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Natural attenuation in a slag heap contaminated with cadmium: The role of plants and arbuscular mycorrhizal fungi

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ABSTRACT

A field study of the natural attenuation occurring in a slag heap contaminated with high available cadmium was carried out. The aims of this research were: to determine plants colonizing this slag heap; to analyze colonization and morphological biodiversity of spores of arbuscular mycorrhizal fungi (AMF); to determine spore distribution in undisturbed samples; to know mycelium and glomalin abundance in the rhizosphere of these plants, and to investigate glomalin participation in Cd-stabilization. Forming vegetal islands, 22 different pioneering plant species from 11 families were colonizing the slag heap. The most common plants were species of Fabaceae, Asteraceae and Poaceae. Almost all plants were hosting AMF in their roots, and spores belonging to *Gigaspora*, *Glomus*, *Scutellospora* and *Acaulospora* species were observed. Micromorphological analysis showed that spores were related to decomposing vegetal residues and excrements, which means that mesofauna is contributing to their dispersion in the groundmass. Mycelium mass ranged from 0.11 to 26.3 mg/g, which contained between 13 and 75 mg of glomalin/g. Slag-extracted total glomalin was between 0.36 and 4.74 mg/g. Cadmium sequestered by glomalin extracted from either slag or mycelium was 0.028 mg/g. The ecological implication of these results is that organisms occupying vegetal patches are modifying mine residues, which contribute to soil formation.

1. Introduction

Natural attenuation refers to the naturally occurring processes in soil and groundwater that degrade and dissipate pollutants, which include inorganic and organic contaminants. This *in situ* process comprises biodegradation, dispersion, dilution, adsorption, volatilization, and chemical or biological stabilization or destruction of contaminants [1]. A variety of other terms have also been used to describe natural attenuation including natural restoration, intrinsic remediation, intrinsic bioremediation, passive bioremediation, spontaneous bioremediation, and bioattenuation [2]. The natural attenuation process is driven by microorganisms and plants occurring naturally in polluted soils, which may participate mainly in metal sorption. Kamnev and van der Lelie [3] drew attention to the role played by soil microorganisms in the processes underlying plant-based methods of remediation (phytoremediation) of heavy metal contaminations, including phytostabilization. This may be applied to natural or man-induced remediation. Phytoremediation is gaining popularity in reducing metal load in the contaminated medium due to it is environmentally friendly, less destructive to soil biota, and a cheap alternative.

The dominant beneficial forms of fungi colonizing roots of most of the plants are the arbuscular mycorrhizal fungi (AMF), which are obligate symbionts widely distributed among higher plant species [4]. Moreover, Rillig [5] suggested that these fungi are functional components of terrestrial ecosystems world-wide. Several benefits from AMF to the plant–soil system have been described, both in agroecosystems and natural ecosystems; however, little is known about their role in heavy metal-polluted soils [6].

Arbuscular mycorrhizal fungi are integral, functional parts of plants roots, playing a central role in ameliorating metal toxicity in their hosts [7]. Some authors suggested that populations of arbuscular mycorrhizal fungi are the key factor in soil development and successful plant establishment. Evidence shows that the extra-radical mycelium (EM) appears to be involved in binding and sequestrating metals [8–9]. The last authors showed that this binding is at cell wall level. Recently, a new factor of presumably great importance in these processes is glomalin, a glycoprotein copiously produced by all AMF tested [10]. González-Chávez et al. [11] showed that this protein, extracted from polluted soil or from hyphae, strongly and irreversible sequestrates metals such as Cu, Cd, Zn. Therefore, AMF may stabilize metals in the soil, reduce their availability and decrease the risk of toxicity to other soil microorganisms and plants growing in polluted substrates. Although some

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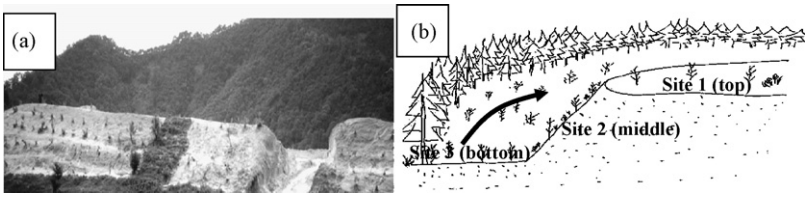

Fig. 1. (a) A view of the slag heap and (b) location of sampling sites.

information on the role of mycorrhizae in these conditions has been gained, it is still poorly understood how natural invading plants cope with the contaminants. For instance, why vegetation colonization is often producing uneven and irregular islands or patches, and the relationship with AMF activity.

In general, studies related with AMF are carried out in bulk soil samples; therefore, natural distribution and relationship between fungal organisms and soil components are little known. According to Bullock et al. [12], micromorphology is a useful tool that uses undisturbed samples to localize soil components and the mutual relationships between them. The application of this technique may permit elucidate the participation of AMF and the relationship within vegetation irregular islands.

Research carried out in Temascaltepec, Mexico shows that efforts to remediate this disturbed and contaminated area have been made; these however have been unsuccessful. Several reforestation practices with *Pinus* species have been attempted but only two trees have been able to establish themselves at the base of the heap. Instead, some invasive herbaceous plants have been the first colonizers [13]. Many of these plant species are recognized in the literature as hosts of AMF in natural or agricultural soils. Hence, the aims of this study were: (1) to determine plants colonizing this slag heap, (2) to find out whether plants colonizing this slag heap were colonized by AMF and the spore morphological biodiversity of AMF, (3) to analyze *in situ* distribution of spores in soil thin sections, (4) to know the abundance of fungal mycelium and glomalin content in the rhizosphere of these plants, and (5) to investigate the participation of the glomalin in the stabilization of Cd in this contaminated substrate.

2. Materials and methods

The present study was carried out on slag heaps (wastes from the silver mine La Guitarra) located at Temascaltepec in the south west part of the State of Mexico (19°03′5.8″LN, 100°04′23.7″). The climate is temperate slightly cool, with rain season in summer. The mine residues have been deposited on the river bank of the Verde River. The slag heap is surrounded by pine and sacred fir forests were the dominant species are *Pinus* sp., *Abies religiosa*, *Buddleia* sp., *Quercus* sp. and *Alnus* sp. Camprubí [14] reported that the mineralogical composition of residues is mainly by: quartz and calcite, with secondary minerals such as: pyrite, bornite, chalcopyrite.

2.1. Slag characterization

Thirty rhizospheric slag samples from three different positions of a heap were collected (Fig. 1): Site 1 (top), Site 2 (middle part) and Site 3 (bottom of the heap). Labelled waste samples were processed as follows: field-moist soil samples were air-dried in a soil processing room, and passed through a 2 mm sieve and stored in plastic bags. Slag samples were chemically characterized. pH was determined in water and 10 mM calcium chloride ($CaCl_2$) solution at 1:2.5 ratio [15]. Organic matter (OM) was analyzed by Walkley and Black procedure [16]. Cation exchange capacity (CEC) by the ammonium acetate method [15]. Available Zn, Mn, Cu, Ni, Cd and Pb were quantified by using an absorption atomic spectrometer (PerkinElmer 3110, Norwald, CT, USA) after extraction with 0.05 M diethylenetriaminepentaacetic acid (DTPA), 0.01 M triethanolamine and 0.01 M $CaCl_2$ [17]. Mineralogical composition was determined in 16 mine residues samples by X-ray diffraction using random powder mounts of the fine residues fraction (<50 μm). Colour of mine residues was determined by using Munsell chart.

2.2. Root colonization and morphological biodiversity of spores of arbuscular mycorrhizal fungi in field slag samples

Spores were separated from 10 g of the 30 slag samples by sieving and decantation method [18]. Permanent slides using polyvinyl alcohol-lacto-glycerol (PVLG) were prepared with the spores found at the sites, for determination of the morphological diversity. Microphotographs were taken as records.

Roots of plants growing at the three positions mentioned at the slag heap were carefully washed with tap water and rinsed with distilled water, cut into 1-cm segments with a steel blade and processed for root colonization. The roots were cleared and stained to determine AMF colonization as recommended by Phillips and Hayman [19]. Fifty root segments per root sample were mounted on slides in 50% aqueous glycerol and examined under a compound microscope (Nikon Alphaphot 2 YS2) at 100× magnification. Each segment was observed at three different microscopy fields so 150 visual observations were recorded per root sample. Colonization frequency (the presence or absence of fungal structures in the stained root segments) was determined and expressed as percentage of fungal colonization.

2.3. Micromorphological analysis

Eight undisturbed oriented soil sampled (10 cm × 8 cm × 6 cm dimensions) were collected in vegetal patches in the Site 3. In laboratory, samples were air-dried and then impregnated with unsaturated polyester resin (HU-543) and monostyrene (7:1 ratio). Once the resin hardened, the blocks were horizontally and vertically sectioned. Thin sections (30 μm thickness, 7.5 cm × 5 cm) were prepared and mounted on glass according to Murphy [20]. Spores and soil components (excrements, organic matter residues and groundmass) were described under a polarized microscope (Olympus BX51) using the concepts and terminology of Bullock et al. [12] and Stoops [21]. Spores and excrements were horizontally counted by the line counting method using polarizing microscope in eight thin sections. Values were expressed as frequency of the total spores number related to excrements, residual organic matter with different degree of preservation and groundmass in a thin section.

2.4. Quantification of mycelium from slag samples

Mycelium was obtained from 10 g slag of 30 rhizosphere samples in the same three positions of the slag by sieving and decantation method [18]. Three replicates per sample were

performed. Direct collection of the mycelium was done under stereo-microscope using very fine forceps. The mycelium was dried for 36 h at 65 °C and weighed. Biomass was expressed as milligram of mycelium per gram of slag.

2.5. Glomalin extraction from slag and mycelium samples

Glomalin extraction (the easily extracted glomalin EEG and total glomalin TG) from slag and mycelium samples was performed as described by Wright and Upadhyaya [10].

2.6. Cd content in glomalin

Cadmium was quantified in dried glomalin extracted from slag samples and mycelium. Glomalin samples were digested with concentrated HNO_3 for 3 h and heated to 115 °C. Cd concentration was determined by AAS using a PerkinElmer 3110 (Norwald, CT, USA) instrument. Analytical reagents grade chemical were used throughout. Glassware and polyethylene containers were cleaned with 1:1 laboratory reagent grade hydrochloric acid, rinsed three times with distilled water, then three times with distilled–deionised water before use. Internal control samples were included to check the quality of analysis, and certificated standards (PerkinElmer) were used for the calibration of the AAS. Pearson correlations were run for the parameters considered in Sections 2.4–2.6.

3. Results and discussion

The study on this slag was performed because site characterization plays an important role in setting the constraints on the natural attenuation process [2]. In order to define any potential or current threats to human health or the environment, site characterization must be carried out. This research regarded the extent of contamination and the effect on plant and mycorrhizal fungal populations.

3.1. Characterization of the slag

pH values were uneven; at Site 1 they ranged from 4.3 to 7.5; in Site 2 from 5.0 to 6.5 and at the Site 3 from 4.3 to 6.1. Organic matter content also varied between study sites from less than 1 to 119.4 g/kg. CEC varied from less than 2.6 to 23.3 $cmol_c$/kg and was related with OM content, since it is the dominant colloid present in the plant colonizing patches. The mine colour residues without OM was 5Y 8/8; although in the different analyzed samples the colour widely varied (5Y 7/6 to 10YR 3/3) (Table 1). In the case of the vegetal patches located at Site 3, where more detail study was made, the pH, the OM content, and colour were very consistent (5.6, 37.6 g/kg, 10YR, respectively). Accordance to these characteristics, these mine residues have formed an A horizon and have tendency to develop a Mollic or Umbric epipedon [22]. A horizon is an important link between soil and plants, since most plant roots and nutrients are within [23]. This demonstrates that plants colonization plays an important role in soil development (Fig. 2).

Additionally, the colour change in 5 units in value and chrome, which indicates that melanisation process, is taking place in very short time (approximately in 4 years). Melanisation, the change in soil colour by OM addition or by transformation of dark organic compounds [24], occurs in longer time. For example, Shafer et al. [25] reported that in newly created mine soils 30 years were required for C to build up the top 10 cm in levels comparable to undisturbed soil.

Mineralogical analysis showed that in all samples (16) quartz was the unique mineral in these residues (Fig. 3). This fact drives their rehabilitation management less complicated, because there are not minerals such as pyrite, chalcopyrite etc., as reported by Camprubí [14]. Cadmium was the single toxic metal found in high concentrations in the slag heap. Available Cd concentration varied at each of the three positions analyzed (Table 1). The lowest available Cd concentrations (<0.12 mg/kg) were found at Site 1, while in Sites 2 and 3 the concentration ranged between 0.25 and 0.7 mg/kg. Besides metal concentration, Cd availability in soils is currently affected by pH, OM and clay contents [26,27]; however, in these substrates, DTPA-extractable Cd was not higher at low pH, because apparently low pH might allow Cd transport. In addition no clay minerals have been introduced to the slag, so CEC is mainly associated to OM. After silver extraction by flotation procedure, slag residues in suspension are conducted by water through pipes and poured on the top of the tailing heap. Then water poured at the top passed all the way down through the heap. The Cd high concentration, especially at the Site 3 suggests that Cd is lixiviated down the heap as result of the dumping process; however, some experiments should support whether leaching of Cd is occurring.

Cadmium movement through lixiviation may represent a risk to the *Pinus* forest established around the waste heap. Sánchez-Guzmán [28] reported slopes between 10 and 100% on soils around the heap, which may also increase the risk of the dispersion of Cd contained in the residues.

3.2. Plants growing in the slag heap

Mining substrates do vary considerably in their physical and chemical nature but they are likely to inhibit natural colonization of most plant species for many years [29]. However, in the present research, 22 different pioneering plant species from 11 families were found growing on the slag heap (Table 1), most of them common on the surrounding plant communities including not native plants, such as *Phytolacca icosandra*. In almost all cases, these plants are growing only in fertility islands. On Site 1, plants were present in few island patches involving two or three plant species associated. For example, *Dalea obreniformis*, *Melampodium divaricatum*, *Galinsoga parviflora* or *Trifolium goniocarpum*, *Lopezia racemosa*; however, *P. icosandra* and *Crotalaria longirostrata* were always found independently grown alone. On Site 2 plants formed an irregular border line. On Site 3 plants were more abundantly distributed surrounding the slag heap. Members of the Fabaceae, Asteraceae and Poaceae families were the most common. Six species of Fabaceae and five species of Asteraceae were established in the three sites of this slag heap. Plants such as *Tagetes micrantha*, *Eleusine indica*, *D. obreniformis*, *Rhynchelytrum repens*, *L. racemosa*, *Crussea longiflora*, *Aschynomene villosa*, *Jaegeria hirta* and *Anagallis arvensis* invaded the substrate with the highest available Cd levels (0.50–0.70 mg DTPA-available Cd/kg slag). Yuan et al. [30] reported that native plants, such as *R. repens*, are species that should be used to rehabilitate areas, because of their ability to colonize hash environments and participate in soil formation. Additionally, Lubke et al. [31] reported that especially legumes promote soil development and increases of soil nitrogen by their nitrogen-fixing capability. Carrillo-González and González-Chávez [32], and Freitas et al. [33] reported the presence of members of genera of Fabaceae and Asteraceae and *Euphorbia* in metal-polluted sites. For example, *Tagetes lunulata*, *Dalea bicolour* and *Euphorbia* sp. have also been found in mine refuse in Portugal.

These plants showed poor Cd accumulation, therefore these invasive plants behaved as excluder species. The highest Cd concentrations found in leaves tissues were for *A. villosa* 0.52 mg/kg; *C. longiflora* 0.56 mg/kg; *A. arvensis* 0.62 mg/kg and *L. racemosa* 0.70 mg/kg, collected at the low and middle part of the tailing heap. The concentration in the other plant samples ranged from traces up to 0.48 mg/kg. However, the number of plant species colonizing the

Table 1
Plant species and arbuscular mycorrhizal fungi growing at three different positions of a slag heap contaminated with cadmium

Site position and No.	Plant host	Family	Colour	pH H_2O[a]	OM (g/kg)	CEC ($cmol_c$/kg)	Mycorrhizal infection (%)	Genus of AMF found	Mycelium in slag (mg/g)	Available Cd in slag (mg/kg)
Top/Site 1	*Dalea* sp.	Fabaceae	10YR 3/4	7.5	115.0	22.6	+(31)	*Glomus*, *Acaulospora*	0.87 ± 0.14	<dl
	Melampodium divaricatum (Rich. In Pers.) DC. In DC.	Asteraceae	10YR 3/4	7.5	119.4	23.3	nd	*Glomus*	0.77 ± 0.42	<dl
	Galinsoga parviflora Cav.	Asteraceae	10YR 4/4	7.1	1.2	<2.6	+(29)	*Glomus*	1.08 ± 0.08	<dl
	Trifolium goniocarpum Lojac.	Fabaceae	5Y 8/8	4.6	1.4	<2.6	+(35)	No spores observed	0.22 ± 0.07	0.04
	Eragrostis intermedia Hitchc.	Poaceae	2.5Y 7/6	4.4	<1.0	<2.6	–	No spores observed	0.11 ± 0.02	<dl
	Phytolacca icosandra L.	Phytolaccaceae	2.5Y 7/6	4.8	<1.0	<2.6	–	No spores observed	0.11 ± 0.09	0.04
	Lopezia racemosa Cav.	Onagraceae	5Y 8/8	5.6	<1.0	<2.6	+(37)	No spores observed	0.80 ± 0.63	<dl
	Crotalaria longirostrata Hook & Arn.	Fabaceae	5Y 8/8	4.9	<1.0	<2.6	nd	No spores observed	0.60 ± 0.23	<dl
	Phytolacca icosandra L.	Phytolaccaceae	5Y 8/8	5.0	1.3	<2.6	nd	No spores observed	1.01 ± 0.53	0.12
Middle/Site 2	*Lopezia racemosa* Cav.	Onagraceae	5Y 8/6	5.4	23.0	2.85	+(26)	*Glomus*	1.31 ± 0.33	0.28
	Bidens odorata Cav.	Asteraceae	10YR 4/6	5.3	13.5	6.6	+(5)	No spores observed	1.87 ± 1.04	0.28
	Tagetes micrantha Cav.	Asteraceae					+(44)			
	Rhynchelytrum repens (Willd.) Hubb.	Poaceae	5Y 7/8	5.7	10.0	5.9	+(30)	No spores observed	2.52 ± 0.68	0.30
	Dalea obreniformis (Rydb.) Barneby	Fabaceae	5Y 6/8	6.6	6.7	5.0	+(32)	No spores observed	nd	nd
	Aschynomene villosa Poir.	Fabaceae	5Y 7/8	4.9	10.0	4.0	+(24)	No spores observed	4.08 ± 0.11	0.26
	Eleusine indica (L.) Gaertn.	Poaceae	5Y 8/8	5.5	9.0	3.8	+(5)	*Glomus*	5.98 ± 0.01	0.52
	Tagetes micrantha Cav.	Asteraceae	5Y 7/8	5.5	9.5		+(44)	*Scutellospora*	3.94 ± 0.44	0.22
	Euphorphia ocymoidea L.	Euphorbiaceae	5Y 7/8	5.1	17.0	<2.6	+(27)	No spores observed	2.48 ± 0.44	0.24
	Crusea longiflora (Willd.ex Roem. & Schutt) Anderson	Rubiaceae	5Y 6/8	4.9	5.0	4.6	+(16)	No spores observed	2.12 ± 0.10	0.56
Bottom/Site 3	*Crotalaria rotundifolia* J.F. Gmel.	Fabaceae								
	Anoda cristata L.	Malvaceae	5Y 6/8	5.4	2.7	5.6	+(20)	No spores observed	3.13 ± 0.60	0.28
	Dalea obreniformis (Rydb.) Barneby	Fabaceae	10YR 5/4	5.5	20.0	<2.6	+(41)	*Scutellospora*, *Glomus*	5.32 ± 2.3	0.42
	Sida rhombifolia L.	Malvaceae	10YR 4/3	5.9	35.0	3.0	+(46)	*Scutellospora*	10.96 ± 2.1	0.26
	Anagallis arvensis L.	Primulaceae	10YR 3/3	5.6	45.0	6.0	+(30)	No spores observed	9.27 ± 6.5	0.62
	Aschynomene villosa Poir.	Fabaceae	10YR 4/6	5.5	35.0	8.6	+(31)	No spores observed	nd	0.32
	Lopezia racemosa Cav.	Onagraceae	10YR 4/8	5.6	35.2	10.4	+(32)	No spores observed	26.3 ± 4.0	0.70
	Jaegeria hirta (Lag.) Less	Asteraceae	10YR 4/6	5.8	47.1	8.7	+(36)	*Glomus*, *Scutellospora*	23.2 ± 10.3	0.48
	Cuphea procumbens Ort.	Lythraceae	10YR 3/3	5.8	54.2	10.8	+(38)	*Acaulospora*, *Glomus*, *Scutellospora*	13.6 ± 3.09	0.42
	Aschynomene villosa Poir.	Fabaceae	5Y 6/7	5.7	25.0	12.0	+(33)	*Scutellospora*, *Acaulospora*	4.14 ± 1.25	0.26
	Ipomoea pedicellaris Benth.	Convolvulaceae	10YR 3/6	6.1	29.8	12.2	+(27)	No spores observed	7.79 ± 3.85	0.78
	Dalea obreniformis (Rydb.) Barneby	Fabaceae					+(33)	No spores observed		
	Crotalaria rotundifolia J.F. Gmel.	Fabaceae	5Y 6/8	5.5	13.5	7.8	+(33)	No spores observed	1.03 ± 0.46	0.12
	Melampodium divaricatum (Rich. In Pers.) DC. In DC.	Asteraceae	10YR 4/4	5.4	<1.0	<2.6	+(40)	No spores observed	1.37 ± 0.34	0.60

[a] In water 1:2.5 soil:solution ratio; OM = organic matter; CEC = cation exchange capacity; dl = below to detection limits; nd = not determined.

Fig. 2. Rhizosphere activity and developed soil from mine residues (Site 1).

tailing heaps was very limited compared to the number of species commonly found in undisturbed forest of these areas; for instance, Sánchez et al. [34] reported around 76 families and 222 genera.

3.3. Arbuscular mycorrhizal fungi present on the slag heap contaminated with cadmium

Almost all the pioneer species growing in this area were colonized by AMF. This fungal colonization may be of great importance for the establishment of the early plant community structure and largely responsible for the colonization of such habitats as suggested by some authors [35].

Roots of all the collected plants presented colonization by native AMF, which was lower than 50% (5–46%). These low levels of colonization agree with earlier reports [36]. Pawlowska et al. [37] found plants with low colonization levels dominating polluted sites and plants with high colonization levels dominating non-polluted locations. Regvar et al. [36] suggested that this low colonization in plants growing in highly metal-polluted sites appears to be a competitive advantage.

In arid and semiarid soils, Camargo-Ricalde and Esperón-Rodríguez [38] showed that some plant species create resource islands, rich in soil organic matter, nutrients and spores of AMF. These appear to be important factors that allowed the establishment of plants in the Site 3 of this study, even where the highest levels of Cd were observed. These authors suggested that AMF may be involved in plant species survivorship and establishment. In the present research, spores from *Gigaspora*, *Glomus*, *Scutellospora* and *Acaulospora* were observed in the rhizosphere of the plants colonizing the slag heap (Fig. 4). This fact reflects a high diversity of genera of AMF in this polluted site, which contrasts with previous publications reporting low diversity of AMF in polluted soils [37]. For instance, Khan [35] found *Glomus*, *Scutellospora* and *Acaulospora* in the rhizosphere of plants growing in a non-polluted site. Whereas in a Cr contaminated site only *Gigaspora* spp. was found, suggesting a selection pressure or the effect of the toxicity.

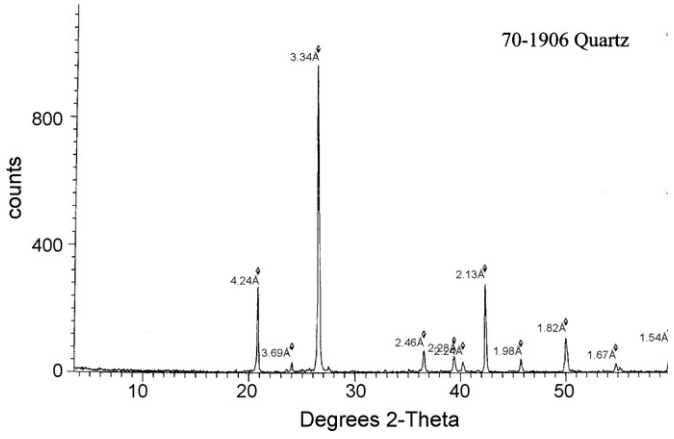

Fig. 3. Spectrum of the mineralogical composition of the slag.

In the present work, in some plants rhizosphere, members of *Glomus* were abundantly observed; in addition, *Scutellospora* was also very profuse. In the rhizosphere of *D. obreniformis*, *J. hirta*, *A. villosa* and *Cuphea procumbens*, from Site 3, more than one fungal genus was observed.

Leake et al. [39] stated: "the loss of diversity is characterized by the virtual elimination of certain key genera such as *Acaulospora* and *Scutellospora*, both of which tend to be abundant in undisturbed communities and the dominance of communities by a single species, *Glomus mosseae*". Additionally, these authors commented that due to large functional differences at the genus level, a diverse AM genera is worthy important in the soil. Fungal diversity in rhizosphere plants deserves more attention, as this information may help to understand functional diversity of these fungi in polluted soils.

Some authors have suggested that the introduction of AM fungal inoculum into areas devoid of symbiotic organisms may be a possible strategy for accelerating the process of vegetation of the wastes [6,35]. However, from the results of this work, fungal introduction is not necessary as this slag heap had a diverse AMF population, represented by members from different AM fungal genera. Instead, vegetating with seedlings inoculated with native AMF in the nursery, and transplantation near to the established plants in order to increase the AM fungal propagules in the area, may favour the revegetation as islands or patches. Moreover, organic matter additions may speed up patch formation. Khan [35] proposed fungal strains collected from long term heavy metal-polluted areas could be often the most efficient way for phytoremediation practices. Thus use of AMF, organic matter additions and revegetation may increase natural remediation of this site (accelerated natural attenuation).

3.4. Micromorphological analysis

The resistance structures of AMF (spores or sporocarps) were strongly related to vegetal residues in decomposition and excrements (Fig. 5; Table 2). From 732 resistance structures of AMF, 65% were single spores and 35% were sporocarps. This is the first report showing evidence of the *in situ* relationship between AMF and the soil components, especially with mesofauna. Several spore morphotypes were observed (from single spores to loose or compacted sporocarps); however, any spore type was related with specific soil component (Table 2). Spores were widely distributed in the groundmass of the slag residues (Fig. 5) and dispersed by mesofauna activity (Fig. 6). Fungal dispersion by mesofauna may be the ecological relevance as fungal structures distribute readily fungal inoculum for the colonization of the pioneer plants in the mine residues. Therefore fungal and mesofauna activity may be related, but more research need to be done to clarify this relationship.

Due probably to heavy-walled large resting spores, these appeared complete and healthy. These results from undisturbed samples confirm the high distribution of spores in the slag as observed in altered slag samples.

Rabatin and Stinner [40] concluded that spores are an important food source for a wide range of soil animals, which affect AMF in two different ways: (1) affecting the density or distribution of external hyphae (mainly by grazing) or (2) by ingesting and dispersing spores. An example of this is given by Jonas et al. [41], who showed that AMF are consumed by Colembolla.

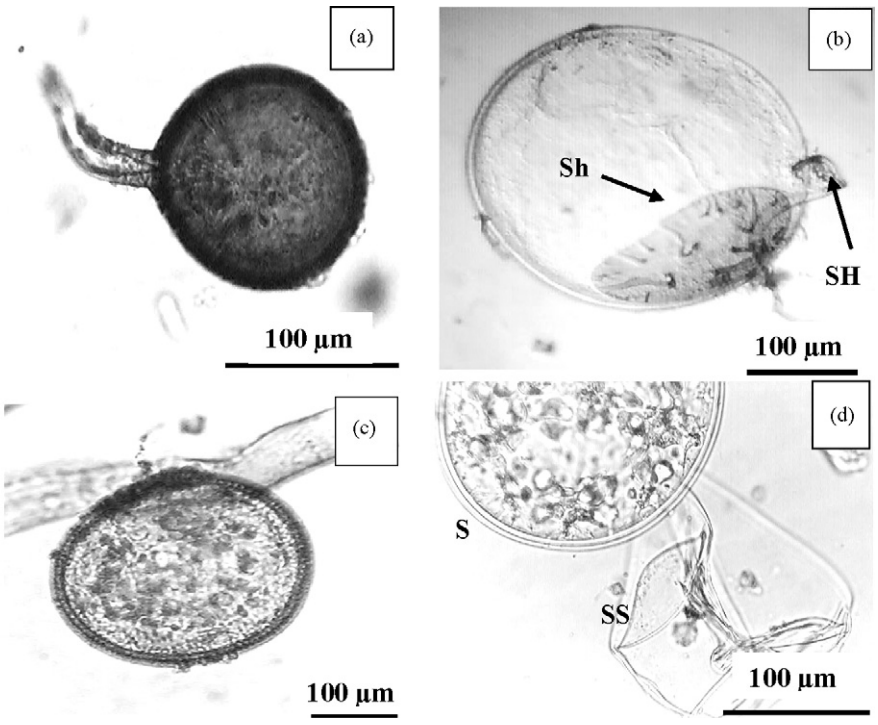

Fig. 4. Spores of different arbuscular mycorrhizal fungi found in the slag heap contaminated with cadmium. (a) Spore of *Glomus*, (b) spore of *Scutellospora* showing a typical shield (Sh) and swollen suspensor hypha (SH), (c and d) spores (S) of *Acaulospora* showing a sporiferous saccule (SS).

In accordance to morphology, thin sections also allow us to observe at least 10 different kinds of mesofauna excrements in mine residues, which are involved in structure development of soil. Some examples are shown in the Fig. 6. This reflects a very active and intimate biological participation of AMF, mesofauna population and plant roots in the vegetal patches. Furthermore, micromorphology analysis showed physical changes in the slag residues due to aggregation process related mainly with mesofauna, which are acting in the soil formation (Fig. 7). Barois et al. [42] also observed that the process of soil formation was related with faecal pellets from fauna, probably Enchytraeids and Acari, living in the rooted soil below *Mulhembergia macroura* grass, the dominant grass. Since few hyphae were observed in the aggregates, glomalin should be weakly involved in this early stage process. This information is relevant due to relatively little knowledge has been reported about the interaction between mesofauna and AMF, which have

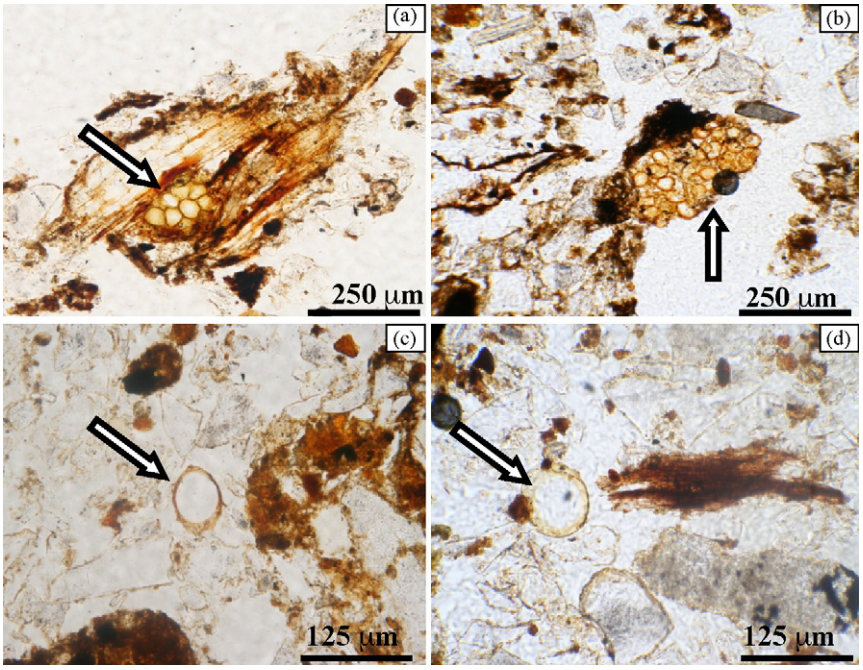

Fig. 5. Spores distribution in thin section of soil developed from mine residues in Temascaltepec, Mexico State. (a) Sporocarps in root tissue residues; (b) sporocarps in groundmass; (c and d) single spores in groundmass, PPL.

Table 2
Frequency of resistant fungal structures related to mesofauna excrements, organic matter or groundmass in eight thin sections from vegetal patches of mine residues

Association of spore or sporocarps	Frequency (%)								Average (%)
	1	2	3	4	5	6	7	8	
Excrements	35	28	32	25	55	40	35	23	34.12 ± 10.1
Organic matter	28	27	38	42	35	29	43	42	35.50 ± 6.7
Groundmass	25	15	28	18	32	16	19	22	21.80 ± 6.0
Total									91.42

an important effect on plants. Then, this kind of interaction influence plant–soil processes and more research should be followed in order to know the type of mesofauna population implicated in this process.

It was also observed that roots were surrounding by quartz grains, which may contribute in soil aggregation; however, this phenomena was observed in small frequency (<5%). It signifies that some roots are able to adhering quartz probably by mucigel or cementing components.

3.5. Fungal mycelium in the slag heap

Leake et al. [39] concluded that the extra-radical mycelium of AMF is a key fungal structure. There is accumulated evidence of its importance in biogeochemical cycles, soil microbial ecology, plant community and agroecosystem functioning. In heavy metal-polluted soils, the EM has been involved in metals binding and sequestration [8,9]. In this research the remarkable fact was the presence of abundant mycelium (Table 1) from AMF and other filamentous fungi in the rhizosphere of plants present in this area. The rhizosphere from different plants promoted abundant mycelium production, for instance, *L. racemosa* and *J. hirta*, from Site 3, presented the highest amount of mycelium (>20 mg/g dry slag). Mycelium production may be related to the type of AMF and other fungi involved in the rhizosphere, the level of metals in the slag or plant root exudation imposed by slag conditions. Leung et al. [7] reported that more hyphal production (12–24 mg) was observed in the rhizospheres of *P. vittata* and *C. dactylon* from mine sites than those from non-polluted sites (5–17 mg).

Two types of mycelium were observed (Fig. 8a and b). The first one was bifurcated, hyaline with coenocytic hyphae, and the second one was lineal or bifurcate, brown and septate. The most abundant was the first one. In some cases, spores, presumably from AMF, bound to the EM were observed (Fig. 8c). Rillig [5] pointed out that the EM from AMF represents a considerable, often prevailing component of soil microbial biomass. Drew et al. [43] showed evidence that AMF may produce more EM in the soil primarily to increase the probability of locating and colonizing a new host plant. Püschel et al. [44] also observed that an established EM has an enhanced potential to colonize roots of plants, even if these belong to species usually not hosting AMF. These findings should be studied in more detail in order to reveal whether the EM plays a role to increase plant establishment in this kind of substrates.

The amount of mycelium extracted varied among the slag samples according to the three different slag heap positions studied. At the top of the slag (Site 1), between 0.11 and 1.08 mg mycelium/g slag with an average of 0.62 ± 0.4 mg/g was quantified. In contrast, in the middle part, Site 2, it was between 1.31 and 6.0 mg/g with an average of 3.13 ± 1.0 mg/g. The highest amounts of mycelium were found at the slag heap bottom, Site 3 (1.03–26.3 mg/g) with an

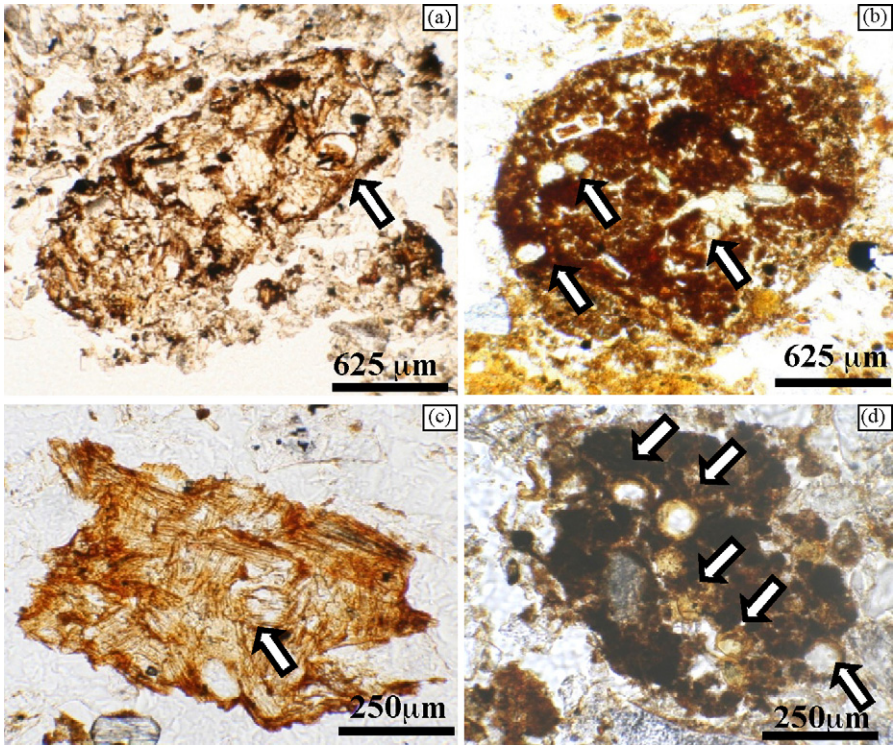

Fig. 6. Mesofauna excrement with spores (arrows) in soil developed from mine residues. Temascaltepec, Mexico State, PPL.

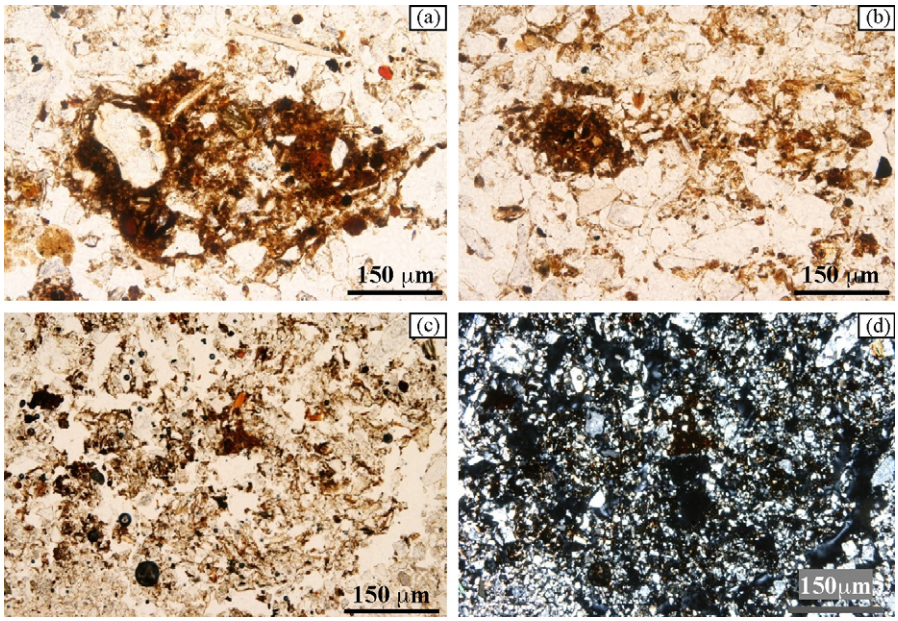

Fig. 7. Development of soil structure in mine residues. (a) Mesofauna excrements and inorganic matrix, PPL; (b) disintegrated excrement, PPL; (c) microaggregates structure, PPL; (d) as part (c) but in XPL.

average of 10.29 ± 9.7 mg/g. Similar to spore morphological diversity, abundance of EM was higher in this site, which presented the higher contents of Cd (Section 3.1.) and a positive correlation between mycelium and available Cd was observed ($r = 0.5872$, $p \leq 0.5$). Mycelium production may be important in Cd sequestration, because AMF-hyphae biomass may account between 54 and 900 kg/ha as mentioned by Zhu and Miller [45]. Preliminary results show that mycelium could be more important than glomalin to sequester Cd (unpublished results); however, in the present research Cd sequestration by mycelium was not determined.

The level of organic matter may influence the amount of mycelium present in the sampled sites. Labidi et al. [46] observed that the application of compost enhanced the production of AM mycelia. It is possible that these changes in organic matter in the slag may favour to soil formation.

The AMF diversity and EM abundance may be another evidence of microbial activity influencing in soil formation process. This is through their effect on plants and other microorganisms such as bacteria, which are important in the early stages of soil formation [47]. For example, a diverse community of AMF has been

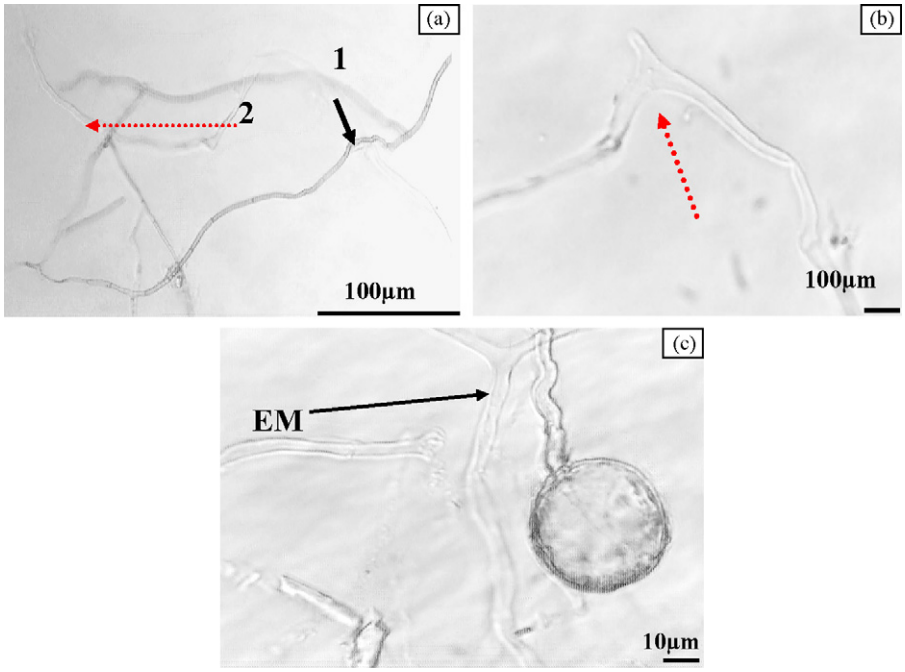

Fig. 8. Mycelium observed in rhizosphere samples from plants growing in Temascaltepec in a slag heap containing high Cd concentrations. (a) Two types of mycelium observed, (b) most abundant mycelium observed with characteristics such as: bifurcation, hyaline-colour, and non-septate, (c) external mycelium (EM) containing young spores of AMF.

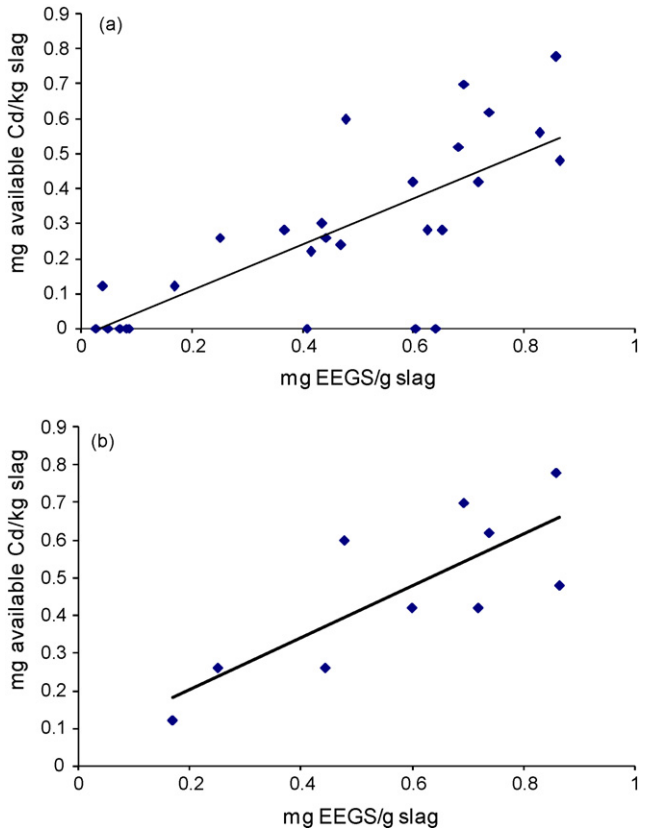

Fig. 9. Relationship between easily extracted glomalin (EEGS) extracted from slag and DTPA-available cadmium found in three sites into slag heap at Temascaltepec, Mexico state. (a) All sites were considered, (b) only the most contaminated site was considered.

related to higher plant diversity and stability of plant community structure [48]. Abundant EM, spread through the rhizosphere, creates an effective substrate that allows new colonization of roots of surrounding plants or emerging seedlings, then increasing plant establishment in the soil [44,49]. In addition, the EM has strong influence on bacterial population [50,51]. These observations open an interesting research area where the participation of AMF on the soil formation process needs to be studied further, giving higher priority to the EM of AMF, since this fungal structure functions as network of power and influence in the soil [39].

3.6. Glomalin content

Since plants growing in the slag residue were mycorrhizal, spores were widely distributed and mycelium was abundant, it resulted interesting to study glomalin production in this slag and its Cd sequestration capacity.

3.6.1. Easily extracted glomalin from the slag (EEGS)

The content of EEGS in the slag heap profile was found between 0.06 and 2.3 mg/g of slag. At Site 1, it was highly variable (with an average of 0.22 ± 0.22); while at Site 2, it was 0.54 ± 0.14 and at Site 3, it was 0.56 ± 0.24 mg of EEGS/g. These valued are in accordance to these reported by Chern et al. [52], which ranged from 0.007 to 2.9 mg/g soil.

A positive correlation between EEGS and mycelium amount was found ($r = 0.4831$, $p \leq 0.5$). Similarly, EEGS was positively correlated ($r = 0.7374$, $p \leq 0.5$) with the slag-available Cd (when considering the three slag positions; Fig. 9a). However, this correlation was stronger ($r = 7823$, $p \leq 0.5$), when just the bottom positions (the most polluted sites in the slag heap) were considered (Fig. 9b). In contrast, to these results, Vodnik et al. [53] did not find correlation between EEG and the concentration of Pb or Zn in the soil. They concluded that deposition of new glomalin into the soils was not affected by the rate of heavy metals.

3.6.2. Total glomalin extracted from slag (TGS)

The amounts of TGS were between 0.36 and 4.74 mg/g slag through the heap profile. The average of TGS at the top was 3.40 ± 0.49 mg/g, in the middle position was 0.92 ± 0.29 and at the bottom was 1.86 ± 0.94. No correlation was observed between TG and available Cd in the slag. Levels of TG values are in accordance to those found by González-Chávez et al. [11], who reported 1.19 mg/g of glomalin from polluted soil. However, the value from TGS and EEG are low compared with a recently research published by Vodnik et al. [53]. These authors extracted glomalin from Zn and Pb polluted soils with an older organic matter from rendzic leptosols on dolomite. They reported that EEG concentration ranged from 1.18 to 4.72 mg/g and TG in soils varied between 3.3 and 67.2 mg/g. According to Rosier et al. [54], in soils containing high amounts of organic matter, glomalin contents may be overestimated when using the Bradford total proteins test. Therefore, it is possible that in the soils studied by Vodnik et al. [53] the high levels of organic matter (5.4–21.2%) or slow glomalin decomposition may partially contribute to elevated levels of glomalin. In the present work, glomalin contents is hardly over-estimated since organic matter content varied between traces −12% at the top, 0.27–1.70% in the middle part and between traces −5.42% at the bottom of the slag. The organic material is not a recalcitrant substrate, due to the young stage of the soil formation and it is related only with the incipient rhizospheric substrate and the superficial layer as observed in the Fig. 2.

3.6.3. Glomalin extracted from the mycelium (GEM)

The amount of glomalin extracted from the mycelium was much higher than that extracted from the slag. Glomalin content did not follow a trend between the different slag heap positions analyzed. At the top of the slag heap glomalin was quantified between 13 and 75 mg of glomalin per gram of mycelium with an average of 37 ± 19 mg glomalin/g mycelium. In the middle part, it was between 7 and 72 mg of glomalin/g mycelium (average of 28 ± 16 mg/g) and at the bottom, it ranged from 2.5 to 75 mg/g (average of 22 ± 20 mg/g). The amount of glomalin extracted from the mycelium and available Cd were not correlated. These values are higher than those found by González-Chávez et al. [11]. These authors in an *in vivo* experiment with *G. mosseae* BEG 25 colonizing sorghum and feed with nutrient solution containing between 0.5 and 20 mM of Cu, reported production of glomalin ranged from 2 to 7 mg of glomalin/g mycelium.

In the present research, glomalin concentration was higher in the EM than in the slag. This may be explained because this protein is copiously produced in active hyphae [10]. The high concentrations of mycelium found in vegetation patches may show its fungal activity reflecting high glomalin production. It is possible that glomalin in the patches may accumulate by via hyphal turnover and release from dead mycelium as mentioned by Driver et al. [55]. This result is in accordance with the recent information published by Purin and Rillig [56] and González-Chávez et al. [50], who reported that glomalin is mainly located in the layers of the EM.

3.6.4. Cd content in glomalin extracted from slug (Cd-GES) and from mycelium (Cd-GEM)

The EM has high potential to accumulate Cd and other metals [11]. For example, Janoušková et al. [57] pointed out that the EM accumulated 10–20 times more Cd per unit of biomass than tobacco roots. Cadmium immobilization by the EM has been reported by

Joner et al. [8] and Janoušková et al. [57]; however, little research has done in relation to metal sequestration by glomalin extracted from polluted soils or mycelium.

In this research, Cd content in glomalin extracted from slag or from mycelium was not significantly different. GES contained up to 0.029 mg of Cd/g, while GEM sequestered up to 0.027 mg of Cd/g. These values are according to the first results shown that glomalin, a recalcitrant soil protein, is a fungal component that sequestrates heavy metals [11]. These authors reported that glomalin extracted from two polluted soils (containing between 0.03 and 0.20 mg Cd/g soil determined by DTPA-available Cd) bound between 0.020 and 0.080 mg Cd/g of glomalin. Higher values of metal sequestration were observed by Chern et al. [52]. They reported between 0 and 0.34 mg Cd/g protein in sediments containing from 18 mg Cd/g sediment, when the permissible limits are between 8.9 and 9.3 mg Cd/L of sediment. Additionally, other metals such as Fe, Pb, Mn and Zn may be sequestered by glomalin [52,53].

Recent unpublished research also showed sequestration between 0.004 and 0.012 mg Cd/g by glomalin extracted from hyphae of the fungus *G. mosseae* BEG 25 growing between 0.5 and 2 mg/L of $Cd(NO_3)_2$. Cd-stabilization by fungal components represents a way to diminish environmental risk of this metal. Modifying the substrate physically and chemically to render metals less toxic allows metal stabilization, which permits plant growth [29,57]. Cadmium occupies the seventh place of the top ten priority hazardous substances listed by the American Agency for Toxic Substances and Disease Registry (ATSDR) [3].

Glomalin, produced by the EM, is a component of heavy metal sequestration and may contribute to the reduction of the bioavailability of these contaminants at the soil. González-Chávez et al. [11] showed that glomalin may irreversibly sequester heavy metals, hence reducing the concentrations of free metals in the soil.

There is abundant information related to the amelioration of AMF with the toxicity of heavy metals in plants; however, little research has been directed to the importance of the EM and the glomalin in the sequestration of HM and the importance of these fungal structures in the remediation of metal mine sites or heavy metal-polluted soils. Results from this research show that mycelium and glomalin were both actively abundant in this slag heap. The large amounts of mycelium observed in this research suggest the necessity of future studies related to modification and stabilization of this substrate (slag) since participation of AMF in the aggregation and stabilization of soil has been fully recognized [11,5]. In addition, information related to the role of the AMF in polluted soils is an important factor which may have strong ecological implications.

4. Conclusions

This field study shows information related with AM fungal component-plant developed in a slag heap polluted with high available concentrations of Cd as a first characterization of this site.

- Natural attenuation by plants, AMF and mesofauna is occurring in this slag heap under study. In the vegetal patches, plants are modifying some important properties such as: organic matter, colour, pH and structural development and forming A horizons.
- More research should be addresses to identify mesofauna species participating in soil formation as they were strongly related with soil structure development and spore dispersion.
- Different genera of AMF (*Glomus*, *Gigaspora*, *Scutellospora*, *Acaulospora*) were colonizing roots and rhizospheric soil of almost all plants established in the slag heap.
- Arbuscular fungi are closely interacting with roots, decomposed organic matter and mesofauna population. As a result these fungi are involved in residue transformation and soil formation.
- Mycelium of AMF and from other fungi was abundantly produced in vegetal patches.
- Glomalin was involved in Cd-stabilization.
- In order to continue restoration or rehabilitation of this heap, enhancement of mycorrhizal development by optimizing substrate conditions such as organic matter additions and introduction of AMF-native mycorrhizal plants should be done.

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