REVISTA ARGENTINA DE
MICROBIOLOGÍA

BRIEF REPORT

Arbuscular mycorrhizal fungi in heavy metal highly polluted soil in the Riachuelo river basin

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Abstract   The Riachuelo river basin (RRB) is considered one of the most polluted environments in the world. Knowledge of arbuscular mycorrhizal fungi (AMF) adapted to this extremely polluted environment is important for the establishment of future soil restoration projects. This work aims to make a first list of AMF species present on the RRB. Soil and root samples were randomly taken in an area of approximately $1500\,m^2$, mycorrhization percentages were evaluated. AMF species were detected by molecular and morphological techniques. Sixteen AMF morphological species and 64 molecular species were reported in this work. *Dominikia iranica, Funneliformis constrictum, Funneliformis mosseae, Rhizophagus intraradices, Rhizophagus irregularis* and *Septoglomus viscosum* were detected by both techniques while *Claroideoglomus* sp. was only detected by pyrosequencing. The list of species reported in this work represents the first description of the RRB AMF community.
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PALABRAS CLAVE
Hongos micorrícicos arbusculares;
Metales pesados;
Suelo contaminado;
Metagenómica

Hongos formadores de micorrizas arbusculares en el suelo extremadamente contaminado con metales pesados de la cuenca del río Riachuelo

Resumen   La cuenca del río Riachuelo (CRR) es considerada uno de los ambientes más contaminados del mundo. Conocer los hongos formadores de micorrizas arbusculares (HFMA) adaptados a este ambiente extremadamente contaminado es importante para el establecimiento de futuros proyectos de restauración de suelos. Este trabajo se propuso hacer una primera lista de especies de HFMA presentes en la CRR. Se tomaron muestras de suelo y raíces al azar en un área de aproximadamente 1500 m^2 y se evaluaron los porcentajes de micorrización. La identificación de especies de HFMA se basó en técnicas moleculares y morfológicas. Se detectaron 16 especies morfológicas y 64 especies moleculares de HFMA. *Dominikia iranica*, *Funneliformis constrictum*, *Funneliformis mosseae*, *Rhizophagus intraradices*, *Rhizophagus irregularis* y *Septoglomus viscosum* se detectaron mediante ambas técnicas, mientras que *Claroideoglomus* sp. solo fue detectado por pirosecuenciación. La lista de especies reportada en este trabajo representa la primera descripción de la comunidad de HFMA de la CRR.

One of the most important concerns raised by the world society is the progressive chemical degradation of soils by contamination as a consequence of human activities. The Matanza and the Riachuelo are lowland rivers that run through the Pampean plain along the South metropolitan area of Buenos Aires (Argentina). The Matanza-Riachuelo basin is the most important water system in this area (233 800 Ha), covering 14 municipalities of Buenos Aires province and the city of Buenos Aires. According to the last census (2010), about 8 million people live in this area (https://www.indec.gov.ar, last accessed: February 2019). The lower portion of the Riachuelo river basin (RRB) is the most physically, chemically and biologically degraded environment of the Matanza-Riachuelo river basin. The high population and industrial density were the causes of the extreme deterioration of the basin water, air and soil. Nowadays this area is considered one of the ten most polluted environments in the world[2]. A wide range of inorganic and organic pollutants can be found in the area. Among them, the most commonly found are: As, Cd, Cr, Cu, Hg, Fe, Ni, Pb, Zn and Mn[12].

Successive attempts have historically been made to restore the environment of the RRB, with particular emphasis placed on the aquatic environment ecosystem (http://www.acumar.gob.ar/eje-ambiental/, last accessed: February 2019)). For the edaphic environment, tasks of landscape restoration were carried out: the towpath space was recovered (previously occupied by industries and precarious housing), visible wastes were removed, and native plants were planted. No attempt to remedy the basin soil has been made yet.

Among the available technologies to remediate polluted soils, bioremediation involves lower costs and minimal environmental alterations. Arbuscular mycorrhizal fungi (AMF), belonging to *Glomeromycota* Phylum, were previously found in soils with high contents of heavy metals (HM), proving that they have established mechanisms of resistance or tolerance to these adverse conditions[9]. AMF are strict biotrophs,

needing to colonize a host root to get access to carbohydrates and complete their life cycle. In exchange, fungi improve host tolerance to biotic or abiotic stressful conditions among other benefits. The vast majority of plants are capable of establishing a symbiosis with AMF, increasing the volume of explored soil by roots through the establishment of hyphal networks. They also contribute to soil aggregation improving the edaphic structure, being considered key microorganisms for the productivity and diversity of ecosystems[14]. Based on this fact, the symbiosis establishment could be considered a plant strategy to improve its status in highly polluted environments. Recently it was confirmed that AMF colonization improves the efficiency of rhizoremediation because of the uptake of contaminants by fungal hypha and their transport to the plant tissues[9].

Knowing the AMF species adapted to the extremely polluted environment of the RRB is important for future soil restoration projects. Species characterization by the traditional approach (based on propagule traits) has many challenges: the degraded conditions of spores in the field, the presence of non-sporulating species or the difficulty of distinguishing between functional and morphological diversities, among others. Second-generation sequencing technologies have been increasingly considered to be useful and complementary tools to elucidate the diversity of AMF in environmental samples[4].

The aim of this work was to make a preliminary list of the AMF species presenting the RRB by molecular and morphological techniques, and to evaluate the degree of colonization of the plant roots growing there.

Sampling took place in the lower portion of the RRB (*Avellaneda*, 34°39′27.36″S, 58°22′34.37″W) in September 2013. Twenty-five soil cores (10 cm depth) were randomly taken in an area of 1500 m^2 and then homogenized into a unique sample in order to remove the effects of field heterogeneity[1]. The soil sample was divided and stored at 4 °C for subsequent physicochemical analyses, evaluation of HM

content and identification of AMF species by molecular and morphological techniques.

A survey of the local flora was carried out and plants were collected from the towpath (0.5–15 m from the water edge). Seventeen plant species were identified: *Festuca arundinacea*, *Paspalum distichum*, *Sorghum halepense*, *Taraxacum officinale*, *Senecio bonariensis*, *Chamaemelum nobile*, *Trifolium* sp., *Cyperus eragrostis*, *Dichondra microcalyx*, *Salix babylonica*, *Morusnigra*, *Ricinus communis*, *Commelina erecta*, *Hydrocotyle bonariensis*, *Conium maculatum* and *Juncus pallescens*.

Roots of herbaceous mycorrhizal plants were sampled to evaluate their mycorrhizal status. They were cleared in KOH (10%) and stained with trypan blue in lactic acid (0.02%)[11]. Thirty root segments (1 cm) per plant were examined under a light binocular microscope (model: Olympus BX51) in order to estimate the percentage of AMF colonization[6]. Mycorrhization frequency was calculated as the percentage of root segments containing AMF hyphae, arbuscules, coils or vesicles; mycorrhization intensity was estimated by sorting out the root segments in percentual intervals of AMF intraradical cover. The percentage of arbuscules, coils and vesicles, were separately registered, considering arbuscules and coils as characteristic structures of an active symbiosis and vesicles as storage structures[3]. Mycorrhization frequency reached high values (72.2%, SD: 31.7); however, the intensity of AMF colonization did not (38.2%, SD: 19). Arbuscules and coils were more commonly found than vesicles, being detected in 86.5% (SD: 26.2) and 31.1% (SD: 23) of the mycorrhizal roots, respectively. Intraradical AMF spores were observed, though in a very low proportion. Typical mycelium and structures of *Glomus tenue* were observed associated with *J. pallescens* roots. AMF colonization was not only frequent but also in an active stage due to the high percentage of arbuscules and coils. Mendoza et al.[8] have also reported the presence of one of these mycorrhizal plants (*H. bonariensis*), not only in the lower portion of the basin but also in the middle and upper portions. The authors observed that *H. bonariensis* plants were always colonized by AMF.

Physicochemical analyses of the soil and the evaluation of HM contents were conducted at the Instituto de Geocronología y GeologíaIsotópica (INGEIS, CONICET) and at the Comisión Nacional de Energía Atómica (CNEA, Regional Cuyo unit) (Table 1; supplementary material). HM can be naturally found in the soil or originated as a result of anthropogenic activities. Many of these heavy metals are essential micronutrients for plant growth; however, when exceeding certain concentrations, these elements may become toxic and hinder plant growth. High contents of Fe, Zn, Pb, Cu, Cd, Sr, Ni, Cr and Mn were found in the RRB soil (Table 1; supplementary material). However, according to the Argentine legislation (Law 24051: Hazardous waste in Argentina, decree 831/93, Annex II, Table 9), only Cd, Cr and Mo exceed the levels considered suitable for agricultural and residential use of the soil. Other authors have previously reported similar levels of soil contamination across the Matanza-Riachuelo river basin by many of the HM reported in this paper[8,12].

Twelve pot trap cultures were established to facilitate the taxonomical identification of AMF spores. One liter pots were were filled with a sterile (100 °C for 1 h, three consecutive days) perlite:soil mixture (2:1) as growing substrate and 10 g of the RRB sampled soil containing AMF propagules and mycorrhized root segments as inoculum. Sorghum, tomatoes, peas and clover were used as host plants. Cultures were monitored every two months for eighteen months. AMF propagules were isolated from 100 g (dry weight) of RRB soil and from trap culture substrate by wet sieving and decanting method. Spores that appeared healthy were manually selected and removed using a micropipette under a stereomicroscope (model: Olympus SZ61). All propagules were mounted on slides containing polyvinyl alcohol-lactic acid-glycerol (PVLG) and a mixture of PVLG-Melzer reagent. Spore and subcellular structure characteristics were observed under a light binocular microscope (model: Olympus BX51). Taxonomic classifications, whenever possible, were made in accordance with the descriptions available at the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM) website (http://invam.wu.edu, last accessed: February 2019) and at Dr. Blaszkowski's website (http://www.zor.zut.edu.pl/Glomeromycota/, last accessed: February 2019). Taxonomic assignments were done according to the Index Fungorum (http://www.indexfungorum.org/names/names.asp, last accessed: February 2019).

Propagules of 15 different AMF species were isolated from RRB sampled soil and pot trap cultures and classified as: *Acaulospora* sp., *Dominikiairanica*, *Entrophospora infrequens*, *Funneliformis constrictum*, *Funneliformis mosseae*, *Gigaspora* sp., *Rhizoglomus microaggregatum*, *Rhizophagus* sp., *Rhizophagus clarus*, *Rhizophagus diaphanus*, *Rhizophagus intraradices*, *Rhizophagus irregularis*, *Sclerocystis sinuosa*, *Scutellospora pellucida* and *Septoglomus viscosum*.

DNA was isolated from 0.25 g (dry weight) of RRB soil (previously sieved through a 2 mm mesh) in triplicate. The MoBio Power Soil DNA isolation kit was used, following the manufacturer's instructions (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). Amplification was performed on a Fast Start High Fidelity PCR system (Roche Applied SCIENCE, Mannheim, Germany) following the manufacturer's instructions. PCR conditions are detailed as follows: 95 °C (5 min); 30 cycles of 95 °C (45 s), 57 °C (45 s), 72 °C (60 s); 72 °C (4 min). AMV4.5F/AMDGR oligonucleotides were chosen because of their specificity to amplify AMF DNA in environmental samples[4]. Primers were specifically designed for 454 pyrosequencing with the Genome Sequencer-FLX (GS-FLX) 454 Titanium System (Roche Applied Science, Mannheim, Germany). Amplification products were purified using AMpure Beads (Agencourt) in order to remove primers, impurities and small spurious products. Amplification and 454 amplicon pyrosequencing were performed at the Instituto de Agrobiotecnología de Rosario (INDEAR), following Roche 454 software manuals.

Treatment of the obtained sequences was carried out according to Colombo et al.[4] MOTUs were defined at 97% (97% dataset) and 90% (90% dataset) of sequence similarity. The first percentage was chosen in accordance to the traditional definition of microbial 'species'. The second and lowest percentage was chosen due to the possible overestimation of AMF richness when considering a 97% similarity, as a high genetic diversity was observed within a single individual in comparison with other organisms, probably due to the asexual and multinucleated nature of AMF[14]. Representative sequences from each MOTU were

compared with 18S rRNA published sequences on the Maarj-AM(specific database for AM fungal) and the NCBI (National Centre for Biotechnology Information) databases using the nr/nt BLAST (Basic Local Alignment Search Tool) algorithm. Only sequences with query coverage and similarity values higher than 97% (*E*-values equal or close to zero) were considered. After MOTU identification, non-*Glomeromycota* sequences were removed from the data sets. Taxonomic assignments of *Glomeromycota* sequences were made according to the Index Fungorum. Whole analyzed sequences are available as FASTQ at the NCBI Sequence Read Archive database (accession number: SRR2968218).

A total of 179 286 sequences (mean length: 204.8 pb, SD: 16.1 pb) were obtained and grouped into 217 MOTUs (97% dataset) or 92 MOTUs (90% dataset). According to the best BLAST hit in MaarJAM and the NCBI databases, 46.13% (64 MOTUs) and 48.41% (12 MOTUs) of the total reads belonged to *Glomeromycota* sequences, at the 97% and 90% datasets respectively (Table 2; supplementary material).

In order to know if the sequencing effort was enough to cover the entire AMF diversity, rarefaction curves were constructed with Quantitative Insights Into Microbial Ecology (QIIME) pipeline by randomly selecting a series of different sized subsets of reads from the data set. Each sampled subset was replicated ten times by the program. Rarefaction curves reached the asymptotes in both cases (Fig. 1).

Both datasets presented AMF MOTUs that could only be classified at the Phylum level. MOTUs belonging to the Order *Glomerales* were always the most abundant (95.9% and 91.1%), while Order *Diversisporales* sequences were only registered at 97% dataset (0.03%). AMF sequences of genera *Claroideoglomus* (36.1%), *Dominikia* (26.5%) and *Rhizophagus* (14.2%) were the most representative ones at 97% dataset whereas 15.8% of *Glomerales* sequences could not be classified at the genus level (Table 2; supplementary material). At 90% dataset the genus *Claroideoglomus* was also the most abundant (34.3%), followed by *S. viscosum* AMF species (3.1%). No sequence of the genus *Rhizophagus* was detected; however, 53.8% of the *Glomerales* sequences were not identified at the genus level in this dataset (Table 2; supplementary material). The higher abundance of *Glomerales* could be related to a higher tolerance of
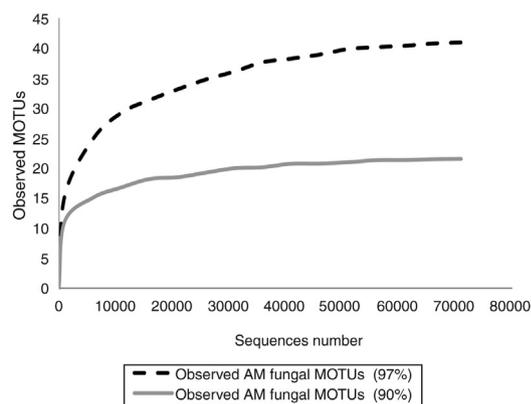

Figure 1   Accumulation curves on the occurrence of AMF MOTUs obtained by 454 amplicon pyrosequencing of RRB soil metagenomic DNA, when defining 97% (dotted black line) or 90% (solid grey line) of sequence similarity.

this order to extreme conditions due to their life history strategies, characterized by short life cycles, a great investment in spore production and the capability to interconnect different mycelia through hyphal bridges[14].

In order to check the right taxonomic assignment of MOTUs, phylogenetic analyses were performed using the maximum likelihood distance method. *Glomeromycota* sequences from both molecular data sets were aligned using the ClustalW program. Bootstrap analysis with 1000 replicates was used to test the confidence of the branches. Phylogenetic trees were constructed and edited by the Tree Explorer of the MEGA 6.06 program. Both trees resulted in consistent ordinations of MOTUs. While the statistical supports of the branches were not always high, phylogenetic groups were well established, with the exception of the sequences belonging to *F. constrictum* and *S. viscosum* and the phylogenetic group formed by *R. intraradices* and *R. irregularis* sequences, which could not be distinguished in this analysis (data not shown).

Fifteen morphological species and 64 MOTUs of AMF were reported in this work. These results represent the first description of the AMF community of the extremely polluted RRB soil. Mendoza et al.[8] detected AMF spores in the lower portion of the basin in Avellaneda city; however, they were not taxonomically identified. *D. iranica*, *F. constrictum*, *F. mosseae*, *R. intraradices*, *R. irregularis* and *S. viscosum* were the only species detected by both assessed techniques. The 90% dataset failed to identify MOTUs at the species level, except for *F. mosseae* and *S. viscosum*. Contrary to our expectations, AMF richness estimated by the 90% dataset was much lower than that detected by the morphological technique. Hence, the use of 90% similarity among sequences to delimitate MOTUs when studying AMF communities would not be advisable.

Claroideoglomus sp. MOTUs were abundantly detected by the pyrosequencing technique; however, spores were not recovered from the soil. This could be attributed to the non-sporulation of *Claroideoglomus* mycelium under the particular environmental conditions of RRB. Panwar et al.[10] observed a variation in the sporulation dynamic associated to physico-chemical-climatic conditions of soil. However, *Claroideoglomus* spores were not recovered from the trap cultures either. In turn, *E. infrequens* and *R. microaggregatum* spores were recovered from RRB soil samples but their sequences were not. In a previous work, Colombo et al.[4] reported the same inconsistency. The authors related the molecular undetectability of these species to the difficulty to isolate DNA from very small spores (*R. microaggregatum*) or to the low number of propagules in the soil (*E. infrequens*).

Many of the AMF species described in this work had been previously morphologically or molecularly detected in soils with high HM concentrations. Spores of *F. mosseae* were found in strongly contaminated soils with Pb, Zn and Cd in Shipham (England) and with Pb, Zn, Cu, Ni and Cd in Braunschweig (Germany), where *Claroideoglomus claroideum* was also abundantly found[5]. We detected *F. mosseae* in RRB soils by morphological and molecular techniques; however, spores of *C. claroideum* were not isolated in this sampled site. The genus *Claroideoglomus* was only detected by the metagenomic analysis of soil, but this technique failed to differentiate sequences at the species

level. *R. intraradices*, *R. clarus* and *C. claroidedum* had been previously described in soil contaminated with Cd, together with *Funneliformis geosporum*, *Funneliformis coronatum* and *Diversispora versiforme*[13]; however, these three latter AMF species were not present in the sampled RRB site. Spores of *R. intraradices* were also found in soil containing high concentrations of Zn[7]. Typical *Gigasporaceae* auxiliary cells and *G. tenue* characteristic mycelium and vesicles were observed in association with root segments sampled in Ni contaminated soils[15].

Knowing AMF species of the RRB would facilitate future isolation and conservation works of these fungal strains in order to use them in bioremediation assays.

Conflict of interest

Authors have no conflict of interest.

Funding

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ram.2019.05.001.

References

1. Alvarez R, Alvarez CR. Soil organic matter pools and their association with carbon mineralization kinetics. Soil Sci Soc Am J. 2000;64:184–9.

2. Bernhardt A, Gysi N. The world's worst 2013: the top ten toxic threats. Cleanup, progress and ongoing challenges. Blacksmith Institute, New York and Green Cross Switzerland, Zurich; 2013.

3. Cabello MN. Hydrocarbon pollution: its effect on native arbuscular mycorrhizal fungi (AMF). FEMS Microbiol Ecol. 1997;22:233–6.

4. Colombo RP, Fernández Bidondo L, Silvani VA, Carbonetto MB, Rascovan N, Bompadre MJ, Pérgola M, Cuenca G, Godeas AM. Diversity of arbuscular mycorrhizal fungi in soil from the Pampa Ondulada, Argentina, assessed by pyrosequencing and morphological techniques. Can J Microbiol. 2014;60:819–27.

5. del Val C, Barea JM, Azcon-Aguilar C. Diversity of arbuscular mycorrhizal fungus population in heavy-metal-contaminated soils. Appl Environ Microbiol. 1999;65:718–23.

6. Declerck S, Strullu DG, Plenchette C. *In vitro* mass-production of the arbuscular mycorrhizal fungus *Glomus versiforme*, associated with Ri T-DNA transformed carrot roots. Mycol Res. 1996;100:1237–42.

7. Hildebrandt U, Regvar M, Bothe H. Arbuscular mycorrhiza and heavy metal tolerance. Phytochemistry. 2006;68:139–46.

8. Mendoza RE, García IV, de Cabo L, Weigandt CF, Fabrizio de Lorio A. The interaction of heavy metals and nutrients present in soil and native plants with arbuscular mycorrhizae on the riverside in the Matanza-Riachelo River Basin (Argentina). J Sci Total Environ. 2014;505:555–64.

9. Miransari M. Hyperaccumulators, arbuscular mycorrhizal fungi and stress of heavy metals. Biotechnol Adv. 2011;29:645–53.

10. Panwar V, Meghvansi MK, Siddiqui S. Short-term temporal variation in sporulation dynamics of arbuscular mycorrhizal (AM) fungi and physico-chemical edaphic properties of wheat rhizosphere. Saudi J Biol Sci. 2011;18:247–54.

11. Phillips JM, Hayman DS. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc. 1970;55:158–60.

12. Rendina A, Bargiela M, de Cabo L, Arreghini S, de Iorior AF. Formas geoquímicas de metales pesados en sedimentos del Rio Matanza y principales afluentes. Rev Mus Argent Cienc Nat. 2001;3:33–9.

13. Renker C, Blanke V, Buscot F. Diversity of arbuscular mycorrhizal fungi in grassland spontaneously developed on area polluted by a fertilizer plant. Environ Pollut. 2004;135:255–66.

14. Martins Teixeira L, Siqueira Carvalho M da G, Facklam RR. Enterococcus. En: Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA, editors. Manual of Clinical Microbiology, 9th edition. Washington DC, ASM Press, 2007, p. 430-42.

15. Turnau K, Mesjasz-Przybylowicz J. Arbuscular mycorrhiza of *Berkheya coddii* and other Ni-hyperaccumulating members of Asteraceae from ultramafic soils in South Africa. Mycorrhiza. 2003;13:185–90.