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Differential responses to high soil chromium of two arbuscular mycorrhizal fungi communities isolated from Cr-polluted and non-polluted rhizospheres of *Ricinus communis*

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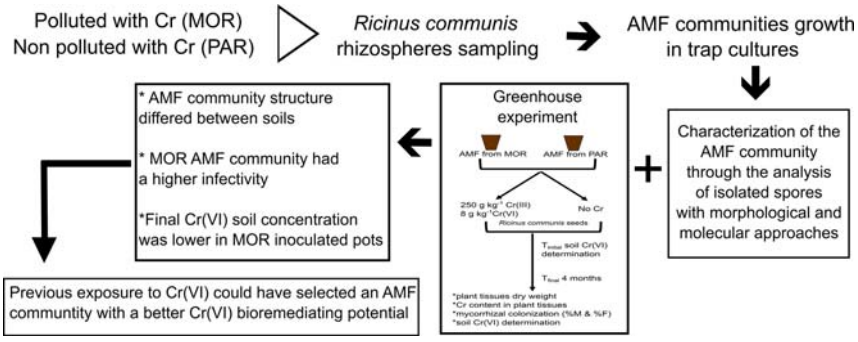
HIGHLIGHTS

- Acaulosporaceae and Gisgasporaceae morphotypes were only observed in the culture traps from non-Cr polluted *Ricinus communis* rhizospheres.
- Paraglomeraceae ribotypes were detected only in the culture traps from a Cr-polluted *Ricinus communis* rhizospheres.
- AMF community from a Cr-polluted *R. communis* rhizosphere had a higher infectivity than the communityisolated from a non Cr-polluted rhizosphere.
- Previous exposure to Cr(VI) could have selected an AMFwith a better Cr(VI) bioremediation potential.

GRAPHICAL ABSTRACT



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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are symbionts in roots of 70–90% of terrestrial plants, and are present even in highly perturbed sites like soils polluted with potentially toxic elements (PTE). Plants and indigenous AMF present in polluted soils may play an important role in PTE uptake (phytoextraction) and complexation (phytostabilization). The present work was addressed to: i) analyze the spore community structure of AMF associated to *Ricinus communis* rhizospheres from a chromium (Cr) polluted site (MOR) and a non-Cr polluted site (PAR); ii) analyze whether plant growth and mycorrhizal colonization of *R. communis*, and Cr(VI) remaining concentration in soil, differed in both rhizosphere soils when exposed to a mixture of Cr(III)/Cr(VI) polluting concentration in a greenhouse experiment. Culture trap from both rhizosphere soil were established previously to the study to propagate AMF. The community structure was analyzed with morphological and molecular approaches. The spore community structure differed in MOR as related to PAR rhizosphere soils: Acaulosporaceae and Gigasporaceae morphotypes were only observed in PAR and Paraglomeraceae ribotypes were detected only in MOR. Finally, a greenhouse experiment was designed to study the response of both communities when grown in association with *R. communis* and exposed to polluting concentrations of Cr(III)/Cr(VI) (250/8 μg g^{-1} soil d.w.). The mycorrhizal colonization was higher in roots grown in presence of MOR than in presence of PAR AMF community, exposed or not to Cr, and the final Cr(VI) concentration in soil was lower in the pots with MOR AMF community than in the pots with PAR AMF community (6.9 ± 0.2 and 14.3 ± 1.5 μg Cr(VI) g^{-1} soil d.w., respectively). Our results suggest that the previous exposure to polluting concentration of Cr could have

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selected AMF species with a higher infectivity and thus more likely to better contribute to reduce the pollutant concentration in the soil.

1. Introduction

Industrial development has helped to improve human quality of life during the last decades. However, inappropriate industrial waste handling and final disposal has contributed to soil and water contamination. Anthropogenic pressure generated by industrial activities triggers drastic deterioration of soil, producing a marked loss of stability, degradation and even desertification (Barea et al., 2007). Potentially toxic elements (PTE) such as As, Cu, Cd, Cr, Pb and Zn, are one of the most common pollutants, mostly near industrial sites (Järup, 2003; Baena and Huertos, 2008; Meier et al., 2012, Gil-Cardeza et al., 2014); they could become a risk to human health as they can enter to the food chain *via* agricultural products or contaminated drinking water. High concentrations of PTE in soil can lead to changes in the structure and/or functioning of microbial communities, (Krishnamoorthy et al., 2015) and have detrimental effects on ecosystems and human health.

Chromium is used in several industrial processes (*i.e.* leather tanning, alloy and stainless steel production). Chemistry of Cr is quite complex; it is found in soils in two oxidation states, Cr(III) and Cr(VI). Chromium(III) is non-toxic and not readily absorbed by plants; in contrast, Cr(VI) is highly toxic; it is a Class A carcinogen by inhalation and an acute irritating agent to living cells (James, 1996; Khan, 2001; Dhala et al., 2013). Chromium(VI) is water soluble in the full pH range, while Cr(III) is prone to be adsorbed on soil surface or precipitate as chromium hydroxide in a slightly acidic or alkaline environment (James, 1996; Khan, 2001; Dhala et al., 2013). Hexavalent Cr exists in neutral-to alkaline soils principally as a chromate anion (CrO_4^-) or as moderately-to-scarcely soluble chromate salts (*e.g.* $CaCrO_4$, $BaCrO_4$, $PbCrO_4$) (James, 1996; Dhala et al., 2013). In laboratory experiments, it was demonstrated that a variable fraction (typically <15%) of Cr(III) added to soils oxidized to Cr(VI). The extent of oxidation was proportional to the level of easily reducible Mn(III,IV) hydroxides and oxides in the soil and also to the form of Cr(III) added (soluble *vs.* insoluble; organically complexed *vs.* inorganic forms) (James, 1996).

Chemical reduction of Cr(VI) to Cr(III) is the most common remediation strategy developed until now. Though the reduction–remediation strategy offers a rapid solution, it is very expensive for a large scale treatment and it does not assure that re-oxidation of Cr(III) to Cr(VI) will not occur (James, 1996; Panda and Sarkar, 2012). In contrast, phytoremediation, which uses higher plants and the associated soil microbes as decontaminating agents, is a less expensive, long-lasting and eco-friendly strategy to decontaminate PTE polluted soils (Meier et al., 2012; Dhala et al., 2013; Ali et al., 2013). The establishment of vegetation on polluted soils also helps to prevent erosion and metal leaching. In the case of Cr, whose different redox states have different degree of toxicity, plants convert highly hazardous Cr(VI) to the relatively less toxic Cr(III) (Ali et al., 2013).

Arbuscular mycorrhizal fungi (AMF), which develop mutualistic associations with the roots of most terrestrial plants, allow plants a greater absorption of nutrients and water due to an increase in soil exploration area by the extraradical mycelium (ERM) (Azcón-Aguilar et al., 1999; Parniske, 2008). AMF association with plants contributes to promote plant growth even under conditions of environmental stress such as high temperatures, low water availability or soil polluted with PTE (Hassan et al., 2014). With regard to the later, communities of AMF adapted to soils polluted with PTE have attained a great tolerance to PTE toxicity and could therefore stimulate plant growth in contaminated sites (Karimi et al., 2011; Ferrol et al., 2016). The presence of high levels of PTE could become a selection factor acting on microbial communities, promoting those species able to tolerate high concentrations of PTE. These communities could, then, have the potential to be used as inoculants for site restoration as they could favor phytoextraction and/or stabilization and/or mycorrhizostabilization processes of the PTE (Göhre and Paszkowski, 2006; Gil Cardeza and Gómez, 2014; Ferrol et al., 2016). In particular, an enhanced Cr uptake by the plant was detected in the presence of AMF (Davies et al., 2001; Arias et al., 2010). Recent studies conducted *in vitro* with *Rhizophagus irregularis* associated to *Medicago truncatula* plants (Gil Cardeza et al., 2017) or associated to transformed carrot roots (Wu et al., 2015, 2016) demonstrated that the ERM uptakes and translocates Cr to roots. These data support that the use of AMF as bioremediation agents could enhance the phytoremediation capacity of plants to detoxify the environment of Cr(VI).

Previous research revealed the presence of AMF in the rhizosphere of *Ricinus communis*, one of the most abundant plants in a Cr(VI) polluted site studied (Gil-Cardeza et al., 2014). That study proposed that Cr mycorrhizostabilization could be a predominant mechanism used by *R. communis* to diminish Cr(VI) soil concentration, and thus suggested the need of further research to shed more light on the potential of native AMF to contribute the Cr phytoremediation process. Moreover, the application of *R. communis* for phytoremediation purpose has been suggested as a good alternative since it is a non-edible shrub of wild as well as cultivable nature with an important industrial oil yielding application (Bauddh et al., 2015). Thus, the present work was addressed to: i) analyze the community structure and abundance of spores from AMF associated to *R. communis* rhizospheres from a Cr-polluted site and a non-Cr polluted site; ii) analyze whether the plant growth, mycorrhizal colonization and Cr absorbed in plant tissues of *R. communis* differed between both AMF communities (from a Cr-polluted and a non-Cr polluted site) when exposed to a mixture of Cr(III)/Cr(VI) polluting concentrations in a greenhouse experiment. Initial and remaining Cr(VI) concentration in soils was also analyzed. Culture traps from both rhizosphere soils were established previously to the study to propagate AMF. We expected that the remaining Cr(VI) soil concentration will be lower in the pots with the AMF community from the Cr-polluted site.

2. Materials & methods

2.1. Soil sampling

Samples were collected at a 0–25 cm depth from *R. communis* rhizospheres (soil just below the plant and adhered to the root) from a Cr-polluted and a non-Cr polluted site. Five replicates were taken randomly from each rhizosphere at a distance of 1–3 m from the water flow. The soils samples were stored in the dark at 4 °C until the analyses (*i.e.* 2 months); after that trap cultures were made. The Cr-polluted area (MOR) was located in the margins of Morón stream, belonging to Morón borough (34°39′0″S, 58°37′0″W), an industrial/urban area with a highly dense population. Preliminary analysis determined 444 μg total Cr and 10.5 μg Cr(VI) g^{-1}soil d.w. (Gil-Cardeza et al., 2014); both concentrations were higher than the limit allowed by Argentinean regulations (250 and 8 μg of total Cr and Cr(VI), respectively; Regulative order 831/93 law 24051). The non-Cr polluted area (PAR) was an undisturbed site in the margins of Paraná River (32°52′40″S, 60°40′38.2″W), Argentina, where Cr concentrations were 40 and 0 of total Cr and Cr(VI) μg g^{-1} soil d.w., respectively. Soil organic matter was 78 ± 4 and 57 ± 1 g kg^{-1}soil d.w.; pH was 7.7 ± 0.1 and 6.7 ± 0.1 and water content was 179 ± 2 and 155 ± 2 g kg^{-1} soil d.w., for MOR and PAR, respectively. The intensity of root colonization in the whole root system (M%) in *R.*

communis roots was 51 ± 13 in MOR (Gil-Cardeza et al., 2014) and 13 ± 6 in PAR.

2.2. Trap culture setup

In order to increase the number of fresh, viable AMF spores, trap cultures were made with the rhizosphere soils sampled. Trap culture involves the development of a host plant with the ability to establish mycorrhiza profusely, allowing the increase in the number of AMF spores in relation to the initial inoculum at the end of the plant life cycle. *Sorghum bicolor* has been reported as a species which produces high amount of spores in non-polluted and PTE polluted trap soils (Del Val et al., 1999). Trap cultures were established by sowing 100 *Sorghum bicolor* seeds previously disinfected with 1% hypochlorite solution during 5 min, in pots (2 L) previously filled with a mix (1:1) of rhizosphere soil from *R. communis* from MOR or PAR and autoclaved sand. The trap cultures were grown for 3 months under greenhouse conditions (mean 25 °C-14/10 h light/dark photoperiod), were regularly irrigated and then left without irrigation for a month in order to favor the sporulation process before harvest. Shoots were harvested separately, and soil with roots were transferred to plastic bags, air-dried, and stored in a refrigerator until the analysis (2 months). The intensity of root colonization in the whole root system (M%) was 65 ± 8 and 59 ± 7 and the number of spores per g of soil was 8 ± 2 and 7 ± 1 in MOR and PAR trap cultures, respectively.

2.3. Analysis of the AMF spore community from the trap cultures

2.3.1. Morphological characterization of AMF spores

AMF spores were extracted (one extraction per pot; n = 4) from the trap cultures soil (MOR and PAR). The technique of wet sieving, decantation and sucrose gradient centrifugation was used (Oehl et al., 2003). For each extraction the number of AMF spores was determined by observation under stereomicroscope. Then, in order to analyze diversity of AMF spores, the assembly was done in polyvinyl alcohol-lactic acid-glycerol (PVLG) and observed under optical microscope (400×). The taxonomic classification was made at the family level, based on the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM), and considering taxonomic characters of the three most abundant AMF families (Acaulosporaceae, Gigasporaceae and Glomeraceae) which account for 70% of the species described so far: supporting hyphal, spore wall (color, consistency, layers, presence of ornamentation), presence of sporogenous sac, presence of germination shield.

2.3.2. Molecular characterization of AMF spores

Spores were extracted from the trap cultures soil (MOR and PAR) as indicated in the section before (one extraction per pot; n = 4). For DNA extraction, single AMF spores were transferred to a clean, sterile watch glass. All debris were removed under a dissecting microscope and sterile distilled water was replenished several times until only healthy spores remained. Single spores were selected and transferred together with 2.5 μL of sterile distilled water to sterile PCR tubes using a 10 μL tip (Lee et al., 2008). The spores were broken using the tip as a pestle, placed in a water bath at 94 °C for 4 min to denature DNAse and then placed on ice. All samples were stored at −20 °C until PCR amplification (1–5 months).

PCR amplification was made as previously reported by Lee et al. (2008). A nested PCR that amplified the 5′ end of the Small SubUnit ribosomal RNA gene (SSU rRNA) was made.

A first PCR was carried out using universal fungal primers NS1-NS4 (5′ GTA GTC ATA TGC TTG TCT C 3′-5′ CTT CCG TCA ATT CCT TTA AG 3′; Eurofins Genomics) and a second PCR with AMF specific primers AML1-AML2 (5'ATC AAC TTT CGA TGG TAG GAT AGA 3′ - 5′GAA CCC AAA CAC TTT GGT TTC C 3′; IDT). Both PCRs were carried out using 1 U of Taq polymerase μL^{-1} (Invitrogen), 0.2 mM dNTPs (Promega), 1.5 mM $MgCl_2$ and 0.5 μM of each primer in a final volume of 20 μl. The first PCR was performed as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, 40 °C for 1 min, 72 °C for 1 min, followed by a final extension period at 72 °C for 10 min. Products from this PCR were diluted 1/10 with distilled sterile water and 5 μL of the dilution were used as template for the second PCR. The second PCR was performed as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, followed by a final extension period at 72 °C for 10 min. Two microliter of the PCR products were analyzed performing a gel electrophoresis in 1.5% agarose with SYBR Green (Invitrogen) at 100 V for 30 min. Bands were observed under UV light and were considered positive those with an approximately size of 800 bp. Positive PCR products were cleaned with a kit (AppBiotech, Argentina) following manufacturer's indications. The cleaned amplicon was sequenced using Sanger capillary electrophoresis method by the Instituto Nacional de Tecnología Agropecuaria (INTA, Argentina). The DNA sequences were first analyzed manually with Sequencher program and then a Neighbour Joining (NJ) phylogenetic tree was constructed using a 1000 replicates bootstrap method with MEGA 6 software. Known AMF species 5′ end SSU rRNA sequences were used as reference AMF DNA and two non AMF species were used as outgroup (Lee et al., 2008; Krüger et al., 2012).

2.3.3. Relative abundance of AMF spores

Based on the morphological characterization, relative abundance was calculated by relating the number of spores belonging to each family to the total number of spores.

2.4. Greenhouse experiment

2.4.1. Greenhouse experiment establishment

A greenhouse experiment (Fig. 1) was established in order to evaluate plant growth and, mycorrhizal colonization of *R. communis* plants, when exposed to MOR or PAR AMF community in presence or absence of a mixture of Cr(III)/Cr(VI) polluting concentrations (250/8 $\mu g\ g^{-1}$ soil d.w.). Prior to the start of the experiment, sterile vermiculite was mixed with the soil from each trap cultures in order to obtain a final concentration of 5 AMF spores per gram of soil (Cornejo, personal communication). The vermiculite/trap cultures soil mixtures were placed into pots (550 g; 20 pots per rhizosphere soil) and Cr(III) as CrO_3 and Cr(VI) (as $K_2Cr_2O_7$) were added to half of the pots (10 pots per soil) to achieve a final concentration of 250 μg Cr(III) and 8 μg Cr(VI) g^{-1} soil d.w. (limits allowed for residential areas as mentioned before); controls pots received the same volume of water. The pots were left until the water evaporated (2 weeks approximately). Thus, the treatments were: the AMF community from the polluted area with and without Cr(III) and Cr(VI) addition (MOR + Cr and MOR No Cr, respectively), and the AMF community from the non-polluted area with and without Cr(III) and Cr(VI) addition (PAR + Cr and PAR No Cr, respectively). Then, *R. communis* seeds, previously disinfected with a 1% hypochlorite solution, were sowed and plants were grown for 4 months under greenhouse conditions (mean 25 °C-14/10 h light/dark photoperiod) and were regularly irrigated. At the end of the experiment the plants were harvested and the following parameters were analyzed: i) plant tissues dry weight, ii) total Cr in plant tissues and iii) intensity (%M) and frequency (%F) of the mycorrhizal colonization. Concentration of Cr(VI) in soils was determined at the beginning and at the end of the experiment. A scheme of the experimental design is shown in Fig. 1.

2.4.2. Plant tissues dry weight

The plants were harvested at the end of the experiment. Five plants per treatment were randomly selected, shoots were separated from the roots and the tissues were dried at 80 °C for 48 h. The dry weight was determined with an analytical balance.

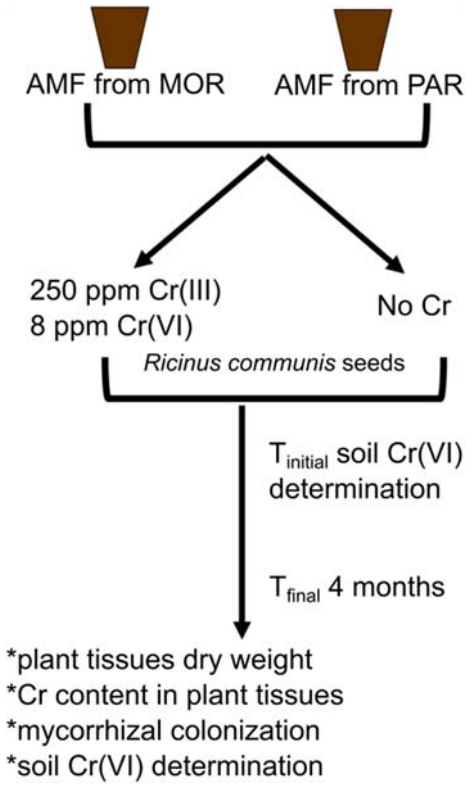


Fig. 1. Greenhouse experiment scheme. AMF communities isolated from *R. communis* rhizospheres from a Cr-polluted site (MOR) and from a non-polluted site (PAR) after 4 months of trap culture with *Sorghum bicolor* as the host plant. The culture traps soils were mixed with sterile vermiculte to obtain 5 AMF spores g^{-1} soil.

2.4.3. Total Cr in plant tissues

Five plants per treatment were randomly selected and total Cr was extracted from root and shoot ashes with an acid solution of 2 N HCl/1 N HNO_3 (1 g ashes: 20 mL acid solution) (Gil-Cardeza et al., 2014). The ashes were obtained by incinerating the dried plant tissues in a muffle at 500 °C for 5–6 h. Total Cr was determined by atomic absorption spectrophotometry (Khan, 2001). Cr concentration in plant tissue was expressed as μg Cr g^{-1} dry weight (DW).

2.4.4. Mycorrhizal colonization

Mycorrhizal colonization in *R. communis* roots was determined by observation of stained roots adapted from Phillips and Hayman (1970) under a light microscope at 400× magnification. Briefly, roots were clarified with KOH 2.5%; *R. communis* roots were incubated for 20 min in the autoclave. Acidification was carried out by incubation of roots overnight at room temperature. After acidification, roots were stained with cotton blue (0.5 g methyl blue: 1 L acid glycerin) for 15 min at 90 °C. The intensity and frequency of the mycorrhizal root colonization (%M and %F, respectively) was estimated as follows (Trouvelot et al., 1986):

$$\%M = \left(95 * n^{\circ}5 + 70 * n^{\circ}4 + 30 * n^{\circ}3 + 5 * n^{\circ}2 + 1\right)/\text{total } n^{\circ}$$

$$\%F = n^{\circ} \text{ of root pieces positive for AMF/total } n^{\circ}$$

where n° counts for the number of root pieces (20 per root) that have a certain degree of colonization. Thus, n°5 > 90%, n°4 > 50%, n°3 < 50%, n°2 < 10% andn°1 < 1%.

2.4.5. Soil Cr(VI) concentration

Total Cr(VI) was extracted from soil with an alkaline solution ($NaOH/Na_2CO_3$; pH = 12) at time 0 and within the first week after ending the experiment in order to minimize changes in Cr redox status (1 g dry soil: 50 mL alkaline solution). Total Cr(VI) extraction is made in an

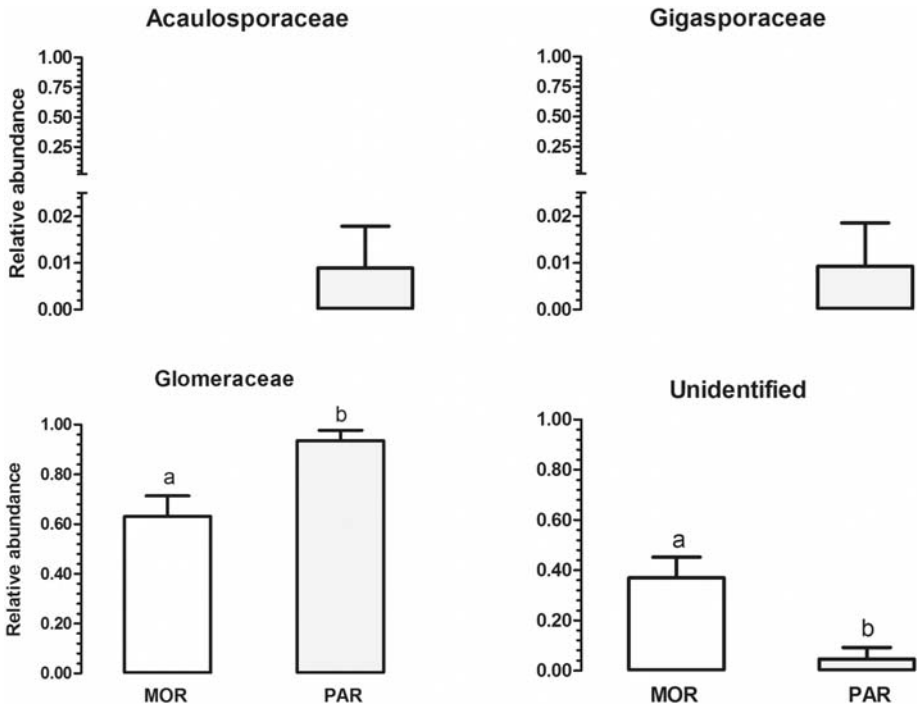


Fig. 2. Relative abundance of AMF families isolated from *R. communis* rhizospheres from a Cr-polluted site (MOR, white bars) and from a non-polluted site (PAR, grey bars) after 4 months of trap culture with *Sorghum bicolor* as the host plant. Results are expressed as means ± SEM (n = 4). Values with different case letters in a column do differ significantly at P ≤ 0.05 (t-student).

extreme alkaline environment to avoid the changes in Cr oxidation states (James et al., 1995). Cr(VI) was analyzed by diphenylcarbazide (DFC) method. The soil extract turns to pink when DFC is added if Cr(VI) is present, and Cr(VI) concentrations is determined photometrically (OD 540 nm) (James et al., 1995).

2.5. Statistical analysis

All the analyses were conducted using INFOSTAT (Di Rienzo et al., 2011) free edition. Plant tissues dry weight and mycorhizal association, %M and %F, were analyzed by two-way ANOVA, and multiple comparisons of medias were made in the interaction with Bonferroni post-test ($\alpha = 0.05$). Differences between AMF spores abundances and between Cr(VI) soil concentration medias were analyzed by T-student. Mycorrhizal colonization (%M) and plant tissues dry weight were transformed by Arcsen and natural logarithm, respectively, in order to meet the homoscedasticity and normality assumptions.

3. Results

3.1. AMF spore community from the trap cultures

3.1.1. Morphological characterization and relative abundance of spores

Morphotypes isolated from trap cultures performed from rhizosphere soil from MOR and PAR were mostly the same as morphotypes found in the original rhizosphere, where Glomeraceae was the most abundant family. An Acaulosporaceae morphotype observed in MOR original rhizosphere was absent in the trap cultures (data not shown). Several Glomeraceae morphotypes were observed in the trap cultures: 5 morphotypes with a relative abundance of 0,63 ± 0,08 in MOR trap cultures and 4 morphotypes with a relative abundance of 0,94 ± 0,04

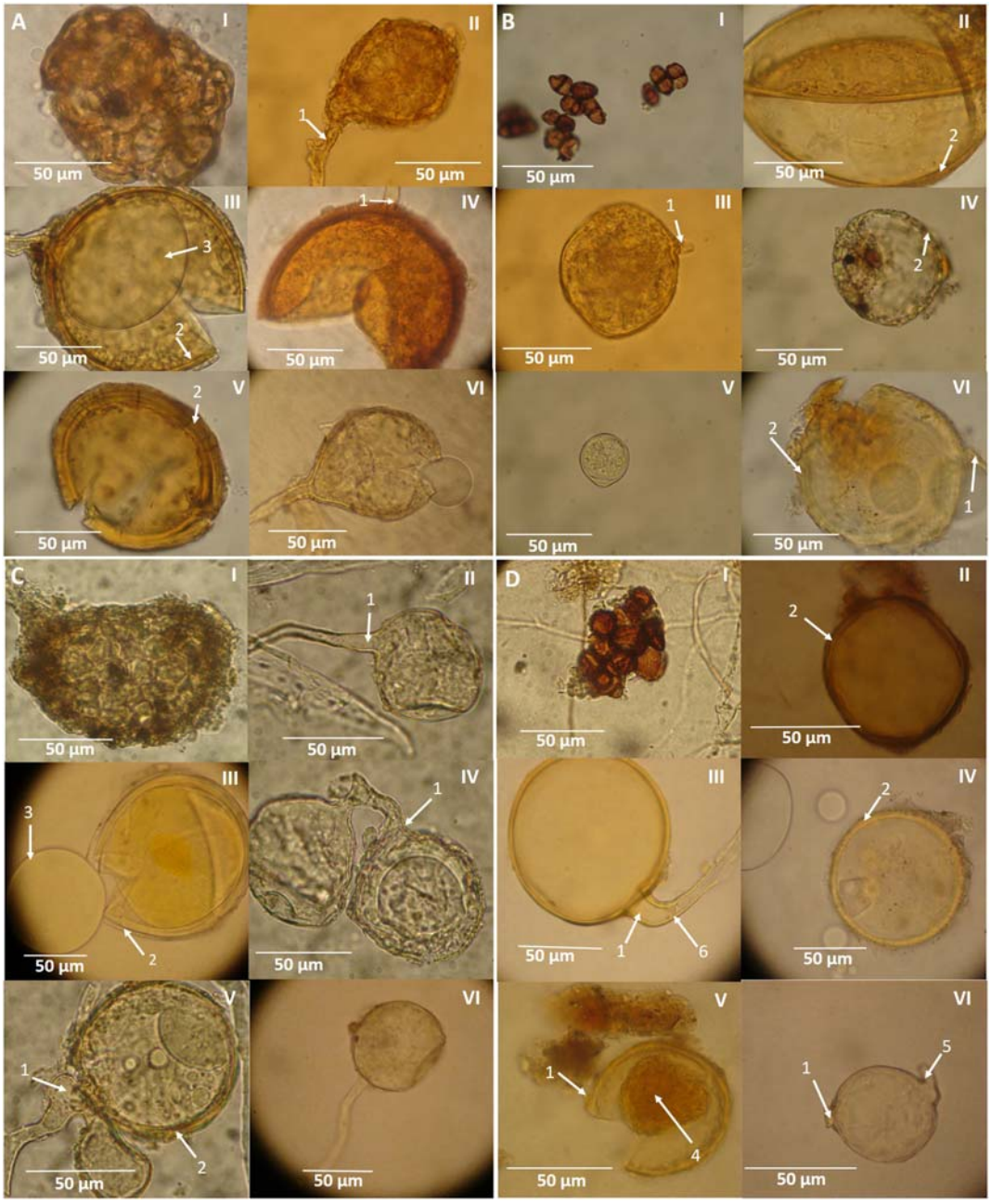
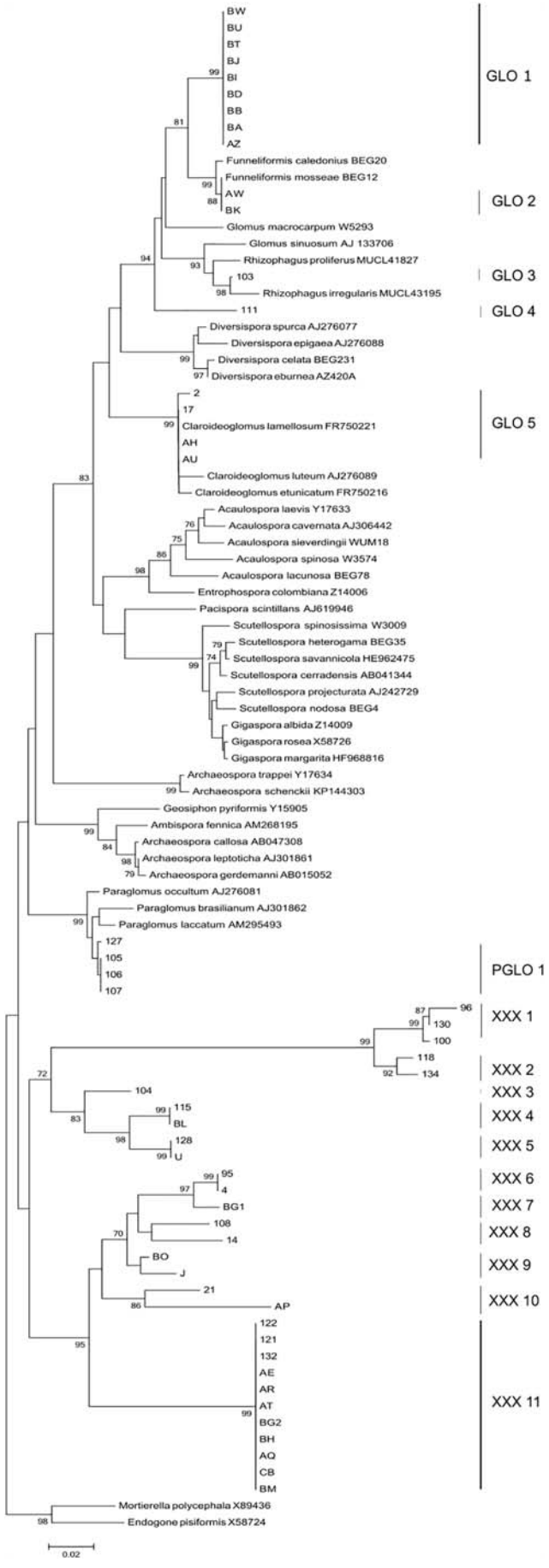


Fig. 3. AMF spores isolated from *R. communis* rhizospheres from a Cr-polluted site (MOR, A and C) and from a non-polluted site (PAR, B and D) after trap culture with *Sorghum bicolor* as the host. 1: Sustentation hypha. 2: Spore wall. 3: Lipid drop. 4: Germination shield. 5: Saccule neck. 6: Septum.



in PAR (Fig. 2 Glomeraceae panel). In contrast, only 1 Acaulosporaceae morphotype and 1 Gigasporaceae morphotype were observed in PAR trap cultures both with a relative abundance of 0,009 ± 0,009 (Fig. 2, Acaulosporaceae and Gigasporaceae panel). It was not possible to classify all the morphotypes observed into a Glomerycota family so they were classified as "unidentified": 15 morphotypes in MOR and 6 in PAR trap cultures, with a relative abundance of 0,37 ± 0,08 and 0,05 ± 0,05, respectively (Fig. 2, unidentified panel). Total AMF spores number was 8 and 7 spores g^{-1}soil in MOR and PAR trap cultures, respectively.

In MOR trap cultures single spore size varied from 50 to 300 μm in diameter, the shape from globose to subglobose and the spore wall structure was variable between each morphotype (Fig. 3A II–VI). The cytoplasmic content was dense, granular or hyaline. Other spores, irregular in shape with a visible hypha just in some few cases were associated forming sporocarps, globose to irregular in shape with a peridium brown-yellowish in color. In PAR trap cultures sporocarps were tight clusters without a peridium and hyphae, small spores in size, ovoid to irregular in shape, dark brown in color (Fig. 3B I). Single spores (Fig. 3B II–VI) varied from 50 to 150 μm in diameter, the shape from globose to subglobose and the cytoplasmic content was tightly granulose or hyaline. Layers of the spore wall varied in thickness and color, mostly brown to brown-yellowish. Number of sporocarps was higher in MOR trap cultures than in PAR trap cultures (14,6 *vs.* 2,72%, respectively) (Fig. 3A I and C I). The spores that classified in the Gigasporeceae family had an internal structure which could correspond to the germination shield; the spores had a diameter of 60 μm, and the spore wall a thin and hyaline external layer and a yellow-brown inner layer (Fig. 3D V). The spore morphotype classified in Acaulosporacea family had a diameter of 30 μm and a membranaceous, thin and hyaline spore wall attached to the neck of the saccule (Fig. 3D VI).

3.1.2. Characterization of spores by SSU rRNA gene partial sequence

Amplicons from the expected size were obtained (*i.e.* ~800 bp for Glomerycota species, Lee et al., 2008). Each positive amplicon was sequenced and a Neighbour joining phylogenetic tree was constructed (Fig. 4). The analysis revealed 10 monophyletic ribotypes; five belong to Glomeraceae (GLO1-GLO5), one to Paraglomeraceae (PGLO1) and eleven that did not group with previously identified AMF species (XXX1-XXX11; Fig.4). AMF species cultured from the rhizosphere soil from MOR grouped exclusively in GLO3, GLO4, XXX1, XXX2, XXX3, XXX6, XXX8 and PGLO1, while AMF from the rhizosphere soil from PAR grouped exclusively in GLO1 and GLO2, XXX7 and XXX9 ribotypes (Table 1). The rest of the rybotipes (GLO5, XXX4, XXX5, XXX10 and XXX 11) contained AMF species from both cultured rhizospheres (Table 1). Three GLO ribotypes could be identified as AMF genre: GLO2 as *Funneliformis*, GLO3 as *Rhizophagus* and GLO5 as *Clareidoglomus* (Fig. 4).

3.2. Greenhouse experiment

The dry weight of *R. communis* shoots and roots was not affected by the addition of Cr(III) and Cr(VI) or by the origin of the AMF community (Fig. 5A and B). Total Cr content from each replicate was below the limit of detection so pooled samples were analyzed. Total Cr content in the pooled tissues were: 87 and 97 μg Cr g^{-1} DW of shoots and 1545 and 1840 μg Cr g^{-1} DW of roots in *R. communis* plants grown with PAR and MOR AMF communities, respectively.

Arbuscular mycorrhizal structures were observed in all roots of *R. communis* (Fig. 6). The AMF structures were mostly hypha, vesicles and spores; arbuscules were occasionally found. The highest mycorrhizal colonization (M%) was determined in roots grown in presence of

Fig. 4. Neighbour-joining tree of AMF partial SSU rRNA gene amplified from spores isolated from *R. communis* rhizospheres from a Cr-polluted site (MOR, numbers) and from a non-polluted site (PAR, letters) after 4 months of trap culture with *Sorghum bicolor* as the host. Bootstrap values were estimated from 1000 replicates. Chromium polluted amplicons are represented in by numbers; non-Cr polluted amplicons are represented with letters. Only support values above 70 are shown at the branches.

Table 1
Ribotype richness of AMF spores isolated from *R. communis* rhizospheres from a Cr-polluted site (MOR) and from a non-polluted site (PAR) after trap culture with *Sorghum bicolor* as the host.

Ribotype	*R. communis* rhizosphere cultivated in trap culture	
	MOR	PAR
GLO 1	–	9
GLO 2	–	2
GLO 3	1	–
GLO 4	1	–
GLO 5	2	2
PGLO 1	4	–
XXX 1	3	–
XXX 2	2	–
XXX 3	1	–
XXX 4	1	1
XXX 5	1	1
XXX 6	2	–
XXX 7	–	1
XXX 8	2	–
XXX 9	–	2
XXX 10	1	1
XXX 11	3	8

GLO, Glomeraceae; PGLO, Paraglomeraceae; XXX, unidentified.

AMF isolated from MOR regardless Cr(VI) soil concentration (Fig. 6A, white columns). The lowest M% was determined in the roots grown in the Cr(VI) polluted pots and in presence of the PAR AMF community (Fig. 6A, grey columns). The Cr(III)/Cr(VI) addition reduced the frequency of AMF (Fig. 6B, No Cr *vs.* + Cr).

Initial (Fig. 7A) and final Cr(VI) (Fig. 7B) soil concentrations were highest in PAR soils that contained the non-poll AMF community, compared to MOR soils containing the poll AMF community.

4. Discussion

4.1. AMF spore community

Arbuscular mycorrhizal fungi richness was higher in MOR trap cultures (20 morphotypes and 13 ribotypes) compared to PAR trap cultures (12 morphotypes and 9 ribotypes). Contrastingly, Khan (2001) reported a lower level of diversity of AMF morphotypes in Cr-polluted rhizospheres, in comparison to non-Cr polluted. There is not a clear consensus about the effect of PTE in AMF diversity. Some studies have reported a lower richness of AMF morphotypes/ribotypes (Sánchez-Castro et al., 2017, Wei et al., 2014, Hildebrandt et al., 2007, Del Val et al., 1999) while other researchers reported a higher richness in PTE polluted soils as compared to non-polluted PTE soils (Krishnamoorthy et al., 2015; Wu et al., 2010). Glomeraceae family was the most abundant in both trap cultures, according to the morphological characterization (0.630 and 0.936, MOR and PAR, respectively). This observation is in agreement with most of the AMF diversity surveys around the world in PTE polluted sites (Sánchez-Castro et al., 2014; Krishnamoorthy et al., 2015; Wu et al., 2010; Del Val et al., 1999). The richness in Glomeraceae family achieved with the morphological characterization was higher in MOR than in PAR (5 and 4 morphotypes respectively) while it was the same between both soils when the DNA sequenced was analyzed (3 and 3 ribotypes). In the unidentified group the richness was always higher in MOR than in PAR with both characterization methods (15 and 6 morphotypes and 9 and 6 ribotypes, in MOR and PAR trap cultures, respectively). AMF species that belonged to the Acaulosporaceae and Gigasporaceae family were only detected with the morphological approach in PAR trap cultures while an AMF specie that belonged to the Paraglomeraceae family was detected with the molecular approach in MOR trap cultures. These differences suggest that the two approaches used in this study to characterized AMF communities were complementary. The number of morphotypes was higher when the community was analyzed with the morphological technique as compared to the number of ribotypes obtained with the molecular characterization (20 *vs.* 13 for MOR trap cultures and 12 *vs.* 9 in PAR trap cultures). Some inhibitors like phenolic compounds could have occurred in some of the DNA extracted from the spores and thus not have been amplified. In fact, 40% of the total spores/DNA samples isolated amplified after the nested PCR reaction.

The phylogenic tree was constructed only with DNA sequences from characterized AMF species as reference; no uncultured sequences were added. With this approach it was possible to classify 3 Glomeraceae ribotypes to genera level and very closely related to 3 AMF species: *Funneliformis mosseae*, isolated from PAR trap culture; *Rhizophagus irregularis*, isolated from MOR trap culture and *Clareidoglomus lamellosum*, with spores isolated from both cultured rhizosphere soils. The presence of *R. irregularis* was reported by several authors in sites polluted with PTE: Krishnamoorthy et al. in a site polluted with As, Cd and Zn (2015), Schneider et al. in an As polluted site (2013) and Zarei et al. in a Pb and Zn polluted site (2010). Pawlowska and Charvat (2004) reported that the hyphal density in the root compartment of *R. irregularis* associated to transformed root *in vitro* increased when the ERM, from the hyphal compartment, was exposed to increasing concentrations of Cd, Pb or Zn, suggesting that *R. irregularis* could adapt to PTE polluted environments. In accordance to this hypothesis, the phylogenetic tree analysis showed that spores isolated from MOR were closely related to *R. irregularis*.

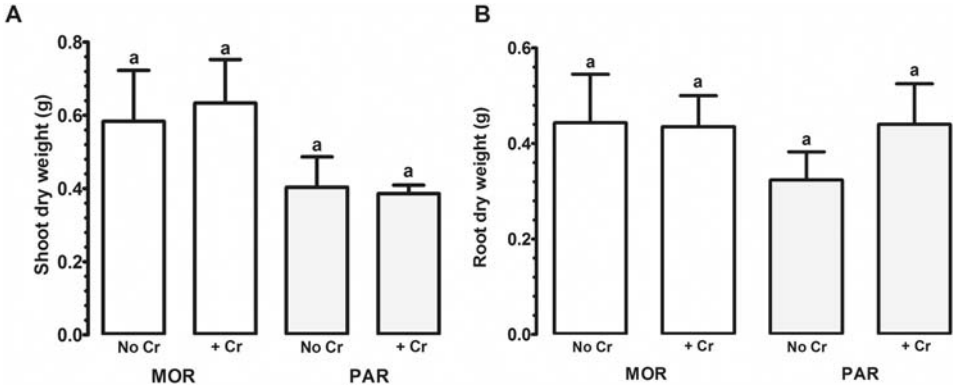


Fig. 5. Dry weight of *Ricinus communis* shoots (A) and roots (B) after 4 months of growth with an AMF community isolated from a Cr-polluted soil (MOR) or with an AMF community isolated from a non-Cr polluted soil (PAR) with or without the addition of polluting Cr concentration. The AMF communities were isolated from *Ricinus communis* rhizospheres derived trap cultures using *Sorghum bicolor* as the host plant. Values with the same lower case letters in a column do not differ significantly at $P \leq 0.05$ (two-way ANOVA, Bonferroni post-test in the interaction). n = 4. No Cr: no addition of Cr; +Cr: addition of Cr to a final concentration of 250 Cr(III) and 8 Cr(VI) μg Cr g^{-1} soil.

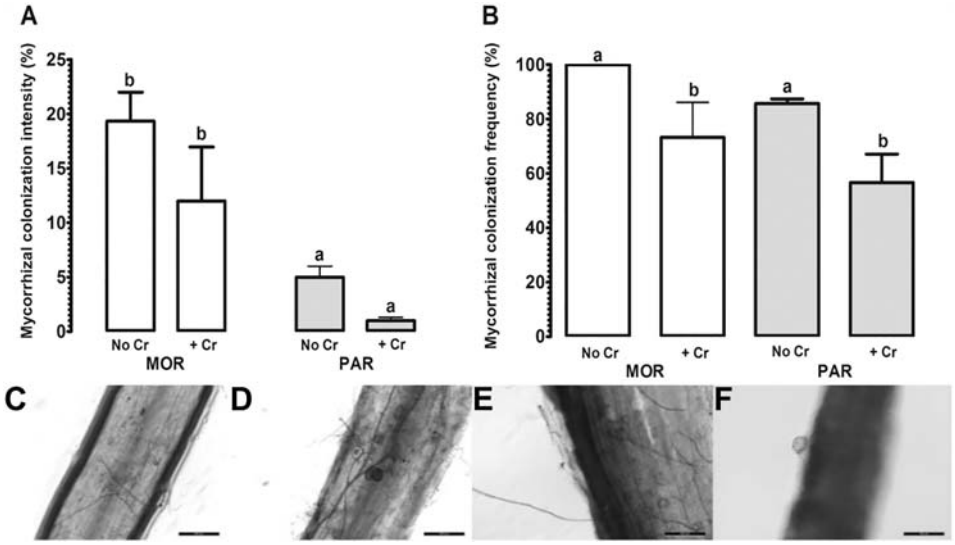


Fig. 6. Mychorrhizal root association in *Ricinus communis* after 4 months of growth with an AMF community isolated from a Cr-polluted soil (MOR) or with an AMF community isolated from a non-Cr polluted soil (PAR) with or without the addition of polluting Cr concentration. The AMF communities were isolated from *Ricinus communis* rhizospheres derived trap cultures using *Sorghum bicolor* as the host plant. Values with the same lower case letters in a column do not differ significantly at $P \leq 0.05$ (two-way ANOVA, Bonferroni post-test in the interaction). n = 4. No Cr: no addition of Cr; +Cr: addition of Cr to a final concentration of 250 Cr(III) and 8 Cr(VI) μg Cr g^{-1} soil. A: Mycorrhizal colonization intensity B: Mycorrhizal colonization frequency. C, D, E and F: AMF structures observed in *R. communis* roots stained with cotton blue, C: MOR with No Cr, D: MOR +Cr, E: PAR with Cr, F: PAR + Cr.

4.2. Greenhouse experiment

As expected, in accordance with a previous *in situ* study (Gil-Cardeza et al., 2014), total Cr concentration in the aerial part of *R. communis* was lower than those reported for hyperaccumulator species of Cr (1000 μg g^{-1} DW, Ali et al., 2013). *Ricinus communis* roots grown in presence of PAR AMF community had a %M below 5% whereas %M in roots grown in presence of MOR AMF community was above 10%. The higher %M observed in roots inoculated with MOR AMF community would indicate that the prolonged exposure to Cr could have selected species with a higher infectivity. As regards to that, Pawlowska and Charvat (2004) found that the presymbiotic hyphal extension of a *R. irregularis* culture derived from an unpolluted environment increased with Cd and Pb concentrations when cultured *in vitro*. No increase was observed in the presymbiotic hyphal extension of a *F. mosseae* culture. Spores that closely related to *R. irregularis* were found in MOR trap cultures and so could partially explain the higher %M. In agreement, spores that closely related to *F. mosseae* were only found in PAR trap cultures. Nevertheless, the addition of Cr had a negative effect on AMF colonization in both AMF communities, as shown by the lower %F detected in roots from MOR and PAR pots exposed to the polluting concentrations of Cr, as compared to non Cr exposed control roots.

A mixture of Cr(III)/Cr(VI) was added to half of the pots to obtained an initial concentration of 250Cr(III)/8 Cr(VI) μg g^{-1} soil d.w. However, initial Cr(VI) concentration was 10 times higher than the Cr(VI) concentration added to the soils (80 and 60 μg Cr(VI) g^{-1} soil d.w., MOR and PAR respectively). The pots were a closed system and since oxidation-reduction reactions can occur between Cr(III) and Cr(VI) redox species, likely the higher Cr(VI) initial concentration can be attributed to oxidation of part of the Cr(III). Moreover, the addition of Cr(III) as a soluble form (CrO_3) could have favor the oxidation of Cr(III) to Cr(VI) (James, 1996). Final Cr(VI) concentration in soil was lower in the pots that contained the AMF from MOR than in the soils from the pots that contained the AMF from PAR (6.9 ± 0.2 and 14.3 ± 1.5 μg Cr(VI) g^{-1} soil d.w., respectively). At the end of the experiment, the pots inoculated with MOR AMF community could be considered as non Cr(VI) polluted, since Cr(VI) final concentration in the pots was below the allowed limit by Argentinian legislation (8 μg Cr(VI) g^{-1} soil). On the other hand, in pots inoculated with PAR AMF community Cr(VI) soil concentration remained above the allowed limit.

The soils used for the greenhouse experiment were derived from trap cultures previously made to propagate both AMFs communities, so it is possible to assume that the soils were enriched in AMF, as compared to other soil microorganisms. Thus the differences in Cr(VI) soil

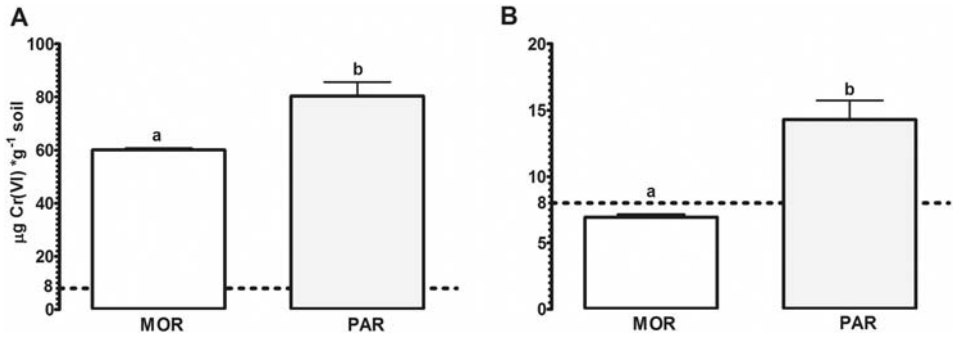


Fig. 7. Initial (A) and final (B) total Cr(VI) soil concentrations. Final Cr(VI) total concentration was determined in soils after 4 months of growth with an AMF community isolated from a Cr-polluted soil (MOR) or with an AMF community isolated from a non-Cr polluted soil (PAR). The AMF communities were isolated from *Ricinus communis* rhizospheres trap cultures using *Sorghum bicolor* as the host plant. T-student, different lettersindicate significant differences ($P < 0.05$). Dashed lines: the allowed value for residential areas in Argentina (Regulative order 831/93 law 24051) n = 5.

final concentration could be attributed to the differences in %M between both treatments, MOR and PAR, strongly suggesting that the previous exposure to Cr(VI) selected an AMF community (MOR) able to diminish more efficiently Cr(VI) concentration in soil. The low Cr content in the aerial tissues and the presence of AMF structures in *R. communis* roots points mycorrhizostabilization as the most likely bioremediation mechanism of the *R. communis*/AMFsystem.

As far as we know, there are no previous reports about the characterization of an AMF community adapted to a Cr(VI)-polluted site. Our results strongly suggests that the exposure of an AMF community to Cr(VI) selected AMF species, *i.e. R. irregularis*, able to tolerate polluting concentrations of this PTE and thus with a good potential to be used as bioremediation agents. Based on the knowledge of the composition of the AMF spore community analyzed in the present study, future research should be focused on evaluating the bioremediation capacity of single morphotypes.

Conflict of interest

The authors of the manuscript entitled "Differential response to an artificial soil Cr polluting concentration of two Arbuscular mycorrhizal fungi communities isolated from a chromium polluted and non-polluted rhizospheres" declare no conflict of interest.

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