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Proteomic Analysis of Eucalyptus Leaves Unveils Putative Mechanisms Involved in the Plant Response to a Real Condition of Soil Contamination by Multiple Heavy Metals in the Presence or Absence of Mycorrhizal/Rhizobacterial Additives

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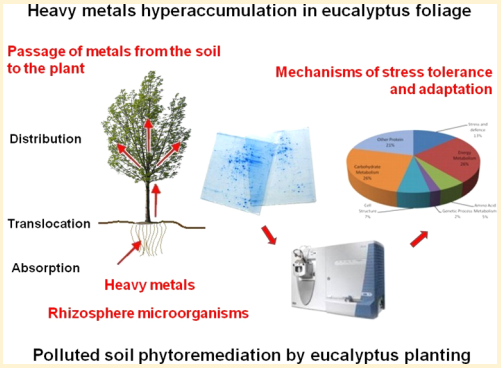
Supporting Information

ABSTRACT: Here we report on the growth, accumulation performances of, and leaf proteomic changes in *Eucalyptus camaldulensis* plants harvested for different periods of time in an industrial, heavy metals (HMs)-contaminated site in the presence or absence of soil microorganism (AMs/PGPRs) additives. Data were compared to those of control counterparts grown in a neighboring nonpolluted district. Plants harvested in the contaminated areas grew well and accumulated HMs in their leaves. The addition of AMs/PGPRs to the polluted soil determined plant growth and metal accumulation performances that surpassed those observed in the control. Comparative proteomics suggested molecular mechanisms underlying plant adaptation to the HMs challenge. Similarly to what was observed in laboratory-scale investigations on other metal hyperaccumulators but not on HMs-sensitive plants, eucalyptus grown in the contaminated areas showed an over-representation of enzymes involved in photosynthesis and the Calvin cycle. AMs/PGPRs addition to the soil increased the activation of these energetic pathways, suggesting the existence of signaling mechanisms that address the energy/reductive power requirement associated with augmented growth performances. HMs-exposed plants presented an over-representation of antioxidant enzymes, chaperones, and proteins involved in glutathione metabolism. While some antioxidant enzymes/chaperones returned to almost normal expression values in the presence of AMs/PGPRs or in plants exposed to HMs for prolonged periods, proteins guaranteeing elevated glutathione levels were constantly over-represented. These data suggest that glutathione (and related phytochelatins) could act as key molecules for ensuring the effective formation of HMs-chelating complexes that are possibly responsible for the observed plant tolerance to metal stresses. Overall, these results suggest potential genetic traits for further selection of phytoremediating plants based on dedicated cloning or breeding programs.



INTRODUCTION

Pollution caused by heavy metals (HMs) represents one of the most important alterations of the biosphere.[1,2] Living organisms cannot biodegrade HMs; they can only transform the metal oxidation/complexation status. For most species, Zn, Ni, Cu, Mn, and Se are essential micronutrients; these metals become toxic when present in high concentrations. Conversely, Cd, Cr, and Pb are noxious to organisms even at low levels. Thus, animals/plants have developed homeostatic mechanisms to control HMs concentration in their body parts.[3]

In plants, HMs enter the root system via ion carriers or channels.[4] Within plant cells, HMs exert their toxicity by replacing essential cations, by generating reactive oxygen species (ROS), or by binding to thiol moieties, thus ultimately damaging molecular functions.[5] To limit damage, absorbed metals are generally trapped within the cell wall, subjected to excretion or compartmented into subcellular districts.[6] HMs internalization can also occur within the cytoplasm through the formation of complexes with phytochelatins, reduced glutathione, or heat shock proteins (HSPs).[7,8] All mechanisms mentioned above highly modulate metal toxicity to plants.[9]

Plant metal uptake is highly influenced by soil microorganisms. Arbuscolar mycorrhizae (AMs) and plant growth-promoting rhizobacteria (PGPRs) can regulate metal levels in the soil[10] and facilitate plant development by favoring nutrient acquisition, by synthesizing growth-stimulating substances and/or by conferring increased tolerance to HMs toxicity.[11−14] These microorganisms can also mutually interact to inhibit plant pathogens.[15] Since AMs/PGPRs can also regulate the

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accumulation of HMs in plant tissues, they have been proposed in conjunction with plants for the phytoremediation of polluted sites.

Plant response to HMs-induced stress has been widely investigated.[5,7,16−18] To understand plant response to metal challenges and to unravel related tolerance mechanisms, transcriptomic technologies have been used.[19] However, gene expression changes are often not reflected at protein level, thereby hindering real effectors that actively participate in plant adaptation. Despite being a comparatively recent research field, ecotoxicoproteomics has gradually been applied to study the plant response to different HMs, becoming an established technology for environmental investigations.[20−22] In these studies, tissues from metal-tolerant/accumulator plants were subjected to differential two-dimensional electrophoresis (2-DE) experiments for quantitative protein evaluation with respect to control from counterparts grown in noncontaminated soils.[23−25] Prototypic examples are investigations performed on vegetables, trees, and herbs exposed to MHs, which were proved to accumulate in specific plant districts.[23−28] These studies provided insights into the mechanisms of metal toxicity to organisms as well as the plant response to counteract it. On the basis of these investigations, some plants were suggested as candidates for phytoremediation of contaminated soils, emphasizing the importance of mycorrhizae in improved performances in localized accumulation of pollutants.[25,29] The above-mentioned studies were performed on a laboratory scale and describe the proteomic changes in plants following the addition to the pot of a single metal. This condition does not generally represent a real situation present in a contaminated soil, where multiple HMs occur together at high concentrations.

Eucalyptus rostrata, *E. cladocalyx*, *E. melliodora*, *E. polybractea*, and *E. viridis* have been preliminarily shown to be effective in soil phytoremediation, with accumulation of HMs in their foliage.[30,31] The aim of this study was to characterize the adaptation properties of *E. camaldulensis* and to accomplish an ecotoxicoproteomic investigation on this plant in a real condition scale, by describing growth and accumulation performances, together with leaf proteomic changes, of shrubs harvested for 6 months in an industrial, metal-contaminated soil, in the absence/presence of AMs/PGPRs. Plants grown in a neighboring noncontaminated area for the same period were used as a control. To obtain additional insights into the adaptation mechanisms underlying eucalyptus metal tolerance and stress response, analysis was extended to the leaves of plants grown *in situ* for 30 years.

MATERIALS AND METHODS

Investigated Area. This study was conducted in the former Zn smelter "Pertusola Sud" property, Crotone, Italy.[32] The full site covers approximately 50 ha and is divided into different working sectors. Experiments were carried out on the byproduct accumulation sector (about 5 ha), which was originally used for the disposal of industrial waste, and on another noncontaminated, neighboring sector.

Experimental Design, Plant Material, and AMs/PGPRs Inoculation. *Eucalyptus camaldulensis* Dehnh. plants were 6-month-old, selected genetic clones that were transplanted into the soil of the areas of interest, grown under controlled conditions (Supporting Information Figure S1) and grouped accordingly.

Group 1. Sixty control plants that were grown for 6 months in the soil of the noncontaminated sector and were fed only with a nutrient solution containing phosphate (added as 70 g of $NaH_2PO_4{\cdot}2H_2O$ for each plant) 3 times a week.

Group 2. Sixty plants that were grown for 6 months in the soil of the byproduct accumulation sector previously inoculated with a cocktail of AMs/PGPRs (see below); these were fed with the nutrient solution reported above (3 times a week).

Group 3. Sixty plants that were grown for 6 months in the soil of the byproduct accumulation sector and were fed with the nutrient solution reported above (3 times a week).

Group 4. Ten plants that were grown for 30 years in the soil of the byproduct accumulation sector and were fed for 6 months as mentioned above.

For group 2, the contaminated soil was mixed beforehand with various AMs/PGPRs at 10% v/v ratio. AMs and PGPRs were selected on the basis of their known effects on plant biomass, toxicity symptoms, metal/nutrient uptake, and their possible mutual interaction.[33−37]

In the late morning, leaves were collected randomly from plants of the same group; they were washed with water, 70% ethanol, dried, and stored at −80 °C until use for metal/proteomic analyses.

Physicochemical Analysis of the Soil Samples before AMs/PGPRs Addition. Soil samples were analyzed as described previously.[32] In brief, they were dried at 25 °C for 2 days, sieved to <2 mm and measured for their particle size with a Bouyoucos hydrometer (Canadawide Science, Canada). For each sample, a soil/water slurry at 1/2.5 or 1/2 ratio was analyzed to determine pH and electrical conductivity values, respectively. In parallel, soil samples were dried at 40 °C, for 24 h, ground to powder, weighed into 10 mg aliquots, and added to 18% HCl to remove carbonates. After drying overnight, corresponding carbon and nitrogen content was determined with a CHN1500 instrument (Carlo Erba, Italy). Soil samples were also analyzed for metal content. They were dried at 105 °C, for 24 h, and treated with 18% HCl in a MARSXpress microwave oven (CEM, USA). After mineralization, samples were filtered through 0.45 μm polytetrafluoroethylene devices, diluted, and finally analyzed for metal content with a Vista MPX instrument for ICP−OES (Varian Inc., USA).

Identification of the Soil Microorganisms Originally Present in the Ground Samples. Soil samples (10 g) from contaminated/noncontaminated areas were aseptically collected in sterile bags containing 90 mL of sterilized peptone water (Oxoid-Thermo, USA) and ground with a mechanical Stomacher 400 homogenizer (International PBI, USA). Isolates were differentiated to species level by amplifying 16S (bacterial) and 18S rRNA (fungal) with PCR.[38]

Biometric Measurements. Growth performance was estimated by measuring plant height and diameter. Data were used to calculate the growth rate percentage as follows

$$\text{growth rate } (\%) = [(x_1 - x_0)/x_0]100$$

where x_0 and x_1 correspond to the measurement of the plant stem diameter/height at the time of planting and harvesting, respectively.

Analysis of Metal Content in Plant Tissue Samples. Plant samples were analyzed for metal content as reported

Table 1. Physicochemical Characteristics of the Soil Samples Used in This Study[a]

sample	sand (%)	clay (%)	silt (%)	pH	EC (mS/cm)	OC (%)	total N (%)
contaminated soil	62	19.3	18.7	8.0 ± 0.2	1.34 ± 1.0	1.46 ± 0.33	0.10 ± 0.02
noncontaminated soil	60	17.2	16.3	8.2 ± 0.2	1.36 ± 0.8	1.32 ± 0.20	0.14 ± 0.02

[a]Notation: OC, organic carbon; N, nitrogen; EC, electrical conductivity. Data shown are the mean values ± standard deviation (SD).

above. Before analysis, plant tissues were dried at 105 °C, for 24 h, and treated with 18% HCl in a MARSXpress microwave oven (CEM, USA).[32]

Protein Extraction. Protein mining was performed using the TCA/acetone precipitation procedure followed by a phenol/ammonium acetate-based extraction/precipitation step to remove contaminants;[39−41] a separate experiment was performed for each biological replicate (3 in number for each condition). Briefly, 10 frozen leaves (from 6 plants) were finely ground in a mortar with liquid N_2; the resulting material was lyophilized. Experimental details are reported in the Supporting Information.

Two-Dimensional Electrophoresis. 2-DE was performed according to a standard method.[42] Experimental details are reported in the Supporting Information. For quantitative analysis, each biological replicate was analyzed in technical triplicate in order to achieve nine gels per single experimental condition. Gel images were acquired and analyzed as reported in the Supporting Information.

Mass Spectrometric Analysis. Spots were excised from gels, alkylated, and digested with trypsin, as previously reported.[41] Digest aliquots were subjected to a desalting/concentration step and analyzed by nanoLC-ESI-LIT-MS/MS with a LTQ XL mass spectrometer (Thermo, USA),[43] as reported in the Supporting Information. Raw data from nanoLC-ESI-LIT-MS/MS were searched by the MASCOT search engine (version 2.2.06, Matrix Science, UK) against an updated *Eucalyptus* EST database from NCBI (2012/07/09). Database searching was performed as reported in the Supporting Information.

RESULTS

Physicochemical Characterization of the Soil Samples. Soil samples taken from contaminated and noncontaminated areas were characterized for parameters influencing metal mobility.[44] In both cases, the soil was classified as a slightly alkaline sandy loam, containing poor levels of nutrients and organic matter (Table 1). As expected, the polluted site showed higher metal concentration values than the nonpolluted site, and exceeding those acceptable for an industrial area (Table 2). In particular, Zn and Cu showed the highest values, followed by Pb, Cd, and As. Trace elements were also observed in the contaminated site as a result of the smelting activities.[32]

Microbial Characterization of the Soil Samples. Before placing eucalyptus *in situ*, bacteria and fungi present in the contaminated and noncontaminated soil were characterized (Table 3). Both soils showed the occurrence of *Bacillus* and *Rhizopus* subspecies, demonstrating a general similarity in the representation of soil microbes; conversely, *Pseudomonas*, *Actinobacteria*, and *Trichoderma* subspecies were only observed in the noncontaminated site.

A prevalence of *Bacillus* subspecies was observed in both cases. Under stress conditions, these bacteria produce endospores that remain dormant for long periods. They are able to stimulate plant growth by functioning as a nitrogen-fixing bacteria facilitating nutrient uptake.[45] In parallel, *Aspergillus*, *Eurotium*, *Rhizopus*, and *Trichoderma* fungal subspecies were identified in both soils. These fungi can survive in extreme environmental conditions; they were isolated in industrial waste effluents containing high HMs levels.[46]

Table 2. Range, Mean, and Standard Deviation (SD) Values of Total Concentration of Trace Elements in the Collected Soil Samples[a]

	contaminated soil		non-contaminated soil	
	range	mean values ± SD	mean values ± SD	D. Lgs 152/06[b]
As	5.22−432	152 ± 130	n.d.	50
Cd	19.4−797	388 ± 263	n.d.	15
Cu	31.6−7233	2540 ± 1878	17.2 ±0.12	600
Ge	n.d.−6.42	0.53 ± 1.85	n.d.	
Hg	2.04−114	24.5 ± 32.4	n.d.	5
In	n.d.−55.1	17.2 ± 16.6	n.d.	
Pb	49−13502	5359 ± 3701	n.d.	1000
Sb	3.85−107	47.3 ± 33.9	n.d.	30
Ti	0.50−56.1	26.3 ± 16.6	n.d.	10
Zn	1329−133874	49788 ± 37722	89.8 ±2.3	1500

[a]Range and mean values are reported as metal content (mg)/kg of soil. Not detected, n.d. [b]Permissible threshold values in industrial soils.

Table 3. Bacteria and Fungi Identified in the Contaminated and Noncontaminated Soil Samples. Data Related to the Contaminated and Noncontaminated Soil Samples Are Indicated with Asterisks and Circles, Respectively

bacterial species	fungal species
Bacillus sp. CNE 9*°	*Aspergillus niger* RFW-5*
Bacillus subtilis DmB4*	*Eurotium amstelodami* CBS 518.65*
Bacillus pumilus LLS-M1−17*°	*Aspergillus nomius* isolate P.tri.Iso14*
Bacillus pumilus S1*°	*Rhizopus oryzae* TY.GF1*°
Paenibacillus lautus DAN9*°	*Trichocomaceae* sp. LM276*
Bacillus subtilis HU61*°	*Eurotium repens* WB13*
Bacillus sp. DU29(2010)*	*Trichoderma reesei*°
Uncultured γ proteobacterium clone M0027_096*	
Pseudomonas sp.°	
Actinobacteria sp.°	

Addition of AMs/PGPRs to the Contaminated Soil and Evaluation of the Plant Performances. AMs are beneficial symbionts for most plants and interact with soil bacteria.[10−14] The latter promote the functioning of mycorrhizae-plant symbiotic mechanisms, facilitating the fungal binding to roots, improving uptake of soil nutrients, producing siderophores/phytohormones, and promoting plant resistance to pathogens. Different *Bacillus* and *Paenibacillus* spp. have these beneficial effects and were used to generate AMs/PGPRs additive cocktails.[33−36]

On the basis of their specific properties and mutual interaction abilities, different AMs/PGPRs were pooled in a

Table 4. Bacteria and Fungi Inoculated into the Polluted Soil of the "Pertusola Sud" Property, Crotone (Italy), where Group 2 Plants Were Grown

rhizosphere microflora						
		taxonomy				
kingdom	type of symbiosis	phylum	family	species	function	reference
Fungi	Ectomycorrhiza	Basidiomycota	Sclerodermataceae	*Pisolithus tinctorius*	improves plant fitness	
	Arbuscular Mycorrhiza (AMs)	Glomeromycota	Acaulosporaceae	*Entrophospora colombiana*	unknown	
			Glomeraceae	*Glomus clarum*	unknown	
				Glomus etunicatum	effect on plant biomass; P uptake; reduction of toxicity symptoms; Ni accumulation in roots and shoots.	74
				Glomus intraradices	As, Cu, and Zn uptake	47,77
Bacteria	Plant growth-promoting rhizobacteria (PGPRs)	Firmicutes	Bacillaceae	*Bacillus licheniformis*	Ni uptake; protects plant from Ni toxicity	75
				Bacillus licheniformis, Bacillus thuringiensis	improves metal phytoextraction, increases Se, Cd, and Cr uptake, ACC deaminase, production of siderophores	76
				Bacillus megaterium	facilitates Cu uptake; promotes plant biomass	78
				Bacillus subtilis	forms a protective endospore allowing the organism to tolerate extreme environmental conditions	79
			Paenibacillaceae	*Bacillus polymyxa*	nutrient uptake	60
				Paenibacillus azotofixans	nutrient uptake, production of siderophores	

cocktail (Table 4) and added to a separate area of the contaminated site; this district was used for transplanting group 2 plants. Group 1 was grown in a noncontaminated area, while group 3 was grown directly in the contaminated soil, without the addition of AMs/PGPRs. Group 4 plants have been present on the contaminated soil since the beginning of its industrial misuse (about 30 years). Groups 1−3 were used for comparative experiments on growth performances; leaves from groups 1−4 were used for comparative metal accumulation and proteomic experiments.

Elevated HMs levels in the soil determined a reduction in plant growth, compared to the control (Figure 1, groups 1 and 3). This phenomenon had been expected and was in line with metal toxicity effects on plant performances.[47] The addition of AMs/PGPRs to the contaminated soil allowed a recovery of the eucalyptus growth performances, both in terms of stem diameter and height values (Figure 1, groups 2 and 3). Growth performances of plants present in the contaminated soil to which AMs/PGPRs had been added were even better than the control (Figure 1, groups 1 and 2). These results demonstrate the beneficial effect of this AMs/PGPRs cocktail in promoting growth and ameliorating metal toxicity in plants, even if they are grown on a contaminated, industrial site.

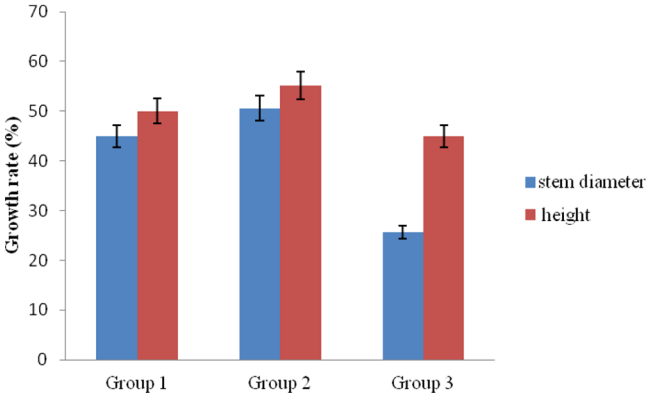

Figure 1. Stem diameter and height increase of *E. camaldulensis* plants after 6 months. Data refer to the plants belonging to groups 1, 2, and 3.

Ability to accumulate metals in the foliage was then comparatively evaluated for the different groups; corresponding As, Cd, Pb, and Zn content was measured to this end. In general, plant growth in the contaminated soils for different times (either in the presence/absence of AMs/PGPRs) showed a higher concentration of these metals in their leaves than counterparts grown in the noncontaminated soil, as expected for accumulator species (Figure 2, group 1 with respect to groups 2, 3, and 4). Relative quantitative amounts of the different metals present in the foliage of the plants grown in the contaminated areas reflected those measured in the corresponding soils (Figure 2, Table 2). Similar metal concentration values were ascertained in the leaves of plants grown in the contaminated soil for 6 months or 30 years (Figure 2, groups 3 and 4). Conversely, a significant increase in metal accumulation was observed when plants were grown in the contaminated soil in the presence of AMs/PGPRs (Figure 2, group 2 with respect

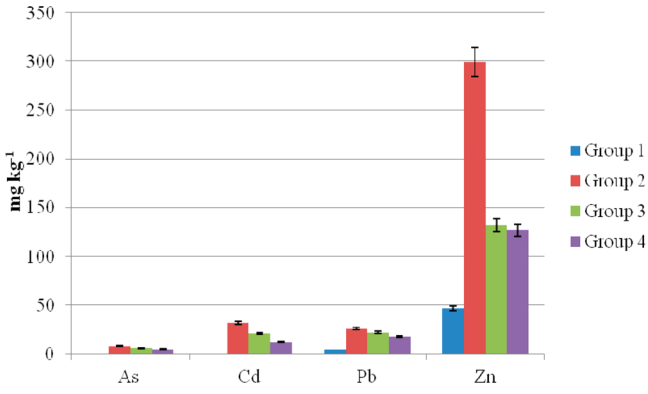

Figure 2. Metal content in the foliage of *E. camaldulensis* plants after 6 months or 30 years of growth under different environmental conditions, i.e., metal contamination in the absence/presence of AMs/PGPRs. Data expressed as metal (mg)/leaves (kg) refer to the plants belonging to groups 1, 2, 3, and 4.

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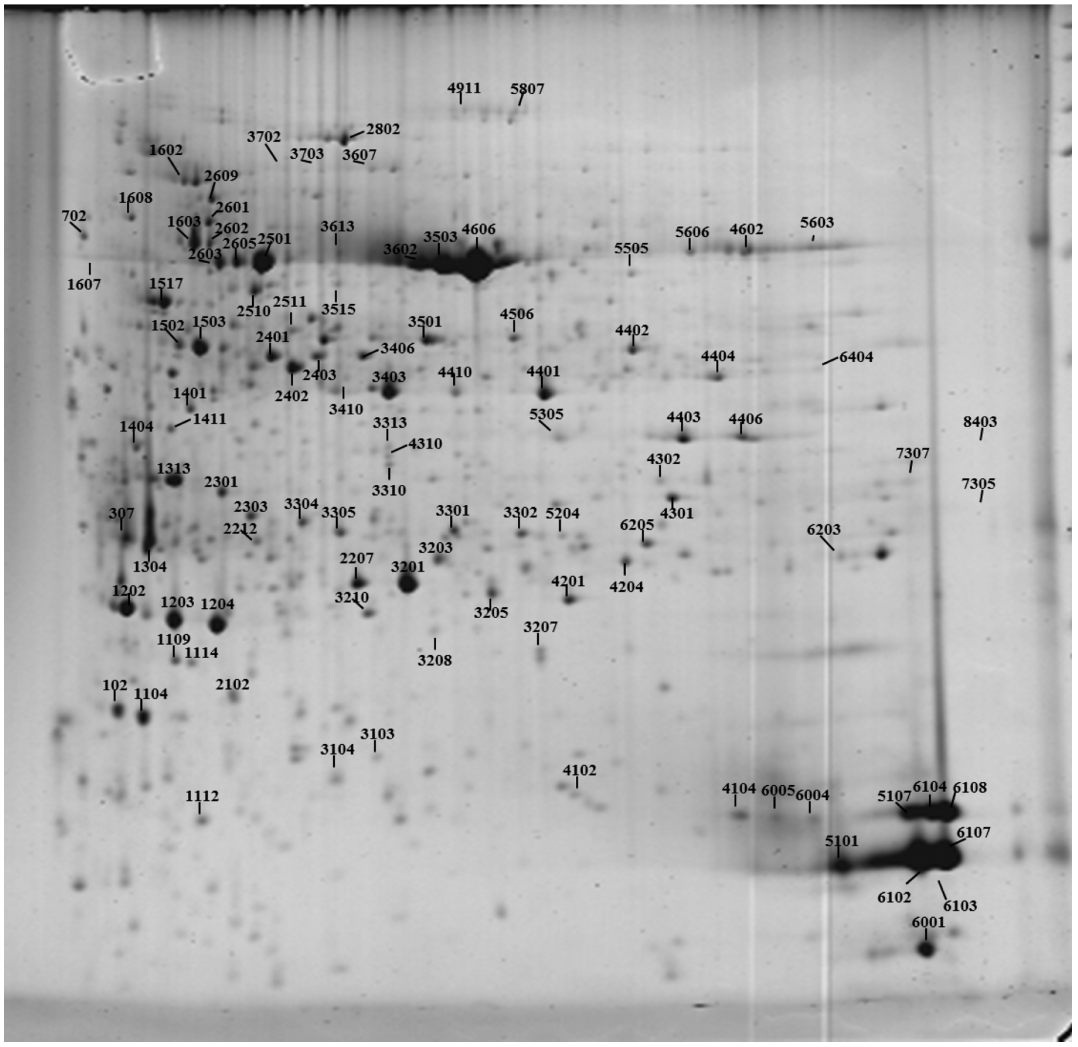

Figure 3. 2-DE proteomic map of *E. camaldulensis* leaves after staining with colloidal Coomassie Brilliant Blue G. Data for control plants are shown. Differentially represented or constant spots further submitted to nanoLC−ESI-LIT-MS/MS for protein identification are indicated and numbered in line with Supporting Information Table S1.

to groups 3 and 4); this trend was evident for Zn, the most abundant metal detected in the foliage.

Data reported in Figure 2 on *E. camaldulensis* confirmed and extended to other HMs preliminary studies on the Al, Pb, and As-accumulating properties of other eucalyptus subspecies.[30,31] Moreover, they extended to real-condition-scale (i.e., a Zn smelter site) information on the positive effect of AMs/PGPRs soil additives in improving the metal accumulating properties of *E. camaldulensis*, confirming preliminary data from greenhouse experiments on *E. globolus* growth in the presence of Cu, *Glomus deserticola, Trametes versicolor*, and *Coriolopsis rigida*.[48]

Differential Leaf Proteomics of Plants Exposed to Various Environmental Challenges. Leaves are responsible for plant carbon uptake and transpiration, but they can also behave as a metal accumulation site. To gain a better understanding of the mechanisms of plant tolerance, detoxification, and stress response toward metals, proteomic studies on leaves of plants grown in the presence of HMs are relevant, both in terms of basic knowledge and for applications in phytoremediation/phytoextraction.[23−29,47] *E. camaldulensis* leaves are generally recalcitrant to proteomic analysis.[49] Literature analysis shows that the TCA/acetone-based procedure has been widely used for plant proteomics,[47,50] as well as in studying organisms exposed to metal pollutants.[51−54] The phenol-based method has the potential for recalcitrant tissues to generate better purified samples, yielding highly resolved proteomic maps.[55] It has also been used to analyze plants subjected to HMs-related stress.[26,56,57] After different trials on *E. camaldulensis* leaves, we observed the best results by sequentially combining both protocols (data not shown).

2-DE resolved eucalyptus proteins within 10−150 kDa and 3−10 units of molecular mass and pH range, respectively. A representative gel for group 1 leaves is shown in Figure 3. Average proteomics maps showed 654 ± 59, 601 ± 51, 619 ± 53, and 672 ± 63 spots for groups 1, 2, 3, and 4, respectively (Supporting Information Figure S2). The degree of similarity between the gels from plants grown in contaminated soils and the control was 88% (group 2/group 1), 92% (group 3/group 1), and 99% (group 4/group 1). Image analysis of 2-DE gels revealed 83 spots as differentially represented between various experimental groups ($p < 0.05$). These spots plus selected constant spots (24 in number) were subjected to nanoLC−ESI-LIT-MS/MS analysis, which characterized corresponding proteins from *Eucaliptus* subspecies (Supporting Information Table S1). Eight spots contained multiple components as a result of a concomitant protein migration in 2-DE.

Differentially represented proteins in *E. camaldulensis* were categorized into different functional classes (Figure 4). The

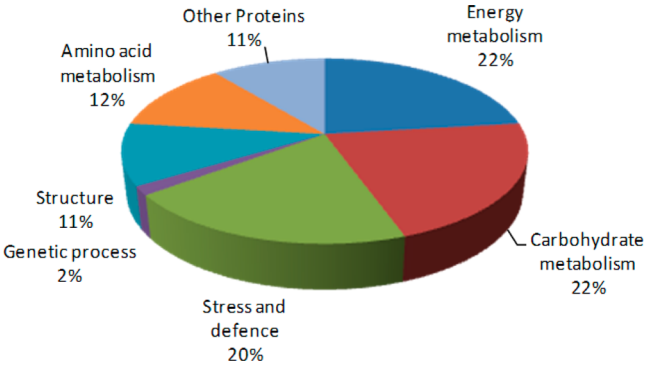

Figure 4. Functional distribution of the differentially represented proteins as identified after leaf proteomic analysis of the plants belonging to groups 1−4.

most represented components were proteins involved in energy metabolism (22%), carbohydrate metabolism (22%) and stress/defense (20%). They will be discussed in detail in the following sections. Among the components classified as other proteins (11%), the putative uncharacterized protein corresponding to EST379478285 is worth mentioning since it showed the largest quantitative differences in groups 2−4 with respect to the control. Although it has not yet been related to a specific function in any other species, this protein contains structural elements predicted to bind to RNA.

Proteins Associated with Photosynthesis and Energy Metabolism. Unlike plants that are sensitive to HMs and similar to other metal hyperaccumulator organisms challenged during *in vitro* experiments,[23−25,58] eucalyptus grown in the contaminated areas showed an over-representation of enzymes involved in photosynthetic electron transfer and the Calvin cycle in its leaves. In particular, thylakoid-membrane located electron transport chain cytochrome b6-f complex Fe−S subunit 1 (spots 102, 1104), ribulose biphosphate carboxylase large chain (RuBisCo LSU) (spots 3503, 3602), ribulose bisphosphate carboxylase/oxygenase activase (spots 1502, 1503), and sedoheptulose-1,7-bisphosphatase (spot 1401) presented augmented levels in the presence of HMs. Other proteins involved in thylakoid membrane stabilization, namely lumenal 17 kDa, 29 kDa, and At1g03610 proteins (spots 1112, 3305, 6103), also increased their concentration in group 3. Amounts of RuBisCo LSU returned to basal values when AMs/PGPRs were present, while a further increase in concentration was observed under this condition for the proteins mentioned above, together with an over-representation of other species involved in photosynthetic and energy metabolism activity, namely oxygen-evolving enhancer proteins 1, 2-1, and 3-2 (spots 1313, 2207, 5107, 6104, 6108), chloroplastic ferredoxin NADP reductase isoforms (spots 4403, 4406, 5305), phosphoribulokinase (spots 2402, 3406) and fructose-biphosphate aldolase (spots 3403, 4401, 4410). These quantitative trends may represent a major reason behind the ability of eucalyptus to accumulate HMs, since the maintenance of high photosynthetic rates (and corresponding elevated ATP, NADH/NADPH levels) is generally advantageous for an active plant stress acclimation. This phenomenon seemed to be enhanced by the presence of AMs/PGPRs, which determined increased growth performances and metal accumulating properties (Figures 1 and 2), similar to what has been observed in other plant species.[59,60] Conversely, a regulator of CO_2 assimilation (carbonic anhydrase−spot 5204), photorespiratory 2-phosphoglycolate phosphatase (spot 1404), and light harvesting chlorophyll a/b binding proteins 6A, 8, and 21 (spots 1203, 1204, 3201, 307, 1304) were down-represented in both conditions or in group 2, respectively.

The higher photosynthetic and energy metabolism activity observed for eucalyptus grown in the contaminated areas did not find a parallel in an increased ATP synthase activity. In fact, ATP synthase subunit β (spots 1603, 2601, 2602, 2603, 2605) was down-represented in the presence of HMs, but rose to control levels when AMs/PGPRs were present. In contrast, mitochondrial ATP synthase subunit d (spots 1109, 1114) greatly increased its concentration in the contaminated areas either in the absence or presence of AMs/PGPRs, probably as a result of an increased local ATP requirement. Unlike plants that are sensitive to HMs and similar to other metal hyperaccumulator organisms challenged during *in vitro* experiments,[23−25] eucalyptus growth in the contaminated areas did not show the need to remobilize energy and reductive power from other metabolic pathways (glycolysis, tricarboxylic acid cycle, and polysaccharide degradation). Indeed, enolase (spots 2602, 3515), enolase 2 (spot 2501), phosphoglycerate kinase (spot 2511), triosephosphate isomerase (spot 3305), and NADP-dependent malate dehydrogenase (spot 2511) showed reduced levels in the presence of HMs, which in some cases returned to control values in the presence of AMs/PGPRs. Alternately, glyceraldehyde-3-phosphate dehydrogenase (spot 6404), transketolase (spot 3607), and malate dehydrogenase (spot 6404) did not change their concentration in any of the conditions that we evaluated. The down-representation in group 3 of the chloroplast stem-loop binding protein of 41 kDa (spot 4404) and glucose-1-phosphate adenylyltransferase small subunit 1 (spot 2510) was in line with the above-mentioned considerations on energy remobilization. The first protein is involved in the regulation of heteroglycans and monosaccharide mobilization,[61] while the second one plays a key role in starch biosynthesis/degradation;[62] both proteins returned to control levels in the presence of AMs/PGPRs.

On the other hand, a different quantitative trend was observed for other enzymes that were associated with carbohydrate metabolism but also with cell structure function, namely UTP-glucose-1-phosphate uridylyltransferase (spots 3602, 3613) and UDP-arabinopyranose mutase 1 (spots 3501, 4506). Both were over-represented in plants from all contaminated areas. Since these enzymes are involved in callose deposition in the cell wall and in the biosynthesis/degradation of noncellulosic polysaccharides therein, it is plausible that their quantitative profile may be associated with a reshaping of the cell wall, as a result of metal compartmentalization in this district.

In general, our data on photosynthetic and energy metabolism proteins from eucalyptus growth in a real situation of contaminated industrial soil closely reflected other observations on hyperaccumulator plants realized in a laboratory environment.[25] It should be noted that most of the differentially represented proteins that we detected in plant growth in the contaminated areas for a limited period of time (in the absence/presence of AMs/PGPRs) returned to control values when very protracted times (30 years) were considered. This observation suggests that eucalyptus eventually sets up additional energetic mechanisms to reduce the detrimental

effects caused by prolonged exposition to HMs. In line with this hypothesis is the unique over-representation in group 4 of (i) ATP synthase subunit β (ATP synthesis), (ii) enolase, enolase 2, triosephosphate isomerase (glycolysis), (iii) carbonic anhydrase (assimilation of CO_2), (iv) protein thylakoid formation 1. This variable effect of HMs over extended times was also mentioned in other studies on metal-sensitive plants.[63] In contrast, constant over-representation of electron transport chain cytochrome b6-f complex Fe−S subunit 1, ribulose bisphosphate carboxylase/oxygenase activase, sedoheptulose-1,7-bisphosphatase, mitochondrial ATP synthase subunit d, UTP-glucose-1-phosphate uridylyltransferase, UDP-arabinopyranose mutase 1 in groups 2−4 suggests that these proteins are essential factors for eucalyptus, associated with indispensable pathways that allow the plant to survive in a metal-contaminated environment.

Proteins Associated with Stress and Defense. Some HMs are directly involved in redox reactions leading to the production of toxic ROS, while redox inactive-HMs may indirectly trigger oxidative stress by depleting major thiol-containing antioxidants and enzymes, disrupting electron transport chains, inducing membrane lipid peroxidation, and altering protein structure (as result of amino acid oxidation).[2,5,18,24] The ability of a plant to tolerate and accumulate HMs is generally associated with their ability to cope with these toxic molecules; accordingly, these organisms have developed several antioxidant defense mechanisms comprising both enzymatic and nonenzymatic effectors.[18]

Similarly to what has been observed in other *in vitro* studies on plants sensitive to HMs and metal hyperaccumulators challenged with Cd, Cu, As, and Al ions,[23−25,58,64] eucalyptus growth in the contaminated area showed an over-representation of various detoxifying enzymes involved in reducing ROS concentration and stress tolerance.[65] In particular, increased levels of Mn-superoxide dismutase (spot 4201), Fe-superoxide dismutase (spot 3210), L-ascorbate peroxidase (spot 2212), catalase 2 (spot 4602), and aldehyde dehydrogenases (spot 5606) were observed in group 3. With the exception of catalase 2 (over-represented in all cases), proteins mentioned above returned to basal/reduced levels in groups 2 and 4. A similar quantitative trend was also observed for proteins assisting the functional recovery of oxidized/misfolded proteins, such as 2-Cys peroxiredoxin BAS1 (spot 1202), 17.1 kDa class II HSP (spot 3104), probable mediator of RNA polymerase II transcription subunit 37c (also known as HSP2 70 kDa) (spot 1602), chaperone protein ClpC (spot 2802), and chaperonin 60 subunits α and β (spots 1608 and 2609, respectively). Some of the proteins are also involved in important physiological functions such as enzyme activation or redox sensoring.[66] These data suggest that eucalyptus overexpresses a number of the antioxidant defense/protective machineries coded in its genome to face metal stress, which return to normal expression values when AMs/PGPRs are present or plants are well-adapted to growth under metal-stress conditions (exposure for prolonged periods of time). Also in these cases, additional protective mechanisms/factors have to be claimed for eucalyptus to reduce the detrimental effects due to metal challenge, whether it is derived or not from the concomitant presence of AMs/PGPRs.

On the other hand, plants growth in all contaminated areas showed a reduction of pathogenesis-related proteins (PRs), namely remorin (spot 4302), and 26 kDa endochitinase 2 (spot 7305). PRs are pathogen-responsive components that have also been associated with metal-induced stress in various HMs-sensitive plants,[23,24,58] possibly as a result of common signaling pathways that are activated under both stress conditions through senescence-related processes. Their variable representation after a metal challenge has also been reported in other HMs hyperaccumulator plants,[25] suggesting that eucalyptus, as well as these plants, does not use these pathways intensively to cope with metal stress. Conversely, constant over-representation of protein SGT1 homologue (spot 1517) was observed in all experimental conditions associated with metal stress. This protein has been associated with plant basal disease resistance to bacterial blight, possibly acting as a positive regulator of basal defense.[67] Its constant induction in groups 2−4 suggests its pivotal role in the survival of eucalyptus in a metal-contaminated environment.

Proteins Involved in Glutathione Metabolism. Our proteomic analysis also revealed the differential representation of a number of plant proteins associated with the biosynthesis of glutathione and, ultimately, of low molecular mass chelators. The latter compounds strongly interact with HMs and minimize metal binding to thiol groups of functionally important proteins, limiting the corresponding toxicity.[68] Phytochelatins are the best characterized example of chelators that are synthesized from glutathione.[7] They form complexes with HMs in the cytosol, facilitate metal transport into vacuoles, and are associated with tolerance mechanisms toward corresponding stresses.[2] Similarly to what has been observed in other *in vitro* studies on plants sensitive to HMs and hyperaccumulators challenged with metals,[23−25,58] eucalyptus growth in the contaminated area showed an over-representation of various enzymes involved in the biosynthesis of glutathione amino acid constituents and in the conjugation of glutathione with endogenous/xenobiotic compounds. In particular, cysteine synthase (spots 3310, 3410), glutamine synthetase isozyme 2 (spots 2401, 2403), and serine hydroxymethyltransferase (spot 5603) were induced in groups 2 and 3, indirectly suggesting an overproduction of glutathione in eucalyptus exposed to HMs in order to directly bind to metals, to limit metal-associated oxidative stress, and to fuel phytochelatin biosynthesis.[8,63,69] Similarly, glutathione-S-transferase (spot 3205) and glutathione-S-transferase U17 (spot 6205) were over-represented in the presence of HMs, returning to basal levels when AMs/PGPRs were present. Glutathione-S-transferase (GST) isoforms mediate the conjugation of glutathione with endogenous/xenobiotic substances,[70,71] which are then transported into the vacuole.[72] Interestingly, GTSs were also shown to be involved in the direct conjugation of toxic metals with glutathione in yeast.[73] A better characterization of these enzymes would be necessary in the future to analyze their specific function in plant response to HMs-associated stress.

DISCUSSION

As verified in preliminary experiments performed in greenhouses, metal mines, or metal production plants, various eucalyptus subspecies are able to grow on Al, Pb, and As-contaminated sites, accumulate these metals in their foliage, and thus have been proposed as effective hyperaccumulator organisms for phytoremediation of polluted soils.[30,31] In this study, we extended this observation to *E. camaldulensis* plants that were grown in a Zn smelter site presenting elevated concentrations of different metals. Plants harvested in the contaminated soil were still able to grow, although with reduced

performances with respect to counterparts present in a noncontaminated site, and accumulated various metals in their leaves. The addition of AMs/PGPRs to the contaminated soil promoted eucalyptus growth and determined development performances even better than those observed in control plants. It also favored metal hyperaccumulation within plant foliage, as already observed in the case of *E. globolus* cultivated in a greenhouse in the presence of Cu and selected AMs/PGPRs.[48]

Comparative proteomic analysis of the corresponding leaves showed the molecular mechanisms underlying plant adaptation to the various metal challenges. Similarly to what has been observed in *in vitro* studies on other metal hyperaccumulator organisms but not on plants sensitive to HMs,[23−25] eucalyptus growth in the contaminated areas showed an over-representation of enzymes involved in photosynthetic electron transfer and the Calvin cycle. The addition of AMs/PGPRs generally increased the activation of these energetic pathways, suggesting the existence of signaling mechanisms to address the energy/reductive power requirement derived from the concomitant, augmented plant growth performances or sensing the presence of increased metal concentrations in the foliage and of microrganisms (and their helpful metabolites) close to the plant roots. Most of the differentially represented proteins that we detected in plant growth for 6 months in the contaminated areas returned to control values when prolonged time periods were considered, implying the hypothetical activation of additional energetic mechanisms to partially satisfy the organism's physiological requests.

An over-representation of various enzymes involved in limiting ROS concentration or indirectly associated with glutathione metabolism also occurred in HMs-exposed eucalyptus, similarly to what has been observed for plants sensitive to HMs or other metal hyperaccumulators evaluated on a laboratory scale.[23−25] These data suggest that eucalyptus uses a number of antioxidant defense/protective machineries to face metal stress. While most antioxidant enzymes and chaperones returned to almost normal expression values in the presence of AMs/PGPRs or in plants used to growth under metal-stress conditions, proteins ensuring proper amino acid levels to synthesize elevated glutathione concentrations were constantly over-represented in all conditions. This observation suggests that glutathione (and deriving phytochelatins) could act as key molecules for ensuring the effective formation of HMs-chelating complexes, which could possibly be responsible for the observed plant tolerance to metal stresses.[2] Further studies are needed to clarify this issue and to evaluate the corresponding phytochelatin levels. No massive activation of general stress pathways was observed, unlike what has been reported for other HMs-sensitive plants.[23−25]

In conclusion, this study confirms the positive application of *E. camaldulensis* in managing metal-polluted areas. Similarly to other cases, our results confirm that the plant accumulator performances are enhanced when AMs/PGPRs are present in the soil, thus suggesting their use as additives for environmental applications. In general, eucalyptus plants inoculated with AMs/PGPRs seem to show a physiological behavior resembling that of counterparts that are well-adapted to growth in the contaminated site. Notwithstanding a number of common proteomic signatures between both plant groups, specific protein representation differences were also observed. These findings indicate the need for further investigations in the near future into the impact of long-term plant adaptation with respect to short-term response, and the specific role/mechanisms of soil microorganisms in conferring tolerance to HMs toxicity.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedure, materials, and methods. Table and additional figures as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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