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ENVIRONMENTAL MICROBIOLOGY

Arbuscular Mycorrhizal Fungi, *Bacillus cereus*, and *Candida parapsilosis* from a Multicontaminated Soil Alleviate Metal Toxicity in Plants

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Abstract We investigated if the limited development of *Trifolium repens* growing in a heavy metal (HM) multicontaminated soil was increased by selected native microorganisms, bacteria (*Bacillus cereus* (Bc)), yeast (*Candida parapsilosis* (Cp)), or arbuscular mycorrhizal fungi (AMF), used either as single or dual inoculants. These microbial inoculants were assayed to ascertain whether the selection of HM-tolerant microorganisms can benefit plant growth and nutrient uptake and depress HM acquisition. The inoculated microorganisms, particularly in dual associations, increased plant biomass by 148% (Bc), 162%, (Cp), and 204% (AMF), concomitantly producing the highest symbiotic (AMF colonisation and nodulation) rates. The lack of AMF colonisation and nodulation in plants growing in this natural, polluted soil was compensated by adapted microbial inoculants. The metal bioaccumulation abilities of the inoculated microorganisms and particularly the microbial effect on decreasing metal concentrations in shoot biomass seem to be involved in such effects. Regarding microbial HM tolerance, the activities of antioxidant enzymes known to play an important role in cell protection by alleviating cellular oxidative damage, such as superoxide dismutase, catalase, glutathione reductase, and ascorbate peroxidase, were here considered as an index of microbial metal tolerance. Enzymatic mechanisms slightly changed in the HM-adapted *B. cereus* or *C. parapsilosis* in the presence of metals. Antioxidants seem to be directly involved in the adaptative microbial response and survival in HM-polluted sites. Microbial inoculations showed a bioremediation potential and helped plants to develop in the multicontaminated soil. Thus, they could be used as a biotechnological tool to improve plant development in HM-contaminated environments.

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Introduction

Heavy metals (HMs) in soil can cause detrimental effects on ecosystems [1]. High concentrations of metals such as zinc, nickel, mercury, cadmium, or copper cause environmental pollution because they have a strong persistence. The use of plants for bioremediation purpose is based on their ability to tolerate HMs. Thus, AMF symbiosis and/or saprophytic beneficial microorganisms may contribute to phytoremediation via strategies such as HM sequestration or accumulation, keeping metal concentrations in the plants below critical values and improving plant growth and nutrition [2–5]. Plant and microorganisms living in HM-polluted soils are often adapted to such detrimental conditions, and the use of such organisms to remediate HM-contaminated soils is an attractive possibility for the elimination or transformation of the contaminants [6]. At high concentrations, HMs may be toxic to plants and soil microorganisms, reducing growth. However, adapted and HM-tolerant soil microorganisms can enhance plant growth and nutrition, by acting as plant growth-promoting agents and also by interacting with metals through bioaccumulation or biotransformation processes [7]. Thus, plants have

been used successfully in bioremediation strategies in association with rhizosphere bacteria and AMF [8].

An important component of the soil rhizosphere is the community of mycorrhizal fungi. They are obligate symbionts that benefit plant nutrition and improve plant tolerance of abiotic stresses, such as those produced by HM pollution [2, 3, 9]. Also, AMF-colonised root cells accumulate reactive oxygen species (ROS) in response to stress. In AMF, genes with putative roles in the alleviation of antioxidant stress have been described [10, 11]. This indicates that AMF are able to make colonised plants more HM-tolerant and protect against the oxidative stress produced by HMs [2]. In general, the transfer of saprophytic rhizosphere microorganisms from one environment to another causes a metabolic imbalance, instantaneously producing O_2^- and free radicals.

The production of phytohormones by microorganisms is related to the improvement of plant growth [12]. The microbial effectiveness may be mediated in part by changes in the production of IAA.

The toxicity of HMs to bacteria is due, in part, to oxidative stress [13, 14, 15]. The antioxidants may be involved directly in the adaptive microbial response for survival in HM-contaminated soil. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), or glutathione reductase (GR) are distributed widely in aerobic microorganisms, but to the best of our knowledge, no previous studies have investigated the role of these antioxidant activities in the stress tolerance of rhizosphere microorganisms (yeast and/or bacteria) from HM-contaminated soil. Biochemical responses in organisms against environmental stress can be regarded as early warning indexes of stress in the environment. Thus, we hypothesised that antioxidant microbial responses to HMs may suggest variations in the level of HM tolerance, depending on the microbial adaptation.

This study aimed to provide information about the importance of microbial antioxidants in the range of microbial adaptation and/or tolerance to HM contamination. As well, changes in cell metabolic processes leading to microbial production of proline and indole-3-acetic acid (IAA) in an HM-contaminated medium were also determined. Proline and betaine have been found to increase the activity of enzymes involved in the antioxidant defence system [16, 17].

Proline may be accumulated in cells under stress conditions. This free amino acid may act as an osmolyte, as well as regulating cytosolic pH, scavenging hydroxyl radicals, regulating the NAD/NADH ratio, and stabilising proteins [18]. Proline may also act as a chelating agent for some HMs. Many enzymatic activities and low-mass molecules of living organisms possess antioxidant capabilities and can protect cells against adverse effects of ROS [19]. Thus, they have been considered as biomarkers of environmental pollution [20].

Here, we have studied whether the inoculation of selected, HM-adapted microbial strains (*Bacillus cereus* (Bc), *Candida parapsilosis* (Cp), and/or arbuscular mycorrhizal fungi (AMF)) was effective in improving plant growth and nutrition in a multicontaminated soil. The microbial ability to decrease metal translocation from the soil to the plant shoots was also evaluated. No previous information is available on the potential of yeast to contribute to phytoremediation when associated or not with AMF. Since this yeast was isolated from a polluted soil, we consider it a good and well-adapted candidate for our purpose. Microbial antioxidant enzymes, such as superoxide dismutase, catalase, glutathione reductase, and ascorbate peroxidase, as well as proline and IAA production, were also determined, in the presence and absence of HMs, as an index of microbial HM tolerance.

Materials and Methods

Two independent experiments were carried out in this study. In experiment 1, in a bioassay under greenhouse conditions, we tested the effectiveness of autochthonous microorganisms (AMF, *B. cereus*, and *C. parapsilosis*) in single or in dual inoculation to increase plant growth and nutrition in the natural multicontaminated test soil.

In the experiment 2, once tested the microbial effectiveness increasing HM plant tolerance (in terms of growth), autochthonous bacterial and yeast strains assayed were grown in axenic nutritive culture medium, with or without extract from the HM-contaminated test soil, to determine HM tolerance. For that, changes or maintenance of proline and IAA production and antioxidant activities by microbial cells in noncontaminated or HM-contaminated medium were determined. In the cell-free medium, the remaining metals after the microbial growth were also tested.

Selection and Identification of Metal-Tolerant Microbes

The bacterial and yeast strains were isolated from the above-mentioned soil following serial soil dilutions, 1 g of homogenised soil was suspended in 100 mL of sterile water (dilution 10^2), and this suspension was further diluted to reach dilution 10^4 to 10^7. The suspension was sown on agar plates [21]. The most abundant cultured bacterial and yeast strains were selected.

The autochthonous mycorrhizal inoculum, also coming from the multicontaminated soil, was a mixture stable over time of a morphologically distinct fungal *Glomus* species, the *Glomus mosseae* strain being the most abundant AMF spore in this soil [2].

Total DNA from bacterial or yeast isolates selected in this study was obtained [22]. Identification of the bacterium was carried out by 16S ribosomal DNA (rDNA) cloning and sequencing [7]. Database searches for 16S rDNA sequence similarity unambiguously identified the HM-tolerant bacterium as *B. cereus* (accession AY795568).

For the identification of the yeast strain, specific primers designed for D1/D2 region of the large ribosomal subunit (26S rDNA) were used in polymerase chain reaction (PCR) with DNA isolated from the yeast [23, 24]. Primer sequences were: NL-1 (5′-GCATATCAATAAGCGGAG GAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAA GACGG-3′). The small subunit of 5.8S rDNA (ITS1/ITS4 region) was also used for a more effective identification [23, 24]. Primer sequences were: ITS1 (5′-TCCGTAGGT GAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGA TATG-3′). The 26S and 5.8S genes were amplified by the PCR technique, which yielded about 600 bp in each DNA fragment. These DNA fragments were cloned and sequenced [7] which allowed identification of the yeast strain used in this study as *C. parapsilosis* (accession AJ749821).

Experiment 1

This greenhouse experiment consisted of a factorial design with two inoculations factors: (1) mycorrhizal fungus inoculation treatments non-AMF and AMF and (2) bacterial or yeast inoculants. These two factors were combined to give six treatments: (1) single mycorrhizal inoculation with autochthonous AMF inoculum, (2) single bacterial inoculation with autochthonous bacterial isolate from multicontaminated soil, (3) single yeast inoculation with autochthonous yeast from multicontaminated soils, (4) dual AMF/bacterial inoculation, (5) dual AMF/yeast inoculation, and (6) untreated control.

Four surface-sterilised seeds of *T. repens* were sown in each pot. The seedlings were tinned to two per pot 2 weeks after emergence.

The multicontaminated test soil used in the greenhouse experiment is described in Table 1. The soil was air dried, sieved to less than 2 mm, and mixed with quartz sand (<1 mm) at a soil/sand (1:1, *v*/*v*). The test soil came from Gorguel in the province of Murcia (Spain). Pots were filled with 500 g of the soil/sand (1:1, *v*/*v*) mixture.

For inoculation purposes, appropriate pots were sprinkled with 1 mL (10^8 cell per millilitre) of this bacterial or yeast strains grown in the corresponding nutrient medium (nutrient broth, 8 gL^{-1} for bacteria and yeast extract–peptone–dextrose (YPD) for the yeast) at 28 °C for 24–48 h (bacteria) and for 72–96 h (yeast).

The inoculum AMF was bulked in an open pot culture of red clover and consisted of soil, spores, mycelia, and infected root fragments. Five grams of inoculum were added to appropriate pots at sowing time.

The nonmycorrhizal treatments received the same amount of autoclaved inoculum together with a 2-mL aliquot of a filtrate (<20 μm) of the AMF inoculum to provide a general microbial population free of AMF propagules. A suspension (1 mL^{-1}) of the diazotrophic bacterium *Rhizobium leguminosarum* bv. *trifolii* (10^8 cell per millilitre) was sprinkled over the seeds of all pots at the time of sowing.

Plant Growth Conditions

The plants were grown in a greenhouse under the following conditions: 16–8 h day/night cycle, 21–15 °C, and 50% relative humidity. The photosynthetic photon flux density was 500 μmol $m^{-2}s^{-1}$ as measured with a light metre (LICOR, model LI-188B). Water lost was replaced daily by top watering with tap water.

Parameters Measured

Biomass Production, Nutrient, and Metals Shoot Concentrations

At harvest (3 months after planting), the root system was separated from the shoot, and dry weights were measured after drying in a forced draught oven at 70 °C for 2 days. N, K, and P concentrations in shoots were determined (after Kjeldahl digestion), flame photometry and colorimetry [25], respectively.

Al, Mn, Cu, Cd, Mo, Zn, As, and Ni concentrations were determined after wet digestion of the air-dried plant samples with HNO_3+H_2O_2 by inductively coupled plasma atomic emission spectrometry [26].

Symbiotic Development

The percentage of mycorrhizal root length infected was estimated by observation of fungal colonisation after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (*v*/*v*) [27]. Quantification was carried out using the grid-line intersect method [28]. Nodulation was checked visually.

Experiment 2

Microbial HM Bioaccumulation, Proline and IAA Production, and Antioxidant Activities

In the second experiment, the bacteria and yeast strains were assayed in axenic medium with and without (control) an extract (soil/water 1:1, *v*/*v*) of contaminated test soil to evaluate IAA, proline, antioxidant activities SOD, CAT, APX, and GR), and metals accumulation by

Table 1 Chemical, biochemical, microbiological, and physical characteristics of the test soil

pH (H_2O)	7.7	Total N (g kg^{-1})	0.22
EC (1:5, dS m^{-1})	2.5	Available P (μg g^{-1})	1.00
Aggregate stability (%)	48.7	Water soluble C (μg g^{-1})	41.00
Water soluble carbohydrates (μg g^{-1})		10.00	
	Total		Soluble
Fe (mg kg^{-1})	139,045	Fe (mg kg^{-1})	0.540
Mn (mg kg^{-1})	8,300	Mn (mg kg^{-1})	0.190
Al (mg kg^{-1})	19,385	Al (mg kg^{-1})	0.410
Zn (mg kg^{-1})	47,695	Zn (mg kg^{-1})	1.710
Pb (mg kg^{-1})	8,555	Pb (mg kg^{-1})	0.170
Cu (mg kg^{-1})	168	Cu (mg kg^{-1})	0.114
Cd (mg kg^{-1})	52	Cd (mg kg^{-1})	0.028
Ni (mg kg^{-1})	34	Ni (mg kg^{-1})	0.036
As (mg kg^{-1})	475	As (mg kg^{-1})	0.019
Cr (mg kg^{-1})	31	Cr (mg kg^{-1})	0.015

each microorganism. Treatments were replicated five times.

Bacterial and yeast strains were grown in 250-mL flasks containing 50 mL of the respective growing medium in which water (controls) or soil extract (soil/water 1:1, *v*/*v*) were the liquid components of the appropriate medium (nutrient broth 8 gL^{-1} (bacteria) and YPD (yeast)) in a shake culture for 24–48 h (bacteria) and 72–96 h (yeast) at 28 °C.

Metals Bioabsorption by Bacterial and Yeast Strains

Metals (Fe, Mn, Zn, and Cd) in the remaining supernatant, after bacteria or yeast culture medium centrifugation, were also determined [29].

Production of IAA and Proline by the Cells of Bacteria and Yeast Strains

The production of IAA by the bacteria and the yeast was determined using the Salper reagent [30]. Three millilitres of fresh Salper reagent were added to free-cell supernatant and kept in complete darkness for 1 h, and the optical density at 535 nm was measured in each treatment. A standard curve was prepared for IAA (Sigma, USA). The proline was estimated by spectrophotometric analysis at 515 nm [31].

Antioxidant Enzymatic Activities

Method for the extraction of enzymes microbial cells were homogenised as described by Aroca et al. [32] in a cold mortar with 4 mL 50 mM phosphate buffer (pH 7.8) containing 1 mM EDTA, 8 mM $MgCl_2$, 5 mM DTT, and 1% (*w*/*v*) polyvinylpolypyrrolidone. The homogenate was centrifuged at 27,000×*g* for 15 min at 4 °C, and the supernatant was used for enzyme activity determination. Total SOD activity (EC 1.15.1.1) [33] was measured on the basis of SOD's ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25 °C. CAT activity (EC 1.11.1.6) was measured according to Aebi [34]. Consumption of H_2O_2 (extinction coefficient of 39.6 $mM^{-1}cm^{-1}$) at 240 nm for 1 min was monitored. The reaction mixture consisted of 50 mmol L^{-1} phosphate buffer (pH 7.0) containing 10 mmol L^{-1} H_2O_2 and 100 μl of enzyme extract in a 2-mL volume. APX activity (EC 1.11.1.11) was measured in a 1-mL reaction volume containing 50 mmol L^{-1} potassium phosphate buffer (pH 7.0), 0.1 mmol L^{-1} hydrogen peroxide, and 0.5 mmol L^{-1} ascorbate. The H_2O_2 was added to start the reaction, and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate for ascorbate [35]. GR activity (EC 1.20.4.2.) was estimated by measuring the decrease of absorbance at 340 nm due to the oxidation of NADPH [36]. The reaction mixture (1 mL) contained 0.1 M HEPES–NaOH 100 mmol L^{-1} (pH 7.8), 1 mmol L^{-1} EDTA, 3 mmol L^{-1} $MgCl_2$, 0.5 mM oxidised glutathione, 150 μl enzyme extract, and 0.2 mmol L^{-1} NADPH was added and mixed thoroughly to begin the reaction. The results were expressed in micromole NADPH oxidised per gram fresh weight per minute, and the activity was calculated from the initial speed of reaction and the molar extinction coefficient of NADPH (ε_{340}=6.22 mmol $L^{-1}cm^{-1}$). Total soluble protein amount was determined using the Bradford method [37] and bovine serum albumin as standard.

Statistical Analyses

Data were subjected to analysis of variance using microbial inoculation treatments as sources of variation. Post hoc

comparisons with Duncan's test were used to compare treatment means. Percentage values were arcsin transformed before statistical analysis.

Results

Shoot and root dry weights were increased by the microbial inoculants, particularly by *B. cereus* (shoot) and *C. parapsilosis* (roots) when combined with AMF (Fig. 1). These microorganisms also had a beneficial effect on the contents of foliar nutrients (N, P, and K). The greatest N, P, and K acquisition occurred in plants inoculated with both *B. cereus* and AMF (Fig. 2).

Most of the inoculation treatments were effective in decreasing HM concentrations and increasing nutrient contents in the shoot biomass. In general, AMF-colonised plants had lower Mn, Cu, Cd, Zn, and Ni concentrations, particularly when coinoculated (Table 2). This microbial effect on tissue concentration differed according to the element involved, the Al, Mn, and As concentrations being depressed to a greater extent in coinoculated plants.

Plants inoculated with AMF and *B. cereus* or *C. parapsilosis* showed the greatest symbiotic development (AMF colonisation and nodulation; Fig. 3). In the absence of microbial inoculants (control roots), neither mycorrhization nor nodule production was observed, in spite of the use of nonsterilised natural soil (Fig. 3).

The IAA production by *C. parapsilosis* was higher than by *B. cereus*, independent of the presence of metals in the culture medium (Fig. 4). Nevertheless, metals decreased (by approximately one half) the IAA production (Fig. 4). In the same way, *C. parapsilosis* also accumulated more proline than *B. cereus* under all growing conditions. Metals in the soil extract increased (*B. cereus*) or decreased (*C. parapsilosis*) proline production (Fig. 4).

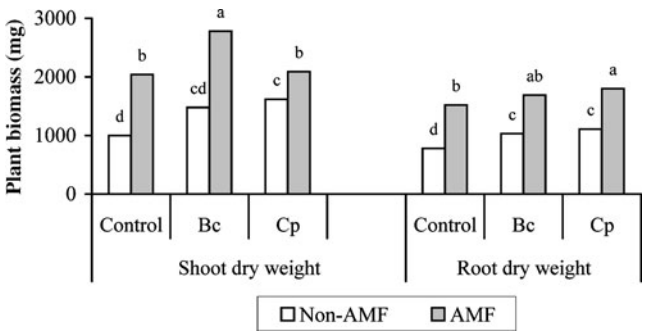

Figure 1 Effect of single or dual inoculation with autochthonous arbuscular mycorrhizal fungi (*AMF*), *B. cereus* (*Bc*), or *C. parapsilosis* (*Cp*) on plant biomass (shoot and root dry weight, milligram). Values having a common letter are not significantly different ($P<0.05$) as determined by Duncan's multiple range test ($n=5$)

Figure 5 shows the antioxidant enzyme activities (CAT, SOD, APX, and GR) of these microorganisms grown in a medium with or without metals. The CAT and GR activities were higher in *C. parapsilosis* than in *B. cereus*, and for both, the presence of metals decreased these activities. In contrast, the highest SOD activity was found in *B. cereus*, and this activity did not change in the presence of metals. In the case of APX, the microbial reaction to the presence of metals was different. While the APX activity of *C. parapsilosis* did not change, that of *B. cereus* was increased by the metals.

Metal (Fe, Mn, Zn, or Cd) bioaccumulation differed for these plant growth-promoting rhizobacteria microorganisms (Fig. 6). The bacterial sequestration of Fe and Cd was higher than for Mn or Zn. By contrast, *C. parapsilosis* accumulated much more Mn and Zn than the bacteria. This indicates the specific bioabsorption abilities of each one of these strains. The bioaccumulation of metals by these two microorganisms ranged from 2% (Mn and Zn) to 17% (Cd) in the case of *B. cereus*, and from 1% (Fe) to 40% (Zn) in the case of *C. parapsilosis* (Fig. 6).

Discussion

In this study, we evaluated the interactions between HM contamination and beneficial rhizosphere microbes adapted to the multicontaminated soil conditions and their effects on plant development. The inoculated, HM-resistant *B. cereus*, *C. parapsilosis*, and AMF were able to grow and to improve plant development under such polluted conditions. Metal-resistant microorganisms are isolated from metal-rich biotopes. These selected microorganisms were the most abundant ecotypes in the multicontaminated test soil. Toxicity of HMs to microorganisms is due, in part, to oxidative stress [14]. Microbial cells have specific enzymes and low-molecular mass compounds possessing antioxidant activities which can protect cells against the adverse effects of reactive oxygen species produced by environmental stresses [38]. The performance of the plants and their ability to reach optimum growth on the multicontaminated soil seemed to be dependent upon the activity developed by these selected microbial groups. A relevant effect of the inoculated microorganisms was the improvement of nodulation and AMF colonisation—which was not observed in the control, noninoculated plants. This effect of the inoculants may have contributed to the improved nutrition and development of AMF-inoculated plants in this soil.

It seems that different mechanisms were functioning in the stimulation of plant growth by *B. cereus* and *C. parapsilosis* following their inoculation. Sequestration of Fe and Cd was greater for *B. cereus* than for *C. parapsilosis* while *C. parapsilosis* accumulated more Mn and Zn than *B.*

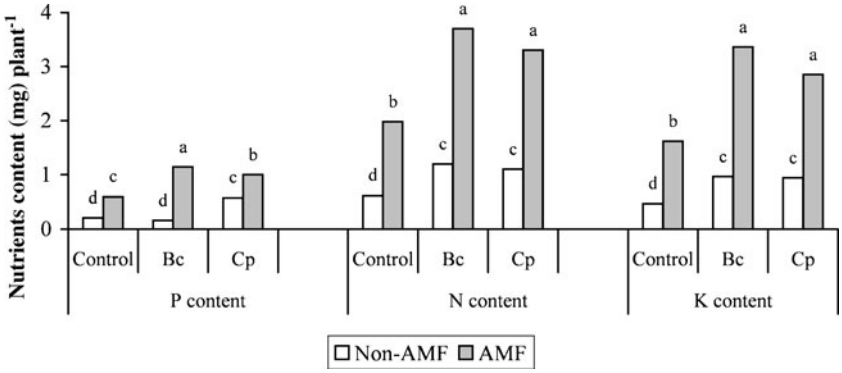

Figure 2 Effect of single or dual inoculation with autochthonous arbuscular mycorrhizal fungi (*AMF*), *B. cereus* (*Bc*), or *C. parapsilosis* (*Cp*) on nutrients content (milligram per plant). Values having a common letter are not significantly different ($P<0.05$) as determined by Duncan's multiple range test ($n=5$)

cereus. In general, Gram-positive bacteria accumulate higher HM contents than Gram-negative bacteria because of differences in the structure of their cell wall. Nevertheless, in the cytosol, chelators such as glutathione or metallothionins bind metals, and HM transporters collaborate to actively reduce the levels of metals by pumping them out of the cytosol. Thus, selected microorganisms are able to tolerate a wide range of metal concentrations in the soil.

For bacteria of the genus *Bacillus*, transport of metal–citrate complexes across the cytoplasmic membrane into the cells has been described [39]; this is an active process mediated by designated transporter proteins [40]. This and other mechanisms may be involved in the effect of the inoculation of *B. cereus* observed in this study. Metals may be adsorbed onto the surface of bacteria and bound to the exopolysaccharides secreted by many microorganisms. In fact, one important microbial mechanism of plant growth stimulation may be based on soil detoxification via microbial metal accumulation [29]. Nevertheless, IAA production by these microorganisms is an additional factor that also contributed to the plant growth stimulation observed here [12].

The metal-adapted microorganisms isolated from HM-polluted soil appear to have multiple strategies that provide low sensitivity, enabling them to survive under such conditions [41]. *B. cereus* and *C. parapsilosis* produced differing quantities of IAA and differed in their ability to accumulate particular metals, but their effectiveness with respect to plant performance and symbiotic development (nodulation and AMF colonisation) was quite similar. Additional microbial processes seem to be functioning concomitantly in the plant stimulation effect of these saprophytic microorganisms, particularly with regard to HM tolerance [42]. The microbial metal sequestration and/or IAA production observed could be an effective mechanism for increasing not only plant growth but also AMF and rhizobial development. The advantages of a healthy and well-developed community of both of these beneficial symbionts include better plant nutrition in HM-polluted environments [43].

Table 2 Effect of single or dual inoculation with autochthonous mycorrhizal fungi (AMF), *B. cereus* (Bc), or *C. parapsilosis* (Cp) on metals (Al, Mn, Cu, Cd, Mo, Zn, As, and Ni) concentration

Treatments	% Al	% Mn	% Cu	% Cd
C	161.0a	123.0b	10.7a	1.14a
AMF	178.0a	114.0bc	9.5b	1.12a
Bc	113.0c	127.0b	7.7c	0.91b
Cp	181.0a	146.0a	11.4a	0.93b
AMF + Bc	86.9d	100.2c	9.6b	0.92b
AMF + Cp	139.7b	97.4c	9.7b	1.0ab
	% Mo	% Zn	% As	% Ni
C	0.84b	173.2a	19.0a	2.93a
AMF	1.07a	147.2b	19.7a	1.06c
Bc	0.98b	152.5ba	15.6b	2.40a
Cp + Bc	1.16a	161.0a	18.8ba	2.45ab
AMF + Bc	0.96b	150.0b	11.0c	0.94c
AMF + Cp	0.95b	142.8b	12.3c	1.13c

Values having a common letter are not significantly different ($P<0.05$) as determined by Duncan's multiple range test ($n=5$)

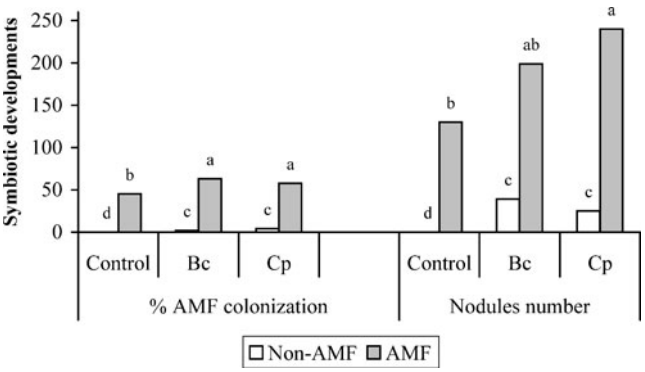

Figure 3 Effect of single or dual inoculation with autochthonous arbuscular mycorrhizal fungi (*AMF*), *B. cereus* (*Bc*), or *C. parapsilosis* (*Cp*) on symbiotic developments (percentage of AMF colonisation and nodule number). Values having a common letter are not significantly different ($P<0.05$) as determined by Duncan's multiple range test ($n=5$)

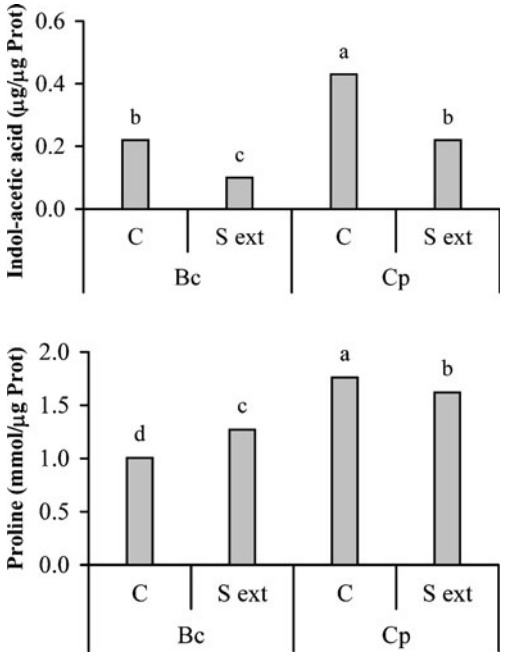

Figure 4 Indol-3-acetic acid (microgram per microgram Prot) and proline production (nanomole per microgram Prot) by *B. cereus* (*Bc*) and *C. parapsilosis* (*Cp*) in medium with or without (*C*) multi-contaminated soil extract (*S ext*). Values having a common letter are not significantly different ($P<0.05$) as determined by Duncan's multiple range test ($n=5$)

The results show that native AMF in this natural, multicontaminated soil limited the colonisation by the inoculated AMF. Also, nodulation was greatly depressed, and it was even absent in noninoculated soil. These

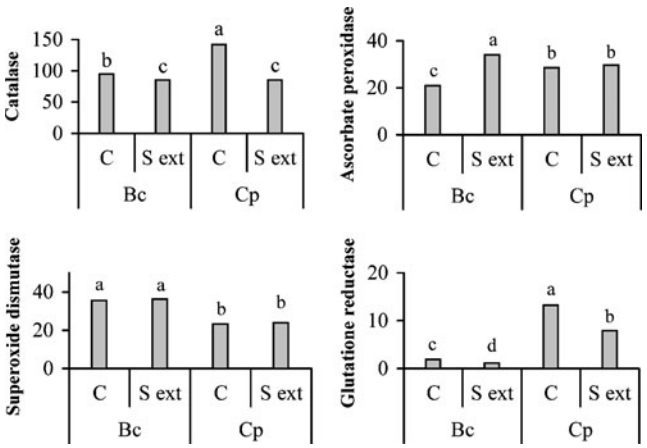

Figure 5 Catalase (nanomole per minute microgram Prot), ascorbate peroxidase (nanomole per minute microgram Prot), superoxide dismutase (nanomole per minute microgram Prot), and glutathione reductase (nanomole per minute microgram Prot) by *B. cereus* (*Bc*) or *C. parapsilosis* (*Cp*) in medium with and without (*C*) multicontaminated soil extract (*S ext*).Values having a common letter are not significantly different ($P<0.05$) as determined by Duncan's multiple range test ($n=5$)

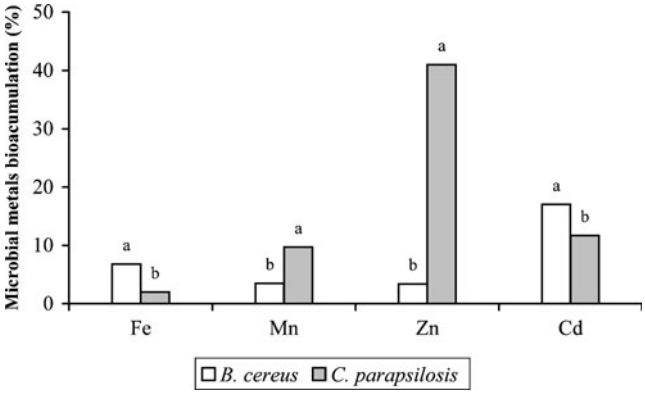

Figure 6 Effect of inoculation with autochthonous *B. cereus* or *C. parapsilosis* on metals bioaccumulation (percentage) from multi-contaminated soil extract. Values having a common letter are not significantly different ($P<0.05$) as determined by Duncan's multiple range test ($n=5$)

symbiotic groups were scarce in the natural, HM-polluted soil, but in this experiment, the soil was diluted (1:1, by volume) with sand. The soil dilution, the limited time of plant growth (3 months), and the use of only one host plant (clover) may explain the paucity of symbiotic structures in the control plants.

The values of both of these symbiotic parameters were important for N, P, and K nutrition of plants in this soil [3]. The inocula applied increased both values and thus attenuated the detrimental effect of HMs on plant nutrition and development. Metal concentrations in plants were affected by the microorganisms applied, and the microbial activity regarding metal acquisition differed according to the metal and/or inoculant involved. This indicates that each biological treatment affected in a specific way the translocation value for each metal.

Nevertheless, the AMF inoculation, particularly in association with *B. cereus* or *C. parapsilosis*, greatly decreased the metal concentrations in clover shoots, as was observed previously for AMF and *Brevibacillus brevis* in a soil contaminated artificially with a single metal [3, 29].

One interesting and new result reported in this study is the intrinsic ability of microorganisms such as *B. cereus* and *C. parapsilosis* to cope with HM toxicity and to tolerate the stressful conditions in the multicontaminated test soil.

Mycorrhizal symbioses and/or *B. cereus* help colonised plants to attenuate the adverse effects of metal-induced oxidative stress, by regulation of antioxidant activities [3]. ROS are capable of causing considerable cellular damage through peroxidation of membrane lipids.

Antioxidant enzymes, such as SODs, CATs, and APXs, are distributed widely in aerobic microorganisms. However, the metabolism of antioxidant enzyme activities in *B. cereus* and *C. parapsilosis* is not completely understood

in relation to the ability of these microorganisms to resist HM stress. *B. cereus* and *C. parapsilosis* have differing levels of antioxidant activities and also respond differently to HMs. These differing antioxidant responses suggest variations in the level of tolerance, depending on the microbial adaptation. SOD activity was higher in *B. cereus* than in *C. parapsilosis* but CAT, APX, and GR were lower in *B. cereus*. The soil extract containing soluble metals significantly decreased CAT and GR in both microorganisms, but SOD did not change and APX increased. Nevertheless, these results suggest that, for microbial cells, antioxidative enzymatic activities are not the only mechanism involved in sustaining their growth under HM stress.

We hypothesised that the metal resistance of microorganisms can be ascribed partially to the antioxidative enzyme metabolism. Thus, we expected that the less adapted microbial group would not be able to attenuate oxidative stress. Nevertheless, *C. parapsilosis* maintained its SOD and APX activities without any change in the presence of metals—although it was not able to maintain its CAT and GR activities. Similarly, the presence of metals decreased the CAT and GR activities in *B. cereus* culture and increased APX, while SOD activity did not change. These results indicate that SOD has a nonsignificant role in the defence against the oxidative stress induced by HMs and that changes in CAT, APX, or GR would be useful markers for the microbial strategies of HM tolerance. Nevertheless, the protective action of these enzymes is variable, and their particular contribution to the protection against oxidants may be different in each microorganism involved. The results indicate that these microorganisms possess very effective antioxidant systems. The balance between the SOD and APX and CAT activities is critical for suppressing toxic ROS levels in cells [44]. Nevertheless, more studies need to be performed to determine if these microorganisms decrease, in part, the effects of metal stress by changing the metabolism of ROS. Hence, no information is available on whether microbial HM tolerance is related to the regulation of ROS-scavenging enzymes in the associated plants. In this study, the metals in the growth medium did not change the SOD activity in *B. cereus* or *C. parapsilosis*. This may be an indication of cellular metal tolerance. However, SOD, CAT, and GR did not increase when the microorganisms were subjected to HM stress.

In response to stress, *C. parapsilosis* cells accumulated higher concentrations of free proline than *B. cereus*, which may indicate greater chelation of HMs [45]. Reduced HM (Cu, Cr, Ni, Zn)-dependent lipid peroxidation and K^+ efflux from *Chlorella vulgaris* pretreated with exogenous proline have been demonstrated [46]. Increased levels of glutathione in Cd-stressed cells support the synthesis of phytochelatins for Cd complexation. Proline is probably an important compatible solute and also a component of the cellular antioxidative network involved in alleviation of stress-dependent oxidative effects. It has been reported [47] that proline can participate in the scavenging of ROS such as hydroxyl radicals and singlet oxygen [48]. Recently, it was stated [49] that free proline, known to accumulate in plant tissues during abiotic stresses, could contribute to the scavenging of free radicals produced under a variety of abiotic stresses, in addition to its main role as an osmoprotectant under osmotic or water-deficit stresses. In this study, the fact that *C. parapsilosis* exhibited higher proline accumulation may be related to its greater Mn and Zn accumulation compared with *B. cereus*.

ROS may act in plants as signalling molecules, to control defence reactions in intermediate stress conditions [50]. Thus, in plant cells, ROS may be regulated by specific mechanisms to ensure cell functioning under varied environmental conditions. SOD usually acts as the first line of defence against ROS, and subsequently, CAT, APX, and GR detoxify the H_2O_2 produced by SOD. But in plant roots, the maintenance of the correct balance between SOD, CAT, and APX is crucial in order to determine the steady-state level of ROS [44]. In this study, antioxidant enzyme activities were determined in microbial cells and not in plant roots.

Microorganisms are involved in plant nutrient acquisition, root development, and metal absorption from the growth medium, but all these processes could be regulated differently according to the resistance and intrinsic stress tolerance of the microorganism involved.

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