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Response of three arbuscular mycorrhizal fungi to simulated acid rain and aluminium stress

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Abstract

Simulated acid rain (SAR) combined with higher concentration of aluminium (SAR+Al) influenced the ecophysiology of three arbuscular mycorrhizal fungi (AMF) in both the germination and symbiotic phases of their life cycle. *Acaulospora tuberculata*, an isolate from the soil with low pH, exhibited a higher tolerance to environmental stress as compared to *Glomus mosseae* and *G. fistulosum*. This higher tolerance may be related to the edaphic conditions of soil of the isolate origin. The histochemical staining of the alkaline phosphatase and NADH-diaphorase activities in the extraradical mycelium (ERM) of the AMF proved to be more sensitive indication of negative effects of the SAR or SAR+Al stress compared to commonly measured parameters of the AMF such as mycorrhizal colonisation or growth of the ERM.

Additional key words: *Acaulospora tuberculata*, alkaline phosphatase, *Deschampsia flexuosa*, extraradical mycelium, *Glomus fistulosum*, *Glomus mosseae*, maize, NADH-diaphorase, *Zea mays*.

Introduction

Plant growth, survival and reproduction under stress conditions can be significantly improved by arbuscular mycorrhizal fungi (AMF) (for review see, *e.g.*, Sylvia and Williams 1992). The chemistry of the soils exposed to the acid rain emissions is

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Abbreviations: AMF - arbuscular mycorrhizal fungi; ALP - alkaline phosphatase; ERM - extraradical mycelium; NADH-D - nicotinamide adenine dinucleotide diaphorase; NS - nutrient solution; SAR - simulated acid rain; SDM - shoot dry mass.
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changed as soil pH decreases, and the acidifying processes, inducing Al toxicity, lead to nutritional disharmony and may adversely influence plant growth and also deteriorate the symbiotic AMF population (Danielson and Visser 1989). Nevertheless, even in severely polluted soils, some symbiotic associations of grasses with AMF were found (Vosátka *et al.* 1991).

The AMF can protect plants against stress of high and toxic concentrations of heavy metals in the soil (Galli *et al.* 1994), reduction of heavy metals toxicity for mycorrhizal plants was already demonstrated (Dueck *et al.* 1986). The findings about the role of AMF in heavy metals uptake and their interaction with a host plant still remain rather controversial and the mechanisms of protective effects of the AMF towards host plant are still unknown. For example, decrease of both heavy metal uptake and accumulation in the plant tissue of mycorrhizal plants was observed by some authors (Dueck *et al.* 1986, Schüepp *et al.* 1987, Koslowsky and Boerner 1989), while the others reported increase of the uptake, particularly with increase of soil acidity (Killham and Firestone 1983). Dehn and Schüepp (1989) suggested that the AMF can serve as a barrier for translocation of heavy metals into the aboveground part of a plant. Another reason for the higher tolerance of mycorrhizal plants to heavy metals could be an improvement of P nutrition and increase of biomass, what leads to reduction of relative concentration of heavy metals in plant tissues and consequently to reduction of heavy metals toxicity (Haselwandter *et al.* 1994).

A great ecophysiological variability of AMF species may be expressed in their tolerance, adaptability and also function under high concentrations of heavy metals (Weissenhorn *et al.* 1994, Griffioen *et al.* 1994) or extreme level of pH (Sylvia and Williams 1992). Factors affecting the interactions of heavy metals and the AMF were summarised by Haselwandter *et al.* (1994) as follows: host plant species, AMF isolate, level of mycorrhizal colonisation of the roots of host plant, activity of AMF within the roots, concentration of heavy metals in the growing substrate, availability of heavy metal and its interaction with other ions, activity of associative rhizosphere micro-organisms.

Commonly measured parameters of the AMF such as mycorrhizal colonisation or the growth of the ERM, may not indicate sensitively enough changes of vitality and functioning of the AMF as regards plant growth response. Therefore the adoption of protocols for histochemical staining of the ERM may enhance the potential for assessment of stress effects on a metabolic state of the AMF. While the alkaline phosphatase (ALP) activity is supposed to be a marker of functional state of mycorrhizal symbiosis, with respect to P transfer (Tisserant *et al.* 1992), the nicotinamide adenine dinucleotide diaphorase (NADH-D) activity detects general mitochondrial enzymes as a measure of the ERM hyphae vitality (Sylvia 1988).

Two experiments were designed to study whether SAR or SAR combined with aluminium influenced the development of three AMF in two phases of their life cycle: *1*) germination of spores in a presence or absence of a host plant, and *2*) functioning of symbiotic association with a host plant cultivated in a hydroponic system evaluated as the plant growth, mycorrhizal colonisation, growth and ALP and NADH-D activities of the ERM.

Materials and methods

In the first experiment, the effects of simulated acid rain (SAR) and/or SAR amended with Al on spore germination of three AMF were tested in presence or absence of roots of *Deschampsia flexuosa* seedlings. Three factors were tested: AMF species, irrigation and presence of plants. Spores of *Glomus mosseae* (isolate BEG 25), *Glomus fistulosum* (BEG 23) and *Acaulospora tuberculata* (BEG 41) were extracted by wet-sieving from pure pot cultures of the AMF multiplied on maize for 4 months. 15 healthy spores were placed between 45 mm diameter nitrocellulose membrane (pore size 0.45 μm) sandwich and put into the plastic slide frames and buried in fine sand in plastic Petri dishes of 9 cm diameter. During 6 weeks of incubation the sand in Petri dishes was irrigated twice a week by 5 cm^3 of one of three experimental solutions: *1*) basic nutrient solution (NS), according to Gryndler and Vosátka (1996), *2*) NS simultaneously with SAR consisting of 6.2 mg dm^{-3} NO_3^-, 2 mg dm^{-3} NH_4^+, 22 mg dm^{-3} SO_4^{2-} (pH 3.8), and *3*) the NS+SAR amended with 3×10^{-3} M Al in a form of $Al_2(SO_4)_3$. One half of the Petri dishes was left without a host plant while host plants were planted into the other half. Three weeks old seedlings of *D. flexuosa* from surface sterilised (10 % of NaOCl for 10 min) seeds were pre-cultivated in sterile sand and then put between membranes in the slide frames to allow the roots to grow in close vicinity of the spores.

Six Petri dishes were cultivated in each treatment (90 spores per treatment in total). After incubation the slide frames were opened and the percentage of germinated spores (the spores with at least one developed germination tube longer than 5 mm) was evaluated under stereomicroscope (magnification ×120).

In the second experiment, the effects of the SAR or SAR+Al on the maize growth, development of mycorrhizal colonisation and the length of extraradical mycelium (ERM) and enzymes activity in the three AMF were evaluated. Maize seeds were surface sterilised in 10 % NaOCl for 10 min, rinsed with deionised water and pre-germinated in sterilised sand for 3 d in a thermostat at temperature of 22 °C. Maize plants (7 per treatment) were cultivated in plastic tubes (20 cm in length, 5 cm in diameter) sealed with gauze at the bottom and filled with coarse sand and vermiculite mixture (1:1; v:v) and inserted into 4-dm^3 plastic boxes covered by the lid with openings for tubes. Each tube except for non-mycorrhizal controls was inoculated with 2 g of the AMF inoculum consisted of spores, colonised root fragments and the ERM. Hydroponics solutions having the same composition as in the first experiment (NS, NS+SAR, and NS+SAR+Al) were renewed ones a week in each box. After 11 weeks the plants were harvested and shoot dry mass (SDM) was measured. Parts of root systems were stained by trypan blue in lactoglycerine (modified from Phillips and Hayman 1970) and mycorrhizal colonisation was quantified by modified grid-line intersect method (Giovannetti and Mosse 1980) using a grid inside the eyepiece and counting colonisation in 20 cm of root subsamples at the intersects (magnification ×160).

The ERM length and ALP and NADH-D activities of the ERM were estimated in the substrate sampled from the middle part of each cultivation tube. The substrate was mixed and a 5 g sub-sample was placed into a beaker with 50 cm^3 of distilled

H_2O and agitated at high speed using a magnetic stirrer for 1 min. One cm^3 of supernatant was pipetted onto a membrane filter (24 mm diameter, 0.45 μm pore size) and vacuum filtered. The membrane filter was then cut in half and both halves were put onto a microscope slide. 0.3 cm^3 of ALP staining solution (Tisserant *et al.* 1993) were added to one of the halves whilst the second received 0.3 cm^3 of the NADH-D staining solution (Sylvia 1988). Cover slips were placed on the membrane filters and filters were incubated at 28 °C for 14 h in the dark. The mycelium was then counterstained with 0.05 % of acid fuchsin solution in lactoglycerol for ALP stained samples and with 0.01 % trypan blue solution in lactoglycerol for NADH-D staining. This was done by allowing both counterstains to seep under the cover-slips covering the membranes. The total length of mycelium and the proportion of mycelial length which contained either a black precipitate (ALP activity) or red precipitate (NADH-D activity) was evaluated under the microscope *Olympus BX60* using a grid inside the eyepiece (magnification ×400). The total length of mycelium was expressed in mm of hyphae per 1 g of dry substrate, and the enzyme activity of hyphae was expressed as a proportional ratio (%) of the active hyphae and total hyphae lengths.

All data were analysed by *ANOVA* and Duncan's Multiple Range test when normally distributed or by non-parametric analysis (Kruskal-Wallis and Connover test).

Results

In the first experiment, the germination of both *Glomus* spp. was negatively influenced by NS+SAR treatment, regardless the presence of plant, the combined stress (NS+SAR+Al) showed almost detrimental effect on spore germination (Fig. 1). The spores of *A. tuberculata* were not negatively influenced by NS+SAR itself and

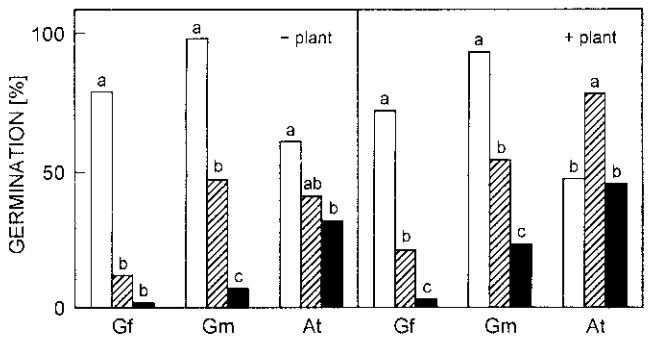

Fig. 1. Spore germination of *Glomus fistulosum* (Gf), *G. mosseae* (Gm), and *Acaulospora tuberculata* (At) incubated between nitrocellulose membranes without (-plant) or with (+plant) roots of *Deschampsia flexuosa*, irrigated by NS (*open columns*), NS+SAR (*hatched columns*), or NS+SAR+Al (*full columns*). Columns indicated by the same letter represent the means of 6 replicates not significantly different within one fungus and plant treatment, according to Duncan's Multiple Range test, $P < 0.05$.

they even germinated successfully at NS+SAR+Al. Surprisingly, in the treatment where a plant was present, the germination of *A. tuberculata* spores was significantly increased when treated with the NS+SAR compared to the treatment irrigated with NS. The factor of plant presence did not affect germination of *Glomus* spores significantly.

In the second experiment, the irrigation as a factor influenced significantly SDM and enzyme activities of the ERM but not mycorrhizal colonisation and total length of the ERM. The AMF species as a factor significantly influenced all above parameters except SDM (Table 1). The interaction of both factors was significant for mycorrhizal colonisation and the ERM enzyme activities.

Table 1. Shoot dry mass (SDM), mycorrhizal colonisation (Myc. colon.) of the maize roots, total length of extraradical mycelium and its alkaline phosphatase (ALP) and NADH-diaphorase (NADH-D) activities of three arbuscular mycorrhizal fungi *Glomus fistulosum*, *G. mosseae* and *Acaulospora tuberculata* cultivated in hydroponics with NS, NS+SAR, or NS+SAR+Al. Means of 7 replicates followed by the same letter are not significantly different within one parameter according to Duncan's Multiple Range test, $P < 0.05$.

AMF species	Solution	SDM [g]	Myc. colon. [%]	Length [mm g^{-1}]	NADH-D [%]	ALP [%]
Uninoculated	NS	2.18 ab	0	0	0	0
control	NS+SAR	2.67 ab	0	0	0	0
	NS+SAR+AL	0.35 c	0	0	0	0
Glomus	NS	1.44 bc	22.0 b	0.52 d	0.19 ef	0.14 de
fistulosum	NS+SAR	1.69 bc	8.8 cd	0.56 d	0.11 f	0.08 de
	NS+SAR+AL	0.45 c	12.7 bc	0.18 d	0.05 f	0.03 e
Glomus	NS	1.77 bc	40.0 a	5.44 a	2.29 a	1.92 a
mosseae	NS+SAR	3.45 a	38.4 a	4.41 ab	1.36 b	0.33 b
	NS+SAR+AL	0.45 c	43.6 a	3.02 bc	0.71 c	0.13 de
Acaulospora	NS	1.50 bc	11.6 bc	1.07 cd	0.34 d	0.34 b
tuberculata	NS+SAR	2.74 ab	12.2 bc	0.96 cd	0.32 de	0.32 bc
	NS+SAR+AL	0.46 c	15.6 c	1.82 cd	0.82 c	0.18 cd
Irrigation (A)		***	n.s.	n.s.	**	***
AMF species (B)		n.s.	***	**	***	***
A × B		n.s.	*	n.s.	***	***

In the uninoculated control and in the *G. fistulosum* treatments the application of the NS+SAR did not decrease SDM of maize as compared to the treatments grown in NS only. Combined stress of the NS+SAR+Al decreased significantly the SDM in the uninoculated and *G. fistulosum* inoculated treatments. By contrast, the application of the NS+SAR increased the SDM of maize inoculated with *G. mosseae*. The combined stress of the NS+SAR+Al decreased significantly the SDM for plants inoculated with *G. mosseae* and *A. tuberculata* as compared to the NS+SAR treatments.

Mycorrhizal colonisation was the highest for *G. mosseae* comparing to the other two fungi. Mycorrhizal colonisation was the same in all irrigation treatments for

G. mosseae and *A. tuberculata*, while *G. fistulosum* colonisation was negatively influenced by the NS+SAR treatment but not by the NS+SAR+Al.

Total length of the ERM was the highest for *G. mosseae* in the NS and NS+SAR treatments, whilst it was significantly lower for the other two fungi. The total length of the ERM was influenced by irrigation only for *G. mosseae* inoculation when its value was significantly lower in the NS+SAR+Al comparing to the NS treatment but still significantly higher than for the other two fungi.

For both enzymes similar effects of irrigation on their activities were observed for all three AMF. For *G. fistulosum* both enzyme activities of the ERM were not significantly influenced by irrigation at all. For *G. mosseae* the trend of a gradual decrease of the ALP and the NADH-D activities was observed by both the NS+SAR and the NS+SAR+Al treatments. For *A. tuberculata* each enzyme responded differently to an irrigation treatment. The NADH-D activity increased in the NS+SAR+Al treatment as compared to the NS treatment, whereas the ALP activity decreased in the NS+SAR+Al treatment as compared to the NS treatment.

Discussion

Irrigation with SAR or SAR+Al influenced both symbionts of mycorrhiza associations. Three AMF in both the germination and the symbiotic phases of their life cycle were affected and the response of AMF showed high species specificity.

The response of spore germination to the plant presence points to a possible role of root exudates inducing changes in rhizosphere chemistry and saprophytic microflora specifically associated with particular AMF (Azcon 1989). The stimulation of germination of *A. tuberculata* in the presence of plant roots when treated with SAR confirmed previous findings of Vosátka and Dodd (1998), however, the effects on *Glomus* spp. were found less pronounced. We cannot explain those discrepancies on the base of our results since the interaction of environmental factors influencing spore germination is very complex and not completely understood yet. *A. tuberculata*, an isolate from the soil with low pH was found to be more adapted to acidification as compared to two *Glomus* spp. originally isolated from neutral soils. The fact that the soil pH can be decisive for germination of *A. laevis* was previously reported by Hepper (1984). The AMF spores are found to germinate the best at the same pH as at pH of the soil of their origin (Green *et al.* 1976, Daniels and Trappe 1980). Therefore, the tolerance of *A. tuberculata* to lower pH can be related to possible acidophilic nature of the *Acaulospora* genera or to a particular origin of this isolate (Vosátka and Dodd 1998). Some extent of tolerance to acidification and heavy metals can be accounted for the variation of geographical isolate characteristics (Weissenhorn *et al.* 1994). Even though *A. tuberculata* in our experiments was not isolated from the soil contaminated by aluminium but from the soil of low pH, the fungus exhibited a significant level of Al tolerance.

Our findings about the negative effects of SAR and SAR+Al on plant growth confirmed previous findings of Koslowsky and Boerner (1989), who found negative effects of Al on plant growth and mycorrhizal colonisation. Our results suggest that

the mycorrhizal growth response of host plant is not related to extent of either AMF colonisation or ERM growth but rather to metabolic activity of the ERM. That might indicate a higher susceptibility of the extraradical fungal structures to edaphic stress compared to intraradical ones. Decrease in vitality of the ERM following SAR and Al stress can result in reduction of nutrient uptake by the ERM and consequently in growth depressions of a host plant. The SAR treatment itself exhibited a neutral or even a positive effect on plant growth and the AMF development due to nutritional effect of N and S in the SAR solution (Vosátka 1995). The Al amendment showed mostly detrimental influence on these parameters what is in agreement with finding of Killham and Firestone (1983). Different AMF species response to stress was observed as indicated by enzymatic activities of the ERM, *e.g.* a higher NADH-D activity of *A. tuberculata* in the SAR+Al treatment, what again points out to a higher stress tolerance of this species. The histochemical staining of both enzymes in the ERM proved to be a sensitive method for indication of the chemical stress tested.

Generally, our study showed that mycorrhizal association can affect the response of host plants to environmental stresses such as low pH and high Al concentration. Different AMF may differ in their responses to environmental stresses in both germination and symbiotic phases of their life cycle. Ongoing research focuses on comparison of ecophysiology of several AMF species of different genera and their isolates adapted to certain environmental stresses.

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