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The role of arbuscular mycorrhiza in zinc uptake by red clover growing in a calcareous soil spiked with various quantities of zinc

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Abstract

Three pot experiments were conducted to investigate the role of arbuscular mycorrhiza (AM) in Zn uptake by red clover. Plants inoculated with *Glomus mosseae* and uninoculated controls were grown in a sterile calcareous soil in 'Plexiglas' (Acrylic) containers with two nylon net partitions (30 μm mesh) to separate the central root zone from the two outer hyphal zones. The effects of mycorrhiza on plant growth and Zn uptake changed dramatically with increasing Zn addition level (range 0–1200 mg kg^{-1}) in the root zone. With Zn addition levels <300 mg kg^{-1}, added Zn did not affect plant yield and above the critical level plant yield gradually decreased but was always higher for mycorrhizal than for controls. Below the critical Zn application rate (50 mg kg^{-1}), Zn uptake was enhanced while above this level Zn translocation to the shoots decreased. At all Zn addition levels, mycorrhizal colonization increased Zn absorption and accumulation in the roots, and this may help to explain the alleviation of Zn toxicity at high Zn application rates. As expected, AM colonization enhanced P nutrition and hence yield at all added Zn levels studied. Efforts were made to obtain direct evidence for hyphal contribution to Zn uptake by applying both Zn to the hyphal growth zone and additional P to the root zone to avoid the 'growth dilution effect'. The data demonstrate that mycorrhizal hyphae could absorb Zn directly from the soil and then transfer it to the plant roots. The hyphal contribution to Zn uptake by the host plant reached its maximum value at the Zn addition level of 50 mg kg^{-1}, in which Zn uptake via the extramatrical hyphae comprised 22% of total uptake, thus confirming the critical Zn application level found previously.

Keywords: Arbuscular mycorrhiza; Calcareous soil; Red clover; Zinc uptake

1. Introduction

The volume of soil exploited by plant roots can be greatly increased by the external mycelium of arbuscular mycorrhizal (AM) fungi (Bolan, 1991; Marschner and Romheld, 1998), and enhanced absorption of relatively immobile micronutrients such as Zn, Cu and Fe from deficient soils by AM plants has been well documented (Faber et al., 1990; Kothari et al., 1991; Li et al., 1991; Liu et al., 2000). On the other hand, under conditions of high available soil Zn and Cu, the concentrations of these trace elements in shoots have been reported to be lower in mycorrhizal than in non-mycorrhizal plants (Dueck et al., 1986; Leyval et al., 1991; Weissenhorn et al., 1995; Liu et al., 2000). Reduced concentrations of micronutrients in mycorrhizal plants are sometimes attributed to a dilution effect linked to an increase in plant dry matter (DM) yield (Nielsen and Jensen, 1983). However, experimental results cannot always be explained by a dilution effect.

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Some authors have reported adverse effects of AM on plant growth, which they have attributed to the enhanced uptake of Cu, Ni, Pb and Zn with increasing metal supply and decreasing soil pH (Killham and Firestone, 1983; Weissenhorn and Leyval, 1995). In general, the effects of AM fungi on the acquisition of immobile metal nutrients by the host plant are still unclear due to differences in experimental conditions used in different studies.

Although the functions of AM under conditions of micronutrient deficient conditions have been widely studied and are well understood, much information is required on the contrasting conditions of excessive trace elements (Leyval et al., 1997). Elevated concentrations of trace metals such as Zn exist in many agricultural soils from past management practices, and this may represent a risk to environmental quality and sustainable food production. Some studies have shown a positive impact of infection by arbuscular mycorrhizal fungi on the resistance of the host plants to Zn contamination of soils (Heggo et al., 1990; Hetrick et al., 1994; Zhu et al., 2001). The widespread existence of AM fungi in metal contaminated sites has also provided evidence of adaptation and tolerance of microorganisms to toxic metals (Shetty et al., 1995; Pawlowska et al., 1996), and metal tolerant fungi have been isolated (Raman et al., 1993; Griffioen, 1994; Weissenhorn et al., 1993, 1994; Hildebrandt et al., 1999). The feasibility of employing AM in soil re-vegetation and remediation has elicited great interest, and numerous studies have focused on the functions of AM fungi in metal-contaminated soils (Vangronsveld et al., 1996; Leyval et al., 1997; Khan et al., 2000).

The present investigation was carried out to: (1) understand the responses of AM fungi to elevated Zn levels in soil; (2) evaluate mycorrhizal effects on Zn uptake and partitioning in the host plant with soil Zn contamination ranging from very low to heavy; and (3) demonstrate Zn uptake via the mycorrhizal hyphae and estimate the hyphal contribution where the dilution effect due to enhanced plant growth has been eliminated. A total of three experiments were conducted in which a nylon mesh compartment cultivation system was employed for effective separation of the hyphal growth zone from the plant root zone so that mycorrhizal effects could be clearly differentiated.

2. Materials and methods

2.1. Host plants

Seeds of red clover (*Trifolium pratense* L.) were surface sterilized in a 10% (v/v) solution of hydrogen peroxide for 10 min. They were pre-germinated overnight and then ready for sowing.

2.2. Mycorrhizal inoculum

Inoculum of the AM fungus *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe comprised a sandy soil containing spores and maize root fragments. The original inoculum was kindly provided by Professor H.G. Wang of the Institute of Plant Nutrition and Fertilizers, Chinese Academy of Agriculture, and propagated on maize plants grown in a sandy soil for 10 weeks.

2.3. Cultivation media

A calcareous sandy soil of low nutrient status was collected from Panggezhuang village, Daxing County, Beijing and was passed through a 1-mm sieve. The soil had the following properties (DM basis): pH (soil:water ratio, 1:2.5) 7.8, 0.39% organic matter, 0.027% total N, 3.9 mg kg^{-1} 0.5 M $NaHCO_3$-extractable P, and 60 mg kg^{-1} 1 M NH_4OAc-extractable K. The soil extractable trace element concentrations were: 7.6 mg kg^{-1} Fe, 3.6 mg kg^{-1} Mn, 0.80 mg kg^{-1} Cu, and 0.63 mg kg^{-1} Zn. Soil metals were extracted with a 0.005 M diethylene triamine pentaacetic acid (DTPA)–0.01 mol/l $CaCl_2$–0.1 mol/l triethanolamine (TEA) solution (pH 7.30). The soil was sterilized by autoclaving at 120 °C for 2 h and then air-dried.

2.4. Cultivation system

Plexiglas pots were designed to permit separation of soil zones for root and hyphal growth. The pots were rectangular boxes 8 cm high and 8 cm deep. They had three compartments: a central one 2 cm wide separated from two outer ones each 5 cm wide by a nylon net of 30 μm mesh size, which allowed penetration by hyphae but not by roots.

2.5. Experiments

In the first experiment, four Zn addition levels, 0, 50, 100 and 300 mg kg^{-1} were applied to the root compartment as analytical grade $ZnSO_4$ solution mixed thoroughly with the soil. Mycorrhizal treatments received 15 g of inoculum, while non-mycorrhizal plants received an equivalent amount of sterilized inoculum together with a filtrate (0.25 μm pore size) of unsterilized soil to provide a similar microflora except for the absence of the mycorrhizal fungus. The inoculum was mixed uniformly with all of the 135 g soil in the root compartment. All treatments received mineral nutrients added in solution to each compartment at rates of 300 mg N ($Ca(NO_3)_2$), 50 mg P (CaH_2PO_4), and 200 mg K (K_2SO_4) kg^{-1} soil as a basal application. Thirty seeds were sown in the central compartment and 7 days after seedling emergence, 20 seedlings were retained for further growth.

Plants grew under a 25/22 °C temperature regime with a 14 h photoperiod at a light intensity of 250 $\mu mol\,m^{-2}\,s^{-1}$ provided by supplementary illumination. There were two mycorrhizal treatments, four Zn application rates and three replicates, giving a total of 24 pots in a randomized block design.

The second experiment was the same design, except for the high application rates of Zn (0, 600 and 1200 $mg\,kg^{-1}$) applied to the root compartment. Thus, there were two mycorrhiza treatments, three Zn application rates and three replicates, giving a total of 18 pots.

The third experiment was designed to evaluate the direct contribution of mycorrhizal hyphae to Zn uptake by the host plant. Zinc was added to the outer compartment, giving three zinc application rates (0, 50 and 200 $mg\,kg^{-1}$). In order to remove any growth dilution effect of mycorrhizal plants on Zn uptake (a growth dilution effect compared with lower-yielding non-mycorrhizal controls), additional P (300 $mg\,kg^{-1}$) was applied to the root compartments of non-mycorrhizal controls. Furthermore, soils with Zn addition were set up very carefully with a piece of plastic board inserted 1 cm outside the 30 μm nylon mesh barriers to protect the hyphal contribution to Zn uptake from any rhizosphere effects due to the direct involvement of plant roots in Zn diffusion and uptake in the hyphal zones. There were two inoculation treatments, three Zn application rates and three replicates to give a total of 18 pots.

At harvest the shoots and roots were recovered separately, oven dried and weighed. Samples for accessing root colonization were prepared using the acid fuchsin staining-grid intersect method (Kormanik et al., 1979; Giovannetti and Mosse, 1980).

2.6. Elemental analysis

Oven dried plant shoots and roots were milled and dry ashed. Phosphorus was determined colorimetrically, and Zn and other trace metals by atomic absorption spectrophotometry using standard methods. Data were tested by analysis of variance and means were compared by Duncan's multiple range test at the 5% level.

3. Results

3.1. Experiment 1

The results for mycorrhizal infection and plant growth (Table 1) show that no root infection was detected in the non-inoculated plants, while inoculated plants had reached infection rates of >50% of total root length irrespective of the Zn levels in the root zone. Zinc addition made no difference to plant DM yield, while plant growth was significantly improved by mycorrhizal colonization at all Zn levels tested. Shoot yields increased more as a result of mycorrhizal infection than did root yields, as indicated by the increase in shoot/root ratio (Table 1).

Similar trends were observed in P uptake (Table 2). No significant differences in P uptake could be detected among the Zn application levels, but at all Zn levels mycorrhizal plants consistently contained more than twice as much P as the corresponding non-mycorrhizal plants.

Both Zn application rate and AM inoculation had significant effects on Zn uptake by the clover plants (Table 3). Zinc concentrations in both shoots and roots of both the non-inoculated and inoculated plants increased dramatically as Zn levels increased. However, the effects of mycorrhizal colonization on Zn uptake seemed to be quite complex. The roots of mycorrhizal plants maintained much higher Zn concentrations than the corresponding non-mycorrhizal plants, while the shoots of mycorrhizal plants had higher Zn concentrations than those of non-mycorrhizal plants only below the Zn application rate of 50 $mg\,kg^{-1}$, with the opposite trend occurring above this level of added Zn. From the calculated values of root to shoot ratio for Zn uptake, it can be observed that Zn was partitioned to the shoots in non-mycorrhizal plants under the high Zn addition

Table 1
Mycorrhizal infection rates (percentage of total root length infected) and effects of mycorrhizal infection and Zn application rate on red clover shoot and root DM yield

Treatment	Infection rate (%)	Shoot DM ($g\,pot^{-1}$)	Root DM ($g\,pot^{-1}$)	Shoot/root (ratio)
$Zn_0 - M$	0.0 d[a]	1.02 c	0.36 c	2.83
$Zn_{50} - M$	0.0 d	0.93 cd	0.31 cd	3.00
$Zn_{100} - M$	0.0 d	1.03 c	0.38 c	2.71
$Zn_{300} - M$	0.0 d	1.01 c	0.34 c	2.97
$Zn_0 + M$	59.9 a	1.94 a	0.57 a	3.40
$Zn_{50} + M$	50.4 abc	1.85 ab	0.44 ab	4.20
$Zn_{100} + M$	59.1 a	1.87 a	0.50 a	3.46
$Zn_{300} + M$	50.5 ab	1.84 ab	0.45 ab	3.78

[a] Within columns, means with the same letter are not significantly different by Duncan's multiple range test at the 5% level.

Table 2
Effect of mycorrhizal infection on P uptake by red clover

Zn level	P uptake (mg pot^{-1})						+M/−M
	Shoot		Root		Total		
	−M	+M	−M	+M	−M	+M	
Zn_0	1.63 de[a]	5.00 ab	0.32 d	1.60 a	1.96	6.60	2.37
Zn_{50}	1.67 de	5.55 a	0.28 de	1.28 bc	1.95	6.83	2.50
Zn_{100}	1.75 d	5.05 ab	0.27 de	1.40 b	2.02	6.45	2.19
Zn_{300}	1.62 def	4.97 abc	0.31 d	1.22 bc	1.93	6.19	2.21

[a] Within columns, means with the same letter are not significantly different by Duncan's multiple range test at the 5% level.

Table 3
Zn concentration of red clover at different Zn application levels

Zn level (mg kg^{-1})	Zn concentration (mg kg^{-1})				Zn uptake (μg) root/shoot (μg μg^{-1})	
	Shoot		Root			
	−M	+M	−M	+M	−M	+M
Zn_0	44.2 g[a]	54.2 f	146.9 f	163.3 de	1.17	0.89
Zn_{50}	83.2 de	92.2 cd	194.2 cd	206.1 c	0.78	0.54
Zn_{100}	106.4 c	87.8 de	220.2 c	272.2 b	0.76	0.83
Zn_{300}	153.1 a	126.3 b	298.6 b	429.8 a	0.66	0.83

[a] Within columns, means with the same letter are not significantly different by Duncan's multiple range test at the 5% level.

levels, while mycorrhizal colonization resulted in the retention of substantial amounts of Zn in the roots with less translocation of Zn to the shoots.

3.2. Experiment 2

When high rates of Zn (600 and 1200 mg kg^{-1}) were added to the root compartment, not only was there no decrease, but also there was actually an increase in the mycorrhizal infection rate of red clover compared with the pots receiving no applied Zn (Table 4). However, both shoot and root DM yields tended to decrease with increasing Zn level, but the differences between treatments were not significant. Shoot yield increased greatly as a result of mycorrhizal infection, whereas root yield was not affected.

Phosphorus uptake by clover plants was greatly facilitated by AM inoculation but not affected by Zn application rate. In general, the P concentration of mycorrhizal clover was twofold to threefold higher than that of non-mycorrhizal plants (Fig. 1).

With increasing Zn addition rate, the Zn concentrations of both shoots and roots increased markedly irrespective of the inoculation treatments. Shoot Zn concentration declined with mycorrhizal infection and the opposite trend was found for root Zn concentration (Fig. 2).

3.3. Experiment 3

There was a slight but insignificant decrease in mycorrhizal infection rate as the Zn addition increased. No significant difference was recorded in shoot yield between mycorrhizal and non-mycorrhizal plants at any Zn application level in the outer compartments. Both root DM yield and length were reduced by fungal colonization (Table 5). Shoot P concentration was about 0.15% and root P concentration about 0.18%, which

Table 4
Mycorrhizal infection rates and effects of mycorrhizal infection and Zn application rate on red clover shoot and root DM yield

Treatment	Infection rate (%)		Shoot DM (g pot^{-1})		Root DM (g pot^{-1})	
	−M	+M	−M	+M	−M	+M
Zn_0	0 d[a]	45.5 bc	1.46 d	2.23 a	0.44 a	0.42 a
Zn_{600} − M	0 d	50.3 a	1.44 de	2.20 ab	0.41 a	0.44 a
Zn_{1200} − M	0 d	46.1 ab	1.23 def	1.99 abc	0.30 ab	0.41 a

[a] Within columns, means with the same letter are not significantly different by Duncan's multiple range test at the 5% level.

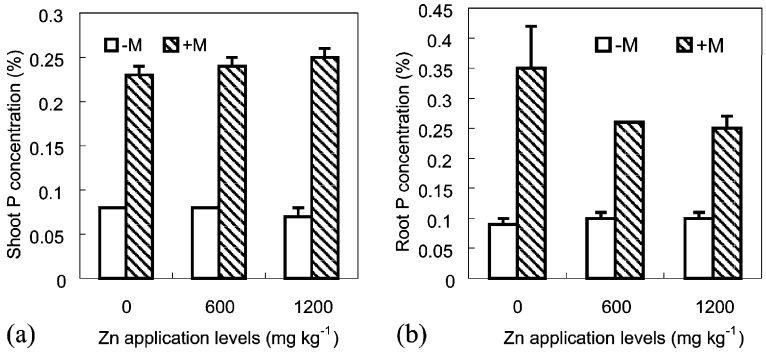

Fig. 1. (a) Shoot and (b) root P concentrations of mycorrhizal (+M) and non-mycorrhizal (−M) red clover plants under different Zn addition levels in the root zone.

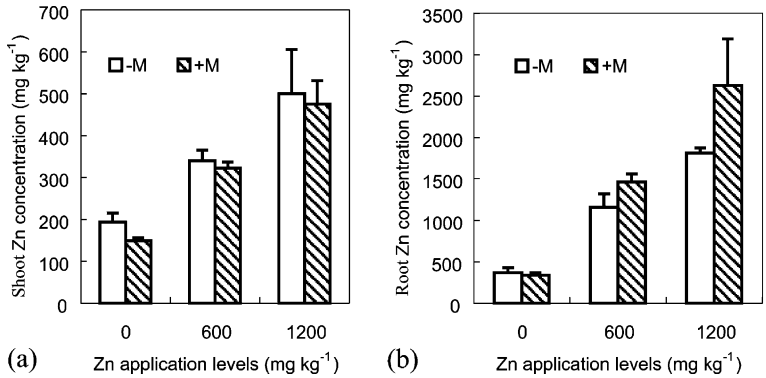

Fig. 2. (a) Shoot and (b) root Zn concentration of mycorrhizal (+M) and non-mycorrhizal (−M) clover plants under different Zn addition levels in the root zone.

Table 5
Mycorrhizal infection rate and dry weight of red clover

Treatment	Infection rate (%)	Shoot DM (g pot^{-1})	Root DM (g pot^{-1})	Root length (m pot^{-1})
$Zn_0 - M$	0.0 d[a]	4.23 a	2.72 ab	167.4 ab
$Zn_{50} - M$	0.0 d	4.00 a	2.54 abc	160.6 a
$Zn_{200} - M$	0.0 d	4.48 a	2.91 a	158.4 ab
$Zn_0 + M$	29.5 a	4.02 a	1.71 def	98.1 de
$Zn_{50} + M$	27.2 ab	4.81 a	2.31 abc	127.0 bc
$Zn_{200} + M$	25.3 ab	4.43 a	1.97 cd	125.4 bc

[a] Within columns, means with the same letter are not significantly different by Duncan's multiple range test at the 5% level.

were sufficient for adequate growth of the host plants under the experimental conditions (data not shown). There were also no significant differences in P concentration between mycorrhizal and non-mycorrhizal plants at any Zn application rate.

Zinc concentrations in non-inoculated plants were not affected by Zn addition rate. Mycorrhizal infection led to a marked increase in Zn concentration of both shoots and roots and this was more pronounced at the elevated Zn levels (Fig. 3).

As the dilution effect on Zn uptake had been eliminated, the increment in Zn uptake by mycorrhizal plants compared with non-mycorrhizal plants should be attributable to hyphal uptake from the outer compartments. Based on this hypothesis, the hyphal contribution to Zn uptake was calculated. At the Zn addition level of 50 mg kg^{-1}, Zn uptake via the extrametrical mycelium amounted to 62 μg, which represented 22% of total Zn uptake by the mycorrhizal plants. At the 200 mg kg^{-1} level, the values declined to 21 μg and 8.1%, respectively.

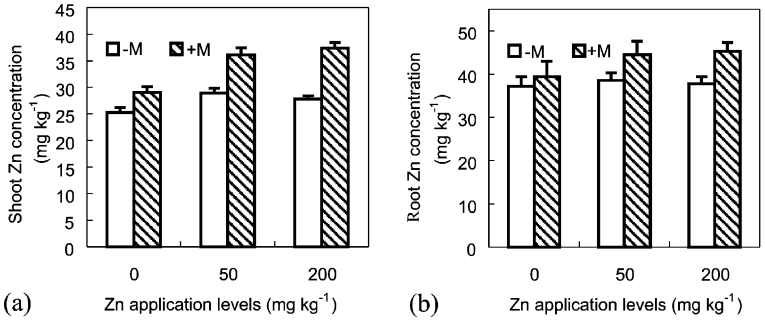

Fig. 3. (a) Shoot and (b) root Zn concentrations of mycorrhizal (+M) and non-mycorrhizal (−M) clover plants under different Zn application levels in the hyphal compartments.

4. Discussion

High levels of colonization by AM fungi were observed both in mine spoils heavily polluted with metals by Diaz et al. (1996) and in agricultural soils contaminated with metals of different origins by Weissenhorn et al. (1995a,b). This leads to the conclusion that metal tolerance has developed naturally in the soil microorganisms. The present study also provides evidence of tolerance to Zn contamination by the AM fungal strain. Even when the Zn addition level was as high as 1200 mg kg^{-1}, the mycorrhizal infection rate did not decrease compared to the control receiving no Zn. This may imply that Zn has no or little effect on spore germination and AM colonization. The fungal strain used did not originate from a metal enriched soil, and the question of whether it would function differently on naturally contaminated soils would require further study.

Under low or moderate Zn application levels, plant growth showed no decrease as Zn addition increased. A slight growth inhibition was observed only at the very high Zn levels above 600 mg kg^{-1}. This may be attributed to the high soil pH value of 7.8, at which a large proportion of the added Zn may have been immobilized and unavailable for plant uptake, leaving a small quantity of available Zn even at the highest Zn application rate studied.

Mycorrhizal infection consistently improved plant yield, especially shoot yield, in all the experimental treatments. This can easily be explained by enhanced P uptake via the extrametrical mycelium. The mycorrhizal plants showed a more than twofold increase in P uptake compared with corresponding non-mycorrhizal plants. Under natural conditions, mycorrhizal plants would colonize polluted sites. Considering the paucity of mineral nutrients, especially P, commonly found together with elevated metal concentrations in these sites (Shetty et al., 1994), the improved P nutrition of host plants might be one of the major mechanisms involved in the alleviation of metal toxicity as a result of mycorrhizal colonization.

Enhanced uptake of Zn at low Zn application levels was found repeatedly in the present study, and the protective effects of AM fungi against Zn toxicity of clover plants under conditions of Zn contamination were also observed. Although plant Zn concentration increased with increasing Zn levels irrespective of inoculation, much Zn was retained in the mycorrhizal roots and translocation to the shoots was inhibited. In contrast, non-mycorrhizal plants experienced higher Zn concentrations in their shoots with a consequent reduction in yield. The high Zn concentrations in mycorrhizal compared to non-mycorrhizal roots indicates that the fungi retain Zn in or on their mycelium (Colpaert and Van Assche, 1992). Direct determination of trace metal concentrations in fungal mycelium was made by Chen et al. (2001) who demonstrated Zn concentrations in AM fungal mycelium of about 10 times those in host plant tissues. Different partition patterns of elements in mycorrhizal and non-mycorrhizal maize were investigated using SIMS analysis by Kaldorf et al. (1999), who found that Zn existed mainly in fungal structures such as vesicles in the root cortex. It can be concluded from these studies that immobilization of Zn in the fungal mycelium is one of the important factors involved in alleviation of metal toxicity by mycorrhizal plants.

In our third experiment, there were no differences in yield between mycorrhizal and non-mycorrhizal plants at any Zn level. This was achieved by applying supplementary P fertilizer to the root compartments of the non-mycorrhizal plants to eliminate the effect of improvement of plant P status exerted by mycorrhizal infection on Zn toxicity alleviation. The mycorrhizal hyphae in the outer compartments were able to grow well even at the added Zn level of 200 mg kg^{-1}, and furthermore they were active and efficient at absorbing P from the hyphal zone and transferring it to the plant, as evidenced by the comparatively high P acquisition by

the mycorrhizal plants compared with non-mycorrhizal controls receiving 300 mg kg^{-1} of additional P. Similar results were also obtained by Li and Christie (2001), and lend further evidence that uninhibited fungal growth under elevated Zn additions is consistent with a high mycorrhizal infection rate. Using culture systems which separate extraradical hyphae from roots, it has been shown that the hyphae can accumulate and translocate ^{65}Zn to a degree that may differ between species (Burkert and Robson, 1994). In the present study the absence of an effect on the Zn concentration of non-mycorrhizal plants and gradually increasing Zn concentrations of mycorrhizal plants with increasing Zn application level support the hypothesis that Zn is taken up and transferred to the host plant via the extraradical hyphae.

Several Zn and Cd tolerant fungal isolates are now available from mycorrhizal research groups (Sambandan et al., 1992; Weissenhorn et al., 1993; Griffioen, 1994), and their functions have been thoroughly studied. Considering that the AM fungus is a non-host specific symbiont, and that the host plant always plays the dominant role in the symbiosis, much attention also should be paid to host plants. More systematic research on the mechanisms involved in arbuscular mycorrhizal metal absorption and transportation processes in plants is necessary for the application of this symbiosis in future soil Rehabilitation programs.

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