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ORIGINAL PAPER

R. Malcová · M. Gryndler · M. Vosátka

Magnesium ions alleviate the negative effect of manganese on *Glomus claroideum* BEG23

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Abstract The phytotoxicity of excessive levels of manganese (Mn), an essential micronutrient, can be alleviated significantly by a high supply of magnesium (Mg) ions to plants. A similar interaction of these two elements in the development of arbuscular mycorrhizal (AM) fungi has been verified in two experimental systems. In in vitro experiments, an outgrowth of hyphae from excised, surface-disinfected root segments colonised with *Glomus claroideum* BEG23 was measured after 5 days incubation in liquid media. When only Mn ions were present in the media at higher concentrations (>0.05 mM), the growth of hyphae from root segments was reduced significantly. Addition of magnesium sulphate to the incubation solution reduced the inhibitory effects of Mn on hyphal growth. Alleviation of Mn toxicity by Mg ions observed in in vitro experiments was verified also for the symbiotic association between *G. claroideum* and maize as a host plant in a hydroponics sand culture experiment.

Keywords Arbuscular mycorrhizal fungi · Proliferation of intraradical hyphae · Ion antagonism · Manganese toxicity

Introduction

Manganese (Mn) is a trace element essential for living organisms as a cofactor activating enzymes of various metabolic pathways (Marschner 1995). It can be detrimental in excess (El-Jaoual and Cox 1998). A negative influence of Mn at high concentration on arbuscular mycorrhizal fungi (AM) has been shown. McGee (1987) found that Mn added to soil inhibited development of AM fungi. High concentrations of available Mn inhibited the growth of AM fungi hyphae from dried root pieces, a significant source of AM infection in soil. Hepper (1979) reported reduction in percentage spore germination after addition of manganese sulphate to water agar, with complete inhibition at 1.4 mg l^{-1} Mn^{2+}.

AM fungi affect Mn uptake by host plants, with effects ranging from increase (e.g. Menge et al. 1978, 1982), no change (e.g. Menge et al. 1978) to decrease (e.g. Arines et al. 1989; Bethlenfalvay and Franson 1989; Pacovsky 1986). Arines et al. (1989) found that the Mn concentration in both shoots and roots of mycorrhizal plants was lower than in non-mycorrhizal controls. With low soil Mn, Menge et al. (1982) found a significantly higher leaf Mn concentration in mycorrhizal than non-mycorrhizal host plants, while in other soils the leaf Mn concentration was lower. Decreased toxicity of Mn in the presence of AM fungi has been reported for soybean (Bethlenfalvay and Franson 1989); however, this effect seemed to be indirect, since Mn concentration in the biomass increased with increasing fungal colonisation. Different conditions in the rhizosphere/mycorrhizosphere of non-mycorrhizal and mycorrhizal plants resulting from differences in root exudation could provide a partial explanation for such contrasting results (George et al. 1994). For example, Kothari et al. (1991) reported that mycorrhizal plants had fewer bacterial Mn reducers in the mycorrhizosphere, lower soil Mn availability and lower Mn uptake than non-mycorrhizal plants.

Mn availability and potential toxicity for organisms depend on soil physical and chemical properties, including the concentrations of other elements (El-Jaoual and Cox 1998). The ionic radius of Mn^{2+} (0.075 nm) lies close to that of magnesium (Mg) (0.065 nm) and, therefore, these ions can substitute or compete with each other (Marschner 1995). Mn can induce deficiency of

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R. Malcová (✉) · M. Vosátka
Institute of Botany, Academy of Sciences of the Czech Republic, 252 43 Pruhonice, Czech Republic
e-mail: malcova@ibot.cas.cz
Tel.: +420-2-67750022, Fax: +420-2-67750022

M. Gryndler
Institute of Microbiology,
Academy of Sciences of the Czech Republic, Vídenská 1083, 142 20 Prague 4, Czech Republic

Mg by both inhibiting uptake and competing at the cellular level (Marschner 1995). Inhibition of Mg uptake by Mn far exceeds the 1:1 competition for specific binding sites at the plasma membrane of root cells. Presumably, Mn also blocks the binding and/or transport sites for Mg (Marschner 1995). Conversely,„ Mn toxicity in plants (Davis 1996) and bacteria (Abelson and Aldous 1950) has been reported to be counteracted by a high supply of Mg. Arines and Vilariño (1989) suggested that physiological antagonism of these two elements was related to mycorrhizal colonisation. Plants heavily colonised by an AM fungus showed a lower Mn:P ratio and a higher Mg:P ratio than less-colonised plants. In the present study, the response of the AM fungus *Glomus claroideum* BEG23 to the presence of Mn and addition of Mg was studied both in axenic conditions (by measuring the growth of hyphae proliferating from colonised root segments) and in an hydroponic sand culture experiment.

Materials and methods

In vitro experiments

To obtain colonised root segments, maize plants (*Zea mays* L.) were grown in a greenhouse in plastic tubes (175 mm in height and 45 mm in diameter) covered with canvas at the bottom and filled with zeolite. Each tube was inserted into a vessel and supplied weekly with 300 ml of distilled water for the first 2 weeks of growth. The plants were then supplied with P2N3 nutrient solution (Gryndler et al. 1992). Each plant was inoculated with 15 g of *G. claroideum* Schenck & Smith (BEG23) soil inoculum containing a mixture of colonised roots, spores and extraradical mycelium (ERM). After a 5-week period, the plants were harvested, the root systems washed and yellow-coloured mycorrhizal roots selected using fine forceps. The root segments from eight plants were used for each in vitro experiment. The collected roots were shaken five times with sterile distilled water for 1 min to remove particles of the substrate. Subsequently, the roots were surface disinfected for 4 h with a mixture of the antibiotics streptomycin (500 mg/l), polymyxin B (500 mg/l), penicillin G (500 mg/l), neomycin (500 mg/l) and rolitetracycline (250 mg/l). The roots were then immersed in a 1:50 solution of sodium hypochlorite (commercial bleach Savo) for 3 min and washed with 1 l of sterile distilled water. The washed roots were transferred to deionised water, cut with sterile surgical scissors into 1- to 2-mm-long segments and washed with sterile deionised water. The root segments were incubated in 30-µl drops of filter-sterilised solutions on the inside of the lids of polystyrene Petri dishes. In five successive in vitro experiments, the incubation solutions contained various concentrations of Mn and/or Mg ions added as the sulphate ($MnSO_4.5H_2O$ and $MgSO_4.7H_2O$, respectively) dissolved in 1 mM BIS-Tris buffer (pH 6.3). The dishes, each containing 16 hanging drops of the medium with the root segments, were incubated in the dark in a humid chamber for 5 days at 25°C. After incubation, the root segments were observed under a microscope (magnification ×63) using an eyepiece equipped with a grid net focal plate. One Petri dish was taken as an experimental unit. The length of AM hyphae per root segment was estimated using a grid-line intersect method and mean percentage of segments bearing proliferating hyphae was calculated. One intersection corresponded to a hyphal length of 0.059 mm. Only non-contaminated root segments were taken into account because of possible interference of the growth of AM hyphae by contaminating saprophytic microorganisms.

Effects of Mn and Mg ions on hyphal growth were investigated in a total of five in vitro experiments. The first three experiments aimed to determine Mn concentrations reducing the growth of AM hyphae, and two further experiments were set up to study the influence of interactions between Mn and Mg ions. Experiment 1 involved four Mn concentrations: 0, 0.1, 1 and 10 µM. Based on the results of experiment 1, Mn concentrations were increased in experiment 2 (0, 0.1, 1 and 10 mM). Experiment 3 aimed to determine the range of inhibitory Mn concentrations and comprised six different concentrations: 0, 0.01, 0.05, 0.1, 0.5 and 1 mM. Experiment 4 was conducted with five Mg concentrations: 0, 0.1, 0.3, 1 and 3 mM added to the basic solution containing 0.1 mM Mn. In the last experiment, the effect of a wider range of Mn and Mg concentrations on hyphal growth was studied, including nine treatments resulting from the combination of three Mn (0, 0.1 and 1 mM) and three Mg (0, 0.15, 1.5 mM) concentrations.

Sand-based hydroponics experiment

An hydroponics experiment was conducted to test the data on Mn/Mg interactions obtained from the in vitro experiments. Maize (*Zea mays* L.) plants were cultivated in a sand-based hydroponics system. Maize seeds were surface disinfected and placed in sand-filled plastic tubes. Each tube was inoculated with 15 g of soil inoculum of *G. claroideum* Schenck & Smith (BEG23). For the first 2 weeks, plants were supplied only with distilled water (2 l per tub). The plants were then supplied with a P2N3 nutrient solution (Gryndler et. al. 1992) with modified Mn and Mg concentrations as described below. The experiment was designed as two-factorial with the following factors: (A) Mn concentration (0, 0.1 and 1 mM), (B) Mg concentration (0.15 and 1.5 mM). Each treatment had twelve replicates. The pH of nutrient solutions was adjusted to 6.3 and the solutions were renewed weekly. The plants were cultivated in a growth chamber under constant conditions (day/night 16/8 h, temperature 23/18°C, RH 60/65%, PAR 330 µmol m^{-2} s^{-1}). During the growth period, plant position in the growth chamber was varied once a week in order to provide all plants with similar light access. After 8 weeks, the plants were harvested and shoot and root dry weights were determined after drying at 80°C. Root samples were taken to evaluate mycorrhizal colonisation. Samples were stained with 0.05% trypan blue in lactoglycerol (Koske and Gemma 1989) and root colonisation was quantified under a microscope by the modified grid-line intersect method (Giovannetti and Mosse 1980) using an ocular grid at a ×100 magnification. The substratum from each tube was homogenised and small aliquots were taken to estimate the length of ERM, using the membrane filtration technique (Jakobsen et al. 1992), and enzymatic activities of ERM. A small aliquot of the substratum was placed in a household blender with 500 ml of distilled water and blended for 30 s. One millilitre of supernatant was pipetted onto a membrane filter (24 mm diameter and 0.40 µm pore size) and vacuum filtered. The membrane filters were then put onto microscope slides and stained with 0.05% trypan blue in lactoglycerol to stain the mycelium retained on the surface. The total length of ERM was assessed using a grid-line intersect method under a microscope using an ocular grid at a ×100 magnification and expressed in metres of hyphae in 1 g of air-dry substrate. The NADH diaphorase and alkaline phosphatase (ALP) activities of ERM were examined by staining in the remaining ERM extracted from the substrate by wet sieving. ALP staining solution (300 µl) (Tisserant et al. 1993) or NADH diaphorase staining solution (Sylvia 1988) was mixed in Eppendorf tubes with the mycelium sample. The Eppendorf tubes were incubated at 28°C for 14 h in the dark. The proportions of ERM length containing either a black (ALP activity) or red precipitate (NADH diaphorase activity) were estimated under a microscope at a magnification of ×400.

Statistical treatment

The data were analysed by one-way or two-way ANOVA with Duncan's Multiple Range Test. The data showing non-conformity to normal distribution were subjected to logarithmic transformation prior to further analysis.

Table 1 Effect of manganese (Mn) on the percentage of root segments bearing proliferating hyphae of *Glomus claroideum* BEG23 and on the length of proliferating hyphae. Means followed by the same letters are not significantly different according to Duncan's Multiple Range Test at $P<0.05$

Mn (mM)	Proliferation of hyphae (% root segments)		Mean length of hyphae (mm per root segment)	
0	57	a	6.1	a
0.01	58	a	7.5	a
0.05	32	b	5.2	ab
0.1	22	b	3.2	b
0.5	1	c	0.0	c
1	4	c	0.0	c
Significance	***	***		

*** Significant effect at $P<0.001$ (one-way ANOVA)

Table 2 Effect of magnesium (Mg) addition to medium containing Mn at 0.1 mM on the percentage of root segments bearing proliferating hyphae of *G. claroideum* BEG23 and on the length of proliferating hyphae. Means followed by the same letters are not significantly different according to Duncan's Multiple Range Test at $P<0.05$

Mg (mM)	Proliferation of hyphae (% root segments)		Mean length of hyphae (mm per root segment)	
0	22	b	3.2	b
0.1	35	ab	6.2	a
0.3	46	a	6.4	a
1	40	a	6.2	a
3	33	ab	1.6	b
Significance	*	**		

* Significant effect at $P<0.05$, ** $P<0.01$ (one-way ANOVA)

Results

In vitro experiments

In experiment 1, there was no effect of Mn on the percentage of root segments bearing proliferating hyphae or on hyphal growth in the Mn concentration range 0.01–10 µM. In experiment 2, both percentage of root segments bearing proliferating hyphae and the length of proliferating hyphae were markedly lower at 0.1 mM Mn than the control (by 94% and 96%, respectively). In experiment 3, the proliferation of hyphae from root segments was reduced at Mn concentrations higher than 0.05 mM and hyphal growth was negligible in the medium containing 0.5 mM Mn. No hyphae grew out of root segments at 1 mM Mn (Table 1).

A significant interaction between Mn and Mg ions was observed. The addition of Mg ions to medium containing Mn at 0.1 mM increased the percentage of root segments bearing proliferating hyphae and improved hyphal growth compared with the same Mn treatment without Mg addition. A high Mg concentration (3 mM), however, reduced hyphal growth (Table 2).

This negative effect of Mg ions on growth was also observed at 1.5 mM (compared with 0.15 mM) (Table 3).

Table 3 Effect of Mn and Mg on the percentage of root segments bearing proliferating hyphae of *G. claroideum* BEG23 and on the length of proliferating hyphae. Means followed by the same letters are not significantly different according to Duncan's Multiple Range Test at $P<0.05$ (*ns* non-significant)

Mn (mM)	Mg (mM)	Proliferation of hyphae (% root segments)		Mean length of hyphae (mm per root segment)	
0	0	43	ab	6.9	a
0	0.15	54	a	7.4	a
0	1.5	14	cd	1.0	c
0.1	0	0	d	0.0	d
0.1	0.15	22	c	1.1	c
0.1	1.5	40	b	2.4	b
1	0	0	d	0.0	d
1	0.15	4	d	0.1	d
1	1.5	10	cd	0.4	cd
Mn (A)		***		***	
Mg (B)		ns		**	
A × B		*		**	

* Significant effect at $P<0.05$, ** $P<0.01$, *** $P<0.001$ (two-way ANOVA)

However, at both concentrations, the addition of Mg ions significantly reduced the inhibitory effect of Mn observed in the absence of Mg ions (Table 3).

Sand-based hydroponics experiment

Mn and Mg significantly influenced the growth of maize plants. Plants supplied with Mn at 1 mM had significantly higher biomass at 0.15 mM than 1.5 mM Mg. At lower Mn concentrations, the difference between the two Mg levels was not significant (Table 4). Considering AM fungal development, both metals affected root colonisation and ERM length. At 1 mM Mn, higher root colonisation and ERM length were observed at 1.5 mM than 0.15 mM Mg. At 0.1 mM Mn, no differences in root colonisation or ERM length were observed between Mg treatments. In the control treatment without added Mn, ERM length was higher at 1.5 mM than 0.15 mM Mg. Both NADH diaphorase and ALP activities were reduced significantly by the addition of Mn to the nutrient solution. However, the effects of Mn/Mg interactions on enzyme activities were not significant (Table 4).

Discussion

The results of the present experiments show that increased Mg concentrations can alleviate Mn inhibition of *G. claroideum*. The significant reduction in the development of the AM fungus at higher Mn concentrations agrees with results published previously (Hepper 1979; McGee 1987). However, Mn concentrations inhibiting AM fungi development cannot be compared directly in studies conducted under different culture conditions. It is thus difficult to define a particular Mn concentration as a threshold above which inhibition of AM fungal develop-

Table 4 The effects of Mn and Mg on the growth of maize plants inoculated with *G. claroideum* BEG23 and on the development of the mycorrhizal fungus. Means followed by the same letters are not significantly different according to Duncan's Multiple Range Test at $P<0.05$ (*ALP* alkaline phosphatase, *ERM* extraradical mycelium, *NADH* nicotinamide adenine dinucleotide, *ns* non-significant)

Mn (mM)	Mg (mM)	Shoot dry weight (g)		Root dry weight (g)		Root colonisation (%)		ERM length (mg^{-1} soil)		NADH-diaphorase (%)		ALP (%)	
0	0.15	1.9	bc	0.7	bc	29	ab	15	b	82	a	40	a
0	1.5	2.0	bc	0.6	c	31	a	20	a	81	a	34	ab
0.1	0.15	1.8	c	0.6	c	23	bc	13	b	75	ab	24	bc
0.1	1.5	2.1	bc	0.8	b	22	c	15	b	61	b	35	ab
1	0.15	3.9	a	1.5	a	7	d	4	c	51	c	20	c
1	1.5	2.3	b	0.8	bc	23	bc	14	b	54	c	26	bc
Mn (A)		***	***	***	***	***	**						
Mg (B)		*	*	***	***	ns	ns						
A x B		***	***	**	**	ns	ns						

*Significant effect at $P<0.05$, **$P<01$, ***$P<0.001$ (two-way ANOVA)

ment occurs. In pot experiments, Mn toxicity is influenced significantly by the substrate (e.g. proportion of clay particles, organic matter, composition of bacterial populations) as well as by plant exudates. The method of measuring the outgrowth of intraradical hyphae from root segments (proliferation) used in the present in vitro experiments avoids problems with metal ion adsorption to soil particles and also reduces the possible chelating of metal ions by root exudates.

Although in vitro proliferation seems to be an appropriate and fast method for studies on interactions between AM fungi and soluble compounds, some variation may exist between root segment material even though plants are cultivated under the same conditions. These discrepancies may arise in experiments conducted throughout the year, where plants producing colonised root segments may differ in their physiology and influence retention of elements within root and fungal tissues. Nevertheless, reduction in hyphal proliferation by increased concentrations of Mn were observed consistently in both the in vitro and in planta sand culture experimental systems. The effects of Mn/Mg interactions were evident in all experiments, regardless of the origin of the experimental material.

The effect of Mg on *G. claroideum* differed between in vitro and hydroponics experiments. In vitro, Mg at 1.5 mM inhibited hyphal growth, but had no effect on root colonisation and no positive effect on ERM growth in the hydroponics sand culture experiment. In contrast, Gryndler at al. (1992) observed increased root colonisation by the same isolate of *G. claroideum* (formerly *Glomus fistulosum*) with 5.8–11.7 mM $MgSO_4$ in the nutrient solution. However, Johnson et al. (1980) found no effect of intensive fertilisation of the soil with Mg on mycorrhizal colonisation and Jarstfer et al. (1998) reported a reduction of mycorrhizal colonisation and sporulation at 11.7 mM $MgSO_4$ in the nutrient solution. The two experimental systems differed in the concentration of Mn at which Mg alleviated Mn toxicity. Mg at 1.5 mM increased hyphal growth in vitro in presence of 0.1 mM Mn but had no effect on root colonisation or ERM length. On the contrary, Mg at 1.5 mM increased root colonisation and ERM length in presence of 1 mM Mn but had no effect on in vitro proliferation of hyphae. Such differences may be attributed to the adsorption of Mn onto substrate particles, absorption by roots, chelating by root exudates or interaction of both ions with other components of the nutrient solution in the hydroponics sand culture experiment. In addition, root segments in vitro are exposed to constant concentrations of Mg and Mn in the incubation medium, whereas concentrations of these ions in the proximity of roots or ERM in hydroponics may vary with time. The threshold value for Mn toxicity probably depends also on the AM fungal structure considered (germinating spores, extraradical or intraradical mycelia) and fungal hyphae in symbiosis with a growing host plant may differ in physiology to fungal hyphae proliferating from excised root segments.

When the effects of these ions on plant growth and fungal development are compared, the biomass of maize plants was highest when supplied with 1 mM Mn and 0.15 mM Mg. In contrast, root colonisation and ERM length were at their lowest with this combination of ions. It may be that host plants are more resistant to excessive Mn levels than are the symbiotic fungus. The increase in biomass of less-colonised plants may also be attributable to reduced expenses for maintaining the symbiosis.

In conclusion, the ability of Mg to alleviate Mn toxic effects on the AM fungus *G. claroideum* was demonstrated in in vitro and hydroponics experiments, although the threshold concentrations for Mn toxicity and alleviation of this effect by Mg differed in the two experimental systems.

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