13 m g^{-1} for both P_o and P_i high levels to 34 m g^{-1} for the lowest P treatment) corresponds with the findings of others (Abbott and Robson 1985, Abbott *et al.* 1984). Thus, the high doses of organic P shows very similar effects on mycorrhiza of *C. villosa* as the amendment with P_i.

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