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Phytoremediation assisted by mycorrhizal fungi of a Mexican defunct lead-acid battery recycling site

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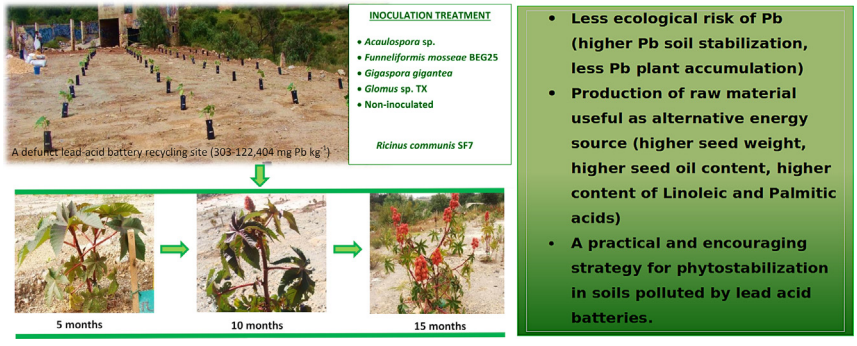
HIGHLIGHTS

- A 15-month field study was followed on a polluted site by lead-acid batteries.
- Fungal treatments influenced soil-pH, -Pb stabilization and Pb shoot accumulation.
- Fungal treatments influenced seed dry weight, oil content, and oil quality.
- *Acaulospora* sp. and *F. mosseae* BEG25 had best field effect on *R. communis*.
- AMF and *R. communis* are an encouraging strategy for Pb phytostabilization.

GRAPHICAL ABSTRACT



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ABSTRACT

A field experiment was conducted during 15 months to study the effects of four arbuscular mycorrhizal fungi (AMF) on the growth of *Ricinus communis* accession SF7. Plants were established on amended soil (vermicompost:sawdust:soil 1:1:1) severely polluted by lead-acid batteries (LAB) located at Mexico State, Mexico. Plants inoculated with *Acaulospora* sp., *Funneliformis mosseae* and *Gigaspora gigantea* had 100% survival in comparison to non-inoculated plants (57%). These same AMF enhanced palmitic and linoleic acids content in seeds of *R. communis*. *Acaulospora* sp. modified rhizosphere soil pH and decreased 3.5 folds Pb foliar concentrations while *F. mosseae* BEG25 decreased three times Pb soil availability in comparison to non-inoculated plants. Spatial changes in Pb soil availability were observed at the end of this research. No fungal effect on P, Ca, Cu foliar concentrations, soluble sugars, proline, chlorophyll or on the activity of two oxidative stress enzymes was observed. Mycorrhizal colonization from the inoculated fungi was between 40% and 60%, while colonization by native fungi was between 16% and 22%. A similar percentage of foliar total phenolic compounds was observed in non-mycorrhizal plants and those inoculated with *G. gigantea* and *Acaulospora* sp. This is the first research reporting effects of AMF on *R. communis* (castor bean) shrubs when grown on a LAB recycling site suggesting the use of *Acaulospora* sp. and *F. mosseae* BEG25 in phytostabilization to ameliorate Pb pollution and decreasing its ecological risk.

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1. Introduction

Lead-acid battery recycling (LABR) is a common industry practice to recover Pb and plastic from aged batteries of motor cars. Due to non-regulated or informal recycling practices occurring in many countries, serious environmental pollution happens when processing and residue disposal is incorrectly carried out (Haefliger et al., 2009; van der Kuijp et al., 2013). Several hazardous contaminants may be released during LABR: Pb, various plastics or hard rubber (ebonite), and sulfuric acid electrolyte solution (UNEP, 2003). Lead is the second among the most hazardous metals in the Priority List of the US Environmental Protection Agency (ATSDR, 2015).

Lead poses a serious concern for human and environmental health due to its high occurrence as a pollutant of water, soil, and air. It has been classified as carcinogenic and mutagenic agent (Alloway, 2013) producing several health damages (gastrointestinal, neurological, cardiovascular, renal, endocrine, *etc.*). Moreover, it cannot be degraded and then it has indeterminate persistence in the environment. Kabala and Singh (2001) mentioned that Pb is persistent and highly immobile in the soil profile and surface concentrations are unlikely to migrate to subsurface layers even after a prolonged period; however, sites, where LABR activity has occurred, will, consequently, continue to pose an exposure risk to local populations (WHO, 2017). Environmental pollution caused by LABR can, therefore, result in severe Pb poisoning in a community, which may continue even after recycling operations have stopped (WHO, 2017). Therefore, attention should be given to environmental remediation in order to mitigate exposure.

Phytoremediation has been suggested as a potential alternative to control risk and toxicity of diverse soil contaminants; however, there are few tests of phytoremediation of polluted LABR sites. In one hand, phytoextraction using synthetic chelates, such as citric acid or nitrilotriacetic acid, have been proposed in order to increase Pb availability to plants (de Araújo and do Nascimento, 2010). However, it may represent a risk mobilizing Pb to subsoil and groundwater and many years are required to decrease significantly total Pb concentrations in the soil. On the other hand, phytostabilization has been proposed as a low cost and environmentally friendly technology to decrease soil bioavailability and environmental damage caused by this and other common and toxic pollutants. Moreover, it reduces aboveground wind and water erosion processes, and the immobilization of metals occurs in the rhizosphere.

It is also suggested that beneficial soil microorganisms may assist phytoremediation of metal polluted soils through different mechanisms such as microbial metal tolerance, their interaction with the pollutants to decrease their soil bioavailability, or with plants to promote host growth and with soil to improve its quality (González-Chávez, 2005). For example, plant growth-promoting bacteria (Rajkumar and Freitas, 2008) or arbuscular mycorrhizal fungi (AMF) have received a lot of attention for assisted phytoremediation of metal polluted soils; however, less information is available for remediation of soils polluted by lead-acid batteries (LAB).

Previous research showed that *Ricinus communis* L. (castor bean) is a plant able to establish in very harsh environments with high concentrations of metals (Ruíz-Olivares et al., 2013; Perea-Vélez et al., 2015) and successfully produce safe raw material for bioenergy purposes (González-Chávez et al., 2015). This plant species is mycorrhizal under field polluted conditions (Ruíz-Olivares et al., 2013) and may slowly help to restore degraded and marginal soils (Rajkumar and Freitas, 2008).

The objective of this research was to evaluate, under field conditions, the feasibility of assisted-phytostabilization and raw material production for biofuel generation by using four AMF separately inoculated in *Ricinus communis* (a non-edible crop with biofuel interest) in a site polluted by Pb due to LABR. Inoculated and non-inoculated plants were transplanted in the field and studied for 15 months.

2. Materials and methods

2.1. Description of the study site

The site is located at Tepetlaoxtoc at the north of Mexico State (Fig. S1). Almost continuous deposits of Neogene and Quaternary occurred; igneous extrusive and basic tobes are observed. A small river draining from the hills feed the area with sediments of feldspar and rock fragments. The present climate of the zone is semi-humid, with a mean temperature of 8 to 14 °C and the average annual rainfall was 683 mm. The wet season (summer) accounts for >80% of the precipitation. Predominant soil type is Phaeozem. Approximately 350 m North to the site, there is a small village in the area called San Andres de Las Peras, while 280 m to the West the Santa Ines area residents are located. To the East, a water creek is found and to the South, a flourishing reforested area is observable (Ruíz-Olivares, 2016). The study site is a defunct lead-acid battery recycling (LABR) area with potential risk for contamination to the environment and human population. This recycling site was functionally operating 20 years ago; until people from the area complained about several health disorders (coughs, headaches, and eyes irritation), and the Mexican Agency of Environmental Protection (PROFEPA) closed the recycling company. This Agency preliminary started chemical soil analysis, found high concentrations of Pb and instructed intervention to reduce the risk from this pollution source.

2.2. Chemical soil characterization

21 superficial soil samples (0–20 cm) were taken into the experimental area (400 m^2) following a systematic sampling from the intersections of 5 × 5 m quadrants. Previous to chemical analysis, soil samples were air-dried in shade, ground, screened through a 2 mm sieve and stored in plastic bags. Chemical characterization consisted of analysis of the following parameters: pH (Orion Research, Model 601, Beverly, MA; Rowell, 1994); electrical conductivity (EC, Conductronic CL35, Puebla Mexico; Rhoades, 1996); both from a 1:2 slurry soil: water solution. Organic matter content (OM) was obtained after incineration of 2 g soil samples in a muffle (FA1730, Thermodyne, Iowa, USA; Dean, 1974). Soluble Na and K were extracted with water (ratio 1:10) and determined by flamometry (PFP7 Jenway, Essex, UK). Sulfate concentrations were analyzed by EPA method (1986) and using a spectrometer (Cary Varian 50, CA, USA) read at 420 nm.

Pseudo-totals metal concentration was quantified in 0.250 g of soil with 6 mL of concentrated HNO_3 and digested by 8 h at room temperature and then 180 °C (1200 W) for 30 min in a microwave (3000 Anton Paar, Austria; EPA, 1994). Diethylenetriamine-pentaacetic acid-extractable (DTPA-extractable) metal concentration was analyzed in a 1:4 soil:extractant solution (0.005 M DTPA, 0.01 M $CaCl_2$, and 0.1 M triethanolamine, pH = 7.3) by Lindsay and Norvell (1978) method. In both cases, samples were analyzed by using a FAAS-Perkin Elmer Analyst 700 spectrometer, USA. Blanks and certified reference materials were included for analyses quality control. An internal reference sample was performed in triplicate with each set of samples. The detection limit (DL) of the method was obtained following the analysis of 10 blank replicates. The arithmetic mean and standard deviation were calculated and DL was determined as three times the standard deviation. The DL for Cd, Cu, Pb and Zn were 0.01, 0.05, 0.07, and 0.01 mg kg^{-1}. Certified standard stock solutions (1000 mg/L) were used for calibrating the instrument used for sample analyses.

Distilled-deionized water was used after preparation (by passing distilled water through a Milli-Q Reagent grade Water System). Before use, all glassware and polyethylene containers were overnight soaked in 10% (v/v) laboratory reagent grade hydrochloric acid, rinsed three times with distilled water and three times with distilled-deionized water. Each all analyses, soil samples were analyzed in triplicate.

Once obtained the DTPA-Pb concentrations, these were used to prepare database and make spatial distribution maps through Natural Neighbor Interpolation at 0, 5, 10 and 15 mat. This method was followed because interpolation is performed using the calculation of weights for neighboring points as summarized by Dumitru et al. (2013) and because the data are uniformly distributed according to Ledoux and Gold (2005). Variograms were obtained following Kriging tools. ArcGIS 10.4 software was used.

2.3. Establishment of the field experiment

After assessing pollution levels and soil chemical traits, a field experiment was established. Preliminary soil preparation was performed; which consisted of mechanical soil leveling with a tractor, 40 × 40 × 40 cm soil excavations at 2.5 m distance each other, and addition of a soil amendment. This consisted of 3 kg of a mixture of soil:vermicompost: sawdust (1:1:1 vol:vol) added to each soil hole previously mentioned.

Ricinus communis L. accession SF7 was selected to conduct this experiment due to its previous selection based on growth performance under other metal polluted soils (Ruíz-Olivares et al., 2013; Perea-Vélez et al., 2015). Seeds of this plant were separately inoculated by adding 300 g of inoculum as a mixture of sand and mycorrhizal propagules (>100 spores, mycelium and root segments of dwarf *Tagetes erecta* L. with >50% of fungal colonization) with one of the four arbuscular mycorrhizal fungi (AMF): *Acaulospora* sp. ZIM (ACA), *Gigaspora gigantea* (GIG), *Funneliformis mosseae* BEG25 (BEG) or *Glomus* sp. (TX). Sowing and fungal inoculation were made in 2 kg black plastic bags with sterile quartz sand. Seedlings of *Ricinus* were kept under greenhouse conditions. A non-inoculated treatment (NI) was also prepared. Watering was performed every third-day using low phosphate (20 μM) Hoagland's nutrient solution (Millner and Kitt, 1992).

One-month-old *Ricinus* seedlings were transplanted to the field into soil holes previously done. The transplant was performed on July 2014. One seedling was settled in each excavation hole. Plants were watered with 2 L of tap water every two weeks.

Three sampling and evaluation points were considered at 5, 10 and 15 months after transplanting (mat) in the field. Soil characteristics were evaluated at 5, 10 and 15 mat. Soil composite samples were collected from the rhizosphere of *R. communis* plants. Soil chemical evaluation was conducted as in the previous chemical soil characterization section.

2.4. Plant evaluation

Similar to soil variables, *R. communis* plants were analyzed to 5, 10 and 15 mat in the field. Plant growth traits (height, number of leaves and internodes, and stem diameter) and plant survival were analyzed. Plant samples were dried at 65 °C to obtain constant weight; grounded and digested in HNO_3 (EPA, 1994, Method 3051A). Then the concentration of P (Kitson and Mellon, 1944) was measured by the colorimetric procedure (vanadomolybdo-phosphoric acid yellow); Ca (after $LaCl_3$ treatment to reduce the chemical interferences), Pb and Cu concentrations were determined by FASS as described above.

Chlorophyll concentration was obtained from a slurry solution obtained from trituration of a 0.02 g of fresh leaves sample with 2 mL of 80% cold acetone, 0.1 g of $MgCl_2$ and sterile sand. This slurry was centrifuged (Hemle Z 323, Germany) for 5 min at 12,000 rpm at 5 °C and absorbance determined at 645 and 663 nm in a spectrometer (UV–VIS; Evolution 300 Thermo) according to Lichtenthaler and Wellburn (1983). Accumulation factors for Pb and Cu were determined according to González and González-Chávez (2006). Quality Assurance and Quality Control was also performed as explained above for soil samples.

Osmoprotectant solutes total soluble sugars were analyzed. Briefly, 0.5 g of fresh *R. communis* leaves and 3 mL 80% ethylic alcohol were heated for 10 min in a water bath. More alcohol (3 mL) was added and the heating procedure was repeated four more times. The extract was dried at 60 °C for 24 h. Sediment was suspended in 1 mL of distilled water and total soluble sugars were analyzed according to Montreuil et al. (1997). Glucose standard in a 0 to 250 mg mL^{-1} concentration was prepared. Six replicates for each leaf sample were performed. Proline concentration was analyzed in 0.1 g fresh *R. communis* leaves mixed at 4 °C with 2.5 mL of sulfosalicylic acid. This homogenized was centrifuged at 14,000 rpm at 4 °C for 5 min. The supernatant was analyzed by Bates (1973) method in a spectrophotometer (UV–VIS Evolution 300, Thermo) at 520 nm.

Total phenolic compounds were determined by colorimetric method Folin-Cioalteau and were expressed as Gallic acid equivalents (Singleton et al., 1999). 50 mg of fresh *R. communis* leaves were mixed with 1.5 mL of 95% ethanol. 50 μL of this extract was combined with 450 μL of distilled water and 250 μL 50% Folin-Ciocalteu reagent and let repose for 8 min. After 1.25 mL of 5% sodium carbonate solution and 2.3 mL of deionized water were added and after 30 min rest absorbance at 725 nm was obtained by using a Spectrometer (UV–VIS Evolution 300 Thermo) and a standard curve made with 98% purity Gallic acid (Sigma-Aldrich).

Super Oxidase Dismutase (SOD) activity was estimated by recording the decrease in optical density of formazone made by superoxide radical and nitro blue tetrazolium (NBT) dye by the enzyme (Dhindsa et al., 1981). 100 mg of the fresh leaf was homogenized, which was previously macerated in liquid nitrogen with 2 mL of 50 mM potassium phosphate buffer (pH 7.5), which included 1 mM EDTA and 2% polyvinylpyrrolidone. 50 μL of the extract was placed in multi-well plates (96), 280 μL of the reaction buffer and 27 μL of riboflavin (2 μM) were added. The plate was exposed to light (30 W lamp) at 30 cm distance for 30 min, and the absorbance by the reaction mixture at 560 nm was read (UV–VIS Evolution 300 Thermo). One unit of SOD was equal to the amount of supernatant that photoinhibited 50% of the formation of NBT by dry mass. Ascorbate peroxidase (APX) activity was determined as described by Jiménez et al. (1997). The enzyme extract was prepared with 100 mg of fresh leaf and macerated with liquid nitrogen. Subsequently, 2 mL of extraction buffer consisting of 5 mM ascorbic acid, 1 mM EDTA and 2% polyvinylpolypyrrolidone (PVPP) was added. The reaction of the assay was carried out at room temperature in 96-well plates, in a reaction volume of 360 μL containing 275 μL of reaction buffer, 50 μL of the enzyme extract and 35 μL of 3% H_2O_2. The reaction was started by adding H_2O_2 and the decrease in absorbance was quantified for 3 min at 290 nm. The enzymatic activity, expressed in U of enzyme per mg of dry matter, was calculated using the molar extinction coefficient for ascorbic acid of $\varepsilon 290 = 5.42 * 106\ L\ mol^{-1}\ cm^{-1}$ (UV–VIS Evolution 300 Thermo).

Finally, mycorrhizal colonization was also quantified according to Giovannetti and Mosse (1980). For analysis at 5 mat, leaves corresponding to the fourth plant internodes, while at 10 and 15 mat, leaves were from the sixth and eighth internodes, respectively. Besides, at 15 mat the number of racemes, length of the first raceme, capsule weight per raceme, the weight of 100 seeds, yield and the content of seeds oil (Ruíz-Olivares et al., 2013) were analyzed. Fatty acids profile in seeds oil (González-Chávez et al., 2015) was also determined. Oil from *R. communis* seeds was saponified, hydrolyzed and methylated with ethylic ether, KOH (Merck analytical grade reagents) and solvents and analytical grade HPLC solvents (methanol, hexane and ethanol). The resultant methyl esters were then analyzed by gas-liquid chromatography, CG-FID (Perkin Elmer Auto System, Spain). An HP-INNOWAX 30 m 9 0.32 mm DI, 0.50 μm column was used with a temperature detector at 300 °C, injection temperature of 260 °C and the column temperature of 150 °C for 4 min, increasing 5 °C/min until 250 °C for 12 min. The injection volume was 2 μL using nitrogen as the mobile phase (1 mL/min). Fatty acid (FA) standards such as stearic, palmitic, oleic, linoleic acids (Sigma-Aldrich) were used.

2.5. Statistical analysis

The field experiment was established under a completely random design with five inoculation treatments (four fungi and a control) and seven replicates each. For the transversal statistical analysis, PROC GLM was used (SAS V.9). In order to compare fungal behaviors along the time (5, 10 at 15 mat) a longitudinal statistical analysis was performed and comparisons were done using the Bonferroni adjustment. ANOVA was done for plant variables only evaluated at 15 mat, the assumptions of the model were checked; for some variables, the logarithm or square root transformation was required in order to get normality. We used the Shapiro Wilks test. The multivariate analysis (principal components -PC-, and cluster analysis with Euclidian distance and average link as the measure of distance and method of grouping) was done to identify the structure within the groups of fungal treatments inoculated in *Ricinus communis*. We used a 50 × 9 matrix for the analysis, in the SAS 9.0 (SAS Institute Inc., 2002). Pearson correlations were also performed when needed.

3. Results

3.1. Soil chemical characteristics

A very high variability in all chemical parameters studied was detected within the site. Soil from the LABR site had alkaline pH, poor OM content and was classified as saline (Table 1). The soil was mainly polluted with Pb and Cu as pseudo-total and DTPA-extractable concentrations were higher to advised threshold concentrations for industrial soils.

Soil characteristics (Table 2) significantly changed during the time of observation (rhizosphere pH, OM, Pb- and Cu-DTPA extractable concentrations); however, effects due to fungal inoculation and observed by longitudinal analysis (taking in account the three analysis points) were only observed on pH (Fig. 1) and Pb-DTPA soil concentrations (Fig. 3a).

The longitudinal analysis (regarding the three analysis points) revealed that rhizosphere of plants inoculated with *Acaulospora* sp., *F. mosseae* BEG25, *and Glomus* sp. had different soil pH compared to this from the native rhizosphere of non-inoculated plants (NI) and this inoculated with the other AMF (Fig. 1). Spatial observations' comparing the field soil concentration of Pb-DTPA extractable at the original pollution status and during the three different study stages (at 5, 10 and 15 mat) is observed in Fig. 2. Significantly lower Pb-DTPA extractable concentration in the rhizosphere of *F. mosseae* BEG25-inoculated plants was observed in comparison to this of the native rhizosphere of NI plants (Fig. 3a). A difference in the concentration of sulfates and Na soil concentrations was not observed during the time of the field experiment (data not shown). In contrast, changes in K soil concentration were observed during the time of experimentation, but not by fungal influence on soil concentrations of K, Na, and sulfates (data not shown).

3.2. Plant evaluation

After 15 mat, plants inoculated with *Acaulospora* sp., *F. mosseae* BEG25 and *G. gigantea* had 100% survival, while plants inoculated with *Glomus* sp. TX survived 86%. In contrast, only 57% of non-inoculated plants survived. In general, plant growth was severely affected by the hostile soil conditions at the LABR site. Involving all treatments, the average height of *R. communis* plants was 59 ± 27 cm; these had 11 ± 13 leaves and 28 ± 5.7 internodes. At 15 mat, plants formed 2 racemes with 37 ± 45 g of seeds per plant and the weight of 100 seeds was 44 ± 7 g. Seed oil content was 43 ± 7%. No statistical differences were found on these variables between each treatment with the different AMF inoculum and control treatment.

Taking into account all treatments, the range of foliar Pb and Ca concentration enhanced significantly from 5 to 15 mat (64 to 159 mg kg^{-1} DW and 1534 to 1772 mg kg^{-1}, respectively). In contrast, foliar P concentration gradually decreased during the time of evaluation (310, 280 and 180 mg kg^{-1} at 5, 10 and 15 mat, respectively). No effect due to fungal inoculation was observed for Ca and P foliar concentration, but mycorrhizal inoculation influenced foliar Pb concentration

Table 1
Initial chemical soil properties of a Pb acid battery recycling site at Tepetlaoxtoc, Mexico State.

Parameter	Values		Classification and threshold concentrations
	Average and sd ($n = 27$)	Range	
pH	8 ± 1	7–10	Alkaline (Jones and Wolf 1984)
EC (dS m^{-1})	4 ± 5	0–13	Saline (Brady and Weil 2008)
OM (%)	0.7 ± 0.3	0.1–1.3	Poor (Fassbender and Bornemisza 1987)
Total concentration (mg kg^{-1})			
Pb	41,893 ± 42,556	303–122,404	800 (TC-IS)[a]; 600 (STS)[b]
Cu	798 ± 900	24–3044	91 (STS)[b]
Zn	195 ± 164	31–574	360 (STS)[b]
Mn	419 ± 242	178–856	NR
Ni	87 ± 63	22–208	20,000 (TC-IS)[a]; 50 (STS)[b]
Cd	7 ± 3	2–13	450 (TC-IS)[a] 22 (STS)[b]
DTPA-extractable (mg kg^{-1})			
Pb	6246 ± 4213	153–12,000	>300 (HCS)[c]
Cu	45 ± 60	1–231	>2.5 (HCP)[d]
Zn	5 ± 3	1–14	>8.0 (HCP)[d]
Mn	3 ± 4	0–11	>50 (HCP)[d]
Ni	2 ± 1	0–5	>100 (HCS)[c]
Cd	0 ± 0	0–1	>5 (HCS)[c]

EC: electrical conductivity, OM: organic matter.
NR = not reported.
[a] Mexican reference for total concentrations industrial soil (TC-IS) according to NOM-147-SEMARNAT/SSA1, 2004.
[b] Suggested thresholds in soils (STS) based on Canadian environmental quality guidelines 2003 for industrial soils, taken from Varun et al. (2012) and proposed by The Canadian Council of the Environment.
[c] High concentrations (HCS) according to Mexican reference for NOM-021-SEMARNAT-2000 for soil fertility, salinity and classification.
[d] High concentrations for plants (HCP) according to Ankerman and Large (1978).

Table 2
Kinetics of soil chemical properties in the rhizosphere of *Ricinus communis* after transplanting in a lead acid battery recycling site at Tepetlaoxtoc, Mexico State.

Values				Significance level	
Parameter	Time (months)	Average	Range	Pr > F (time)	Pr > F (treatment)
pH	5	8.1 a	7.2–8.9	0.0001	0.04
	10	8.2 a	7.4–9.3		
	15	7.7 b	6.8–8.8		
EC (dS m^{-1})	5	1.0 a	0.1–5.2	0.520	0.62
	10	0.9 a	0.2–5.2		
	15	1.0 a	0.3–5.4		
OM (%)	5	0.7 b	0.3–2.2	0.007	0.25
	10	0.8 b	0.4–1.6		
	15	1.0 a	0.4–2.7		
Pb-DTPA[†]	5	3402 b	616–7902	0.0001	0.07
	10	4520 a	319–10,388		
	15	2837 b	519–5720		
Cu-DTPA[†]	5	13 b	5–32	0.02	0.99
	10	15 a	5–36		
	15	16 a	4–42		
K[‡]	5	242 a	81–373	0.0001	0.45
	10	81 c	13–154		
	15	115 b	9–319		

Similar letters for each variable are not significant different ($p < 0.05$) according to longitudinal analysis. Average values and range come from $n = 35$.
[†] DTPA extractable soil concentration (mg kg^{-1}).
[‡] Soluble ion (mg kg^{-1}).

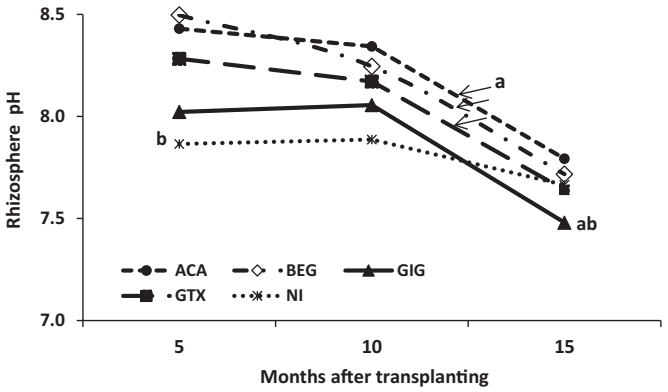

Fig. 1. Influence of arbuscular mycorrhizal fungi on pH of *Ricinus communis* rhizosphere at a lead-acid batteries residue. Different letters represent statistical significance from longitudinal analysis according to all months after transplanting ($\alpha = 0.05$ Tukey test, $n = 35$). Arrows indicate the fungal treatments related to each letter. ACA: *Acaulospora* sp.; BEG: *Funneliformis mosseae* BEG-25; GIG: *Gigaspora gigantea*; GTX: *Glomus* sp. and NI: non-inoculated plants.

(Fig. 3b). Longitudinal analysis (regarding the three analysis points) indicated that plants inoculated with *Acaulospora* sp. have a significant effect on Pb uptake in comparison to control plants.

Foliar Cu concentration, in all plants independently of treatments, was 14 ± 6, 17 ± 8 and 5 ± 4 mg kg^{-1} for 5, 10 and 15 mat, respectively. No effect due to fungal inoculation was observed. Accumulation factors for Cu and Pb were lower than one (0.33 and 0.06, respectively). Therefore, this plant is not an accumulator plant for these two elements; which was also reflected in the absence of these elements in crude oil and cake (data not shown). Plants inoculated with *Acaulospora* sp. had lower accumulation factor (0.03) than control plants (0.13).

In contrast, fungal inoculation significantly enhanced the weight of seeds, seed oil content and fatty acids. All inoculated plants had a higher weight of seeds (from 46 to 50 g per 100 seeds) than those from non-inoculated plants (30 g). The highest content of oil was observed from seeds of plants inoculated with *G. gigantea* (51%), while the lowest oil content in seeds was that from control plants (40%). *Acaulospora* sp. was the main fungus increasing palmitic, oleic and linoleic acids in the seed oil of *R. communis* (Fig. 4) with respect to the control plants. Meristic and stearic acids were similar among treatments.

No clear trend was observed in osmoprotective solutes in leaves of *R. communis* during the study. Including all treatments, total soluble sugars (percentage of dry weight) were 14 ± 2, 20 ± 4 and 12 ± 4 and proline concentration (μM g^{-1} dry weight) was 7 ± 1, 3 ± 0 and 2 ± 1 at 5, 10 and 15 mat, correspondingly. Enzymatic activity from SOD and APX (U g^{-1} dry weight) significantly decreased from 5 to 10 at 15 mat (10 to 2.9 for SOD and 1.71– to 0.6, respectively). Contrary results were obtained for leaf protein (mg g^{-1} dry weight), being 3.2 ± 4 at 5 mat and higher at 10–15 mat (5.9 ± 5 and 4.8 ± 2, correspondingly). Similarly, chlorophyll concentration (total, *a* and *b*) was higher at 10 and 15 mat than this observed at 5 mat. In all these variables significant differences were influenced by time, but not by fungal inoculation.

Total phenolic compounds content in leaves of *R. communis* is presented in Fig. 5. Statistical differences were found at observation times (Pr > F 0.001) and influenced by inoculation treatment (Pr > F 0.035). At 15 mat, control plants had the highest foliar total phenol content (24%), while plants inoculated with *Glomus* sp. TX the lowest value (13%). Mycorrhizal colonization was similar in roots of *R. communis* from all fungal treatments (Fig. 2S). This was in the range of 40% to 60% in inoculated treatments and 16% to 22% of colonization by native AMF in the control plants.

Results from multivariate analysis are shown in Fig. 6. Dispersion of the four fungal treatments on *R. communis* represented in the space, determined the first three principal components (PCs); which together explained 94% of the accumulated overall variation of the thirteen variables studied (Fig. 6a). The PC1 explained 65% of the total difference and revealed to soil extractable DTPA-Pb and foliar-Pb concentrations, mycorrhizal colonization, and seed weight (100 seeds) as main variables responsible for group separation. The PC2 explained 19% of the total variation and was determined mainly by pH, seed oil content, and linoleic acid; while PC3 described 10% of the overall change and was defined largely by total phenolic compounds content and total soluble sugars in leaves (Table S1).

A grouping pattern similar to that obtained with the principal components analysis was observed with cluster analysis (Fig. 6b). A Euclidian distance of 0.7 defined four groups. Control plants were separated from plants inoculated with AMF; while plants inoculated with *G. gigantea* and *Glomus* sp. TX were different from each other and plants inoculated with *Acaulospora* sp. and *F. mosseae* BEG25 clustered together in a single group. Several Pearson correlations were observed among variables studied (Table S2), showing the relationships between AMF-plant-soil variables in the LABR site under study.

4. Discussion

This field study, followed for 15 months after transplanting (mat), shows the beneficial effects of AMF on *R. communis* plants when established in an extremely LABR polluted soil; which was abandoned without receiving any follow-up control measures to remediate the site. These fungi besides enhancing some plant traits (weight of seeds, seeds oil content, fatty acid composition, and foliar total phenolic compounds content), they also modified soil properties such as pH and Pb availability. This study shows that mycorrhizal plants had different patterns of response that control non-inoculated plants; where plants of *R. communis* inoculated with *Acaulospora* sp. and *F. mosseae* BEG25 presented the best plant responses under the adverse field soil conditions observable in the LABR site of study. Hence, suitable AMF-plant interactions may be useful for mycoremediation of sites affected by LABR as pointed out by Kumar et al. (2018) for mine sites rehabilitation.

4.1. Soil chemical characteristics

The study site presented initially severe soil conditions which constrained plant establishment. High soil heterogeneity was observed probably due to uncontrolled recycling activities when solutions were discarded directly to soil from used automobile batteries. Alkaline pH, high EC, low OM content, extremely high concentrations of Pb and Cu were the most common altered soil properties (Table 1).

The average soil pseudo-totals Pb concentration was near to 42,000 mg kg^{-1}; however, the highest value was >122,000 mg kg^{-1}. These Pb soil concentrations have been found in other soils polluted by LAB. For example, Pichtel et al. (2000) observed up to 140,500 mg Pb kg^{-1} soil, while Wasay et al. (2001) found 24,600 mg kg^{-1}. Pseudo-total Pb concentration in soil was higher than maximum level referenced by Kabata-Pendias (2011) for agricultural soils (50–300 mg kg^{-1}), by Nedwed and Clifford (1998) for residential land use in USA (500–1000 mg kg^{-1}), in Canada (375 mg kg^{-1}), in Netherlands (50–600 mg kg^{-1}), in England (500 mg kg^{-1}), and by NOM-147-SEMARNAT/SSA1, 2004 for industrial soils in Mexico (800 mg kg^{-1}).

Average pseudo-total Cu concentration in soil was 798 mg kg^{-1}; being also higher than the maximum concentration for agricultural soils (60–500 mg kg^{-1}; Kabata-Pendias, 2011), Canadian environmental quality guidelines for industrial soils (91 mg kg^{-1}; Varun et al., 2012) and normal soil concentrations (100 mg kg^{-1}; Lindsay and Norvell, 1978). Similarly, extractable DTPA-Pb (6246 mg kg^{-1}) and Cu (45 mg kg^{-1}) concentrations were high in the LABR site (Table 1). These two elements Pb and Cu were originated from acid batteries; which are common constituent compounds of them (reviewed by Ruíz-Olivares, 2016).

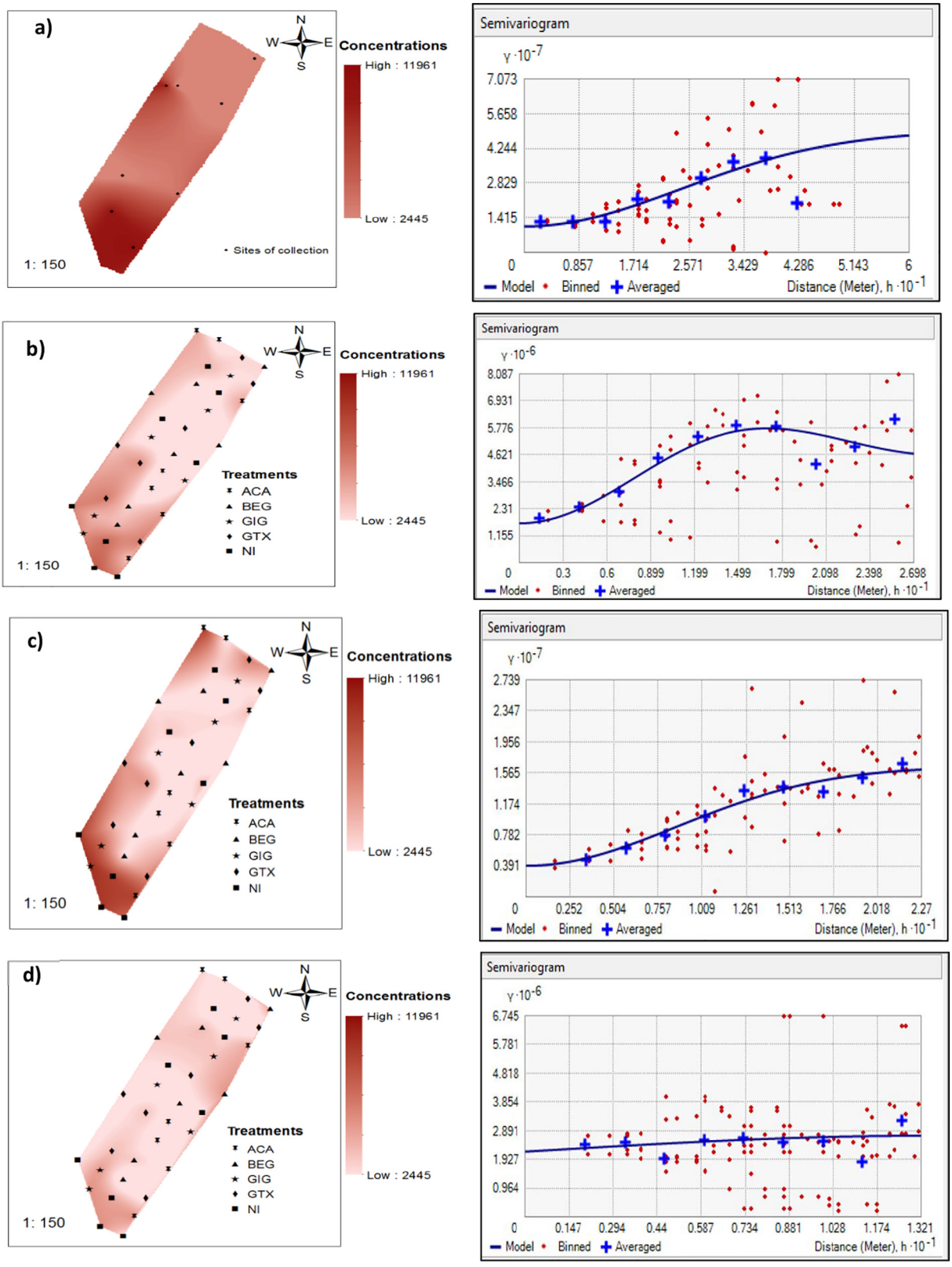

Fig. 2. Spatial representation and semivariograms of DTPA-Pb concentrations before establishing the experiment (a), at five months (b), at ten months (c) and at fifteen months (d) after establishing non- and -inoculated *Ricinus communis* plants in a soil polluted with lead-acid batteries. ACA: *Acaulospora* sp.; BEG: *Funneliformis mosseae* BEG-25; GIG: *Gigaspora gigantea*; GTX: *Glomus* sp. and NI: non-inoculated plants.

Alkaline soil conditions and low OM content are strong limiting factors for plant establishment (Hazelton and Murphy, 2007), as well as high concentrations of heavy metals. Soil chemical characteristics in the rhizosphere of *R. communis* were modified during the field experiment (Table 2). Soil pH and extractable Pb-DTPA concentrations significantly decreased at 15 mat, while OM content increased. These changes are firstly attributable to soil amendment used: vermicompost and sawdust. However, the longitudinal analysis revealed that fungal

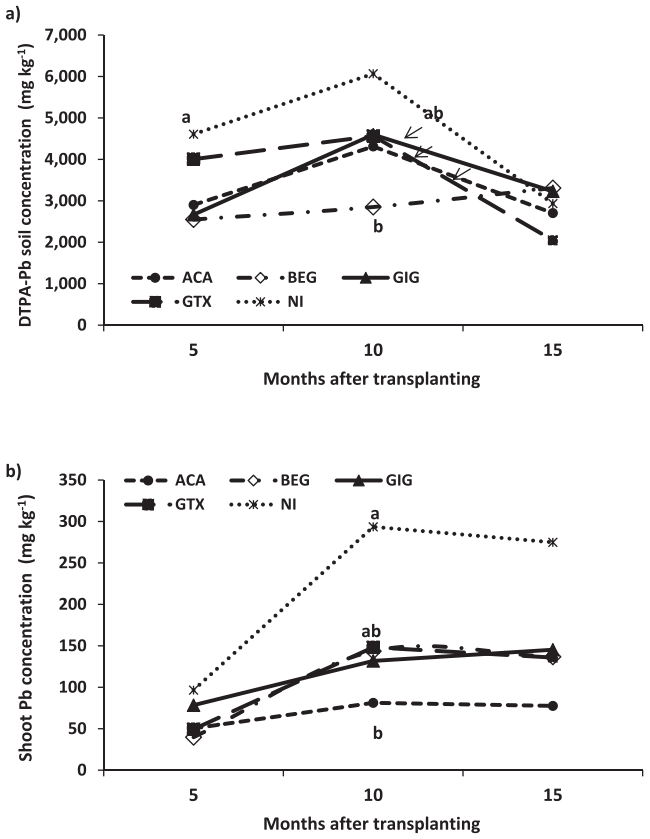

Fig. 3. DTPA-extractable Pb concentration in the rhizosphere of *Ricinus communis* (a) and shoots Pb concentrations of AMF-inoculated plants (b) when growing in a soil polluted with lead-acid batteries. Different letters represent statistical significance from longitudinal analysis according to months after transplanting (Tukey test $\alpha = 0.05$, $n = 35$). Arrows indicate the fungal treatments related to each letter. ACA: *Acaulospora* sp.; BEG: *Funneliformis mosseae* BEG-25; GIG: *Gigaspora gigantea*; GTX: *Glomus* sp. and NI: non-inoculated plants.

inoculation had also an effect on pH ($p < 0.05$; Fig. 1) and extractable Pb-DTPA concentration ($p < 0.05$; Fig. 3a). The organic amendments in phytoremediation (soil organic amendments-aided phytostabilization) are efficient to adsorb and immobilize these and other elements (Impellitteri et al., 2001; Alloway, 2013); hence enhancing soil conditions for plant establishment and growth. Moreover, these improve the OM content, soil fertility, microbial activity and pH (Sunitha et al.,

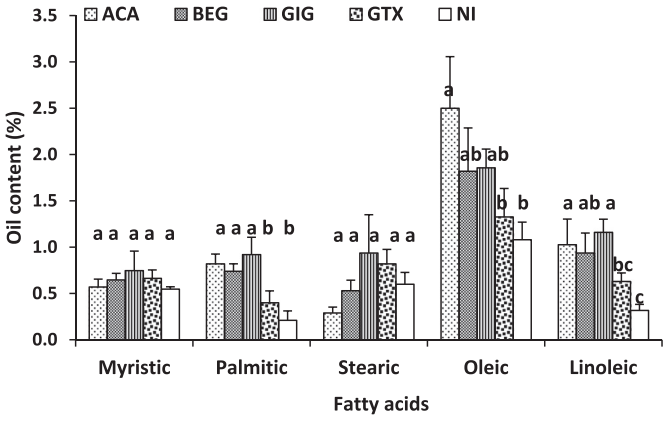

Fig. 4. Oil content in seeds of *Ricinus communis* produced in a soil polluted with lead-acid batteries and influenced by arbuscular mycorrhizal fungi. Different letters represent statistical significance from longitudinal analysis according to months after transplanting ($\alpha = 0.05$ Tukey test, $n = 35$). ACA: *Acaulospora* sp.; BEG: *Funneliformis mosseae* BEG-25; GIG: *Gigaspora gigantea*; GTX: *Glomus* sp. and NI: non-inoculated plants.

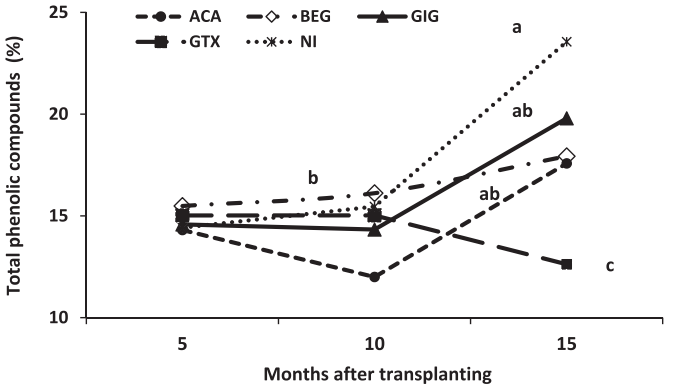

Fig. 5. Effect of four arbuscular mycorrhizal fungi on foliar total phenols of *R. communis* plants growing in a soil polluted with lead-acid batteries. Different letters represent statistical significance from longitudinal analysis according to months after transplanting, average and the standard deviation is presented ($\alpha = 0.05$ Tukey test, $n = 35$). ACA: *Acaulospora* sp.; BEG: *Funneliformis mosseae* BEG-25; GIG: *Gigaspora gigantea*; GTX: *Glomus* sp. and NI: non-inoculated plants.

2014). Gil-Loaiza et al. (2016) also showed that soil amendments allowed plant establishment (mix of *Buchloe dactyloides*, *Festuca arizonica*, *Atriplex lentiformis*, *Cercocarpus montanus*, *Prosopis juliflora*, and *Acacia greggi*) and sustained growth over four years. Their results presented the feasibility for the use of phytostabilization technology in mine tailings.

4.2. Plant evaluation

Plants of *R. communis* were able to grow under the severe soil conditions observed in this LABR site; which contained high pseudo-total and extractable DTPA-Pb and -Cu concentrations. Gomes et al. (2014) observed that liming enhanced soil pH and allowed *R. communis* establishment to grow on a soil polluted with LAB containing high soil Pb concentrations (1811 mg kg^{-1}). Schneider et al. (2016) also showed that *R. communis* can grow on LABR sites with pseudo-total concentrations of Pb (3456 mg kg^{-1}). These authors detected that native AMF colonized roots of this plant species.

In the present study, the longitudinal analysis showed that rhizosphere from *R. communis* plants inoculated with *Acaulospora* sp., *F. mosseae* BEG25, and *Glomus* sp. had higher pH than the one from control plants. García-Sanchez et al. (2017) also observed in mycorrhizal treatments significant increment on soil pH compared to the non-AMF treatments in wheat plants growing on biotransformed dry olive residue. These authors discussed that the inoculation with AMF seems to be the most important factor affecting soil pH, probably as the result of changes in root exudates. Another possible hypothesis arises from recent studies describing the association of soil bacteria in the mycorrhizal plants which modify rhizospheric pH to hydrolyze minerals in the rhizosphere (Jones and Oburger, 2011). In general, little research has been followed to know the fungal effect, or the accompanying microbiota effect on soil pH under field conditions and metal polluted soils. Under controlled experimental conditions, the result of AMF on pH is contradictory. For example, Bago and Azcón-Aguilar (1997) observed strong acidification in the rhizosphere of inoculated plants, but Bago et al. (1996) and Villegas et al. (1996) observed increased pH under *in vitro* conditions.

In the current study, inoculated plants with three AMF showed higher survival than non-inoculated plants; which were also colonized to a lower extent by native AMF from the Pb-polluted soil under study. This high plant survival rates may be due firstly to the lower extractable Pb-DTPA concentrations (Fig. 3a) in the rhizosphere of plants, mainly those inoculated with *Acaulospora* sp., *F. mosseae* BEG25, and *G. gigantea* in contrast with survival rates in plants inoculated with *Glomus* sp. and non-inoculated plants.

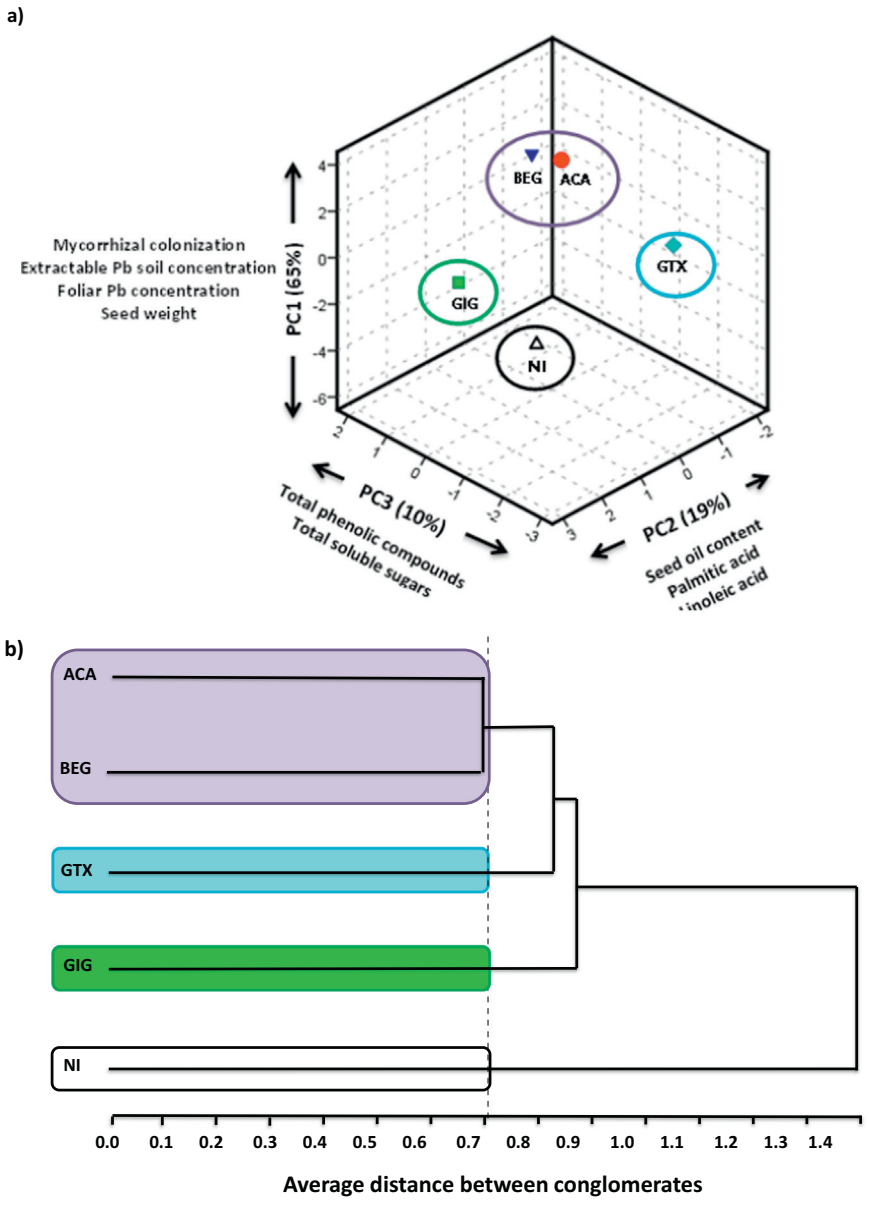

Fig. 6. Principal components analysis (a) and similarity dendrogram (b) of fungal treatments inoculated in *Ricinus communis* plants growing on a lead-acid batteries residue. ACA: *Acaulospora* sp.; BEG: *Funneliformis mosseae* BEG-25; GIG: *Gigaspora gigantea*; GTX: *Glomus* sp. and NI: non-inoculated plants.

At 15 mat, non-inoculated plants accumulated 3.4 fold times higher Pb concentrations in comparison to plants inoculated with *Acaulospora* sp. (Fig. 3b). Control plants had a low Pb accumulation factor; however, this factor decreases (4.3 times) even more by fungal inoculation. The maximum shoot Pb concentration in inoculated *R. communis* plants was 148 mg kg^{-1}, while in control plants Pb concentration was 293 mg kg^{-1}. The concentration of Pb in non-inoculated plants was close to the limit concentration referred as phytotoxic (300 mg kg^{-1}) by Kabata-Pendias (2011), but it is still higher than the 10–20 mg kg^{-1} applied by Vamerali et al., 2010. In contrast, low Pb concentrations (between 50 and 80 mg kg^{-1}) were observable during all growth stages of *R. communis* inoculated with *Acaulospora* sp. Yang et al. (2016) also observed that plant Pb concentrations in mycorrhizal plants were lower than this in control plants when soil concentrations were 1500 mg kg^{-1}. As showed by other authors (Ruíz-Olivares et al., 2013; Perea-Vélez et al., 2015), *R. communis* is a potential plant for remediation of metal polluted soils; which behaves as a non-metal accumulator plant, but not as a metal accumulator or hyperaccumulator as suggested by other authors (Romeiro et al., 2006). Schneider et al. (2016) observed that plants of *R. communis*, growing on soils polluted by LABR, accumulated 624 mg of Pb kg^{-1} of shoots. Plant Pb concentrations do not exceed Pb concentrations of 1000 mg kg^{-1} according to the hyperaccumulation criterion (van der Ent et al., 2013).

Phytostabilization reduces the mobility, availability and therefore the risk of inorganic pollutants without necessarily removing them from the soil (Bolan et al., 2011). Taking into account the extremely high Pb soil concentrations found in this LABR site, *Acaulospora* sp. and *F. mosseae* appear to be good prospects for Pb phytostabilization. In the rhizosphere of *F. mosseae* was observed the lowest extractable soil Pb-DTPA concentrations (Fig. 3a) and plants inoculated with *Acaulospora* sp. had the lowest Pb shoot concentration (Fig. 3b). Similarly, inoculated plants presented lower Pb accumulation factor than control plants. These results are in agreement with the function of AMF in polluted soils: restriction of metal translocation and metal accumulation in mycorrhizal plants by acting as biological filters (Kaldorf et al., 1999; González-Chávez et al., 2002; Zhang et al., 2010). AMF have developed mechanisms to deal with heavy metals such as Pb (González-Chávez et al., 2004 and 2009). Several authors have

mentioned that metal stabilization by AMF can be improved through the application of organic amendments (Alguacil et al., 2008; García-Sanchez et al., 2017). Nowadays, it is recognized that the extra-radical mycelium or spores of AMF are able to immobilize Pb and other heavy metals (Joner et al, 2000; González-Chávez et al., 2004; Vodnik et al., 2008) and decrease translocation of these elements in aerial parts of mycorrhizal plants (González-Chávez et al., 2002; Malcova et al., 2003; González-Chávez and Carrillo-González, 2013; Yang et al., 2015). Hence, higher plant survival, protection against the toxic effect of these elements and enhance of metal plant tolerance are observed in mycorrhizal plants when established in metal polluted soils (González-Chávez et al., 2002).

Interestingly, extractable Pb concentration in soil and plant Pb concentration; together with mycorrhizal colonization represented vectors in the principal component analyses with high value in PC1 (Table S1), thus being the variables explaining 65% of the variance. This is also in accordance to robust Pearson correlations (Table S2) between mycorrhizal colonization and extractable Pb concentration ($r = 0.87$; $Pr > 0.0025$), or mycorrhizal colonization and plant Pb concentration ($r = 0.97$; $Pr > 0.0001$).

Mycorrhizal fungi are beneficial to enhance plant growth, protect plants alleviating cellular oxidative damage in response to Pb stress (Yang et al., 2015, 2016). For example, Yang et al. (2016) in Pb spiked soil, detected higher P concentration in 75 d-old mycorrhizal plants (*Robinia pseudoacacia*, *Trifolium pratense*, *Medicago sativa* and *Lolium perenne*) in comparison to the respective non-inoculated plants at concentrations of 1500 mg Pb kg^{-1}. However, no significant effect was observed on Ca shoot concentrations in none of these plants. In the present study, inoculation by AMF did not influence plant variables such as growth (height, stem diameter, number of internodes and number of leaves) or nutrition (P foliar concentrations). Neither effect on enzymes related to oxidative stress was observed by inoculation with AMF.

According to Kisa et al. (2016), phenolic compounds are one of the stress responses to environmental plant adaptation. For example, enhancement of total phenolic compounds was observed in shoots of corn plants when exposed to Pb, Cd or Cu. However, this increment was dependent on the type of heavy metal and its concentration. These authors also explained that individual phenolic compounds vary among plant species and conditions. Hazzoumi et al. (2015) observed the higher content of total phenolic compounds in non-inoculated plants of *Ocimum gratissimum* L. than plants inoculated with *Rhizophagus intraradices* under non-contaminated soil conditions. In the present research a decrement on total phenolic compounds content (Fig. 5) in plants inoculated with *F. mosseae* BEG25 (1.2 times) and *Glomus* sp. TX (1.8 times) was observed in comparison to control plants. It is not clear why phenolic compounds decreased in plants inoculated with some of the fungi and increased with others under these high Pb soil concentrations. Hence, it results interesting, in future research to try to elucidate the specific phenolic compounds in shoots of *R. communis* under these polluted conditions as this plant variable has the highest positive vector value in the PC3 (0.54; Table S1) and has a robust correlation with shoot Pb concentration ($r = 0.82$, $Pr > F = 0.0071$). This would help to better understanding of non-enzymatic plant mechanisms for scavenging of free radicals induced by heavy metals such as Pb and the participation of AMF.

Similarly, experiments regarding shoot sugars and heavy metals such as Pb should be analyzed more in-depth as these are importantly influencing the three PC analyzed (Table S1) and had negative correlations to pseudo-total and extractable Pb in soil ($r = -0.97$, $Pr > F = 0.001$ and $r = -0.97$, $Pr > F = 0.001$, respectively) and in-plant concentrations ($r = -0.93$, $Pr > F = 0.003$; Table S2). Shoot total sugars are an interesting physiological plant component responding to Pb contamination, although AMF had no significant influence. This would also enhance our knowledge of non-enzymatic plant mechanisms for plant stress to heavy metals.

In the present study, AMF inoculation enhanced the weight of seeds, and seed oil content of some fatty acids of *R. communis* produced under the LABR site (Fig. 4). Apparently, there is no previous published information on the effect of AMF inoculated in plants of *R. communis* when growing on LABR sites, neither regarding the content of fatty acids in oil influenced by metals (González-Chávez et al., 2015). Interestingly, the oil content in seed of *R. communis* under the LABR site, observed in the present study, is in accordance to the one detected in soils polluted by metal mine tailings. Ruíz-Olivares et al., 2013reported that the oil content in seeds from six *R. communis* plant accessions was from 40% to 64%. All these accessions were naturally colonized by native unidentified AMF.

In the case of the content of fatty acids, plants inoculated with *Acaulospora* sp. had 2.3 times the content of oleic acid in seeds; while this fungus and *G. gigantea* enhanced more than three folds the content of linoleic acid. Similarly, three of the four AMF (except *Glomus* sp.) enhanced from 3.5 to 4.3 times the content of palmitic acid in comparison to that of the oil from non-inoculated plants. In the present study, the content of stearic, oleic and linoleic acids was much lower to this reported by González-Chávez et al. (2015) in seed oil of *R. communis* plants naturally growing on soils polluted by mine residues containing high concentrations of metals. These authors found that oleic acid was in the range from 3.0% to 3.8%, while linoleic acid from 3.9% to 4.5% and stearic acid from 0.9% to 1.9% in naturally established plants. Moreover, oil from plants growing in the metal mine tailings had higher linoleic and stearic acid content than those from non-polluted soils. These authors suggested changes in the quality of oil production mainly due to increasing Zn shoot concentrations of this plant. They also observed a contrary effect related to Cu shoots concentrations. In contrast, according to Pearson correlations from the present study, linoleic, oleic and palmitic acids had significant strong negative correlations ($r = -0.94$, $Pr > F = 0.0002$; $r = -0.91$, $Pr > F = 0.0007$ and $r = -0.92$, $Pr > F = 0.0005$, respectively) with extractable Cu-DTPA rhizosphere concentrations (Table S2). In these two studies, none of these two metals (Zn or Cu) had the highest concentration in the soil of shoots of *R. communis*. Hence, according to González-Chávez et al. (2015), it is still needed to elucidate the effect of metals on fatty acids in the oil of the seeds of this plant using a range of concentrations and metals with special emphasis on Zn and Cu. It is highly probable that other environmental and soil factors influence oil quality of *R. communis* growing in metal polluted conditions.

Tlustoš et al. (2007) mentioned that the efficiency of bioremediation is usually better for moderately contaminated soils when compared with extreme pollution. However, our results showed, in a 15 months field experiment on an extremely polluted environment, the beneficial effects of AMF on *Ricinus* plants establishment, oil content and its quality, seed weight and low Pb concentration of *R. communis* plants when grown in a LABR site. Moreover, the effects on pH and less soil Pb availability were also observed. Hence, AMF may be useful for developing phytostabilization techniques that ameliorate sites impacted by LABR activities. Phytostabilization is a feasible friendly environmental alternative to decrease dispersion of contaminants (Bolan et al 2011).

R. communis is a non-edible crop and has several uses. For instance, stems are useful handcraft; seeds are the source of oil for fuel, medicinal and cosmetic applications or use as a lubricant since 4000 years (Rzedowski and Rzedowski, 2001). It is also used in the manufacture of soap and tinctures. The increased behavior of *R. communis* plants due to its inoculation with AMF is relevant, because this renewable raw material may help to solve the constant demand for alternative energy sources (De Lima Da Silva et al., 2006). Biofuel production has several restrictions: it should use infertile soils, avoid destroying natural ecosystems and, avoid competition with food production. Hence, our results support the beneficial effects of AMF associated to *R. communis* to assist remediation on high metal polluted soils, such as this LABR site.

Concluding, the longitudinal analysis revealed that fungal treatments significantly influenced rhizosphere soil pH, DTPA-extractable

Pb soil concentration, shoot Pb concentration and the percentage of phenolic compounds in leaves. While, the transversal analysis showed statistical differences in dry weight of seeds, the rate of seeds oil, and quality of oil (palmitic, oleic and linoleic). *Ricinus communis* responded to inoculation of AMF; however, *Acaulospora* sp. and *F. mosseae* BEG25 had the most notable effect. Our results showed for the first time that the combination of AMF, organic amendments and *R. communis* are part of a practical and encouraging strategy for increasing phytostabilization efficiency of sites polluted by lead-acid batteries.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.10.031.

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