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Taxonomical and functional analysis of four arbuscular mycorrhizal fungi populations obtained from a *Ricinus communis* rhizospheric Cr(VI) polluted soil

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ABSTRACT

In a global context of climate change and loss of biodiversity, phytoremediation appears as a viable strategy to recover polluted soil. Phytoremediation is defined as a strategy to recover polluted soils by means of plants and the associated microorganisms. Arbuscular mycorrhizal fungi (AMF) are one of the most widespread soil microorganisms, they live in symbiosis with 70% of terrestrial plants. In the symbiotic relation, the fungal partner incorporates carbohydrates and lipids facilitated by the plant and the plant incorporates minerals facilitated by the fungal partner. Then, the inclusion of AMF in phytoremediation strategies should become a priority, not only because the presence of AMF will help the plant to adapt to the polluted soil but also because it could enhance the incorporation of carbon to the soil. In addition, the actual context of global biodiversity loss prioritizes the study of local populations in order to promote the incorporation of the local biodiversity to soil management, that includes phytoremediation. In the present work we aimed to taxonomically characterise four AMF populations obtained from *Ricinus communis* rhizospheric Cr(VI) polluted soil. In addition, we aimed to study the symbiosis and the mineral uptake of some elements, including phosphorus and chromium, in *R. communis* plants associated with each AMF population and re-exposed to Cr(VI). We found that three AMF populations grouped near *Rhizophagus* and one near *Paraglomus* accessions and that the four AMF populations were tolerant to the re-exposure to 8 ppm Cr(VI) substrate concentration. Finally, from the mineral content analysis, our results strongly suggest that *Paraglomus* sp., a taxon which appeared earlier in the evolution of AMF, was the population that best adapted to the re-exposure of 8 ppm Cr(VI). Thus, we suggest that future phytoremediation studies should include taxa from this early diverged genus.

1. Introduction

Since the 1800s, human activities have been the main driver of climate change, primarily due to burning fossil fuels (i.e., coal, oil and gas). Industrial activities are one of the principal activities that contributes to climate change (United Nations, 2022). Unfortunately, industrial activities not only pollute because of the burning of fossil fuel, but also pollute the environment with the waste it produces. In this context, phytoremediation appears as a cheap and sustainable strategy not only to recover polluted soil but also to mitigate climate change (Morriën et al., 2017). Phytoremediation consists in the use of plants, and the associated microbiota, to recover polluted soils (Ali et al., 2017; Ferrol et al., 2016). Hence, phytoremediation not only has the capacity to recover polluted soils but also it contributes to the reduction of carbon dioxide (CO_2) since plants transform CO_2 to sugars by photosynthesis (Trivedi et al., 2020). In this sense, the study of the associated

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microbiota has the potential to positively contribute to CO_2 fixation in soil (Morriën et al., 2017).

Potentially Toxic Elements (PTEs) are chemical elements that naturally occur in soil which can become hazardous for biodiversity and life when present in high concentrations (Chiubike and Obiora, 2014). The risks linked with the presence of PTEs depends on their toxicity, bioavailability, the total quantity in the environment and its bioaccessibility. Sites that are close to industrial areas are prone to have high concentrations of PTEs in soil, thus affecting the surrounding ecosystem particularly the chemical, biological and physical soil properties (Schneider et al., 2013). Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Lead (Pd) and Zinc (Zn) are the most common PTEs around industrial sites (Gil Cardeza et al., 2018). Particularly, Cr, is commonly used in leather processing, pigments production, catalysts production (Jacobs and Testa, 2005). Chromium has two oxidation states in soil: Cr(III) and Cr(VI). Chromium (III) has low bioavailability as it precipitates in the soil while Cr(VI), is a class A carcinogen (IARC 1990), highly soluble, mobile and therefore bioavailable. Chromium(VI) can be found as chromate anion (CrO_4^-) or as chromate salts (e.g. $CaCrO_4$, $BaCrO_4$, $PbCrO_4$) in neutral to alkaline soils. Waste from the tanning industry contains a large amount of Cr(III), approximately 40% of the Cr(III) is discarded, and when it is deposited in the soil, up to 15% can be oxidised to Cr(VI) (James, 1996; Saha and Orvig, 2010). Gil-Cardeza et al. (2014) reported the presence of Cr(VI) in soils near Morón river, in Buenos Aires province, Argentina. In the mentioned work they found Cr in a soil fraction associated with glomalin, a protein synthesised by arbuscular mycorrhizal fungi (AMF), strongly suggesting an *in situ* role of AMF in Cr soil immobilisation.

Arbuscular mycorrhizal fungi are one of the most widespread soil microorganisms (Brundett and Tedersoo, 2018). They develop symbiotic associations with 70–90% of land plants species since plants began to grow in the terrestrial environment. Arbuscular mycorrhizal fungi are obligate biotrophs that belong to the *Glomeromycota* monophyletic phylum (Parniske, 2008). Through the symbiotic relation, AMF incorporates sugars and lipids synthesised by the plant (upto 20% of the photosynthesis products may be consumed by AMF as reported by Jakobsen and Rosendahl (1990)), and plants incorporate minerals (i.e. phosphorus) and water (Trivedi et al., 2020). Communication between the symbiotic partners, that allows the exchange of biomolecules, occurs in the arbuscules, a tree-shaped subcellular structure present between the root cell wall and the plasmatic membrane. Another structure that may appear in the symbiosis are vesicles, which are fungal structures that accumulate principally phosphate as polyphosphates (Brundett and Tedersoo, 2018). The nutritional benefits could also appear as a better plant health status (i.e. better defence to pathogens or differential metabolic responses to environmental stress such as the presence of high levels of PTEs). In addition, the consumption of carbohydrates by AMF may improve carbon fixation to the soil. In this sense, the use of AMF in phytoremediation strategies could improve plant establishment in the soils to be restored while contributing to mitigation of climate change (Morièn et al., 2017).

Diversity AMF analysis reports more than 300 species around the world (Öpik et al., 2013). Arbuscular mycorrhizal fungi are ubiquitous organisms, they live in all terrestrial environments, even in hostile environments such as saline soils and PTEs polluted soils (Ferrol et al., 2016). Thus, the study of AMF communities isolated from PTEs polluted sites has the potential not only to improve plant establishment in the soils to be restored but also the potential to promote soil diversity if phytoremediation strategies are designed with the indigenous communities instead of AMF populations from a different origin (Emam, 2015).

In the present work we aimed to characterise taxonomically four AMF populations obtained from *Ricinus communis* L. rhizospheric soil in a Cr(VI) polluted soil (Gil-Cardeza et al., 2014, 2018) and to study the symbiosis and the mineral uptake of some elements, including P and Cr, in *R. communis* plants when re-exposed to Cr(VI). The results of the study are, to our knowledge, the first ones that explores some physiological traits of indigenous AMF populations isolated from a Cr(VI) polluted site. The research on local-indigenous populations promotes the study on diversity, a key factor in order to mitigate climate change. Thus, our results will contribute to the generation of knowledge on AMF diversity, prone to be used in a phytoremediation strategy at the site that also behold mitigation of climate change. We chose *R. communis,* even though it is not a native species, since it was one of the most abundant plant species found in the polluted site and has a fast grow, thus it could be used in a first stage of a phytoremediation strategy (Gil-Cardeza et al., 2014).

2. Material & methods

2.1. Obtention of arbuscular mycorrhizal fungi populations from a Cr(VI) polluted soil

Four AMF populations were obtained from an AMF community sampled from *Ricinus communis* rhizospheric Cr(VI) polluted soil (Gil-Cardeza et al., 2014, 2018). The AMF community was grown continuously for two years under greenhouse conditions using trap culture (TC) technique (host plants: sorghum *(Sorghum bicolor* (L.) Moench), ricinus (*Ricinus communis*) and alfalfa (*Medicago sativa* L.)). AMF populations were obtained by inoculation of fifty (50) morphologically similar (colour, size, shape) spores into roots of three sorghum seedlings of two weeks old. Spores were isolated from the AMF community by the technique of wet sieving and decanting (Oehl et al., 2003). Four different morphotypes were found and were preliminarily named as "transparent" (T), "brown" (Br), "big" (Bi) and "yellow" (Y). Inoculated sorghum seedlings were grown in individual pots of 12 cm diameter (0.25 L) with a mixture of autoclaved sand and clay (4:1) and were fertilised by the addition of 375 mg per pot of slow release fertiliser (1.5 g * L^{-1}) (Basacote 9M®). Five pots were prepared for each morphotype. After 6-7 months one plant per pot was harvested and the root was stained with cotton blue technique for AMF determination (Phillips and Hayman, 1970). Each AMF morphotype was amplified by transplanting one pot of each morphotype into a plastic tray of 35 cm wide by 65 cm long and 5 cm high (approximately 11 L) previously filled with a mixture of autoclaved sand-clay (4:1). Sorghum and ricinus plants were used as host plants; plants were fertilised by the addition of 17 g slow release fertiliser (1.5 g * L^{-1}) (Basacote 9M®). A control tray, without AMF, was also prepared (Fig. 1).

2.2. Taxonomic characterization of AMF populations

Taxonomic characterization of the four AMF populations was performed with data obtained from: i) observation of spore morphology, ii) characteristics of the symbiotic structures in *Ricinus communis* roots, iii) and the analysis of the SSU rRNA DNA sequences obtained from AMF spores. AMF spores were obtained from each tray with the wet sieving and decanting method (Oehl et al., 2003).

2.2.1. Morphological analysis

Approximately, 10 AMF spores from each tray were mounted onto a slide glass with polyvinyl alcohol-lactic acid-glycerol (PVLG) and observed under a light microscope (20–40×). The taxonomic identification was made at the Family level, based on the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM) and the manual "The Glomeromycota: A species list with new families and new genera" (Schüssler and Walker, 2010). The morphological characters evaluated were: supporting hypha, spore wall (colour, consistency, layers, presence of ornamentation), presence of sporogenous sac, presence of germination shield.

For the evaluation of the AMF symbiotic structures, roots of ricinus host plants were harvested from each tray, stained and observed under microscope (Phillips and Hayman, 1970). The symbiotic structures identified were vesicles, arbuscules and auxiliary cells.

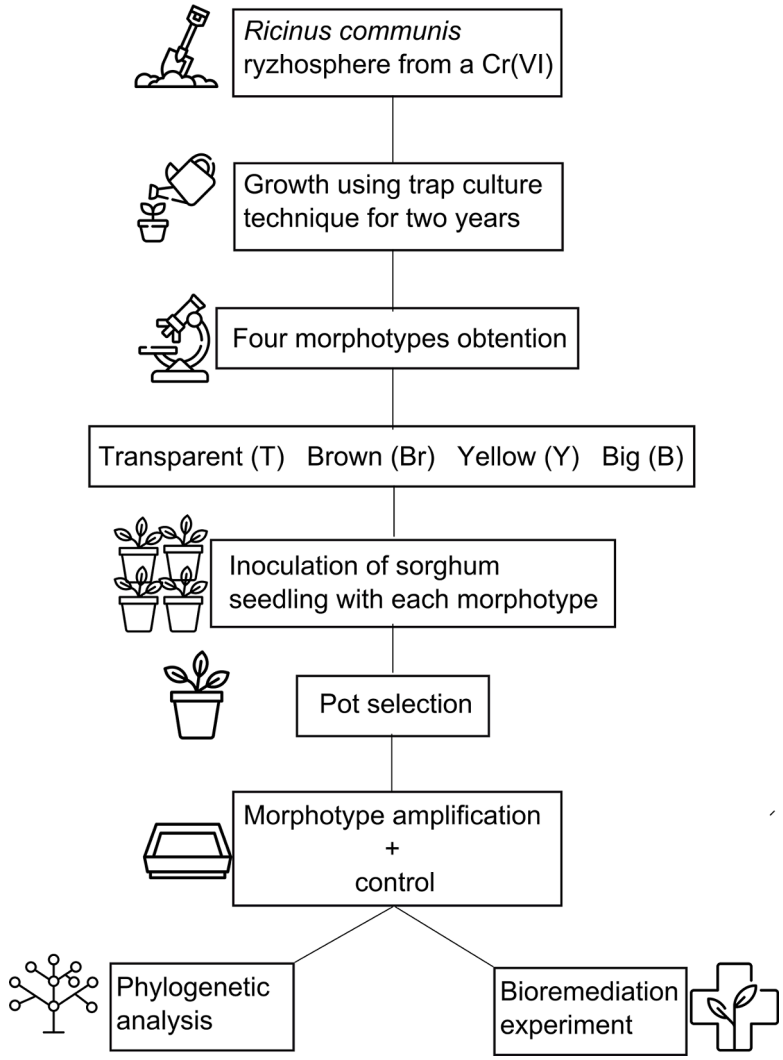

Fig. 1. Methodological scheme for the present study: from AMF population obtention to morphological and phylogenetic characterization.

2.2.2. *Molecular analysis*

SSU rRNA DNA amplification. DNA was extracted from single AMF spores. Single spores were selected under a dissecting microscope and transferred together with 2.5 μL of sterile distilled water to a sterile PCR tube using a 10 μL tip (Lee et al., 2008). Each spore was 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) and performed for the characterization of the AMF community (Gil-Cardeza et al., 2018). 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.

PCR products were analysed performing a gel electrophoresis in 1.5% agarose with SYBR Green (Invitrogen) at 100 V for 30 min and then observed under UV light. Positive PCR products (amplicons of 800 bp size) were cleaned by the precipitation technique. Briefly, the PCR product was precipitated by incubation for 60 min on ice with a potassium acetate solution (3M; Biopack, pro-analysis) and glacial acetic (11.5% w/V, Cicarelli, pro-analysis (99.5% w/w)) followed by a final precipitation step with 2 vol of absolute ethanol (Biopack, pro-analysis (> 99.5% w/w)). The cleaned amplicon was resuspended in 10 ul of sterile de-ionized water and sequenced using Sanger capillary electrophoresis method by the Buenos Aires University sequencing service (UBA, Argentina).

DNA sequences analysis. Forward and Reverse DNA sequences were first edited and analysed with Sequencher v.1.4.1 (Gene Codes Corporation, Ann Arbor, MI USA) to obtain a consensus sequence (700-800 bp size). All sequences were aligned in MEGA 6.06 (Tamura et al., 2013) using pair-wise ClustalW aligment (Larkin et al., 2007). Three consensus sequences were obtained for T, Bi y Y; and two for Br morphotype. Phylogenetic analysis was performed using a 1,000 replicates Bootstrap method with the Maximum Parsimony (MP) and Maximum Likelihood (ML) methods including gaps as informative data; the Tree-Bisection-Regrafting (TBR) algorithm was used for MP (TBR; Nei and Kumar, 2000). Known 5′ end SSU rRNA sequences, obtained from GenBank, were used as reference AMF DNA and two non AMF species were used as outgroups (Lee et al., 2008; Krüger et al., 2012, Gil-Cardeza et al., 2018). Finally, the sequence alignment had 724 bp length out of 59 accessions, including the studied morphotypes and GenBank sequences.

2.2.3. Phylogenetic combined analysis

Molecular and morphological data were combined and the matrix was analysed in TNT program (Tree analysis using New Technology) (Goloboff et al., 2008) using Maximum Parsimony criterion with an heuristic search with 10,000 replicates of Wagner trees followed by TBR branch swapping (holding 10 trees per replicate). To evaluate node support 1,000 bootstrap replicates were calculated and a strict consensus tree was computed from all the most parsimonious trees found.

2.3. Response to Cr(VI) re-exposure of the AMF populations

2.3.1. Experimental design

In order to study the response of each AMF population when re-exposed to Cr(VI) an experiment under semi-control conditions was designed. Twenty *R. communis* seeds, previously disinfected with 1% w/V $Ca(OCl)_2$ (Sigma, technical grade), were seeded on each AMF population tray, including the non AMF control tray. After 3 weeks, three *R. communis* plants were harvested from each tray and the intensity of the AMF symbiosis was determined. The percentage of AMF intensity varied from 12 to 28% between populations; no symbiosis was observed in *R. communis* plants grown in no AMF control trays. Then, at week 4 after the seedling, five *R. communis* plants from each tray were transplanted individually to pots of 0.5 L (5 trays, 5 plants per tray = 25 plants in total). The pots were filled with a mixture of sterile substrate of sand and clay (4:1). Plants were irrigated weekly with 50 ml of low P Hoagland solution (Gil-Cardeza et al., 2021). After an acclimation period of 3 weeks plants were irrigated with a Cr(VI) solution ($K_2Cr_2O_7$; Cicarelli, pro-analysis) in order to obtain an initial Cr(VI) concentration of 8 μg * g $soil^{-1}$ (regulative order 389/93, law 24051). *R. communis* plants were harvested after 3 months of the addition of Cr(VI) and the following determinations were made: total Cr(VI) in substrate; plant tissue dry weight; Chromium (Cr), Phosphorus (P), Iron (Fe), Zinc (Zn) and Copper (Cu) content and concentration in plant tissues; intensity of mycorrhizal association together with the percentage of arbuscules and vesicles.

2.3.2. Total Cr(VI) determination

Total Cr(VI) was extracted by the alkaline method (1 g dry soil: 50 ml alkaline solution, $NaOH/Na_2CO_3$ (Cicarrelli, pro-analysis), pH=12; James 1995). Chromium(VI) concentration was determined in the extracted fraction by diphenylcarbazide (DPC) photometric method (Sigma, reagent e). In presence of Cr(VI) in the medium, the solution turns to pink following addition of DPC. The colour intensity is positively correlated to the concentration of Cr(VI) (James et al., 1995). Thus, for Cr(VI) quantification, a calibration curve was done (i.e. 0.4, 0.8, 1, 2, 4, 6, 8 μg Cr(VI)).

2.3.3. Plant tissues dry weight

Ricinus communis plants were harvested at the end of the experiment (20 weeks old plants). Shoots were separated from the roots and the tissues were dried at 80°C for 96 h. The dry weight was determined with an analytical balance.

2.3.4. Mineral quantification in plant tissue

Dried shoots or roots were grounded separately in a grinder. One hundred mg of each tissue were completely mineralized with the addition of 1 ml HNO_3 (Cicarrelli, pro-analysis (65% w/w)) and incubation for approximately 6 h in a hot water bath (90-100°C). The mineral solutions were diluted with ultrapure water (Millipore, France) to a final volume of 10 mL, and filtered with filter paper N°1 (Whatman, UK) followed by another filtration step with 0.22 μm syringe filter into a 15 mL falcon tube. Chromium (Cr), Copper (Cu), Iron (Fe), Phosphorus (P) and Zinc (Zn) were determined by ICP-MS (NexION 350X, Perkin Elmer). Total mineral concentrations were converted from ppm to mg kg^{-1} and shoots and roots mineral content were determined according to the dry weight of the tissues.

2.3.5. Arbuscular mycorrhizal association

Dry roots were placed in Falcon tubes and re-hydrated for 48 h in deionized water (Gil-Cardeza et al., 2021). The roots were then incubated at 70°C in a water bath for 30 min in 50 mL KOH 10% w/V (Cicarelli, pro-analysis). The KOH was removed and roots were rinsed thoroughly with tap water and rinsed for 5 min with HCl 1% w/V (Cicarelli, pro-analysis (36.5-38% w/w)). Roots were further stained in 25 mL of cotton blue 0.05% w/V (Biopack, suitable microscopy) containing HCl (0.05% w/V) and glicerin (50% w/V; Biopack, pro-analysis (99.5% w/w)) and incubated at 90°C in a water bath for 20 min. The roots were finally rinsed and stored in acid glycerin before observation (Phillip and Hayman, 1970).

For observation, twenty root fragments of ~10 mm length were mounted on microscope slides and examined under a compound microscope at 20-40 X magnifications. The intensity of mycorrhizal association (%I), arbuscule abundance and vesicle abundance in mycorrhizal root system were calculated (%A and %V, respectively). The %I was calculated as follows: (v + 5w + 30x + 70y + 95z)/(v+w+x+y+z), where v, w, x, y, z are the number of root fragments containing an increasing proportion (i.e. v: <1%, w: 1-10%, x: 11-50%, y: 51-90%,z: > 90%) of AMF structures (adapted from Plenchette and Morel, 1996). The %A and %V were calculated with the formula: (100A3 or V3 +50A2 or V2 +10A1 or V1)/mb where A3 or V3, A2 or V2 and A1 or V1 are the number of root fragments containing an increasing proportion (i.e. A3 or V3:>50%, A2 or V2:10-50%, A1 or V1<10%) of arbuscules or vesicles, and mb is the total number of the 20 root fragments containing AMF structures.

2.4. Statistical analysis

All analyses were conducted using INFOSTAT (Di Renzo et al., 2011). Differences between medias were analysed with One-way ANOVA ($p \leq 0.05$). Multiple comparisons between medias was made with the Tukey post test ($p \leq 0.05$). Intensity of the mycorrhizal association (%I) didn't assume the assumptions for homoscedasticity and normality and were transformed with arcsine $\sqrt{}$. Assumptions for homoscedasticity and normality were met for the rest of the data analysed. The relation between total Cr and P plant tissue concentrations and content, with the percentage of vesicles or arbuscules in roots, were analysed with a regression linear analysis. A linear regression was considered significant when the value of the slope differed significantly from zero ($p \leq 0.05$).

3. Results

3.1. Taxonomic characterization of four AMF populations isolated from a Cr(VI) polluted soil

The four AMF populations obtained from a Cr(VI) polluted soil were characterised by a combined approach in which morphology of the spores and of the symbionts radical structures together with the SSU rRNA gene partial sequence were analysed with a Maximum Parsimony method. Morphological characteristics were analysed with a Principal Component Analysis (Infostat software, Di Rienzo et al., 2016). Most explanatory characters obtained in the first two PCA axes were considered for the phylogenetic analysis: hyphae presence, continuity hyphae with wall, hyphae shape, shield, sacculum and auxiliary cells (Table S1). The resulting strict consensus tree is shown in Fig. 2. The analysis revealed two distinct separated groups; Y, Br and Bi populations were grouped near *Rhizophagus* genus accesions while T population closed to *Paraglomus*. Bootstrap values from the AMF populations under study were between 70 and 90 (Fig. 2). AMF populations that were grouped near *Rhizophagus* form a monophyletic clade but could not be clearly separated (i.e.: Y, Br and Bi). Based on the phylogenetic analysis, AMF populations were renamed as Glo 1= Br; Glo 2= Bi; Glo 3= Y; and

Fig. 2. Phylogenetic analysis of four AMF populations isolated from a Cr(VI) polluted soil using spores morphological characteristics, symbiotic roots structures and SSU rRNA DNA sequence data. AMF populations were obtained from an AMF community with the trap culture technique from *Ricinus communis* rhizospheric Cr(VI) polluted soil. Sequence alignment of a fragment of the SSU rDNA (724 sites) and 6 morphological characters on 59 AMF accessions were analysed. The strict consensus tree was constructed by the Maximum Parsimony criterion using TNT programme. Only support values greater than 50 (Bootstrap) are shown in the branches.

Paraglomus sp.= T. In addition, bootstrap values increased when morphologic data was added to the phylogenetic analysis, which in result improved branch resolution (data not shown).

3.2. Response to Cr(VI) re-exposure of the AMF populations

3.2.1. Arbuscular mycorrhizal populations established symbiotic associations with Ricinus communis plants in presence of 8ppm of Cr(VI)

All four AMF populations established symbiotic associations with *R. communis* plants (Fig. 3). As depicted in Fig. 3, the intensity of the symbiotic association was similar between the four populations: total AMF association was approximately 70% whereas arbuscules and vesicles percentage was approximately 40%. No presence of AMF structures was observed in *R. communis* control plants (Fig. 3, No AMF bar). Dry weights of *R. communis* tissues (root and shoot) were similar between AMF populations and between the control (Fig. S1). Total Cr(VI) final concentration in the substrate was below the limit of detection (data not shown).

3.2. Metals and phosphorus concentrations in Ricinus communis plant tissues

Copper (Cu), chromium (Cr), iron (Fe), phosphorus (P) and zinc (Zn) concentrations and total content were determined in roots and shoots of *R. communis* plants associated, or not, with each one of the four AMF populations isolated from the Cr polluted soil.

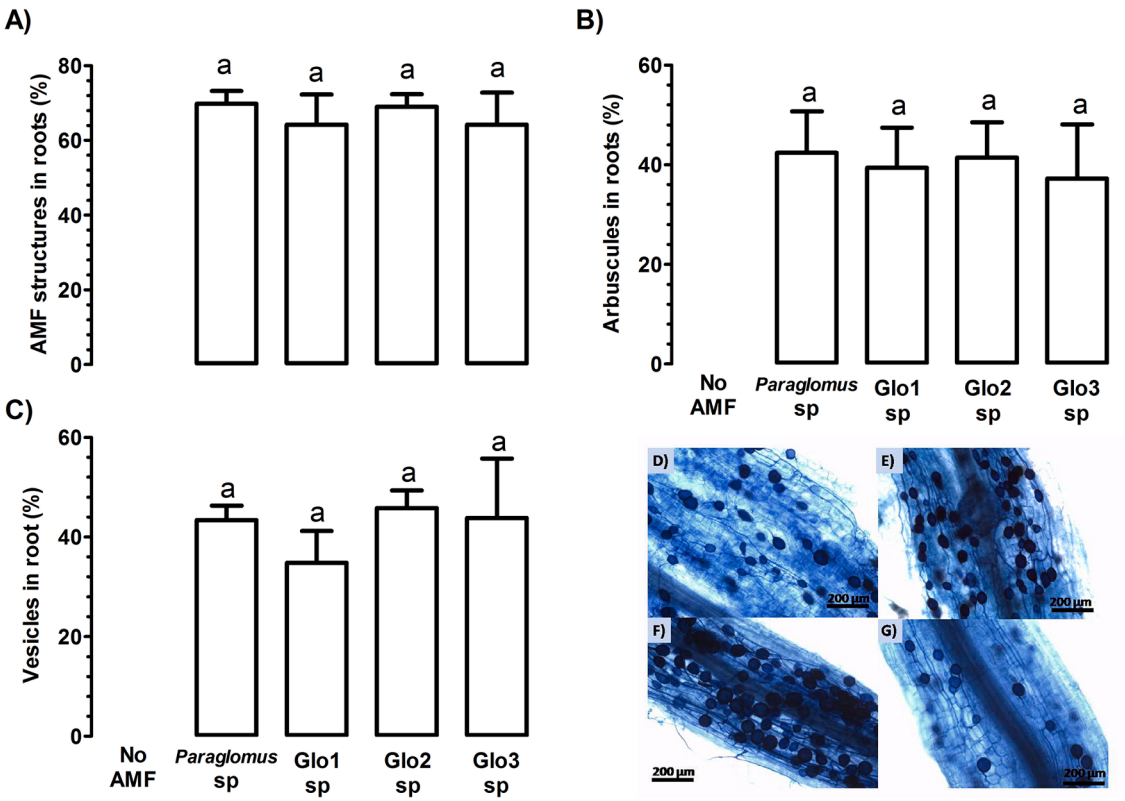

Fig. 3. Arbuscular mycorrhizal fungi association with *Ricinus communis* plants in presence of 8 µg Cr(VI) * g^{-1} of substrate. *R. communis* plants were pre-inoculated with 4 different populations of AMF obtained from an AMF community from *R. communis* rhizospheric Cr(VI) polluted soil. Plants with no AMF were used as controls. Inoculated *R. communis* plants were grown in 0.5 L pots for 3 months in presence of 8 µg Cr(VI) * g^{-1}. After harvest, the presence of AMF structures was evaluated by observation under an optical microscope of *R. communis* roots previously stained with cotton blue. A: Percentage of AMF structures in *R. communis* roots: B: Percentage of arbuscules in *R. communis* roots; C: Percentage of vesicles in *R. communis* roots. Data are expressed as means ± SEM (N = 5). Values with the same lowercase letters in a graph do not differ significantly at $P \leq 0.05$ (one-way ANOVA; multiple comparisons were made by a multiple range Tukey test). Photograph panel. Pictures correspond to cotton blue stained *Ricinus communis* roots associated to a HMA population: D: *Paraglomus* sp.; E: Glo1 sp.; F: Glo2 sp. and G: Glo 3sp.

3.2.1. Chromium

Chromium root content and concentration were similar between the 4 AMF populations and between the non AMF control as well (Fig. 4C and D, respectively). On the contrary, Cr shoot concentration was highest in non AMF control plants, in comparison with AMF inoculated plants, independently of the AMF population analysed (Fig. 4B). In fact, Cr shoot concentration was similar between the four AMF populations (Fig. 4 B). Otherwise, Cr shoot content differed between the AMF population; it was highest in *R. communis* plants associated with *Paraglomus* sp. population and was lowest in plants associated with Glo3 sp. Intermediate values were determined for the non AMF control and Glo1 and Glo 2 sp. Populations (Fig. 4A).

3.2.2. Phosphorus

Root and shoot P concentration and root content were significantly higher in *R. communis* plants associated with each of the four AMF populations, as compared with the non AMF control plants (Fig. 5B, C and D). No differences were observed between the four AMF populations. On the other hand, differences between AMF populations were determined in shoot P content (Fig. 5A). Shoot P content was: highest in *R. communis* plants associated with *Paraglomus* sp. and Glo2 sp., intermediate in Glo1 and Glo3 sp. and lowest in non AMF control plants.

3.2.3. Copper

Copper concentration in *R. communis* tissues, root and shoot, was similar between all the mycorrhizal treatments (Fig. 6, A and D, respectively). Conversely, differences were found when Cu content was analysed (Fig. 7 A and D). Shoot Cu content was highest in *R. communis* plants associated with *Paraglomus* sp. population and lowest in plants that did not establish the mycorrhizal association (Fig. 7A). Root Cu content was highest in three of the four mycorrhizal treatments (*Paraglomus* sp., Glo1 sp. and Glo2 sp.) and lowest in plants with no mycorrhiza (Fig. 7D).

3.2.4. Iron

Iron concentration in shoots was similar between all treatments while root concentration differed (Fig. 6, B and E, respectively). Root Fe concentration was highest in plants associated with *Paraglomus* sp. and lowest in plants associated with Glo2 sp. AMF population and control plants (Fig. 6E). Iron shoot content was highest in plants associated with *Paraglomus* sp. and lowest in those associated with Glo1 sp. and control plants (Fig. 7B). Iron root content was highest in Glo1 sp. and lowest in non mycorrhizal control plants (Fig. 7E).

3.2.5. Zinc

Zinc shoot concentration was similar between all treatments while root concentration was highest in non mycorrhizal control plants (Fig. 6 C and F, respectively). Zinc content in shoot was lowest in control plants and highest in plants associated with Glo2 sp. whereas Zinc content in root was highest in control plants and lowest in plants associated with Glo1 sp. (Fig. 7 C and F, respectively).

3.3. Chromium root concentration was inversely correlated with the percentage of vesicles

The presence of a linear regression between Cr or P concentration or content and the percentage of AMF structures, arbuscules or vesicles, in *R. communis* roots systems was evaluated. No correlation was observed

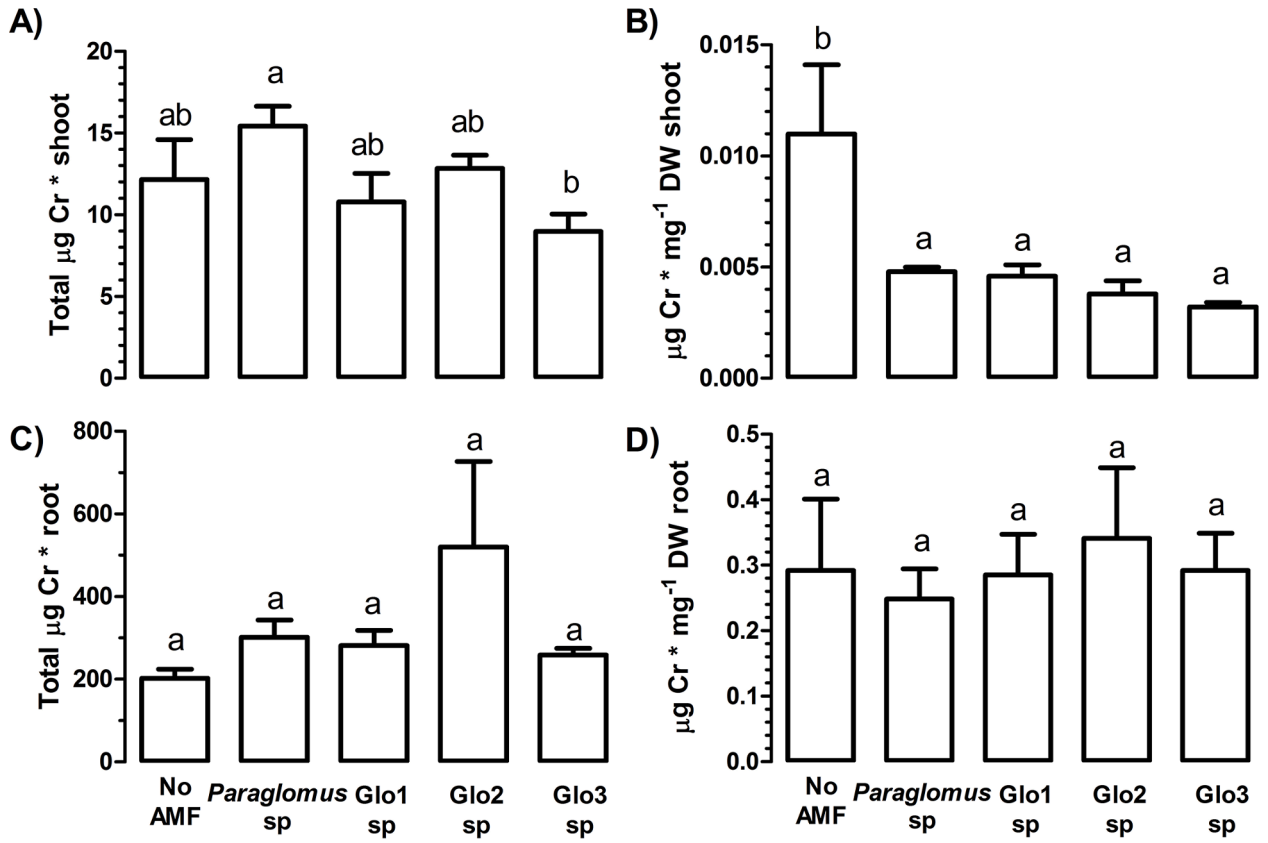

Fig. 4. Chromium content (A, C) and concentration (B, D) in *Ricinus communis* plants tissues grown for three months in presence of 8 µg Cr(VI) * g^{-1} in substrate associated or not to AMF populations isolated from a Cr(VI) polluted soil. Plants were exposed to Cr(VI) after a month of the transplant and were harvested three months after. Data are expressed as means ± SEM (N = 5). Values with the same lower-case letters in a graph do not differ significantly at $P \leq 0.05$ (one-way ANOVA; multiple comparisons were made by a multiple range Tukey test).

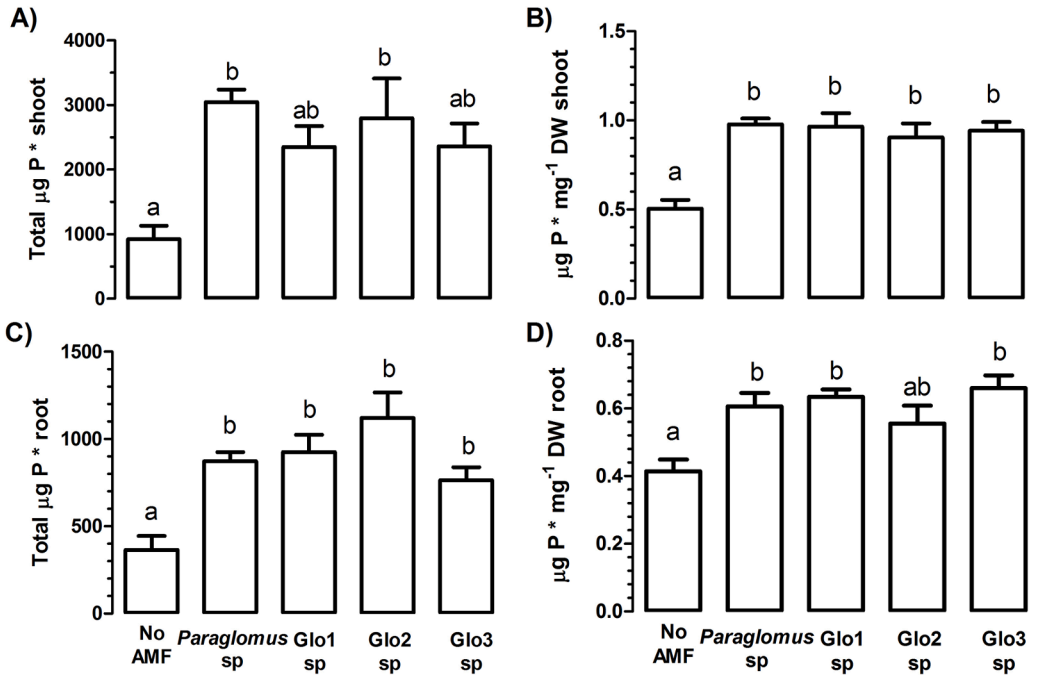

Fig. 5. Phosphorus content (A, C) and concentration (B, D) in *Ricinus communis* plants tissues grown for three months in presence of 8 µg Cr(VI) * g^{-1} in substrate associated or not to AMF populations isolated from a Cr(VI) polluted soil. Plants were exposed to Cr(VI) after a month of the transplant and were harvested three months after. Data are expressed as means ± SEM (N = 5). Values with the same lower-case letters in a graph do not differ significantly at $P \leq 0.05$ (one-way ANOVA; multiple comparisons were made by a multiple range Tukey test).

for most of the combinations analysed (data not shown). Only an inverse linear regression was observed between Cr root concentration and the percentage of AMF vesicles in the root system as depicted in Fig. 8.

4. Discussion

The phylogenetic tree showed that the four AMF populations

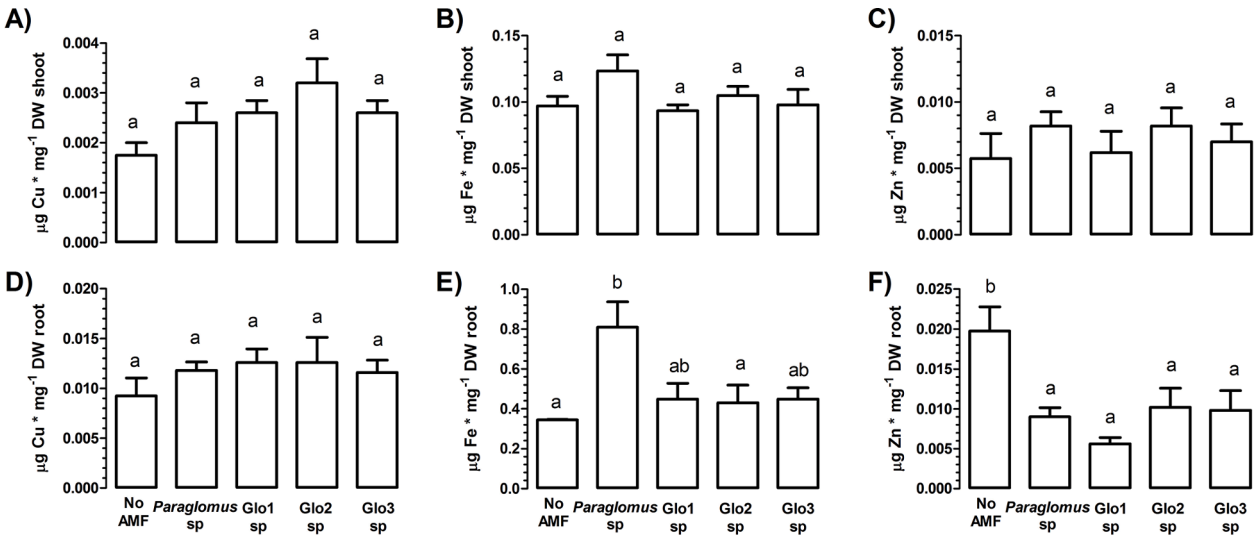

Fig. 6. Copper (A, D), Iron (B, E) and zinc (C, F) concentration in *Ricinus communis* plants tissues grown for three months in presence of 8 µg Cr(VI) * g^{-1} substrate, associated or not to AMF populations isolated from a Cr(VI) polluted soil. Plants were exposed to Cr(VI) after a month of transplant and were harvested three months after. Data are expressed as means ± SEM (N = 5). Values with the same lower-case letters in a graph do not differ significantly at $P \leq 0.05$ (one-way ANOVA; multiple comparisons were made by a multiple range Tukey test).

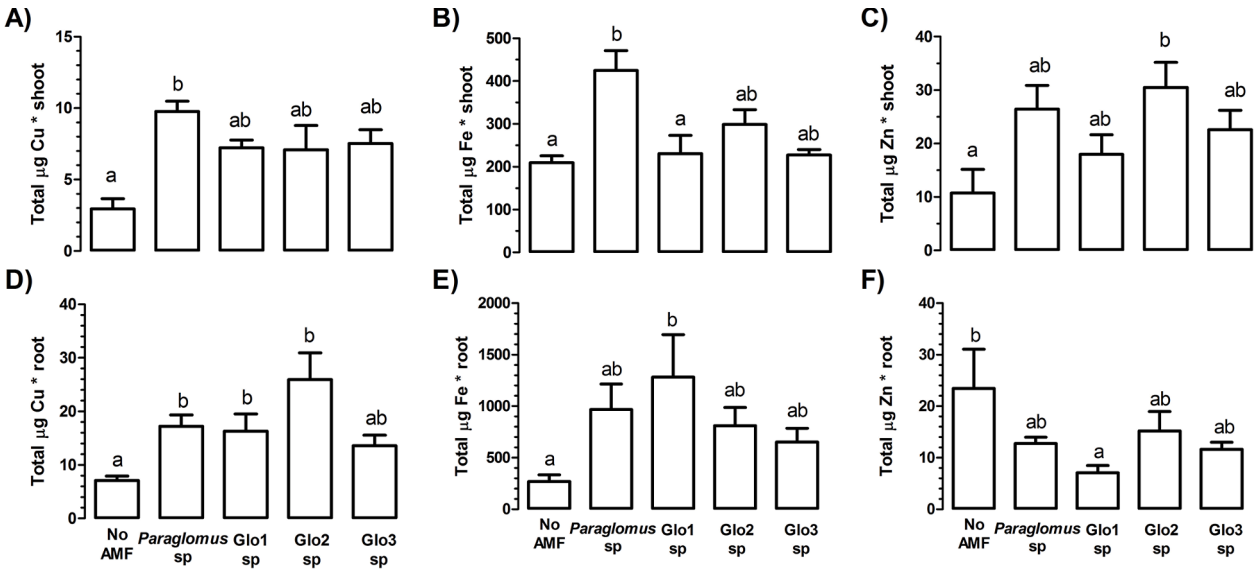

Fig. 7. Copper (A, D), Iron (B, E) and zinc (C, F) content in *Ricinus communis* plants tissues grown for three months in presence of 8 µg Cr(VI) * g^{-1} substrate, associated or not to AMF populations isolated from a Cr(VI) polluted soil. Plants were exposed to Cr(VI) after a month of transplant and were harvested three months after. Data are expressed as means ± SEM (N = 5). Values with the same lower-case letters in a graph do not differ significantly at $P \leq 0.05$ (one-way ANOVA; multiple comparisons were made by a multiple range Tukey test).

obtained for the study belonged to the *Glomeromycota* phylum (Fig. 2). In addition, the phylogenetic analysis showed that 3 of the 4 AMF populations grouped near *Rhizophagus* genus (Glomeraceae) and *Paraglomus* (Paraglomeraceae). This result is in agreement with a previous biodiversity study of the AMF community from where the populations were obtained, in which spores from the Glomeraceae family were the most abundant and Paraglomeraceae spores were also present (Gil Cardeza et al., 2018). Arbuscular mycorrhizal fungi from the Glomeraceae family have a ruderal strategy that consists in a fast recovery from vegetal community and chemist parameters soil changes (i.e. Cr(VI) contamination) (Borda et al., 2020). This could explain the highest abundance of the Glomeraceae family found in this study (3 from 4 AMF populations). Another AMF family that is commonly present in disturbed soils is Acaulosporaceae, since this family have a stress tolerance functional strategy (Borda et al., 2020). However, none of the populations were grouped in Acaulosporaceae family. As previously explained in Gil Cardeza et al. (2018) the absence of Acaulosporaceae family could be due to the trap culture technique since Acaulosporaceae spores were observed in the rhizospheric Cr(VI) polluted soil. This finding highlights the importance of *in situ* analysis in order to report environmental diversity despite the difficulties and limitations of the methodological approach.

It was recently reported, in a meta-analysis published by Marro et al. (2022), that there are a large number of studies for AMF species from the Glomeraceae family, in contrast to a few studies for the rest of the AMF species. In the same study they highlighted the necessity for further studies with the remaining AMF families under stressful conditions. Other meta-analyses also mentioned the numerous studies performed

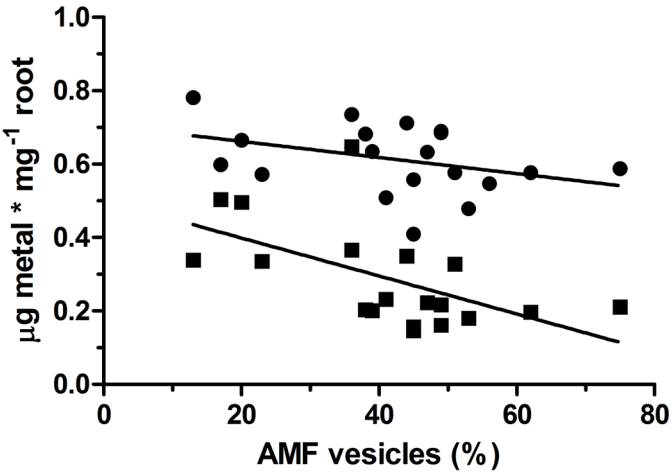

Fig. 8. Linear regression analysis between phosphorus (circles) or chromium (squares) concentration in *Ricinus communis* roots associated with AMF populations isolated from a Cr(VI) polluted soil and AMF vesicles percentages in the root system. Plants were exposed to Cr(VI) after a month of the transplant and were harvested three months after. Each point is an individual pot (N = 24). The linear regression model was tested on each data set. P values: Phosphorus p = 0.1078; Chromium p = 0.0099.

with Glomeraceae species under different stressful conditions (Wang et al., 2019) in contrast with few studies that included Paraglomeraceae family species under the same environmental conditions (Sun et al., 2016; Schneider et al., 2013; Paula et al., 2006). Paula et al. (2006) evaluated the growth of six plant species inoculated with AMF (*Glomus clarum* and *Paraglomus occultum*) in oil polluted soil (Landfarming site/area) and they found that 5 from the 6 plant species responded to AMF inoculation and 3 from the 5 increased their growth (dry weight). However, in the mentioned study *P. occultum* was co-inoculated with *G. clarum* thus it is not possible to attribute the observed response exclusively to *P. occultum.*

All four AMF populations were able to establish the symbiosis with *R. communis* roots in presence of Cr(VI) and the intensity of the symbiosis and the percentage of AMF structures (arbuscules and vesicles) were similar between the four populations (Fig. 3). The same tendency was observed when P tissue concentration and content were analysed; root and shoot P concentration and content were similar between plants associated with AMF, independently of the population. In all cases, P concentration and content were always highest in plants associated with AMF as compared with control plants (Fig. 5), supporting the existing evidence that demonstrates the beneficial effect of AMF symbiosis on P plant nutrition (Brundett and Tedersoo, 2018; Trivedi et al., 2020).

As with P nutrition, Cr shoot concentration was similar between the four AMF populations and differed with the non AMF control plants where Cr shoot concentration was highest, strongly suggesting that the presence of AMF constrained Cr translocation to the shoot (Fig. 4B). The ability of AMF to constrain Cr translocation to shoots has already been reported (Wu et al., 2019). In a recent study, Gil-Cardeza et al. (2021) showed a differential Cr uptake between maize plants associated with *Rhizophagus irregularis* MUCL41833 and maize plants not associated with AMF. In the mentioned study, non mycorrhizal plants depleted more Cr(VI) from the solution than the mycorrhizal counterpart, also suggesting a protective effect of AMF towards Cr accumulation in plants. When Cr shoot content was analysed it was possible to observe differences between the AMF populations (Fig. 4A). Interestingly it was highest in *R. communis* plants associated with *Paraglomus* sp. population. Even though no significant differences were observed between all treatments when plant weight was analysed (Fig. S1), shoot dry weight was almost twice in *R. communis* plants associated with *Paraglomus* sp. as compared to non AMF control plants ((3.1 ± 0.2) g *vs.* (1.8 ± 0.3) g, respectively). This observation could explain the highest Cr content found in shoots of plants associated with *Paraglomus* sp.. Cr root concentration and content were similar between all treatments, including the non AMF control plants. This finding is in agreement with Gil-Cardeza et al. (2021), where Cr root concentration and content was similar between AMF and non AMF control plants despite the differential Cr uptake observed.

Cooper, Fe and Zn are essential micronutrients for plants (Taiz and Zeiger, 2010). Briefly, Cu and Zn are present in several superoxide dismutase enzymes (SOD) which regulate cellular oxidative stress such as the stress generated by the presence of PTEs (e.g. Cr(VI)). Zinc is also involved in gene regulation processes since it is found in the RNA polymerase and with at least 80 enzymatic systems, including DNA repair enzymes (Taiz and Zeiger, 2010). Iron is involved in the catalytic groups of multiple redox enzymes and photosynthetic and respiratory chain proteins (Taiz and Zeiger, 2010). No differences were observed between non AMF control plants and AMF associated plants in either of these three micronutrients when shoot concentration was analysed (Fig. 6A, B and C). When root concentration was analysed differences were observed between non AMF *R. communis* plants and AMF associated plants: Fe was lowest in non AMF roots while Zn was highest (Fig. 6E and F, respectively). Cooper root concentrations were similar between all treatments (Fig. 6D). Several AMF specific gene transporters have been proposed for these three micronutrients, however the role of AMF in its uptake has not been elucidated yet (Ferrol et al., 2016). Since both Fe and Zn are involved in Redox enzymes it is possible that the opposite results obtained when Fe and Zn root concentrations were analysed corresponded to a differential cellular response of the non AMF roots as compared to the AMF roots. The lowest Fe concentration in *R. communis* non AMF control root tissues can be related to a lowest activation of Fe dependent redox enzymes defence mechanism in non AMF roots. In this sense, several studies have shown the activation of several redox enzymes in root tissues of AMF associated plants when exposed to PTEs (reviewed in Khan et al. 2000 and Ferrol et al. 2016). Particularly, an increase of glutathione was shown in AMF Cd exposed maize roots (Zhang et al., 2019) and an increase of SOD was also observed in AMF Cd and Pb exposed *Cajanus cajan* roots (Garg and Agaarwal, 2012). On the contrary, other studies have shown no changes or highest concentration values of redox enzymes in non AMF roots as compared to AMF roots when exposed to PTEs (Merlos et al., 2016; Wu et al., 2018). For example, Wu et al. (2018) reported that an exposure to Cr(VI) did not affect the concentrations of the reactive oxidative stress molecules glutathione in *Medicago truncatula* AMF roots as compared to the non AMF control. In addition, since Zn is also associated to DNA repair enzymes, it is possible to hypothesize that in non AMF roots the DNA repair enzymatic complex was induced, or at least more induced, than in AMF roots, thus suggesting a protective effect of AMF to DNA damage in *R. communis* roots exposed to Cr(VI).

It is commonly assumed that AMF can accumulate PTEs in vesicles and that the accumulation can be a detoxification mechanism (Wu et al., 2019; Parniske, 2008). Vesicles are within the root system so its content is also quantified when mineral or metal concentration in the root is measured. The linear regression analysis made in this study showed an inverse relation between Cr root concentration and the percentage of AMF vesicles in the root system: more vesicles, less Cr root concentration (Fig. 8). Wu et al. (2016) found a positive relation between Cr and P in AMF extraradical mycelium suggesting an active role of P on Cr detoxification. Vesicles accumulate P as polyphosphates (Ferrol et al., 2016; Parniske, 2008) so it is possible to speculate a relation between the inverse correlation found in our study and the role of polyphosphates as a detoxification mechanism. Moreover, an increase in the intensity of AMF mycorrhization was reported when plants were exposed to toxic but sub-lethal Cr(VI) concentrations, such as the 8ppm used in this study (Gil-Cardeza et al., 2021; Wu et al., 2015). However, no non Cr(VI) control was used since the aim of the study was to assess the response of the four AMF populations obtained when re-exposed to Cr(VI). Further

experiments that include exposure to increasing Cr(VI) concentrations should help to elucidate if an increase in Cr(VI) soil or substrate concentration positively correlates with more percentage of vesicles in the root system and if the augmentation continues to correlate inversely with Cr(VI) root concentration.

The present study characterized and explored several physiological responses of four AMF indigenous populations isolated from a Cr(VI) polluted site when associated to *R. communis* plants and re-exposed to 8 ppm of Cr(VI). *Ricinus communis* is a perennial shrub found in the sampled Cr(VI) polluted site (Gil-Cardeza et al., 2014) and is native from Africa, Western Europe and India. So, in order to design a phytoremediation strategy that not only promotes AMF biodiversity but also promotes plant biodiversity, future research should be focus on the relation between these 4 AMF populations and a native plant species (i.e, *Cortadeira selloana* or pampas grass).

5. Conclusions

Morphological, molecular and functional analysis of four AMF populations obtained from a Cr(VI) polluted soil, allowed us to identify Y, Br and Bi populations in the *Rhizophagus*, and T in *Paraglomus* genus. All of them continued to be tolerant to at least 8ppm of Cr(VI) in soil. From the mineral content analysis, including total Cr content in shoot, our results suggest that *Paraglomus* sp. could be the population that best adapted to the re-exposure of 8ppm Cr(VI). According to phylogenetic theories, this taxon appeared earliest in the evolution of the AMF. So, it is possible to hypothesise that this early divergent genus has the ability to establish the symbiosis and grow in hostile environments with high metal or mineral concentrations, such as the primitive land or a PTE polluted soil. Thus, we suggest that future phytoremediation studies should include taxa from this early diverged genus.

CRediT authorship contribution statement

Sehoane Evelin: Formal analysis, Investigation. **Mogni Virginia:** Formal analysis, Writing – original draft. **Pagani Ayelen:** Investigation. **Gil-Cardeza María Lourdes:** Conceptualization, Funding acquisition, Formal analysis, Investigation, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envadv.2023.100343.

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