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Molecular diversity of arbuscular mycorrhizal fungi in relation to soil chemical properties and heavy metal contamination

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The molecular diversity of AMF was found to be influenced by a combination of soil heavy metal and other soil chemical parameters.

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ABSTRACT

Abundance and diversity of arbuscular mycorrhizal fungi (AMF) associated with dominant plant species were studied along a transect from highly lead (Pb) and zinc (Zn) polluted to non-polluted soil at the Anguran open pit mine in Iran. Using an established primer set for AMF in the internal transcribed spacer (ITS) region of rDNA, nine different AMF sequence types were distinguished after phylogenetic analyses, showing remarkable differences in their distribution patterns along the transect. With decreasing Pb and Zn concentration, the number of AMF sequence types increased, however one sequence type was only found in the highly contaminated area. Multivariate statistical analysis revealed that further factors than HM soil concentration affect the AMF community at contaminated sites. Specifically, the soils' calcium carbonate equivalent and available P proved to be of importance, which illustrates that field studies on AMF distribution should also consider important environmental factors and their possible interactions.

1. Introduction

Contamination of soils by heavy metals (HM), resulting from anthropogenic activities such as ore mining and smeltering, is a serious problem in many areas around the world (Gadd, 1993). In addition to physical or chemical soil properties, the quality of HM polluted soils depends on the diversity and activity of biota present (Doran and Linn, 1994; Jeffries et al., 2003). In this context, the arbuscular mycorrhizal fungi (AMF), belonging to the phylum Glomeromycota and associating with native plants, may be important for assessing soil quality in polluted areas (Gaur and Adholeya, 2004).

Glomeromycotan fungi are ubiquitous soil organisms (Treseder and Cross, 2006) and have been repeatedly described in HM polluted sites (e.g. Sambandan et al., 1992; Turnau et al., 2001). Although arbuscular mycorrhizal symbiosis is widespread, the symbiotic functions of AMF species are not equivalent and vary according to the specific AMF isolates, host plants and soil properties (e.g. Hempel et al., 2009; Hildebrandt et al., 1999; Kelly et al., 2005; Redon et al., 2008, 2009). Therefore, the identification of specific phylotypes of AMF and their relationship with soil properties are crucial and provide an important step to understand the ecology of AMF at HM contaminated ecosystems (Gaur and Adholeya, 2004).

Several surveys on the effect of HM on diversity and abundance of AMF have been conducted in HM polluted areas around the globe (e.g. Chao and Wang, 1990; del Val et al., 1999; Regvar et al., 2003; Sambandan et al., 1992; Vallino et al., 2006). Although other soil parameters, such as soil nutrient concentration and pH, may also be highly correlated to AMF distribution (del Val et al., 1999), they have been rarely used to interpret patterns of AMF community composition.

Our study area, adjacent to an open pit mine, is the main source of soil HM pollution in the Anguran region in Iran. In a first survey on AMF, the spore abundance and root colonization parameters of indigenous plants were analyzed on 35 plots along a transect of decreasing HM soil concentration away from the mine (Zarei et al., 2008b). These data showed a strong negative correlation between HM soil concentrations and both AMF spore abundances and root colonization intensities. Morphological spore identification to the

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genus level also suggested that there was a reduced AMF species diversity in highly HM contaminated plots. To test this hypothesis, the AMF diversity in the roots of the indigenous plant species *Veronica rechingeri* was assessed in four selected plots along the same transect using molecular DNA based markers for AMF sequence type identification (Zarei et al., 2008a). The results of this second study clearly showed a decline in the number of AMF sequence types colonizing the roots of *V. rechingeri*, ranging from six types in unpolluted plots to only two types in highly HM polluted plots. The AMF distribution data also revealed AMF sequence types that are adapted to high HM concentrations.

In the present study, we extend our approach by describing the molecular diversity of AMF associated to different dominant plant species (except *V. rechingeri*) in relation to important soil parameters at the Anguran Zn and Pb mining region. These results will give a clearer insight into the combined impact of HM and soil chemical properties on AMF community composition in the Anguran mining region.

2. Materials and methods

2.1. Sampling area, sample collection, and analysis

The Anguran Zn-(Pb) deposit, Zanjan Province, NW Iran (36°40′ N, 47°20′ E; 2950 m a.s.l.), is located within the central Sanandaj-Sirjan Zone of the Zagros orogenic belt. Anguran deposits may represent a new type of low-temperature, carbonate-hosted Zn–Pb ore that is distinct from the Mississippi Valley type and sedimentary-exhalative deposits (Gilg et al., 2006).

In order to study AMF diversity in soil with different levels of Zn and Pb concentrations, the sampling region was divided into areas of high (area 1), medium (area 2), low (area 3), and non-polluted (area 4) levels. The separation between polluted and non-polluted soils was based on recommendation of the Council of the European Communitie (1986) and Vallino et al. (2006). The division of polluted soils into levels of high, medium, and low pollution was based on the variations of Zn and Pb concentration in the study area, their effects on mycorrhizal parameters (Zarei et al., 2008b), and on previous studies on the same topic (Brown et al., 2004; Cao et al., 2004; Long et al., 2003). It has to be noted that the non-polluted area 4 is partially used as an agricultural site, however under extensive management and without irrigation. A twelve plot (100 m × 20 m each) subset from the original 35 plots analyzed in Zarei et al. (2008b) were chosen in order to equally represent each pollution level, with three plots per level. These twelve plots were divided into ten subplots each (20 m × 10 m), for a total of 120 subplots in total. The dominant plant species at each plot were selected, cumulating in three plant species for each level of pollution: area 1 (high HM pollution), *Salvia nemorosa*, *S. officinalis*, and *Nonnea persica*; area 2 (medium HM pollution), *S. officinalis*, *Plantago ovata*, and *S. nemorosa*; area 3 (low HM pollution), *P. ovata*, *S. officinalis*, and *N. persica*; area 4 (unpolluted), *Veronica orientalis*, *Medicago sativa*, and *Triticum aestivum*. In each subplot, one sample consisting of roots and rhizosphere soil of an individual of the dominant plant species was randomly collected, i.e. 120 samples in total. As plant species have the potential to accumulate their own beneficial AMF mycoflora (Klironomos, 2002), this sampling of abundant plant species should give a better overview of the AMF community in each area.

From the ten subsamples of each plot, a composite sample of root and rhizosphere soil was prepared and analyzed in triplicate. Soil chemical properties, AMF spore abundance, and plant root colonization of these samples were determined according to the respective standard methods as described in Zarei et al. (2008b).

2.2. DNA extraction and nested PCR

Fine root samples of each plant species (ten subsamples pooled per plot) were homogenized in liquid nitrogen using a ceramic pestle and mortar. Total DNA was extracted from approximately 20–30 mg of lyophilized roots using the DNeasy Plant Mini Kit following the manufacture's instructions (Qiagen, Hilden, Germany). DNA was eluted in 100 μl elution buffer. Three dilutions (1:1, 1:10, and 1:100) were used as template in a nested PCR for amplification of the ITS region of ribosomal DNA with the primer pair LSU-Glom1/SSU-Glom1 as specific primers for arbuscular mycorrhizal fungi and ITS5/ITS4 as general primers in the first and second reaction of PCR, respectively (Renker et al., 2003). We decided to use this primer system despite its drawback to amplify a certain proportion of non-AMF sequences (Aldrich-Wolfe, 2007; Renker et al., 2003) for several reasons: i) the primer set was widely used in many ecosystems in both plant roots and soil providing a large database of reference sequences for phylogenetic analysis (e.g. Aldrich-Wolfe, 2007; Börstler et al., 2006; Hempel et al., 2007); ii) the primer set amplifies all phylogenetic groups within the Glomeromycota, which is not the case for other widely used AMF primer sets (Helgason et al., 1998); iii) the different levels of phylogenetic resolution represented by the amplified 5.8S and the ITS regions, respectively, allow identification of non-AMF fungi and also provide a sufficient phylogenetic resolution for phylotype delimitation (Redecker et al., 1999); iv) the primer set was previously used in the same study region and was found to provide robust data (Zarei et al., 2008a). In addition, the newly proposed primer sets for AMF (Krüger et al., 2009; Lee et al., 2008) were published after this study was conducted.

For the first step, PCR was conducted as described by Renker et al. (2003) with the following changes: ingredients were taken from the HotStar Plus kit (Qiagen), using 2 μl of 25 mM $MgCl_2$ and 0.8 μl of dNTPs (2 mM each, MP Biomedicals/Q-BIOgene, Heidelberg, Germany) and the denaturation was performed at 95 °C with the initial denaturation phase extended to 15 min. Restriction of the PCR product with AluI (Fermentas, St. Leon-Rot, Germany) to exclude non-AM fungi, and the second amplification step, were carried out as in the original protocol with the exception that ingredients for PCR were taken from a Taq-Core Kit (protocol as implemented by the manufacturer MP Biomedicals/Q-BIOgene). PCR was carried out with a Mastercycler gradient system (Eppendorf, Hamburg, Germany).

2.3. Cloning, RFLP typing, and sequencing

Aliquots of the second PCR products were run on 1.2% agarose gels. The size of each PCR product was estimated by the SYNGENE gel documentation software (SYNGENE, New Castle, England). For each sampling site, the PCR product with the strongest band on the gel and the expected ITS fragment length of AM fungi (500–650 bp) was chosen and purified with the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's recommendations. Purified PCR products of the three respective plant species at each of the four levels of HM pollution were pooled and the four pools were used to construct clone libraries using the pCR4-Topo vector and TOP10 chemically competent *Escherichia coli* following the standard protocol of the TOPO TA Cloning Kit (Invitrogen Life Technologies, Karlsruhe, Germany).

After checking for the presence of ITS inserts using vector-targeting primers M13F and M13R with the same PCR parameters as in the second step of the nested PCR, 60 positive clone check PCR products from each clone library (i.e. 20 clones per initial PCR product) were digested for 1 h at 65 °C with 5 U of TaqI (Fermentas) using 7 μl PCR product in a total volume of 20 μl and visualized by electrophoresis on 2% agarose gels. The size of each restriction fragment was determined with the SYNGENE software. Three to four representatives of each RFLP pattern were selected for purification and sequencing. The corresponding whole length ITS PCR products were purified as described above and sequenced in one direction using an ABI PRISM 3100 Genetic Analyzer and the BigDye Terminator V3.1 Cycle Sequencing Kit, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA).

2.4. Phylogenetic analyses

The closest matches to each sequence were determined using the BLASTN sequence similarity search tool in GenBank (Altschul et al., 1997) and retained as references. Sequences along with reference sequences were aligned using MAFT version 6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). The phylogenetic relations were inferred applying the Kimura-2-parameter model (Kimura, 1980) with the Neighbor Joining (NJ) algorithm (Saitou and Nei, 1987) as implemented in MEGA4 (Tamura et al., 2007). The confidence of branching was assessed by computing 1000 bootstrap resamplings (Felsenstein, 1985).

In accordance with Cullings and Vogler (1998) and Redecker et al. (1999), only the 5.8S subunit gene (160 bp) embedded between the spacers ITS1 and ITS2 was aligned across the complete sequence dataset and used to separate the main AMF groups. Then, inter- and intra-specific species resolutions were obtained by aligning 510 bp (*Glomus* Group A, sensu Schüßler et al. (2001)), 527 bp (*Glomus* Group B), 474 bp (Diversisporaceae) and 513 bp (Acaulosporaceae) of ITS sequences within each sequence cluster separated by the 5.8S alignment. Sequences falling into one clade, including reference sequences and showing sequence identities of at least 92%, were named according to the reference taxa. Other sequences not falling into one clade with a named reference sequence were termed as sequence types of the respective genus and numbered consecutively. If the sequence types were identical with those previously published in Zarei et al. (2008a), the same number was used. The cut off value of 92% was chosen according to Börstler et al. (2006) in order to reflect the natural sequence diversity found within and between the spores of the same AMF species (see also Wubet et al., 2004). DNA sequences of the full ITS region have been deposited at the NCBI GenBank under accession numbers FJ008589–FJ008666.

2.5. Statistical analyses

The diversity of AMF sequence types found at each site was analyzed by rarefaction analysis (Simberloff, 1978) using the analytical approximation algorithm (Hurlbert, 1971) embedded in the Analytic Rarefaction freeware program from Steven M. Holland (http://www.uga.edu/strata/software/Software.html). Based on the assumption that rarefaction curves generally show an exponential rise to an asymptote, the results were fitted to the formula $y = a(1 - e^{bx})$.