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Pb accumulation in spores of arbuscular mycorrhizal fungi

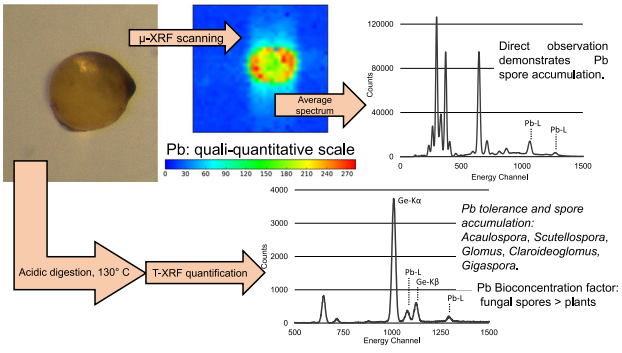
M. Julieta Salazar [a,*], Eugenia Menoyo [b], Valeria Faggioli [c], Jozsef Geml [d], Marta Cabello [e], Judith H. Rodriguez [a], Nicolás Marro [a], Alejandro Pardo [f], M. Luisa Pignata [a], Alejandra G. Becerra [a]

[a] IMBIV-CONICET, Universidad Nacional de Córdoba, Av. Vélez Sársfield 1611, X5016CGA Córdoba, Argentina
[b] GEA-IMASL-CONICET, Universidad Nacional de San Luis, Ejército de los Andes 950, 5700 San Luis, Argentina
[c] Instituto Nacional de Tecnología Agropecuaria, EEA Marcos Juárez, Ruta 12 km 36, 2580 Marcos Juárez, Argentina
[d] Biodiversity Dynamics Research Group, Naturalis Biodiversity Center, Vondellaan 55, 2332 AA Leiden, the Netherlands
[e] Spegazzini Institute, Av. 53 N° 477, B1900AVJ La Plata, CICPBA
[f] Laboratorio de Micología Molecular, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Buenos Aires, Argentina.

HIGHLIGHTS

- A Pb tolerant and accumulator AMF community was identified in polluted soils.
- X-Ray fluorescence was adequate for analyzing Pb accumulation in AMF spores.
- Pb accumulation in spores was directly observed in two Gigasporaceae species.

GRAPHICAL ABSTRACT



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ABSTRACT

Heavy metal (HM) pollution of soils is one of the most important and unsolved environmental problems affecting the world, with alternative solutions currently being investigated through different approaches. Arbuscular mycorrhizal fungi (AMF) are soil inhabitants that form symbiotic relationships with plants. This alleviates HM toxicity in the host plant, thereby enhancing tolerance. However, the few investigations that have addressed the presence of metals in the fungus structures were performed under experimental conditions, with there being no results reported for Pb. The current study represents a first approximation concerning the capability of spores to accumulate Pb in the AMF community present in a Pb polluted soil under field conditions. Micro X-ray fluorescence was utilized to obtain a direct observation of Pb in spores, and the innovation of total reflection X-ray fluorescence was applied to obtain Pb quantification in spores.
The AMF community included species of *Ambisporaceae*, *Archaeosporaceae*, *Gigasporacea*, *Glomeraceae and Paraglomeraceae*, and was tolerant to high Pb concentrations in soil. Pb accumulation in AMF spores was demonstrated at the community level and corroborated by direct observation of the most abundant spores, which belonged to the Gigasporaceae group. Spore Pb accumulation is possibly dependent on the AMF and host plant species.

1. Introduction

Soil contamination with heavy metals (HM) affects the ecosystems due to toxicity and leads to bioaccumulation in diverse organisms

* Corresponding author.
E-mail address: mjulietasalazar@gmail.com (M.J. Salazar).

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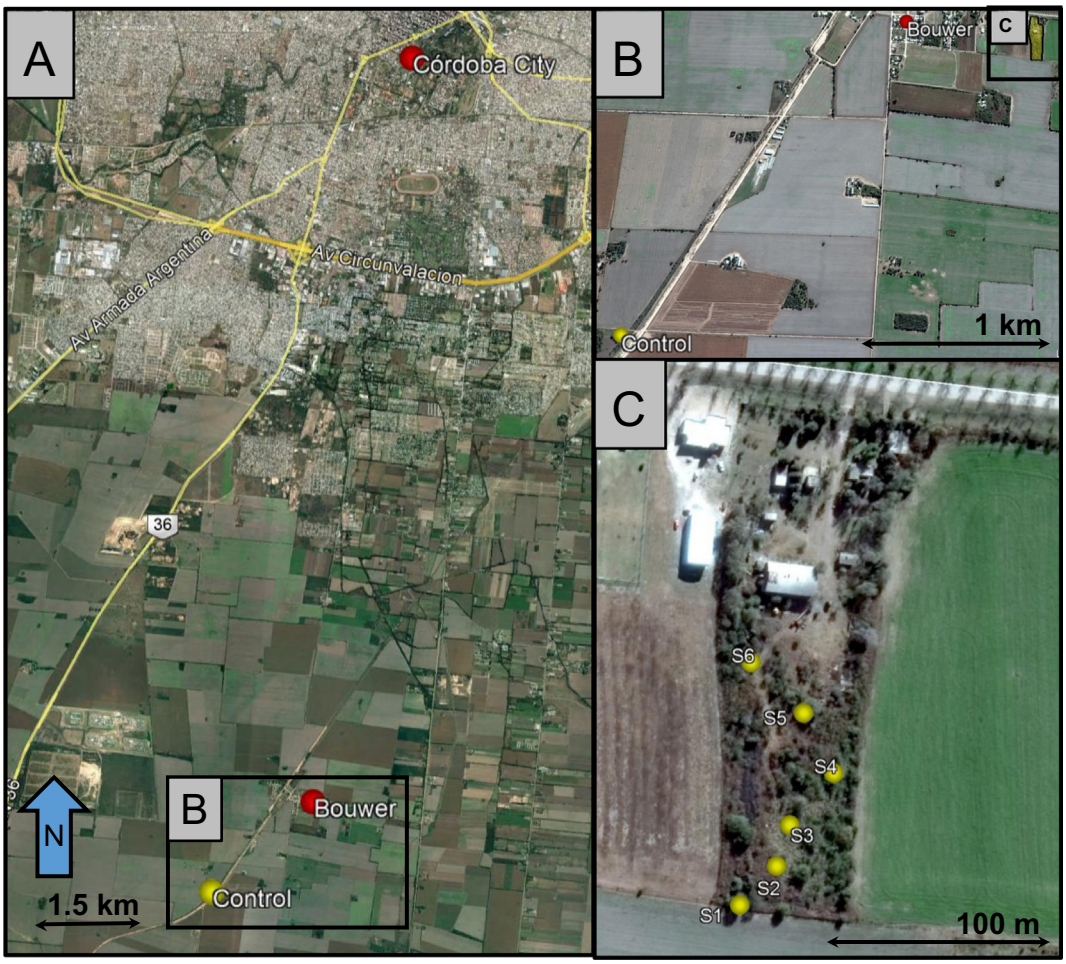

Fig. 1. Rhizosphere soil sampling area in the surroundings of a lead smelter plant located at Bouwer, Córdoba (Argentina).

(Angelova et al., 2004). Pb, in particular, is widely distributed in the environment with exposure producing adverse effects on human health (ATSDR, 2007; García-Lestón et al., 2012).

Soil is also a principal HM sink, with these pollutants being accumulated over time as they are not degradable. Consequently, their remediation requires stable contaminant removal or at least long term immobilization (Jadia and Fulekar, 2009). Conventional methods of soil remediation (excavation, pump and treat, addition of reactants, incineration, vitrification or transportation to dump sites) are expensive, destroy the soil's ability to be used as a productive resource and involve high energy consumption (Aboulroos et al., 2006; Alkorta et al., 2004; Khan and Doty, 2011).

Table 1
Soil physico-chemical parameters and Pb concentration (exchangeable and total fractions) at the sampling area.

Parameter	Sites						
	Control	S1	S2	S3	S4	S5	S6
Physico-chemical parameters							
pH	7.66	7.23	7.43	6.19	6.38	6.3	6.74
Conductivity ($ms \cdot cm^{-1}$)	0.326	0.466	0.239	0.548	0.278	0.231	0.151
Organic matter (%)	2.60	3.67	4.57	7.12	6.76	6.94	4.1
Carbon (%)	1.51	2.13	2.65	4.13	3.92	4.03	2.38
Total Nitrogen (%)	0.148	0.199	0.274	0.399	0.378	0.391	0.237
Carbon/nitrogen ratio	10.19	10.70	9.67	10.35	10.37	10.3	10.03
Extractable phosphorus ($\mu g \cdot g^{-1}$)	69.47	78.10	67.14	49.03	77.57	86.63	14.39
Exchangeable potassium ($\mu g \cdot g^{-1}$)	466.39	536.75	789.13	773.72	480.32	638.23	551.05
Pb pollution characterization							
Total ($\mu g\ g^{-1}$)	14 ± 1	365 ± 23	965 ± 56	89 ± 6	544 ± 33	2938 ± 150	16,186 ± 686
Exchangeable fraction ($\mu g\ g^{-1}$)	0.8 ± 0.3	14 ± 2	92 ± 8	3.7 ± 0.8	29 ± 3	430 ± 23	6556 ± 158
Exchangeable fraction (% of total Pb)	5 ± 1	4 ± 1	10 ± 1	4 ± 1	5 ± 1	15 ± 1	41 ± 2

pH, conductivity and exchangeable potassium were determined according to Jackson and Beltrán (1964), organic matter and carbon were determined according to Walkley and Black (1934), total nitrogen was determined according to Bremner et al. (1996), and extractable phosphorus was determined according to Bray and Kurtz (1945). The exchangeable Pb fraction was extracted with 1 M $MgCl_2$ pH 7, and the total fraction was extracted with pure HNO_3. Results are expressed relative to dry weight.

Table 2
Detection of AMF virtual taxa (VT) of different Glomeromycotina families according to the MaarjAM database obtained from *Bidens pilosa* and *Sorghum halepense* rhizospheric soil in a Pb polluted area (S6).

AMF		Host plant	
Family	VT	*Bidens pilosa*	*Sorghum halepense*
Ambisporaceae	VT283	D	
	VT405	D	D
	VT242	D	
Archaeosporaceae	VT008	D	
	VT009	D	
Gigasporaceae	VT052	D	
	VT041		D
	VT318	D	D
	VT260	D	D
Glomeraceae	VT069	D	
	VT087	D	D
	VT091	D	D
	VT093	D	
	VT096	D	
	VT112	D	
	VT113	D	
	VT130	D	
	VT150	D	
	VT193	D	
	VT195		D
	VT206	D	
	VT217	D	
	VT235	D	
	VT280	D	D
	VT304	D	
	VT332	D	
	VT384		D
Paraglomeraceae	VT281	D	
	VT335	D	
	VT308	D	D
	VT348	D	D
	VT349	D	D
	VT350	D	D
	VT352	D	D
	VT375	D	D
	VT239	D	
	VT238	D	D

Phytoremediation is a viable alternative that uses plants for contaminated soil remediation (Cunningham and Ow, 1996). It presents lower costs and has no destructive impact on soil fertility or structure (Blaylock and Huang, 2000; Jadia and Fulekar, 2009; Schmidt, 2003). However, the success of this technique depends on many factors, with HM bioavailability being one of the most important and is related to several soil and plant parameters, including the microorganisms present (Sarwar et al., 2017). In this context, arbuscular mycorrhizal fungi (AMF) could be a determinant factor. These fungi belong to Glomeromycotina (Spatafora et al., 2016) and develop mutual symbiotic associations with most terrestrial plants, thereby providing a direct physical link between soil and plant roots (Bothe et al., 2009).

Plants colonized by AMF tend to be pioneers at contaminated sites (Khan et al., 2000), emphasizing their role in the accumulation of and tolerance to metals (Gaur and Adholeya, 2004; Hildebrandt et al., 1999). The stimulation of plant growth and a decrease in HM concentration levels have been reported (Andrade et al., 2004; Jankong and Visoottiviseth, 2008) among the benefits of AMF association for plants that grow in contaminated soils with excessive HM.

Most research related to AMF and phytoremediation of HM polluted soils has been focused on the description of AMF tolerant communities (Sánchez-Castro et al., 2017) and the tolerance achieved by plants due to symbiosis and an improvement in phytoremediation efficiency. This has led to host plant tolerance enhancement being widely studied (Cozzolino et al., 2016; Ferrol et al., 2016; Gaur and Adholeya, 2004; Wu, 2017), but the effects on phytoremediation efficiency have been inconclusive. Some scientific findings have indicated an HM uptake enhancement (de Souza et al., 2012) whereas others have shown a reduction (Li et al., 2016) or only an HM improvement in roots (Sheikh-Assadi et al., 2015). In addition, only a few investigations have directly studied the mycorrhizal fungal symbionts, with an ectomycorrhizal fungi species being registered as a Pb accumulator in mantle and rhizomorph (Turnau et al., 2001). Although determinations of HM in spores have been obtained for Zn, Cd (Gonzalez-Guerrero et al., 2008), Cu (Cornejo et al., 2013) and Al (Aguilera et al., 2011), there are no results for Pb in AMF spores reported in the literature.

In summary, the current knowledge framework has been derived from research results obtained by investigating AMF assisted phytoremediation of HM polluted soils, with the main focus being on plant tolerance. Only a few investigations have looked at HM in the fungus itself, and these were only conducted under experimental conditions. Furthermore, these studies did not include Pb as a pollutant. Thus, the aim of the present study was to determine, under field conditions, the capability of the AMF community present in Pb polluted soils to accumulate this HM in spores.

2. Experimental

2.1. Study area

The study area is located in Bouwer, which is 18 km south of Córdoba City, Argentina (Fig. 1). The soil at the site is an Entic Haplustoll

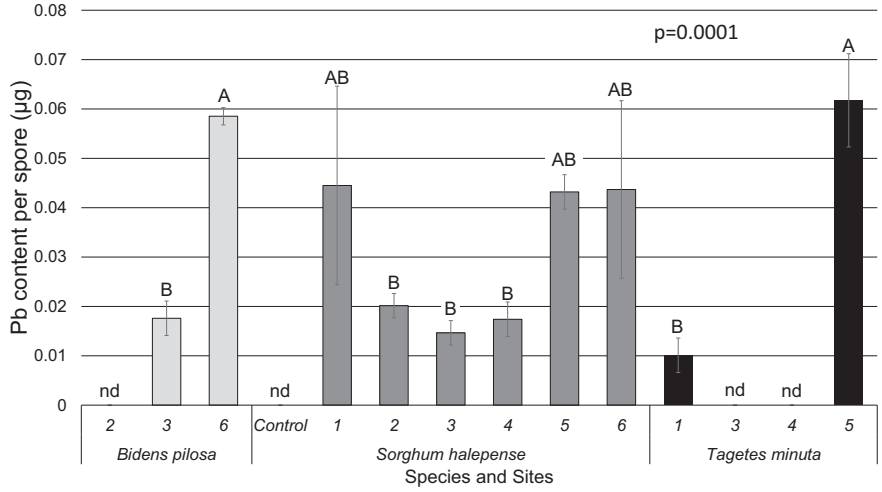

Fig. 2. Pb content per spore for the AMF spore community collected in a Pb polluted area. Mean values, standard deviation and ANOVA comparison. nd signifies that Pb was not detectable. Different letters indicate statistically significant differences with $p < 0.0001$.

according to the American soil taxonomy system (United States Department of Agriculture), and the climate is mild ("Cwa" class according to the Köppen climate classification) with an annual mean temperature of about 15 °C and an average annual rainfall of 500–900 mm (Gorgas and Tassile, 2003). This is an area characterized by a former battery recycling factory that once operated here from 1984 to 2005 (31°33′ 34.02″S; 64°11′9.05″W, whose smelter was closed down due to functional problems associated with a lack of emission control and an inadequate waste disposal. Further information about this area can be found in Salazar and Pignata (2014) and Salazar et al. (2016a).

Rhizospheric soil samples were collected at 6 study sites around the former factory (differing in Pb concentrations) and from a control site (located 2.7 km from the other 6) (Fig. 1). The rhizospheric soil was collected from the three host plant species *Bidens pilosa* L., *Tagetes minuta* L. and *Sorghum halepense* (L.) Pers. Although not all these species were found at all sites, these were the most abundant in the study area and have been previously studied for Pb accumulation in both field and experimental conditions (Cid et al., 2016; Graziani et al., 2016; Salazar and Pignata, 2014; Salazar et al., 2016a; Salazar et al., 2016b; Sosa et al., 2016).

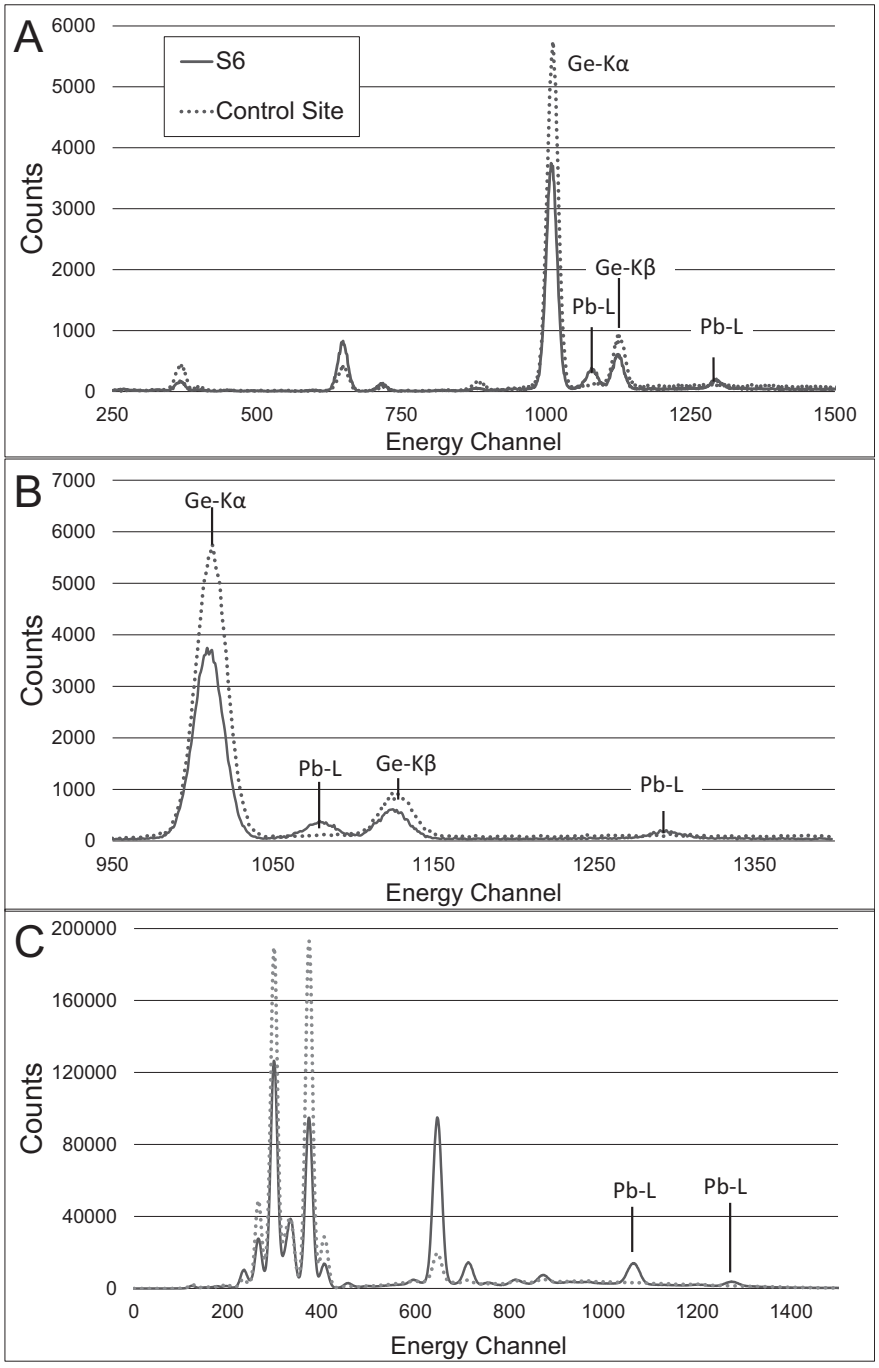

Fig. 3. X-ray fluorescence spectra examples. A) Spectra of two spore pool samples analyzed by TXRF, where the dotted line indicates *B. pilosa* rhizosphere at control site and the continuous line shows *B. pilosa* rhizosphere at S6. B) Amplification of the previous spectra. C) Average spectra for two spores scanned by μXRF, with dotted line indicating *G. decipiens* from *S. halepense* rhizosphere at control site while continuous line shows *G. decipiens* from *B. pilosa* rhizosphere at S6.

2.2. Sampling procedure

For each sampling site, three samples were collected during autumn for each host species present. A stainless steel shovel was employed to remove entire plants and roots with their rhizospheric soil, with the samples being placed in plastic bags. Approximately 50 g of each soil sample was deposited in a sealed plastic bag and maintained at −20 °C for the DNA work. Once in the laboratory, roots and rhizospheric soil were separated by shaking them in plastic boxes and sieving. An aliquot of soil was used to determine the soil physicochemical properties at the sampling sites (Table 1).

2.3. AMF spore isolation

Arbuscular mycorrhizal fungi spores were isolated according to Gerdemann and Nicolson (1963) and Sieverding (1991). Briefly, AMF spores were extracted by wet sieving and decanting 100 g of dry soil, which was then centrifuged in sucrose 80% *W/V*, and the spores were collected from the middle of the solution. A fine sieve (38 μm) was used to collect small spores, and the top sieve (125 μm) was checked for sporocarps and larger spores. Only apparently healthy spores were isolated by direct observation, using a stereomicroscope (Nikon, SMZ745T).

The initial aim was to quantify the Pb in spores for each AMF species separately. However, the high concentration of Pb in the studied soils resulted in a very small number of spores being obtained for each sample. Therefore, if the samples had been subdivided by species, the quantification of Pb would have been at risk of falling below the detection limit. So, it was decided to isolate spores from each soil sample without separating by species, to produce what we have named as "pool samples", which represent the AMF spore community. Pb quantification for these "pool samples" was performed as described in the following section.

2.4. Molecular identification of AMF species

As explained above, although the research scope of this work was limited to the AMF community, we considered that it was of great importance to identify the Pb resistant fungus species. Thus, for the most Pb polluted sampling site (S6) for each host, a molecular identification was conducted. This technique provides results from the DNA, which corresponded not only to spores but also to any fungal structures present in the soil.

2.4.1. Soil DNA extraction, PCR and sequencing

DNA was extracted from 0.5 g of soil samples using Power Soil DNA isolation kit (MO BIO Lab. Inc., USA), following the manufacturer's instructions, and was confirmed by agarose gel electrophoresis (1%), stained with GelRedTM (Biotium) and visualized under UV light.

One microliter of DNA template was added to a 40 μl (final volume) PCR mix containing 25.6 μl of MQ water, 4 μl of 10× buffer, 1.5 μl dNTPs (2.5 mM), 1.5 μl of reverse and forward primers (10 mM), 4 μl MgCl2 (50 mM), 0.5 μl BSA (10 mg/ml) and 0.4 μl BIOTAQ polymerase (5 U/μl). The primers AMV4.5NF and AMDGR (Sato et al., 2005) were used to amplify a part of the SSU (approximately 300 bp), using the following PCR conditions: one cycle of 95 °C for 5 min; then 37 cycles of 95 °C for 20 s, 56 °C for 30 s and 72 °C for 1.5 min; and ending with one cycle of 72 °C for 7 min. Primers were labeled with sample-specific multiplex identification DNA-tags (MIDs). A negative control consisting of MQ water instead of DNA was made and underwent the PCR under the same experimental conditions and was revealed on a gel to be amplicon free. The PCR products were assessed for size distribution and for DNA concentration using a Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA).The products were cleaned using 0.99 Ampure beads (Beckman Coulter, Beverly, MA, USA) to remove short fragments. The amplicons were diluted with MQ water to produce the

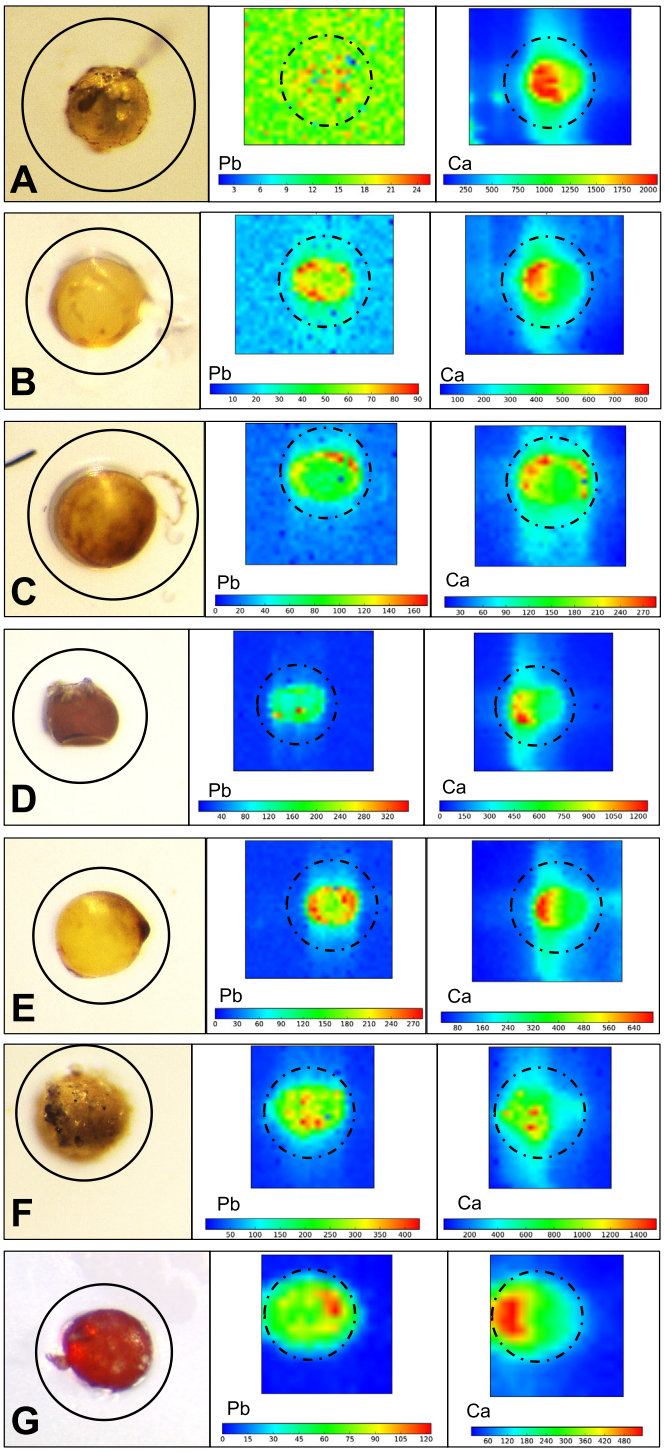

Fig. 4. AMF spore real images and μXRF maps for Pb and Ca. Colour bar indicates the normalized intensity for each element. Continuous line circle in real images indicates the scale size, with the dotted line circle in maps indicating the spore localization. A) *G. decipiens* spore from *S. halepense* rhizosphere collected at control site. Continuous line circle diameter: 0.5 mm. B) *G. decipiens* spore from *S. halepense* rhizosphere collected at the most polluted site (S6). Continuous line circle diameter: 0.4 mm. C) *G. decipiens* spore from *S. halepense* rhizosphere collected at the most polluted site (S6). Continuous line circle diameter: 0.5 mm. D) *S. biornata* spore from *S. halepense* rhizosphere collected at the most polluted site (S6). Continuous line circle diameter: 0.4 mm. E) *G. decipiens* spore from *B. pilosa* rhizosphere collected at the most polluted site (S6). Continuous line circle diameter: 0.4 mm. F) *G. decipiens* spore from *B. pilosa* rhizosphere collected at the most polluted site (S6). Continuous line circle diameter: 0.4 mm. G) *S. biornata* spore from *B. pilosa* rhizosphere collected at the most polluted site (S6). Continuous line circle diameter: 0.4 mm.

same DNA concentration for each sample in the final pool before being sequenced. Paired-end Illumina MiSeq sequencing was carried out at BaseClear (Leiden, The Netherlands).

2.4.2. Bioinformatic work

Two-directional reads were assembled to contigs using Mothur v. 1.32.1 (Schloss et al., 2009). The primers were removed and poor-quality ends were trimmed off based on a 0.02 error probability limit with Geneious Pro 5.6.1 (BioMatters, New Zealand). Subsequently, the sequences were filtered using USEARCH v.8.0 (Edgar, 2010) and the following settings: all sequences were truncated to 200 bp, and sequences with an expected error > 1 were discarded. The remaining sequences were collapsed with USEARCH v.8.0 (Edgar, 2010) into unique sequence types on a per-sample basis while preserving read counts and excluding singletons. These sequences served as the input for OTU clustering at a 97% sequence similarity following Öpik et al. (2010), using USEARCH v. 8.0 (Edgar, 2010 while simultaneously removing putatively chimeric sequences. Representative sequences of the OTUs were subjected to a similarity search against the MaarjAM database (Öpik et al., 2010) using USEARCH. OTUs that did not have at least an 80% similarity over a minimum of 180 bp to any Glomeromycotina sequence in the MaarjAM database were excluded from further analysis.

2.5. Pb analyses in AMF spores

The spore samples were analyzed for Pb using the TXRF and μXRF techniques. The pool samples were used in the quantification by TXRF, while the most abundant spores were isolated for Pb mapping by μXRF. In addition, the latter technique was applied to 6 spores from the control site and 18 spores from S6 (minimum of 3 spores of each species at each site), with the aim of comparing the Pb pattern in spores collected in a natural soil with those from a polluted soil. After mapping, these spores were identified at a specific level following the methodology described in Section 2.3.

2.5.1. Pb quantification in spores

For Pb quantification in samples such as plant tissues or soils, results are normally expressed in μg of Pb per g of sample, so the first step is to extract a specific dry mass to be processed. However, in the particular case of AMF spores, recording the sample mass is complicated by the fact that the low number of spores in the polluted soils implies a reduced spore mass, which would require expensive weighing methods. Additionally, spore content in soil is usually reported by number rather than weight. Consequently, the Pb content in spores was expressed in this study in terms of μg of Pb per spore.

A spore count was first performed for all 42 pool samples (AMF community) Here, *T. minuta* was present at 4 sites, *B. pilosa* at 3 sites, and *S. halepense* at 7 sites; $N = (3 + 7 + 4) \times 3 = 42$). Then, these samples were placed in culture tubes with a screw cap (Hach ©) and digested with 0.5 ml of H_2O_2 (30%) and 1 ml of HNO_3 (37%, from Merk ©) at 130 °C for 24 h with sporadic agitation. When there was no visual evidence of spores, digestion was considered to be complete and an internal germanium standard (10 ppm) was added to the samples. Finally, a volume of 7 μl was pipetted at the centre of the acrylic support.

Standard solutions with known concentrations of Pb were prepared to calibrate the system. All samples were measured for 200 s, using the total reflection set up at the X-ray fluorescence beamline, with a white beam (approximately 0.3 mm wide and 2 mm high) being used for excitation. For X-ray detection, a Ge detector was used with an energy resolution of 148 eV at 5.9 keV and a 0.8 mm collimator in the detector (Sosa et al., 2016), with 42 spectra being obtained.

2.5.2. Pb mapping by μXRF

The spatial distribution of Pb was analyzed by micro scanning XRF mapping. The individually isolated spores were liofilizated and kept in Eppendorf tubes for transportation to LNLS, with each spore being carefully mounted in an ultralene® thin film (4 μm thick), digitally photographed with a scale, and placed in the XRF beamline for micro imaging of Pb. A previous method for plant samples (Tian et al., 2011) was adapted for the AMF spores. The beam was microfocused and the sample was placed at 45° to the incident x-ray beam. Then, the fluorescence yield was detected using a single silicon drift detector, and the elemental distribution maps were collected using a 12 × 22 μm pixel size with a dwell time per point of 200 ms, with the full XRF spectrum data being saved at each pixel. Pb distributions were obtained by windowing in on the elements of interest in the XRF spectra. After scanning, spore samples were detached from the ultralene film using pure water and identified at species level under a compound microscope (Nikon, E200), according to Schüßler and Walker (2010), INVAM (http://invam.caf.wvu.edu/fungi/taxonomy/speciesID.htm), and the Blaszkowski AMF website (www.agro.ar.szczecin.pl/jblaszkowski).

3. Results and discussion

3.1. AMF in Pb polluted soil

The spore families and species identified as virtual taxa (VT) for *B. pilosa* and *S. halepense* rhizospheres from the most polluted soil (S6) are listed in Table 2. This site (S6) presented 5 AMF families: Ambisporaceae, Archaeosporaceae, Gigasporacea, Glomeraceae and Paraglomeraceae, with the last two families mentioned presenting the highest number of VT. Hassan et al. (2011) studied the AMF biodiversity in several HM polluted soils, including sites with Pb, and found that *Glomus* was the most diverse group with its species being dominant, but they only detected two *Gigasporaceae* species. In agreement, several authors have reported *Glomus* domination (Vallino et al., 2006; Bedini et al., 2010; Griffioen, 1994). Vallino et al. (2006) suggested that *Glomus* species are dominant in the polluted ecosystem due to the ability of

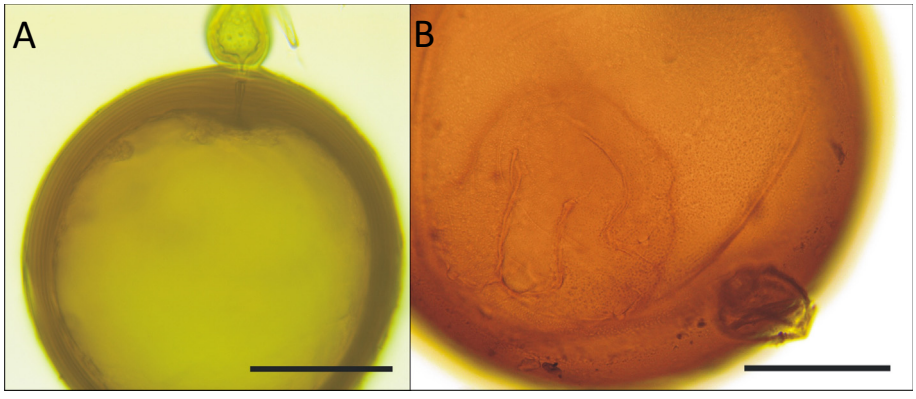

Fig. 5. Real images obtained under a compound microscope for spore identification. Scale bar: 50 μm. A. *Gigaspora decipiens* Hall & Abbott B. *Scutellospora biornata* Spain, Sieverding & Toro.

Glomeraceae to colonize via fragments of mycelium or mycorrhizal roots pieces, while *Gigasporaceae* are only capable of propagation via spores. In agreement with Vallino et al. (2006), during the spore isolation for Pb analyses in the present study the most abundant spores belonged to *Gigasporaceae* despite greater presence of *Glomeraceae* in VT diversity. *Glomeraceae* domination in DNA sequences with spore absence in HM polluted soil was also found by Bedini et al. (2010).

A recent investigation (Schneider et al., 2016) conducted in the vicinity of a recycling battery plant in Brazil found 17 AMF species of different families at the most polluted site (3821 $mg \cdot dm^{-3}$ in the available fraction). Nevertheless, despite Pb being recorded, its concentration in these Brazilian soils was still significantly lower than at Bouwer. In this context, the higher fungal richness found (for both host species rhizospheres) in the present work may indicate that our study site presented a fungal community well adapted to a high Pb concentration in soils. According to Göhre and Paszkowski (2006), this tolerance is more likely due to phenotypic plasticity than to genetic changes.

Our molecular identification results are valuable for two important reasons. First, it is well known that HM pollution in soils has a strong effect on AMF communities and can even completely prevent the colonization of host plants (Vogel-Mikuš et al., 2005). In addition, there is a growing scientific interest in using AMF as a phytoremediation enhancer (Cabral et al., 2015), but there is still little clarity about which species to choose for that purpose, and even less when Pb is present as a pollutant.

According to the above-mentioned authors, *Glomeraceae* is expected to be the most diverse family. However, in our results, this occurred in the case of *B. pilosa* but not for *S. halepense* rhizosphere, with the latter also presenting a lower AMF diversity. Vallino et al. (2006) postulated that mycorrhizal plants work like a microenvironment, with lower pollutant concentrations than in the soil, where *Glomeraceae* species find refuge since they are able to propagate without soil fungal structures such us spores. Thus, *Glomeraceae* have an advantage over other groups that need to develop part of their mycelium in the polluted soil. A previous investigation we carried out in the same study area and sampling site (soil Pb concentration of 1059 $\mu g \cdot g^{-1}$), revealed that *S. halepense* accumulated 3 times more Pb in roots than *B. pilosa* (1407 $\mu g \cdot g^{-1}$ and 448 $\mu g \cdot g^{-1}$ respectively), with a higher Pb concentration found in *S. halepense* roots than in soil (Salazar and Pignata, 2014). In agreement with Vallino et al. (2006), *Glomeraceae* species may have found refuge in *B. pilosa* roots, and that is why we found a higher diversity for this family in the DNA analysis but no spores in the soil. Meanwhile, *Glomeraceae* species may have not found refuge in *S. halepense* roots, since they can accumulate more Pb than soil, and that is why this family is reduced in this host rhizosphere.

3.2. Pb accumulation in AMF spores

We assessed the Pb presence in AMF spores by the two different approaches of TXRF quantification in a spore acid digestion and μXRF mapping (a direct observation of the untreated spore with quali-quantitative results).

The Pb quantification results for spore pools collected from the different rhizospheres at the different sites are presented in Fig. 2, which in itself is a novelty since other scientific research has only provided qualitative results to date. Fig. 3(A and B) presents the X-ray fluorescence spectra examples obtained by TXRF of spore pools with or without Pb (Control site and S6 samples), demonstrating that the method employed has an appropriate detection limit for most of the samples.

Tagetes minuta was found at S1, S3, S4 and S5, with the latter site being more polluted and presenting a significantly higher Pb concentration in AMF spore pools than the others. *B. pilosa* was found at S2, S3 and S6. Here, the most polluted site was S6, which had a significantly greater Pb concentration in AMF spore pools than the other sites (Fig. 2). Nevertheless, the Pb content was undetectable for some samples. In the case of the control site, this could have been due to a very low value of Pb whereas in those cases where the soil was polluted, it may have been due to either a very low value or to a low spore count. *S. halepense* was present at all the sampling sites and the Pb content in spore pool samples was higher in the most polluted soils (S5 and S6) than in the others, except for S1. For future analyses of Pb in AMF spores collected in low polluted soils, it would be desirable to collect more spores or to reduce the digestion volume.

Regarding the Pb content found in spores, the highest concentrations were approximately 0.06 $\mu g \cdot spore^{-1}$. However, the interpretation of this amount is difficult due to a lack of previous data and because the concentration is expressed in μg of Pb per spore. Thus, the concentration in mass terms cannot be compared between spores (μg of Pb per g of spore) and soil (μg of Pb per g of soil) in order to assess accumulation using, for example, a bioconcentration factor. To obtain a more meaningful analysis, we propose an estimation of the average mass of one spore, which can be obtained by using the information (density) provided by the spore isolation method, which is presented in the Supplementary material.

The representative isolated spore maps for Pb and Ca and their correlated real images obtained at the LNLS facilities on the μXRF beamline are shown in Fig. 4; in addition, the real images obtained under the compound microscope (Nikon, E200) during identification are included in Fig. 5. This technique was used for the control and S6 sites, where *T. minuta* was not present. Each spore was individually scanned to obtain fluorescence spectra for each pixel, and then the integrated intensities for Pb and Ca were calculated from these X-ray fluorescence spectra (Fig. 3, C) and normalized by using the intensity of the Compton scattering peak. An elemental mapping for the measurement area was obtained from the normalized intensity of each element, where each map indicates the distribution pattern of one specific element, and the count scales vary in each individual map. The normalized X-ray fluorescence intensities are scaled from red (maximum) to blue (minimum), and the Ca maps are included to show what a typical structural element map looks like as a reference for the optimal image resolution.

The Pb map of the spore isolated from the control site (Fig. 4.a) had a low maximum intensity, which presented more noise than signal for this element. This result indicates that in the presence of the natural Pb concentration in soil, spores did not accumulate detectable levels of Pb. In addition, the appearance of a Pb-free spore is also displayed. At the most polluted site (S6), the most abundant spores were *Gigaspora decipiens* Hall & Abbott and *Scutellospora biornata* Spain, Sieverding & Toro, which were found in both host species *S. halepense* and *B. pilosa* rhizospheres. These were mapped for Pb, and all spores presented detectable levels of Pb, with *G. decipiens* presenting the higher maximum intensity for the latter host species (Fig. 4.E and F compared to Fig. 4.A and B), while *S. biornata* accumulated more Pb in the *S. halepense* rhizosphere (Fig. 4.D compared to Fig. 4.G). These results may indicate that Pb accumulation depends not only on the AMF species, but also on the host species. Although there are no published results specifically related to Pb or HM in this issue, it is well known that colonization by different AMF does not result in the same responses in a single plant species, and in addition colonization by the same AMF does not necessarily result in the same responses in different plant species (Smith et al., 2011). Thus, this might explain the wide differences found in the TXRF quantification results, where spore pools with a high Pb concentration also presented a high variability, making it reasonable to expect variable results for Pb content in spore pool samples.

It is important to highlight that the accumulation of HM in spores is a little studied phenomenon and there are no antecedents for Pb, with the most relevant approaches found in the literature briefly described below. Elemental composition of AMF (*Acaulospora* sp.; *Scutellospora* sp. and *Glomus* sp.) spores from restored sites in Brazil were analyzed by energy dispersive X Ray Spectroscopy (Pagano et al., 2010), with results pointing out that for some elements spore accumulation depends on fungi species. *Glomus* presented a higher accumulation of constituent elements, while *Scutellospora* was the only one that accumulated a

pollutant (Ni). Nevertheless, although the technique employed by these authors provided information about relatively light elements, results about Pb were not reported in this study.

Other authors used a combination of transmission electron microscopy and energy dispersive X Ray Spectroscopy for *Rhizophagus intraradices* (ex. *G. intraradices*), which achieved higher spatial and element detection resolutions (Gonzalez-Guerrero et al., 2008). The fungi hosted in modified root cultures under high Zn, Cd, and Cu exposition were studied, and it was found that essential elements (Zn and Cu) were stored mainly in spores, while the toxic element (Cd) accumulation occurred in hyphae. Nayuki et al. (2014) investigated Cd distribution in *Gigaspora margarita* and *R. irregularis* hyphae, and demonstrated that μ-XRF is an appropriate technique for this purpose. However, the spores were not scanned. Thus, our mapping results are novel in terms of Pb accumulation in AMF spores.

In natural and polluted ecosystems, AMF form extensive hyphal networks in soil, which act as functional extensions of the plant roots by enlarging the accessible soil volume for nutrient uptake and also for pollutant uptake (Göhre and Paszkowski, 2006). This implies that AMF colonization enhances Pb absorption from polluted soils, which has previously been demonstrated in some plants (Sarkar et al., 2018; Chen et al., 2005) where Pb is accumulated in roots. This was attributed to intraradical fungal detoxification, with Pb being accumulated in fungal structures within the root but not in root tissues (Göhre and Paszkowski, 2006). Our results add to the evidence that spores also participate in the detoxifying mechanisms.

3.3. Reflections on the methodology

For the present study, traditional mycorrhizal sampling procedures were applied. This methodology is useful for conducting a field study in Pb polluted soils within a community scale, but reveals few insights on a specific scale. Thus, achieving a better understanding on a specific scale is a main objective for the future, with it being necessary to adapt the sampling procedure. The results presented here and those from the literature (Ferrol et al., 2016; Wu, 2017) indicate that soil pollution causes an important reduction in the AMF spore number, thus sampling procedures need to be able to obtain a larger amount of spores. This would ensure that the measurements are above the detection limit for spores separated by species in Pb polluted soils within a larger pollution gradient.

4. Conclusion

The AMF community present in the most Pb polluted soils from the study area showed a high tolerance to this toxic HM and possessed the mechanisms required to accumulate the pollutant in spores. *Glomeraceae* was the most diverse AMF group present in Pb polluted soil, but the most abundant spores belonged to *Gigasporaceae*, with *B. pilosa* rhizosphere presenting a higher AMF diversity than *S. halepense* rhizosphere and also being a better host plant for the Glomeraceae family.

The direct observation of Pb accumulation in spores was achieved for *Gigaspora decipiens* and *Scutellospora biornata*. Synchrotron radiation was useful for studying Pb and other HM accumulation in AMF spores by the quali-quantitative technique of μXRF and the quantitative technique of TXRF. The amount of Pb accumulated may depend on the AMF species and on the host plant species. For example, *S. biornata* accumulated more Pb than *G. decipiens* in the rhizosphere of *S. halepense*, while *G. decipiens* accumulated more Pb than *S. biornata* or *G. decipiens* in the rhizosphere of *B. pilosa*, with these results being important for revegetation purposes.

Future studies are now necessary to establish the best plant-AMF combinations using a phytoremediation approach, to determine accurately the BCF rather than making an estimation, and to test the phytoremediation efficiency rates of different AMF and host species for different Pb concentrations in soil.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1016/j.scitotenv.2018.06.199. These data include the Google map of the most important areas described in this article.

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