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Effect of manganese on vesicular-arbuscular mycorrhizal development in red clover plants and on soil Mn-oxidizing bacteria

J. Arines, M. E. Porto, and A. Vilariño

UEI de Fisiología Vegetal, Instituto de Investigaciones Agrobiológicas de Galicia, Apdo 122, E-15080 Santiago de Compostela, Spain

Summary. The effect of manganese on the development of vesicular-arbuscular (VA) mycorrhizae and on the population of Mn-oxidizing soil bacteria was studied using red clover as host plant and *Glomus mosseae* or *G. aggregatum* as VA-colonizing fungi. The addition of Mn to the substrate in which plants grew had a detrimental effect on root colonization, *G. aggregatum* being more susceptible than *G. mosseae*. Mn uptake was lower in mycorrhizal than in non-mycorrhizal plants, especially when colonized by *G. mosseae*. The development of mycorrhizae seems to favour the Mn-oxidizing bacteria population.

Key words: Manganese-oxidizing bacteria – Plant Mn uptake – Soil manganese – Vesicular-arbuscular mycorrhizae

Introduction

It is well known that plants with mycorrhizae are able to absorb nutrients in greater quantities than those without, especially under nutrient-deficient conditions. Less well known is the fact that vesicular-arbuscular (VA) mycorrhizae can reduce absorption of nutrients present at high concentrations in the substrate.

Although the subject of research so far, this phenomenon appears to constitute a defence against potentially toxic mineral levels. It is best documented for manganese uptake (Pacovsky 1986; Arines et al. 1989), but even in this case little or nothing is known about the mechanism. Since the dynamics of Mn in the soil depends largely on the activity of oxidizing and reducing microorganisms, one explanation for the uptake-limiting effect of mycorrhizae is that the mycorrhizae increase the activity of oxidizing microorganisms (Mulder 1972; Bromfield 1978, 1979).

Offprint requests to: J. Arines

To investigate this possibility, we grew red clover plants with VA mycorrhizae in substrates with Mn at various concentrations and determined: (a) the colonization by VA mycorrhizae, (b) Mn uptake by the plant, and (c) the size of Mn-oxidizing bacterial populations in the substrate.

Materials and methods

Material

The substrate in which plants were grown was a 1:1 mixture of quartz sand and soil (a sandy loam Cambisol developed over schists). After addition of Mn sulphate solution to a concentration of 90 mg Mn (kg substrate)$^{-1}$, this mixture was stored for 4 months. Shortly before its use for the experiments with mycorrhizae, the concentration of Mn in the substrate was determined by Lakanen and Ervio's (1971) method to be 40 μg g^{-1}.

The VA mycorrhizal fungi used were *Glomus mosseae* (Nicol. et Gerd.) Gerdemann et Trappe and *G. aggregatum* Schenck et Smith. Both were obtained from pure cultures in pots in which *Trifolium pratense* L. was grown.

Experimental design

The sand/soil substrate prepared as above was placed in 1-l pots either untreated (20 pots) or after the addition of *G. mosseae* soil inoculum (20 pots) or *G. aggregatum* soil inoculum (20 pots). Four plantlets of red clover (*T. pratense* L.) were planted in each pot and nutrient solution was added. Pots without mycorrhizal inoculum received nutrient solution with correspondingly more P than the other pots to ensure similar growth of all plants (Pacovsky et al. 1986). After 2 months, the aerial parts of all plants were cut off, and 15 days later four pots of each inoculum group received a dose of 0 (D0), 15 (D1), 30 (D2), 60 (D3) or 120 (D4) mg Mn per pot. The same treatment was repeated 90 and 110 days after the start of the experiment. On day 120, the plants were separated from the substrate and their dry weight, aerial and root Mn concentration, total Mn content, percentage of root colonization by VA fungi and relative abundance of VA mycorrhizal arbuscules were determined. Available Mn, extraradicular VA mycelium and Mn-oxidizing bacteria were determined in the substrate.

Methods

Available Mn was extracted with Lakanen and Ervio's (1971) solution. Exchange Mn was extracted with 1 M $Mg(NO_3)_2$ at pH 7 (Shuman 1985). Mn in all extracts was determined by atomic absorption spectrophotometry (AAS).

Samples of aerial parts and roots of plants were dried and ashed, and the ashes were taken up in hot 5 N HCl. The resulting solution was diluted with distilled water, and Mn determined by AAS. The results were used to calculate Mn concentrations in aerial parts and roots, and also the total Mn content.

The percentage of the root system infected with VA mycorrhizae was determined by Phillips and Hayman's (1970) staining method and by Ambler and Young's (1977) intersection-counting method. The abundance of arbuscules was determined by examining 100 microscope fields with a ×40 objective (Arines et al. 1988).

Extraradicular VA mycorrhizal mycelium was quantified as follows. To a 2-g sample of substrate from each pot was added 2 ml of dispersant (25% Calgon solution) and 10 ml of 0.1% trypan blue solution. The resulting suspension was made up to 100 ml with distilled water, stirred in a hot water bath for 30 min, and poured through a 36-µm-mesh sieve. The material retained was washed under a stream of water and transferred to a 100-ml beaker; the volume was then adjusted with distilled water. The resulting suspension was then heated in a water bath with stirring until its temperature reached 60° C; 0.9 g of agar was then added and the stirring continued for a few minutes longer. A 10-ml sample was taken from the central region of the beaker using a 50-ml syringe, transferred to a 9-cm Petri dish bearing a 5-mm-mesh grid on its bottom, and incubated for 45 min in an oven at 60° C. After removal from the oven, the Petri dish was left at room temperature for the agar to solidify, after which the mycelium was quantified by counting the number of mycelium-grid intersections under a compound microscope at a magnification of ×20–30.

Mn-oxidizing bacterial populations were studied in the substrates treated with Mn doses D0, D2 and D4. Samples were taken from three pots of each 4-pot dose/inoculum group and were stirred for 60 min in sterile flasks with 10 volumes of sterilized water. The resulting extract was diluted 10^4-fold and 50-µl samples were sown in Petri dishes containing Bromfield's (1974) medium with the Mn content increased 4-fold (10 dishes for each set of dose/inoculum conditions). The dishes were sealed with Parafilm and incubated in the dark at 30° C for 20 days. Mn-oxidizing bacteria were detected by adding 3 ml of a 2% solution of benzidine hydrochloride in acetic acid to each Petri dish (Bromfield 1956). The number of colonies was counted, and the surface occupied by the colonies was estimated using tracing paper with a printed grid.

The significance of differences among the results for different treatment groups was estimated by analysis of variance (ANOVA-2 test) and non-parametrical tests. Distributions (of transformed data where appropiate) were deemed normal or non-normal on the basis of the Kolmogorov-Smirnov test.

Table 1. Dry weight per pot of the aerial parts of *Trifolium pratense* plants grown in pots with no inoculum (NI) or with *Glomus mosseae* (GM) or *G. aggregatum* (GA) inoculum, and subsequently treated with various doses of manganese

	Mn dose				
Inoculum	D0	D1	D2	D3	D4
NI	1.6±0.2[a]	1.7±0.2	1.4±0.3	1.5±0.1	1.3±0.1
GM	1.4±0.3	1.6±0.2	1.3±0.1	1.4±0.1	1.1±0.1
GA	1.3±0.2	1.4±0.2	1.7±0.1	1.5±0.1	1.1±0.2

[a] Means ± standard deviations

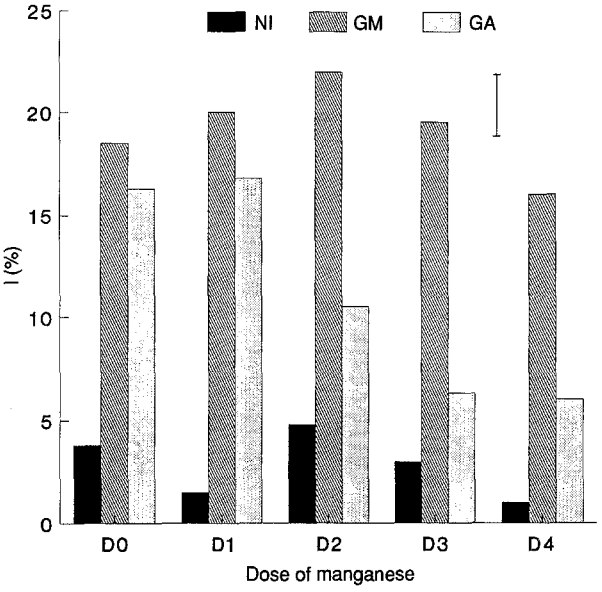

Fig. 1. Percentage of colonization by vesicular-arbuscular (VA) mycorrhizae in the roots of *Trifolium pratense* grown with various inoculum treatments and subsequently treated with various doses of Mn. *Bar* represents the LSD ($P=0.01$) for the effect of manganese (ANOVA-2 test). *D0*, Without Mn; *D1*, 15 mg Mn per pot; *D2*, 30 mg Mn per pot; *D3*, 60 mg Mn per pot; *D4*, 120 mg Mn per pot. *NI*, No inoculation; *GM*, *G. mosseae*; *GA*, *G. aggregatum*; *I*, colonization

Results

Dry weight

One-way analysis of variance of dry weight data for the aerial parts at each Mn dose (Table 1) showed no significant effect of inoculum. Doses D3 and D4 caused evident signs of leaf necrosis, in keeping with which the lowest dry-weight values for aerial parts were those for D4.

Development of VA mycorrhizae

In no case did the proportion of the root system bearing VA mycorrhizae exceed 25% (Fig. 1). Colonization by *G. mosseae* was little affected by Mn treatment, whereas colonization by *G. aggregatum* declined at Mn doses greater than D1. The relative abundance of arbuscules was similarly affected by Mn for both *Glomus* species, falling significantly at doses D3 and D4 (Fig. 2). Although the quantity of external mycelium was low in all pots (between 2 and 16 cm per g soil) the density was increased by Mn in *G. mosseae* especially at low doses (D1 and D2) (Fig. 3).

Mn uptake

The Mn concentration in the plants increased with increasing Mn dosage. Measured concentrations were

Table 2. Concentration of Mn in the aerial parts and roots of *Trifolium pratense* plants grown in pots with no inoculum (NI) or with *Glomus mosseae* (GM) or *G. aggregatum* (GA) inocula, and subsequently treated with various doses of Mn. For a given inoculum, different small letters indicate significant differences ($P=0.01$) between results for different Mn doses

Tissue	Inoculum	Mn dose				
		D0	D1	D2	D3	D4
Aerial parts	NI	103a	686b	1675c	2704d	3382e
	GM	121a	481b	1164c	2300d	3610e
	GA	106a	572b	1136c	2676d	4043e
Roots	NI	332a	2418b	3257b	8737c	12850d
	GM	232a	1763ab	2890b	7332c	14786d
	GA	264a	1806a	3877b	8240c	15110d

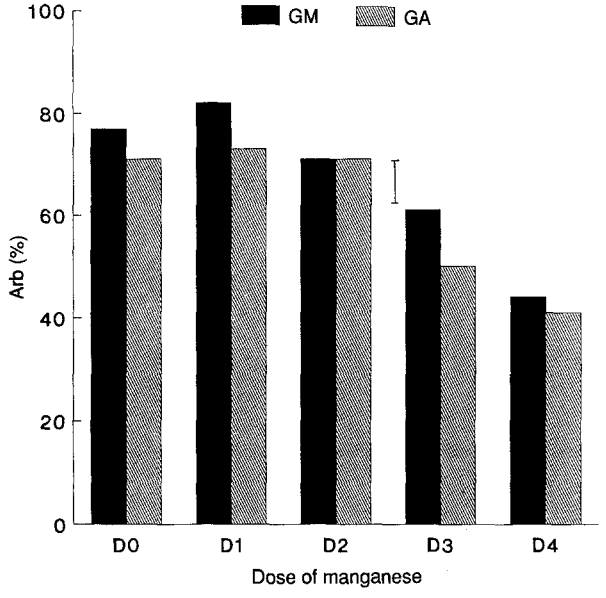

Fig. 2. Relative abundance of arbuscules in the roots of *Trifolium pratense* grown with *Glomus mosseae* or *G. aggregatum* inoculum and subsequently treated with various doses of Mn. *Bar* represents the LSD ($P=0.01$) for the effect of manganese (ANOVA-2 test). *Arb*, Arbuscules. See also legend to Fig. 1

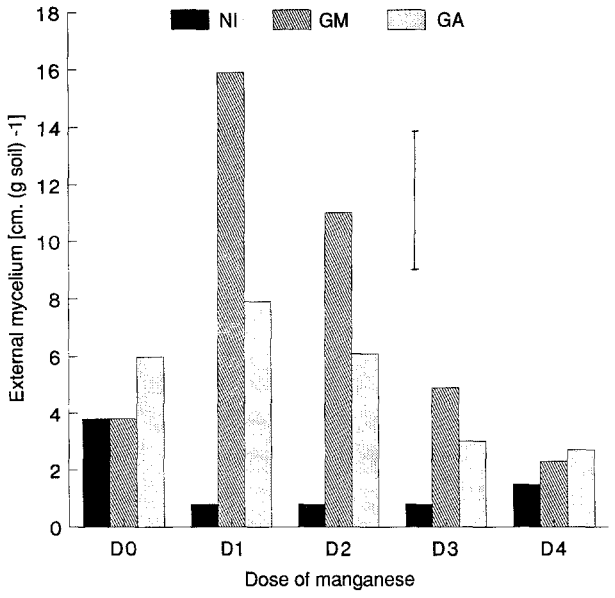

Fig. 3. Density of external mycelium in uninoculated or *G. mosseae*- or *G. aggregatum*-inoculated substrate treated with various doses of Mn. *Bar* represents the LSD ($P=0.01$) for the interaction (ANOVA-2 test). See also legend to Fig. 1

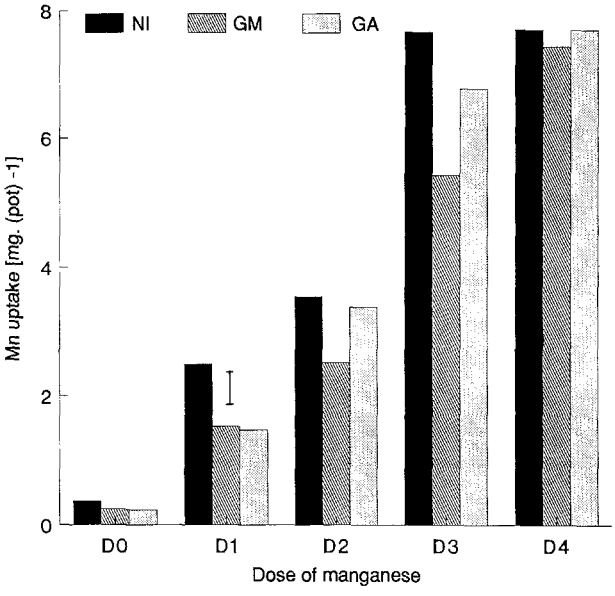

Fig. 4. Total uptake of Mn by *Trifolium pratense* plants grown with various inoculum treatments and subsequently treated with various doses of Mn. *Bar* represents the LSD ($P=0.01$) for the effect of the inocula (ANOVA-2 test). See also legend to Fig. 1

greater in the roots than in the aerial parts (Table 2). For doses D0–D3, the Mn concentration was generally lower in plants with *Glomus* than in those without, and the effect of inoculum type on total Mn uptake overall was statistically significant, plants with *Glomus* having lower Mn contents than those without when treated with doses D1–D3 (Fig. 4); *G. mosseae* reduced Mn uptake more effectively than *G. aggregatum*.

Mn in the substrate

Both available and exchangeable Mn increased with increasing Mn dose (Table 3). Mycorrhizae had no significant effect on the quantity of ammonium acetate-acetic acid/ethylenediaminetetraacetic acid extractable Mn but did affect exchangeable Mn, which for doses D2, D3 and D4 was significantly lower in the presence of mycorrhizae.

Mn-oxidizing bacteria

The presence of *Glomus* increased both the number and surface area occupied by Mn-oxidizing bacterial colonies

Table 3. Concentrations of available and exchange Mn ($\mu g\ g^{-1}$) in the substrate after *Trifolium pratense* plants were grown in pots with no inoculum (NI) or with *Glomus mosseae* (GM) or *G. aggregatum* (GA) inocula, and subsequently treated with various doses of Mn. For a given inoculum, different small letters indicate significant differences ($P=0.01$) between results for different Mn doses; for a given Mn dose, different capital letters indicate significant differences ($P=0.01$) between results for different inocula

Soil Mn	Inoculum	Mn dose				
		D0	D1	D2	D3	D4
Available	NI	50a	116b	182c	335d	544e
	GM	55a	121b	183c	337d	622e
	GA	57a	138b	234c	340d	518e
Exchangeable	NI	3aA	35bA	84cB	190dB	383eB
	GM	4aA	23aA	62bA	161cA	335dA
	GA	4aA	22aA	61bA	170cA	346dA

Table 4. Number of colonies (*n*) revealed by benzidine and the dish surface occupied (S, in cm^2)

Parameter	Mn dose	Inoculum		
		NI	GM	GA
n	D0	2.2± 2.2[a]	8.2± 5.3	38.6±15.5
	D2	2.9± 2.9	5.5± 6.5	22.0±16.5
	D4	3.4± 3.2	8.0± 7.1	31.3±20.3
S	D0	6.4±11.2	22.3±18.3	43.4±13.4
	D2	7.2± 8.3	18.0±20.7	24.3±17.4
	D4	9.5±10.1	32.1±30.5	29.0±18.1

[a] Means ± standard deviations

in cultures of substrate extracts (Table 4). The number of colonies increased more with *G. aggregatum* than with *G. mosseae*, but the results were similar for the surface area occupied by the colonies.

Discussion

The absence of any effect of mycorrhizae on plant growth was probably the result of supplying more phosphorus to the pots with no inoculum.

It is known that high Mn levels decrease infection by mycorrhizal fungi (Hepper 1979; McGee 1987), though the precise dependence of infection on substrate Mn levels is not known. The relatively high level of Mn in the substrate used in these experiments ($40\ \mu g\ g^{-1}$) may have been the cause of the low percentage of colonization observed even in the absence of further doses of Mn (D0).

In interpreting the results of the experiments described here, it should be borne in mind that mature plants with established mycorrhizal colonization (reflected by the D0 pots) were subjected to various doses of Mn; the results do not show the mycorrhizal development on plants grown from the beginning in the presence of different levels of Mn. The percentage colonization by *G. aggregatum* and the abundance of arbuscules were both reduced by Mn dosage. Extraradicular mycelium was favoured by Mn at doses D1 and D2, especially in the case of *G. mosseae*. The colonization by *G. mosseae* was greater than by *G. aggregatum*, particularly at high Mn doses, suggesting that the former is more resistant to Mn; different susceptibilities to the toxic action of Mn among VA fungi have previously been reported by McGee (1987). In contrast, there was no such difference between the two fungi in the abundance of arbuscules, which may be attributed to the faster turnover of arbuscules.

If the symbiosis between fungus and plant is effective in terms of protection against excess Mn, then total Mn uptake should be lower in plants with mycorrhizae (Pacovsky 1986). In this present work, the relatively low colonization levels would not be expected to lead to very great differences between plants with and without mycorrhizae. Nevertheless, total plant Mn content was in fact decreased by both *Glomus* species for doses D1–D3, in keeping with the protective role of VA mycorrhizae noted by Kucey and Janzen (1987) and Arines et al. (1989). The fact that *G. mosseae* decreased Mn uptake more than *G. aggregatum*, is more likely to reflect the greater colonization by *G. mosseae* than a greater efficiency; both species had significant effects on total Mn uptake for doses where they colonized more than 15% of the root system (D1 for *G. aggregatum* and D1–D3 for *G. mosseae*).

Mycorrhizal protection against excessive Mn uptake may be due either to the possessing by fungal hyphae of phytochelatins or similar peptides capable of complexing the metal (Steffens 1990), or to their ability to modify the rhizosphere in a way that reduces the availability of Mn (e.g. by lowering the Mn^{+2} concentration). The latter effect could be brought about via an effect on Mn-oxidizing and/or Mn-reducing organisms, which largely control Mn dynamics in the soil (Mulder 1972). In our experiments, the exchange Mn was lower in mycorrhizal substrates, and the number of Mn-oxidizing microorganisms was greater in the presence of mycorrhizae than in their absence. This suggests that the mycorrhizae in some way favour the growth of such microorganisms, possibly by producing a growth-stimulating exudate.

Further experimentation is required to test this hypothesis and to investigate the possible existence of parallel mechanisms that protect the plant against excessive Mn uptake.

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