Microbial Ecology

Brevibacillus brevis Isolated from Cadmium- or Zinc-Contaminated Soils Improves *in Vitro* Spore Germination and Growth of *Glomus mosseae* under High Cd or Zn Concentrations

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Abstract

In this study we investigated the saprophyte growth of two arbuscular–mycorrhizal fungi (*Glomus mosseae* isolate) under increasing Cd or Zn levels and the influence of a selected bacterial strain of *Brevibacillus brevis*. Microorganisms here assayed were isolated from Cd or Zn polluted soils. *B. brevis* increased the presymbiotic growth (germination rate growth and mycelial development) of *Glomus mosseae*. Spore germination and mycelial development of both *G. mosseae* isolate were reduced as much as the amount of Cd or Zn increased in the growth medium. In medium supplemented with 20 μg Cd mL^{-1}, the spore germination was only 12% after 20 days of incubation, but the coinoculation with *B. brevis* increased this value to 40% after only 15 days. The addition of 20 μg Cd mL^{-1} to the growth medium drastically inhibited hyphal development, but the presence of the bacterium increased hyphal growth of *G. mosseae* from 195% (without Cd) until 254% (with 20 μg Cd mL^{-1}). The corresponding bacterial effect increasing micelial growth ranged from 125% (without Zn) to 232% (200 μg Zn mL^{-1}) in the case of *G. mosseae* isolated from Zn-polluted soil. Mycelial growth under 5 μg Cd mL^{-1} (without bacterium) was similarly reduced from that produced at 15 μg Cd mL^{-1} in the presence of the bacteria. As well, 50 μg Zn mL^{-1} (without bacterium) reduced hyphal growth as much as 200 μg Zn mL^{-1} did in the presence of *B. brevis*. The bacterial effect on the saprophytic growth of *G. mosseae* in absence of metal may be due to the involvement of indole acetic acid (IAA) produced by these bacteria. The Cd bioaccumulation ability exhibited (76%) by Cd-adapted *B. brevis* reduced the Cd damage on *G. mosseae* in Cd-contaminated medium. These capabilities of *B. brevis* isolates partially alleviate the inhibitory effects of Cd or Zn on the axenic growth of *G. mosseae*.

Introduction

A metal-stressed environment can be defined as an external aqueous chemical system that can inhibit cell metabolism and growth by impairing enzyme function, unless the microbial population is able to reduce the toxic effect of the metals [34]. Some fungal and bacterial species, however, developed tolerant traits and can survive and thrive on contaminated environments by adapting mechanisms at the cellular level as constitutive metal tolerance capable of the detoxification and hence tolerance in the presence of excessive amounts of heavy metals. Nevertheless, elevated concentrations of metals in the soil can lead to toxicity and to the inhibition of growth of bacteria and/or arbuscular mycorrhizal (AM) fungi [9, 25, 26].

Resistant bacteria, as adapted *Brevibacillus brevis* and AM fungi as native *G. mosseae*, selected in a previous study [37, 38] are prevalent in soil having toxic metals such as Cd or Zn in high quantities.

Regarding zinc and cadmium, the physiological role of each metal is different. Zn is an essential micronutrient for many cellular enzymes and is required for function of a large number of proteins but it is needed by cells in trace amounts, whereas Cd is not needed by bacterial cells and is very toxic. Nevertheless, elevated concentrations of both essential (Zn) or toxic (Cd) metals can reduce cells biomass or induce growth inhibition as a consequence of toxicity symptoms [14].

In previous studies [37, 38] it was demonstrated that native bacteria isolated from Zn- or Cd-polluted soils interact with autochthonous Zn- or Cd-tolerant AM

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fungi such as *G. mosseae* isolates. The inoculation of both microorganisms tolerant to Zn and Cd enhanced tolerance of colonized-plants to these metals and also the development of AM symbiosis [37, 38]. If *Brevibacillus brevis* is able to survive in a metal-contaminated medium, it probably has the capacity to avoid or detoxify stressed environments. Mechanisms allowing to the bacteria to persist in metal-polluted media include the formation of organic metal-complexing agents [16, 17, 29], precipitation, or redox transformation of metal [34].

Cadmium- or Zn-adapted *Brevibacillus brevis* strains have demonstrated plant-growth-promoting (PGP) and mycorrhiza-helper (MH) effects using Cd or Zn contaminated soils in a microcosm systems. The positive AM symbiotic effects were stimulated by a strain of *Brevibacillus brevis*, where the application of the dual inocula proved very important for alleviating metal toxicity to the plant [37, 38]. To our knowledge, there are no previous studies indicating an interactive role of autochthonous bacteria and AM fungi on plants growing under polluted conditions. The bacterial effect could be due to the ability of rhizosphere bacteria to excrete biologically active substances that can affect the host plant and/or the mycorrhizal fungus [3–5]. Nevertheless, these *Brevibacillus brevis* strains as well as some others microorganisms may possess a range of potential cellular mechanisms (e.g., biosorption and bioaccumulation) that are involved in the detoxification of heavy metals [8]. Thus, these strains not only are able to show tolerance to metals stress but also have the possibility to reduce the metal in the medium. These mechanisms include the binding capacity to metals for chelation of metals in the bacterial cells and other strategies previously reported [34].

The aim of this study was to investigate the effect of different levels of Zn or Cd added to the axenic water-agar medium on the rate of spore germination and hyphal development of *G. mosseae* and to test the role of *Brevibacillus brevis,* metal-tolerant isolates, on the axenic development of the fungus under the polluted conditions assayed. Bacterial mechanisms involved in alleviating metal toxicity were also investigated.

Materials and Methods

Experimental Design. The experiment tested the germination and saprophytic growth of spores of two autochthonous AM fungi: *Glomus mosseae* isolated from Cd-contaminated soils; or *G. mosseae* isolated from Zn-contaminated soils. The spores were assayed alone or in coinoculation with the corresponding autochthonous *Brevibacillus brevis* strain isolated from Cd- or Zn-polluted soils. Plates containing only spores or spore plus bacteria were assayed either without metal or with metal at three levels of Cd (*G. mosseae* and *Brevibacillus brevis* isolated from Cd-contaminated soil) or Zn (*G. mosseae* and *Brevibacillus brevis* isolated from Zn-contaminated soil) in the medium.

All treatments were replicated seven times with a total of 56 plates for each *G. mosseae* (Cd or Zn isolated).

Soil Microorganisms. The soil samples for microbial inocula production were taken from a Cd-treated or Zn-treated long-term field (10 years old) at Nagyhörcsök (Hungary) [19, 40].

From these soils containing the native adapted AMF and bacterial populations both microbes were isolated and cultivated for inocula production. The bacterial strains here selected were the two most abundant cultivable types in original Cd- or Zn-polluted soil (Nagyhörcsök, Hungary).

Indigenous mycorrhizal inocula isolated from the Cd- for Zn-polluted soil (Nagyhörcsöck, Hungary) were identified in each soil sample as *Glomus mosseae* strains (determined morphologically).

The bacterial isolation was carried out following the conventional procedure: 1 g of homogeneized rhizosphere soil was suspended in 100 mL of sterile water (dilution 10^2) and 1 mL of this suspension was serially diluted to reach dilutions 10^4 to 10^7. These were plated in agar nutrient broth medium (8 gL^{-1}) contanining meat extract (3 mL^{-1}) and peptone gelatin (5 gL^{-1}) and cultivated for 48 h at 28°C.

Once selected, the most abundant bacterial type from each Cd- or Zn-contaminated soil were grown in 250-mL flasks containing 50 mL of nutrient broth medium in shake culture for 48 h at 28°C.

Bacterial strains were later identified as *Brevibacillus brevis*, according to the molecular method used. In the appropriate treatments, plates were inoculated with 0.5 mL of the bacterial culture (10^8 cfu mL^{-1}) grown in nutrient broth medium for 24–48 h at 28°C.

The experiments were conducted in 9-cm diameter polyethylene petri dishes containing water-agar (1% Difco Bacto agar) adjusted to pH 7.0 with or without addition of a range of Cd (5, 15, or 20 μg mL^{-1}) as 3Cd $SO_4 \cdot 8H_2O$ or Zn (10, 50, or 200 μg mL^{-1}) levels as Zn $SO_4 \cdot 7H_2O$. Spores of *G. mosseae* were obtained from the rhizosphere of *Trifolium repens* and grown in pot cultures in a greenhouse. Resting spores freshly isolated from respective *G. mosseae* inocula by the wet sieving and decanting technique [2] were surface sterilized with Chloramine T, streptomycin, and Tween 80 mixture for 20 min and then washed five times in sterile water [27]. Spores were transferred by sterile capillary pipettes to petri dishes.

Five surface-sterilized spores of *G. mosseae* were transferred individually to each petri dish, located on the vertices of a pentagon about 3.5 cm on a side. The bacterium was inoculated (0.5 mL having 10^8 cfu mL^{-1}) in the center of the dish, equidistant from the five *G. mosseae* spores.

The incubation was carried out at 25°C in the dark; the plates were sealed with Parafilm to reduce dehydration and contamination risks. Seven replicates per treatment were run.

Measurements. Germination rate and hyphal growth of *G. mosseae* were assessed after 3 weeks incubation. A spore was considered germinated if a germ tube was clearly visible. Hyphal development was determined as described by Hepper and Jakobsen [15].

Molecular Identification of the Bacterial Isolate. Bacterial identification was carried out by 16S rDNA cloning and sequencing as previously described [37]. Database searches for 16S rDNA sequence similarity unambiguously identified both bacteria (Cd- and Zn-adapted) as *Brevibacillus brevis*.

Bacterial Growth under Increasing Cd or Zn Levels in the Medium. The growth of the Cd-tolerant or Zn-tolerant bacteria under increasing Cd or Zn levels was tested in comparison to a *Brevibacillus brevis* from non-polluted soil [Spanish culture type collection, at Burjasot (Valencia)] that was used as a control bacterium for comparison with *Brevibacillus brevis* strains isolated from Cd- or Zn-polluted sites.

Bacterial strains were cultivated at 28°C in nutrient broth (8g L^{-1}) supplemented with 25 or 50 μg mL^{-1} Cd as $3CdSO_4 \cdot 8H_2O$ and 75 or 100 μg mL^{-1} Zn supplemented as $ZnSO_4 \cdot 7H_2O$. The number of viable cells was estimated at 3-h intervals from 0 to 12 h following the conventional procedure: 1 mL of suspension was plated in agar nutrient broth medium (8 gL^{-1}).

Bacterial Capability for Cd or Zn Biosorption. The biosorption study was carried out as described by Kanazawa and Mori [20] with some modifications. Bacteria were grown in 250 mL of nutrient broth until reaching one unit of optical density (600 nm). Then the cells were harvested by centrifugation at 7000 *g* for 30 min, and the bacterial pellet was washed twice with Ringer's solution (NaCl 0.85%, $CaCl_2$ 0.03%, KCl 0.025%, $NaHCO_3$ 0.02%). The harvested biomass was incubated for 1 h at 28°C with a solution containing either 11 μg Cd mL^{-1} as $3Cd\ SO_4 \cdot 8H_2O$ or 267.4 μg Zn mL^{-1} was $Zn\ SO_4 \cdot 7H_2O$.

The suspension was then centrifuged at 7000 rpm and filtered through a 0.45-μm Millipore membrane to separate the biomass from the filtrate. The biomass was dried, weighed, and heavy metals were extracted by nitric acid (24 h). The Cd and Zn contents were determined by atomic absorption spectrometry of both biomass and supernatants.

Production of Indole-3-Acetic Acid (IAA). The production of IAA by the bacteria was measured by the method of Wöhler [39]. The bacteria (*Brevibacillus brevis* strains and a *Bacillus pumillus*, used as control previously selected as an efficient IAA producers [28]) were grown overnight on nutrient broth and then collected by centrifugation at 1000 *g* for 5 min. The bacterial pellet was then incubated at 37°C for 24 h with 3 mL of phosphate buffer (pH 7.5) with glucose (1%) and 2 mL of L-tryptophan (1%). After incubation, 2 mL of 5% trichloroacetic acid and 1 mL of 0.5 M $CaCl_2$ were added. The solution was filtered through Whatman no. 2 filter paper. Three mL of the filtrate were transferred to a test tube and mixed with 2 mL of salper solution (2 mL 0.5 M $FeCl_3$ and 98 mL 35% perchloric acid). The mixture was incubated for 30 min at 25°C in the dark. Then the absorbance of the resulting solution was measured at 535 nm with a Shimadzu UV-1603 spectrophotometer. The calibration curve ranged from 0.5 to 10 μg IAA mL^{-1}.

Statistics. Results in Tables 3 and 4 were subjected to ANOVA. When the main effect was significant, differences among means were evaluated from significance ($P < 0.05$) by Duncan's multiple range test.

Results

The highest number of spores germinated for both isolates of *G. mosseae*, occurred after 7 days of incubation in medium without metal. Percentage of spore germinated and time of germination were positively affected by the inoculation with *Brevibacillus brevis* and negatively affected by the increasing levels of Cd or Zn (Tables 1 and 2). At whatever metal level, the greatest percentage of spore germinated and also the advancement in the germination time were achieved in bacterial inoculated plates. In contrast, the increasing metal level not only decreased spore germination but also delayed the germination time. In both experiments, the highest percentage of spore germination was observed after 4 days in plates without metal and inoculated with *Brevibacillus brevis*. Thus, the presence of bacteria in the axenic medium advantaged 3 days germination time (Tables 1 and 2).

The percentage of spore germination was particularly increased by the bacteria at the highest Cd and Zn levels (Tables 1 and 2). In fact, under 20 μg mL^{-1} Cd, the bacteria increased the germination rate by 233% and advanced the germination process for 5 days (Table 1). In medium having 200 μg Zn mL^{-1}, *G. mosseae* spores germinated after 20 days of incubation but the germination rate was increased by the bacteria by 180% (Table 2). The effectiveness of *Brevibacillus brevis* increasing spore germination was higher under the highest polluted conditions.

Table 1. Effect of *Brevibacillus brevis* on number and percentage of *G. mosseae* spores germinated in axenic medium supplemented with different concentrations of Cd

		Days of spore incubation				
Treatments	*Cd levels (μg mL^{-1})*	*4*	*7*	*10*	*15*	*20*
G. mosseae	0	–	28 ± 4.2 [80%]	–	–	–
	5	–	–	17.5 ± 4.2 [50%]	–	–
	15	–	–	–	13.3 ± 5.2 [38%]	–
	20	–	–	–	–	4.2 ± 2.1 [12%]
G. mosseae	0	32.5 ± 6.3 [93%]	–	–	–	–
	5	–	25.9 ± 4.9 [74%]	–	–	–
+ *B. brevis*	15	–	–	–	18.2 ± 2.8 [52%]	–
	20	–	–	–	–	14 ± 0.19 [40%]

Determinations were done after 4, 7, 10, 15, and 20 days of spore incubation. SEs are given.
(–) No spore germination.

As expected, the greatest hyphal growth from both *G. mosseae* (Cd or Zn isolates) was also observed in medium without metal (Tables 1 and 2). The positive bacterial effect on the enhancement of hyphal growth was strongest in Cd-contaminated medium (Tables 3 and 4).

Presence of Cd or Zn negatively affected hyphal development and the highest Cd or Zn level severely reduced the length of mycelia developed from germinated spore (Tables 3 and 4). However, *Brevibacillus brevis* also had a stimulating effect on this value and increased hyphal development by 95% (without Cd) and by 56% (without Zn) and by 49, 73, or 154% under 5, 10, or 20 μg Cd mL^{-1} and by 25, 65, or 132% under 10, 50, or 200 μg Zn mL^{-1}, respectively as compared to spores that were not inoculated with bacteria. The positive effect of *Brevibacillus brevis* enhancing hyphal development was maintained at whatever metal level, but it was more effective as much the metal increased in the medium. In fact, *Brevibacillus brevis* increased the hyphal development of *G. mosseae* (isolated from Cd-polluted soil) by 95% (in absence of Cd) but the positive effect was higher (154% increase) at the highest amount of Cd in the medium (20 μg Cd mL^{-1}) (Table 1). Similarly, the hyphal development from *G. mosseae* (isolated from Zn-

Table 2. Effect of *Brevibacillus brevis* on number and percentage of *G. mosseae* spores germinated in axenic medium supplemented with different concentrations of Zn

		Days of spore incubation				
Treatments	*Zn levels (μg mL^{-1})*	*4*	*7*	*10*	*15*	*20*
G. mosseae	0	–	26.2 ± 3.8 [75%]	–	–	–
	10	–	–	24.5 ± 4.4 [70%]	–	–
	50	–	–	–	17.5 ± 2.45 [50%]	–
	200	–	–	–	–	10.5 ± 2.1 [30%]
G. mosseae	0	29.4 ± 4.2 [84%]	–	–	–	–
	10	–	–	26.2 ± 3.1 [75%]	–	–
+ *B. brevis*	50	–	–	23.8 ± 3.5 [68%]	–	–
	200	–	–	–	–	18.9 ± 2.8 [54]%

Determinations were done after 4, 7, 10, 15, and 20 days of spore incubation. SEs are given.
(–) No spore germination.

Table 3. Effect of *Brevibacillus brevis* on mycelial (hyphal) growth of *G. mosseae* spores on axenic medium supplemented with different concentrations of Cd after 3 weeks of incubation

Treatments	Cd levels (μg mL^{-1})	Hyphal development (mm $spore^{-1}$)	% of control treatment
G. mosseae	0	18.0 b	100
	5	12.1 b	67
	15	7.0 c	38
	20	2.0 c	11
G-mosseae + B. brevis	0	35.2 a	195
	5	18.0 b	100
	15	12.0 b	66
	20	5.0 c	28

Means followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 4. Effect of *Brevibacillus brevis* on mycelial (hyphal) growth from *G. mosseae* spores on axenic medium supplemented with 3 different concentrations of Zn after 3 weeks of incubation

Treatments	Zn levels (μg mL^{-1})	Hyphal development (mm $spore^{-1}$)	% of control treatment
G. mosseae	0	16.0b	100
	10	12.0 b	75
	50	6.0 c	38
	200	3.0 c	19
G. mosseae+ B. brevis	0	25.0 a	156
	10	15.0 b	94
	50	10.0 bc	63
	200	7.0 c	44

Means followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

contaminated soil) was enhanced by *Brevibacillus brevis* over control by 56% (in absence of Zn) and by 134% when the medium had 200 μg Zn mL^{-1} (Table 2).

Spores growing in plates supplemented with 15 μg Cd mL^{-1} with bacteria produced hyphal growth similar to that of *G. mosseae* spores at 5 μg Cd mL^{-1} without bacteria. Moreover, the negative effect of 5 μg Cd mL^{-1} on mycelial growth seems to be compensated by bacterial inoculation. Mycelial growth from spores growing without Cd reached a similar value to that growing in plates plus 5 μg Cd mL^{-1} in presence of bacteria (Table 3).

Brevibacillus brevis increased the presymbiotic phase of both *G. mosseae* strains. Biosorption of Cd by *Brevibacillus brevis* isolated from Cd-polluted soil was much higher than Zn accumulation by *Brevibacillus brevis* isolated from Zn-polluted soil. The microbial biomass sorption in terms of percentage was 76% in the case of Cd and only 5.6% in the case of Zn. These results indicate that only 24% of Cd remained in the contaminated medium after bacterial growth, whereas nearly the totality of Zn (94%) remained in Zn-contaminated medium after bacterial culture (Table 5).

Autochthonous bacterial strains, isolated from Cd- or Zn- polluted soils, had a higher capacity for growing under high Cd (25 or 50 μg mL^{-1}) or Zn (50 or 100 μg mL^{-1}) concentrations in the medium than the reference nonadapted (from collection) *Brevibacillus brevis* strain, which did not support such metal concentrations and appeared to be very sensitive to the concentrations of these metals used (Fig. 1). Whereas the reference *Brevibacillus brevis* was unable to grow in media supplemented with the applied amounts of Cd or Zn, *Brevibacillus brevis* isolated from a polluted site reached 10^7 or 10^8 cfu after 6 or 9 h in these highly Cd- or Zn-contaminated media (Fig. 1, 2).

Indole acetic acid (IAA) production by autochthonous Cd or Zn *Brevibacillus brevis* was greater than that produced by the reference *Bacillus pumillus* strain (Fig. 3).

Discussion

In previous microcosm experiments using Cd- or Zn-contaminated soils, the AM colonization and extraradical mycelium developed in plants colonized by two *G. mosseae* ecotypes (Cd- or Zn-adapted) were respectively increased in the presence of *Brevibacillus brevis* [37, 38]. These effects could be interpreted as an indirect result (mediated by an increased carbohydrate supply (from a better developed host plant) to the fungus [36]. Nevertheless, according to the results here presented the bacterial stimulating effect found in the AM symbiotic state by Vivas et al. [36, 37] can be also explained by the bacterial effect on the presymbiotic fungal development.

In this study, we found that the highest levels of Zn or Cd did not totally inhibit germination and hyphal growth of *G. mosseae* spores isolated from Zn- or Cd-contaminated areas. Results confirm the previously observed heavy metal tolerance of AMF isolated from polluted sites [11, 12]. The level of protection that the

Table 5. Cd or Zn biosorption by *Brevibacillus brevis* cells (autochtonous Cd- or Zn-adapted bacteria) and that remaining in the supernatant after 1 h of bacterial growth

Bacterial strain	Metal in the Culture medium	Metal cell biosorption		Metal remaining in supernatant	
		μ g^{-1}	%	μg mL^{-1}	%
B. brevis (Cd adapted)	11.1 μg Cd mL^{-1}	8,4	76	2,7	24
B. brevis (Zn adapted)	267.4 μg Zn mL^{-1}	14,8	5,6	252,5	94

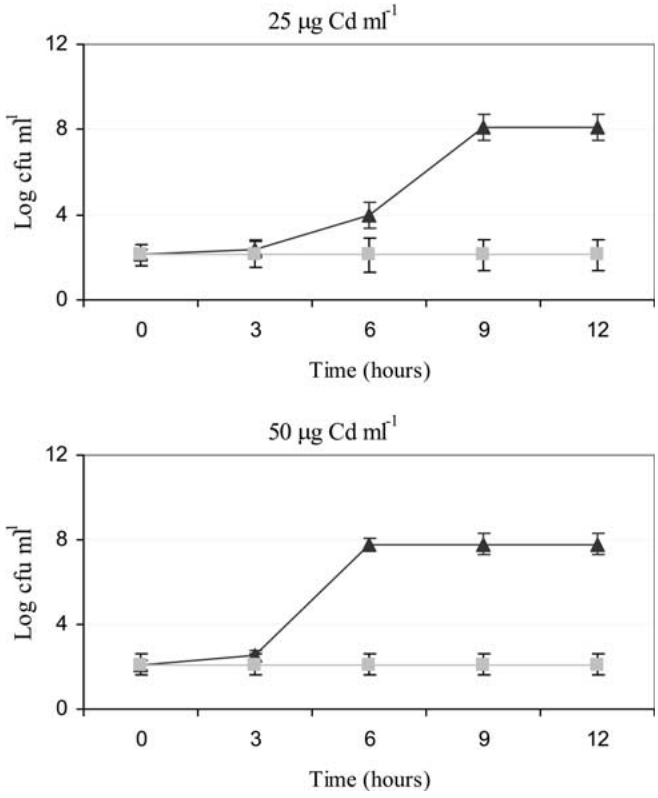

Figure 1. Viable cells (log cfu mL^{-1}) of *Brevibacillus brevis* [autochthonous Cd isolate (traingles) or from collection (squares)] growing in nutrient broth supplemented with 25 or 50 µg Cd mL^{-1} at different time intervals. Vertical bars represent standard errors.

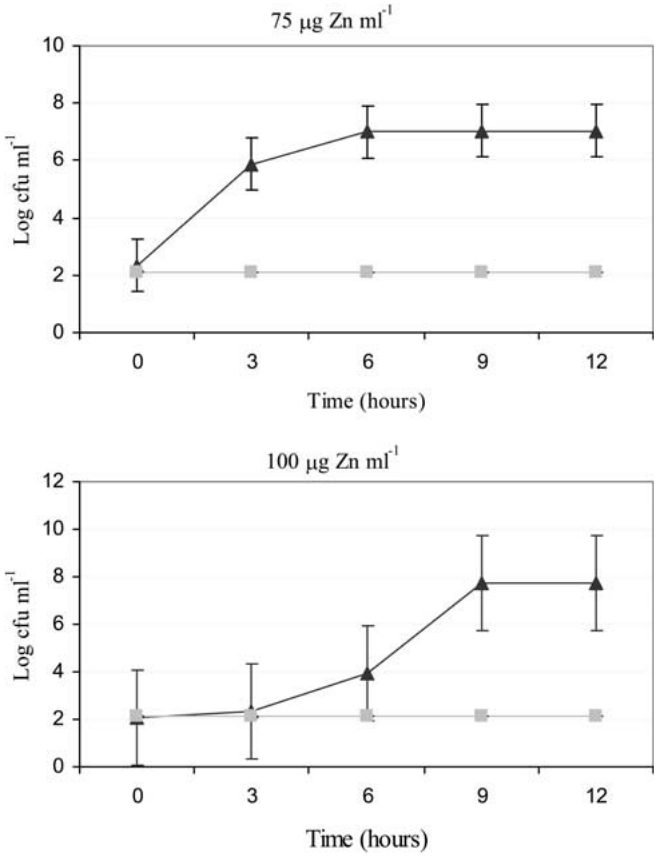

Figure 2. Viable cells (log cfu mL^{-1}) of *Brevibacillus brevis* [autochthonous Zn isolate (triangles) or from collection (squares)] growing in nutrient broth supplemented with 75 or 100 µg Zn mL^{-1} at different time intervals. Vertical bars represent standard errors.

fungus could confer to the associated plants grown in polluted conditions may be related to the level of fungal saprophytic growth. According to these results *G. mosseae* will confer significantly greater plant protection and/or tolerance when associated with the bacterium, and this is in part due to the greater development of the fungus in the saprophytic stage. These results are consistent with the increased development of AM colonization and extraradical mycelium previously observed when both microorganisms are dually inoculated under increasing levels of Cd [37].

This is the first report showing that AM fungal axenic development (germination of spore and/or mycelial growth) under increasing levels of Zn or Cd is enhanced when associated with *Brevibacillus brevis*. In Cd-supplemented medium, this bacterium may attenuate the toxic effect of Cd for the AM fungus or for the colonized plants by its bioaccumulating ability (76%). Results about presymbiotic phase of *G. mosseae* show that the effect of the bacterium decreasing Cd in the medium is particularly important in soils containing the highest quantities of this metal. These results confirm previous results from studies carried out under the microcosm system [37, 38].

Regarding results, bacterial inoculation was a critical factor for reaching maximum growth rates of germination and mycelial development under nonpolluted and particularly under polluted conditions. As results show,

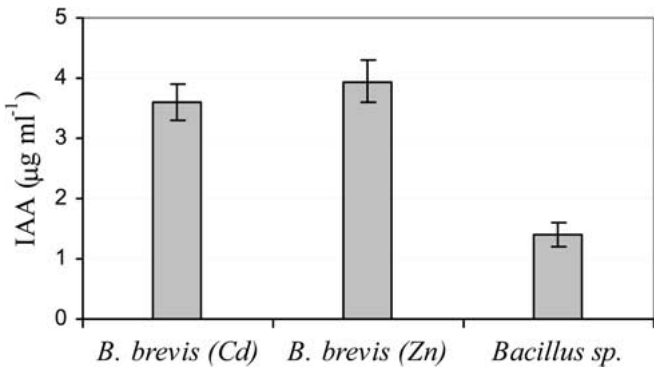

Figure 3. Indole acetic acid (IAA) production by *Brevibacillus brevis* (autochthonous Cd- or Zn-adapted bacteria) and by a reference *Bacillus* strain selected as IAA producer. Vertical bars represent standard errors.

Zn- and Cd-adapted autochthonous *G. mosseae* isolated from polluted sites required this biological influence from bacteria to achieve greater development. In fact, spores of Cd or Zn fungal isolates of *G. mosseae* demonstrated increased mycelial growth, by 195% (without Cd) and by 156% (without Zn), when inoculated with the bacterium compared with uninoculated spores. The bacterial effect was different for germination and hyphal growth values.

Different mechanisms seem to be functioning in the stimulating effect of each *Brevibacillus brevis*, particularly with regard to Cd or Zn alleviation. The high bacterial sequestration observed for Cd is not observed for Zn, but the bacterium always was very effective in increasing the saprophytic fungal development of each *G. mosseae* strain. One mechanism may be based on detoxification by bioaccumulation (Cd). But under Zn contamination, the bioaccumulation mechanism seems not to be the main one responsible for the stimulating effect observed.

Misra [26] reported that Cd resistance has been shown in strains of *Bacillus* closely related to the strain selected in this study. Bacteria of the genus *Bacillus* have been described that transport metal–citrate complexes across the cytoplasmic membrane into the cells [18], which is an active process mediated by the designated transporters protein [22]. This mechanism may be involved in the effect observed in this study.

Kanazawa and Mori [20] isolated Cd-resistant bacteria from metal-contaminated soil and, similarly to this study, determined the growth rate of the bacteria in media with or without Cd. Kanazawa and Mori [21] also found that Cd-adsorbing capacity of Cd-resistant bacteria isolated from heavy metals contaminated soils ranged from 0.8 to 2.9% of their biomass weight. But results of Cd adsorption and uptake showed the existence of a Cd efflux system according to their report [35]. Nevertheless, Scott and Palmer [31] and Scott [30] suggested that most of the Cd was adsorbed on the surface of bacteria and bound to the exopolysaccharides of these microorganisms. The mechanism involved in Cd incorporation in the biomass of *B. brevis* here used has not been yet studied, but the Cd-accumulating ability showed by this bacterium in the present study was much higher than that previously reported [21].

Both bacterial isolates produced similar quantities of IAA and in a higher amount than the *Bacillus* sp. from collection used as reference. In fact, IAA can act as a mycorrhizal growth-promoting compound [6] and as a metals accumulator [10]. Both mechanisms may be involved in the stimulating effect found.

The advantages of a healthy and well-developed mycorrhizal community include better survival and nutrition of plants in stressed environments since such mycorrhizal communities are essential in the establishment of plants [32, 33]. However, native AM fungi in polluted soils produce limited AM colonization and make no significant contribution to the survival or growth of plants in these soils [1]. Thus, the role of AM fungi in reducing Cd or Zn stress in colonized plants has been investigated, and whereas plant root and shoot biomass were drastically decreased by Cd or Zn in non-mycorrhizal plants, the mycorrhizal colonization attenuated the negative effects of such metals for plants. The underlying mechanism of the enhancement of mycorrhizal plant tolerance to heavy metal contamination is the immobilization capacity of intra-and extraradical mycelium to metals in the roots [23]. Regarding Cd acquisition by AM plants, in a previous study we used these Cd-adapted microorganisms as inoculants in a range of Cd levels in soil and we found that Cd in shoot of *Trifolium* plants was substantially reduced by these microbial treatments [37, 38]. Results from the present study justify and can explain these findings.

Despite the fact that little has been previously reported about the relationship between the rate of AM colonization and the tolerance of the host plant to heavy metal toxicity, it is logical to think that AM plants having a well-developed AM colonization (which have the ability for increasing plant nutrition and decreasing metal transport to the plant) have a greatest possibility of resisting stress situations.

It is important to note that adapted AM fungi are able to protect host plants by reducing uptake and translocation of toxic metals into the plant biomass (shoot and/or root). In this perspective the association of AM fungi with *Brevibacillus brevis*, particularly in presence of Cd, could reduce the amount of Cd available for plant uptake since the Cd-adsorbing ability of *Brevibacillus brevis* is very high and it also enhances AM development. Thus, bacterial inoculation not only favored fungal growth and survival by acting as a mycorrhiza helper bacterium (MHB) [13] but also it was able to reduce available Cd in the medium, which also must confer greater protection on the plants.

Data suggest that the capacity of these *Brevibacillus brevis* strains to protect AM spores against the inhibitory effects of high concentrations of Cd or Zn seems to be mainly related to the bioaccumulating ability (in the case of Cd) and to the indole acetic acid production (in the case of Zn) [10].

The ability of Cd resistant *Brevibacillus* strain to remove soluble cadmium from heavily contaminated medium is a very important activity for environmental decontamination. The Cd biosorption achieved by the use of Cd-adapted bacterium may be relevant for improving AM spores germination and hyphal growth.

In previous studies [3, 7] spore germination was apparently unaffected by bacterial inoculation, reaching between 90 and 95% in all cases. Here, the time-course measurements show that *Brevibacillus brevis* successfully

improved the rate and time of *G. mosseae* spore germination and also the subsequent mycelial development under the presence of detrimental agents such as Cd or Zn in the medium. The mechanisms responsible are still unknown but the bioaccumulation of Cd by *Brevibacillus brevis* and the IAA production by these bacteria seem to be involved. The reported bacterial activity is based on the partially alleviation of the inhibitory effect of metals.

These results are indicative of the mutualistic relationship between rhizosphere bacteria and AM fungi. The interaction observed is particularly interesting bearing in mind biology, ecology and practical management of contaminated areas.

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References

1. Allen, EB, Allen, MF (1980) Natural re-establishment of vesicular-arbuscular mycorrhizae following stripmine reclamation in Wyoming. J Appl Ecol 17: 139–147
2. Ann, ZQ, Hendrix, JW, Hershman, DE, Henson, GT (1990) Evaluation of the "most probable number" (MPN) and wet-sieving methods for determining soil-borne populations of endogonaceous mycorrhizal fungi. Mycologia 82: 516–518
3. Azcón, R (1987) Germination and hyphal growth of Glomus mosseae *in vitro*. Effect of rhizosphere bacteria and cell-free culture media. Soil Biol Biochem 19: 417–419
4. Azcón, R (1989) Selective interaction between free-living rhizosphere bacteria and vesicular–arbuscular mycorrhizal fungi. Soil Biol Biochem 21: 639–644
5. Azcón, R (1993) Growth and nutrition of nodulated mycorrhizal and non-mycorrhizal *Hedysarum coronarium* as a results of treatments with fractions from a plant growth-promoting rhizobacteria. Soil Biol Biochem 25: 1037–1042
6. Azcón, R, Azcón-Aguilar, C, Barea, JM (1978) Effects of plant hormones present in bacterial cultures on the formation and responses to VA mycorrhiza. New Phytol 80: 359–369
7. Azcón-Aguilar, C, Díaz-Rodriguez, RM, Barea, JM (1986) Effect of soil microorganisms on spore germination and growth of the VA mycorrhizal fungus *Glomus mosseae*. Trans British Mycol Soc 91: 337–340
8. Berthelin, J, Munier-Lamy, C, Leyval, C (1995) Effect of microorganisms on mobility of heavy metals in soils. In: Huang, PM, Berthelin, J, Bollag, JM, McGill, WB, Page, AL (Eds.) Metals, Other Inorganics, and Microbial Activities (Environmental Impacts of Soil Component Interactions, vol 2), Lewis, Boca Raton, pp 3–17
9. Biró, B, Bayoumi, HEAF, Balázsy, S, Kecskés, M (1995) Metal sensitivity of some symbiotic N_2-fixing bacteria and *Pseudomonas* strains. Acta Biol Hung 46: 9–16
10. Burd, GI, Dixon, DG, Glick, BR (2000) Plant growth promoting bacteria that decreased heavy metal toxicity in plants. Can J Microbiol 46: 237–245
11. del Val, C, Barea, JM, Azcón-Aguilar, C (1999) Assessing the tolerance to heavy metals of arbuscular mycorrhizal fungi isolated from sewage-sludge contaminated soils. Appl Soil Ecol 11: 261–269
12. del Val, C, Barea, JM, Azcón-Aguilar, C (1999) Diversity of arbuscular mycorrhizal fungus populations in heavy-metal-contaminated soils. Appl Environm Microbiol 65: 718–723
13. Garbaye, J (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128: 197–210
14. Hall, JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Experiment Bot 53: 1–11
15. Hepper, CM, Jakobsen, I (1983) Hyphal growth from spores of the mycorrhizal fungus *Glomus caledonius*: effect of amino acids. Soil Biol Biochem 15: 55–58
16. Higham, DP, Sadler, PJ (1984) Cadmium-resistant *Pseudomonas putida* synthezises novel cadmium proteins. Science 225: 1043–1046
17. Higham, DP, Sadler, PJ, Scawen, MD (1985) Cadmium-resistance in *Pseudomonas putida*: growth and uptake of cadmium. J Gen Microbiol 131: 3539–2544
18. Joshi-Tope, G, Francis, AJ (1995) Mechanisms of biodegradation of metal-citrate complexes by *Pseudomonas fluorescens*. J Bacteriol 177: 1989–1993
19. Kádár I, (1995) Contamination of the soil–plant–animal–man foodchain by chemical elements in Hungary. (In Hungarian) Ministry of Environmental Protection and Land Management, Budapest
20. Kanazawa, S, Mori, K (1996) Isolation of cadmium-resistant bacteria and their resistance mechanisms. Part 1. Isolation of Cd-resistant bacteria from soils contaminated with heavy metals. Soil Sci Plant Nutr 42: 725–730
21. Kanazawa, S, Mori, K (1996) Isolation of cadmium-resistant bacteria and their resistance mechanisms. Part II. Cadmium biosorption by Cd resistant and sensitive bacteria. Soil Sci Plant Nutr 42: 731–736
22. Krom, BP, Huttinga, H, Warner, JB, Lolkema, JS (2002) Impact of the Mg^{2+}-citrate transporter CitM on heavy metal toxicity in *Bacillus subtilis*. Archiv Microbiol 178: 370–375
23. Leyval, C, Joner, EJ, del Val, C, Haselwandter, K (2002) Potential or arbuscular mycorrhizal fungi for bioremediation. In: Gianinazzi, S, Schüepp, H, Barea, JM, Haselwandter, K (Eds.) Mycorrhiza Technology in Agriculture, from Genes to Bioproducts, Birkhäuser Verlag, Basel, pp 175–186
24. Leyval, C, Turnau, K, Haselwandter, K (1997) Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. Mycorrhiza 7: 139–153
25. Materson, AM (1992) Effects of agrochemicals and heavy metals on fast-growing rhizobia and their symbiosis with small-seeded legumes. Soil Biol Biochem 24: 435–445
26. Misra, TK (2000) Heavy metals, bacterial resistances. In: Encyclopedia of Microbiology, Vol. 2: 618–627
27. Mosse, B (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J General Microbiol 27: 509–520
28. Probanza, A, Lucas, JA, Acero, N, Gutierrez Mañero, FJ (1996) The influence of native rhizobacteria on european alder (*Alnus glutinosa* L.) growth. I. Characterization of growth promoting and growth inhibiting bacterial strains. Plant Soil 182: 59–66
29. Sakamoto, K, Yagasaki, M, Kirimura, K, Usami, S (1989) Resistance acquisition of *Thiobacillus thioxidans* upon cadmium and zinc ion addition and formation of cadmium ion-binding and zinc ion-binding proteins exhibiting metallothionein-like properties. J Ferment Bioengin 67: 266–273
30. Scott, JA (1990) Sites of cadmium uptake in bacteria used for biosorption. Appl Microbiol Biotechnol 33: 221–225
31. Scott, JA, Palmer, SJ (1988) Cadmium biosorption by bacterial exopolysaccharide. Biotechnol Lett 10: 43–48
32. Shetty, KG, Banks, MK, Hetrick, BA, Schwab, AP (1994) Biological characterization of a southeast Kansas mining site. Water Air Soil Pollut 78: 169–177

33. Shetty, KG, Hetrick, B, Figge, D, Schwab (1994) Effects of mycorrhizae and other soil microbes on revegetation of heavy metal contaminated mine spoil. Environ Pollut 86: 181–188
34. Southam, G (2002) Metal stressed environments, bacteria. Encyclopedia Environ Microbiol : 1893–1901
35. Tynecka, Z (1981) Reduced cadmium transport determined by a resistance plasmid in *Staphylococcus aureus*. J Bacteriol 147: 305–312
36. Vivas, A, Marulanda, A, Gómez, M, Barea, JM, Azcón, R (2003) Physiological characteristics (SDH and ALP activities) of arbuscular mycorrhizal colonization as affected by *Bacillus thuringiensis* inoculation under two phosphorus levels. Soil Biol Biochem 35: 987–996
37. Vivas, A, Vörös, I, Biró, B, Barea, JM, Ruiz-Lozano, JM, Azcón, R (2003) Beneficial effects of indigenous Cd-tolerant and Cd-sensitive *Glomus mosseae* associated with a Cd-adapted strain of *Brevibacillus* sp. in improving plant tolerance to Cd contamination. Appl Soil Ecol 24: 177–786
38. Vivas, A, Vörös, I, Biró, B, Campos, E, Barea, JM, Azcón, R (2003c) Symbiotic efficiency of autochthonous arbuscular mycorrhizal fungus (*G. mosseae*) and *Brevibacillus* sp. isolated from cadmium polluted soil under increasing cadmium levels. Environ Pollut 126: 179–189
39. Wöhler, I (1997) Auxin-indole derivatives in soils determined by a colorimetric method and by high performance liquid chromatography. Microbiol Res 152: 399–405
40. Vörös, I, Biró, B, Takács, T, Köves-Péchy, K, Bujtás, K (1998) Effect of arbuscular mycorrhizal fungi on heavy metal toxicity to *Trifolium pratense* in soils contaminated with Cd, Zn and Ni salts. Agrokém. Talajtan 47: 227–288