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Mycorrhizal colonization affects the elemental distribution in roots of Ni-hyperaccumulator *Berkheya coddii* Roessler

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ABSTRACT

The effect of arbuscular mycorrhizal fungi (AMF) on the distribution and concentration of elements in roots of Ni-hyperaccumulating plant *Berkheya coddii* was studied. Micro-PIXE (particle-induced X-ray emission) analysis revealed significant differences between AMF-inoculated and non-inoculated plants as well as between main and lateral roots. The accumulation of P, K, Mn and Zn in the cortical layer of lateral roots of inoculated plants confirmed the important role of AMF in uptake and accumulation of these elements. Higher concentration of P, K, Fe, Ni, Cu and Zn in the vascular stele in roots of AMF-inoculated plants than in the non-inoculated ones indicates more efficient translocation of these elements to the aboveground parts of the plant. These findings indicate the necessity of including the influence of AMF in studies on the uptake of elements by plants and in industrial use of *B. coddii* for Ni extraction from polluted soils.

1. Introduction

In recent years, plants hyperaccumulating metals have received a lot of attention (Rascio and Navari-Izzo, 2011), not only due to their unique ecological and physiological properties, but also because of their commercial potential in mining metals (phytomining) from low-grade surface ores or mineralized soils that are too metal-poor for conventional mining (Brooks et al., 1998; Chaney et al., 2007, 2000, 1997; Nicks and Chambers, 1998; Sheoran et al., 2009). Since in hyperaccumulators most accumulated metals are translocated to the aboveground parts of the plant, most research has focused on the aboveground organs. However, roots are the prime site of metals' uptake and therefore merit extensive study in the context of hyperaccumulation. Most previous studies on the roots of hyperaccumulators focused on the root physical characteristics, metal transporters in the roots or on the root exudates and their influence on metals' availability and uptake (reviewed by Alford et al., 2010).

Although the role of rhizosphere microorganisms in metal uptake by hyperaccumulating plants has recently been gaining recognition, studies of microorganisms in the roots and rhizosphere, as reviewed by Alford et al. (2010), have been conducted for only less than 10% of known hyperaccumulators. Most studies concerned the rhizospheric bacteria and their effect on metal hyperaccumulation (Abou-Shanab et al., 2003a, 2003b; Belimov and Dietz, 2000; Belimov et al., 2001; Li et al., 2007; Pal et al., 2007). Arbuscular mycorrhizal fungi (AMF), which are the most common symbiotic soil microorganisms, have gained less attention mainly due to the conviction that hyperaccumulating plants do not form mycorrhizal symbiosis. Since 2003, when the presence of AMF in hyperaccumulators has been for the first time reported in four Ni hyperaccumulating plant species from South Africa (Turnau and Mesjasz-Przybylowicz, 2003), an expansion of research on these remarkable interactions took place (Al Agely et al., 2005; Liu et al., 2005; Orłowska et al., 2011; Trotta et al., 2006; Vogel-Mikus et al., 2006; Wu et al., 2009).

The present study focuses on the Ni-hyperaccumulating plant *Berkheya coddii* which, due to high biomass and high Ni accumulation ability, has high potential in economical Ni phytomining (Brooks et al., 1998; Brooks and Robinson, 1998; Harris et al., 2009; Keeling et al., 2003; Robinson et al., 1999, 1997). *B. coddii* forms

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effective symbiosis with AMF, both in the field and under laboratory conditions and the AMF-colonized plants have significantly higher survival rate and better growth in comparison to the non-mycorrhizal ones (Orłowska et al., 2011; Turnau and Mesjasz-Przybylowicz, 2003). We have previously shown that mycorrhizal colonization affected foliar and root elemental concentration and content as well as the translocation of elements from roots to shoots, and this effect depended on the origin of AMF isolates (Orłowska et al., 2011).

In continuation, we report here on study of the localization of elements in lateral and primary roots of *B. coddii* plants inoculated with different AMF isolates, in relation to roots of the non-inoculated plants. By analyzing the lateral roots, which are directly colonized by mycorrhizal fungi, we aimed at studying the accumulation abilities of AMF. Analysis of the primary roots aimed at better understanding the AMF role in accumulation and translocation of elements to the aboveground parts of the plant. Detailed examination of elemental partitioning in roots should help in better understanding the hyperaccumulation mechanism, and how the microorganisms colonizing roots help plants to survive and thrive in ultramafic soil, as well as broaden general knowledge on the AMF influence on the structure and functionality of roots.

2. Materials and methods

2.1. Material

Non-sterilized seeds of *Berkheya coddii* Roessler (Asteraceae), collected from the field at Agnes Mine (South Africa), were germinated on vermiculite/perlite mixture (1:1 v/v) for 7–12 days and then the seedlings were transplanted into 500 ml pots containing soil collected from an ultramafic site at Agnes Mine as a substratum. The substratum contained 650 mg kg^{-1} of Ni (total concentration) and DTPA-extractable concentration of Ni was 160 mg kg^{-1}. The substratum had the mean pH value in H_2O of 5.0, and the following chemical characteristics: mean C content – 3.5%; mean P content (Bray II) – 15 mg kg^{-1}; mean Ca content – 2190 mg kg^{-1}; mean Mg content – 9930 mg kg^{-1}; mean Cr content – 2350 mg kg^{-1}; mean content Mn – 2270 mg kg^{-1}; CEC – 24 (meq/100 g). To eliminate indigenous mycorrhizal fungi, the substratum was pasteurized twice for 12 h at 90 °C, with one day of cooling at room temperature between the heating periods. After pasteurization, the soil was left to stabilize for 2 weeks before starting the experiment.

The plants were inoculated with a commercially available (BIORIZE, Dijon, France, E-1-99/Lav) isolate of *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler originating from non-polluted soil (former *Glomus intraradices* Schenk & Smith) (Krüger et al., 2012; Schüßler and Walker, 2010) or with inoculum containing a variety of fungi originating from the rhizosphere of the Ni-hyperaccumulating plant *B. coddii*, further referred to as "fungi from ultramafic soil": *Diversispora aurantia* ((Blaszk., Blanke, Renker & Buscot) C. Walker & A. Schüßler), *Funneliformis mosseae* ((T.H. Nicolson & Gerd.) C. Walker & A. Schüßler), *Funneliformis coronatum* ((Giovann.) C. Walker & A. Schüßler) and *Rhizophagus irregularis* ((Blaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler). As previously reported (Orłowska et al., 2011), fungi originated from ultramafic soil were more vital and more efficient in increasing plant growth and nutrient acquisition than fungal strain isolated from non-contaminated soil.

The inocula were produced in a pot culture system in association with *Plantago lanceolata* L. *R. intraradices* was propagated in a vermiculite:sand mixture (1:1 v/v), whereas fungi originating from the rhizosphere of *B. coddii* were propagated in a bulk ultramafic soil:sand mixture (1:1 v/v). Pot cultures were maintained for 10 weeks and 50 g of the dry rhizosphere soil from trap cultures containing spores, mycelium and colonized root fragments was used for plant inoculation. The non-inoculated plants (non-mycorrhizal control) received an equivalent amount of autoclaved soil:sand mixture. There were ten replicas of each treatment. The plants were grown in growth room conditions (light intensity of ca. 150 μmol $s^{-1}m^{-2}$; light regime 12/12 h; temperature 22–24 °C) for 15 weeks and they were in vegetative stage at the harvest. The mycorrhizal plants had much higher biomass and survival rate than the non-inoculated ones (Orłowska et al., 2011).

2.2. Preparation of the roots for micro-PIXE analysis

The roots of individual plants were carefully washed in deionized water and immediately frozen by plunging in liquid propane cooled by liquid nitrogen, using the Leica CPC Cryoworkstation, and next freeze-dried in the Leica EM CFD Cryosorption Freeze Dryer (Leica Microsystems AG, Austria). The freeze-drying process followed a long, 208 h programmed cycle. Freeze-dried roots were hand-cut with a steel razor blade. The cross-sections of the main roots and of the thin lateral, potentially mycorrhizal, roots have been prepared from the non-inoculated and inoculated plants.

The sections of the roots of non-inoculated and AMF-inoculated plants were taken from the similar distances from the root tip. The sections of the primary roots were taken from the upper part of the roots, positioned about 2 cm below the shoot. The sections of the lateral roots were taken from the lateral roots of first order because most non-mycorrhizal roots did not have lateral roots of higher order. In these roots, the sections were taken from the middle part of the region carrying the root hairs. The root sections were mounted between two layers of formvar film (0.5% formvar) coated with a thin carbon layer on the measurement side. The micrographs of samples were taken under the light stereo-microscope. Roots' anatomy was observed with a scanning electron microscope (SEM).

2.3. Distribution and quantification of elements by micro-PIXE

Elemental microanalyses were performed using the nuclear microprobe at the Materials Research Department, iThemba LABS, South Africa.

Microanalyses were performed as previously described (Prozesky et al., 1995; Przybyłowicz et al., 2005, 1999). A proton beam of 3 MeV energy was focused to a 3 × 3 μm^2 spot and scanned over the areas of interest, using square or rectangular scan patterns with a variable number of pixels (up to 128 × 128). Particle-induced X-ray emission (PIXE) and proton backscattering spectrometry (BS) were used simultaneously. PIXE spectra were registered with a Si(Li) detector with an additional 125 μm Be layer as an external absorber. The X-ray energy range was set between 1 and 36 keV. BS spectra were recorded with an annular Si surface barrier detector (100 μm thick) positioned at an average angle of 176°. Data were acquired in the event-by-event mode. The normalization of results was done using the integrated beam charge, collected simultaneously from a Faraday cup located behind the specimen and from the insulated specimen holder. The total accumulated charge per scan varied from 1.5 to 7 μC.

Quantitative results were obtained by standardless method using GeoPIXE II software package (Ryan, 2000; Ryan et al., 1990a, 1990b). The calibration of the analytical system was tested by measurements of standards – pure elements and synthetic glasses with known quantities of selected minor elements (internal standards). Quantitative elemental mapping was performed using *Dynamic Analysis* method (Ryan, 2000; Ryan and Jamieson, 1993; Ryan et al., 1995). This method generates elemental images which are (i) overlap-resolved, (ii) with subtracted background and (iii) quantitative, i.e. accumulated in mg kg^{-1} dry weight units. Maps were complemented by data extracted from arbitrarily selected microareas of roots (whole root, epidermis/exodermis, cortex, vascular tissues and pith). Additionally, in lateral roots, the regions of phloem and xylem, and also the part of cortical layer in which mycorrhiza structures occurred, have been selected for analyses. PIXE and BS spectra were employed to obtain average concentrations from these microareas using a full nonlinear deconvolution procedure to fit PIXE spectra (Ryan et al., 1990a, 1990b), with matrix corrections based on thickness and matrix composition obtained from the corresponding BS spectra, fitted with a RUMP simulation package (Doolittle, 1986) with non-Rutherford cross-sections for C, O, N. Matrix corrections done on the basis of BS spectrometry results were essential due to the highly variable thickness of analyzed specimens (the areal density range was between 1 and 7 mg cm^{-2}). Elemental concentrations from these areas are also reported in mg kg^{-1} dry weight.

2.4. Mycorrhizal studies

In the previous study we have shown that the level of mycorrhizal colonization was similar in plants inoculated with *R. intraradices* and with fungi from *B. coddii* rhizosphere (Table 1; Orłowska et al., 2011). To confirm that the chosen root cross-sections of inoculated plants contained the mycorrhizal structures, after PIXE analyses the root cross-sections of lateral roots were stained with one drop of 0.01% aniline blue in pure lactic acid for 3 h and next stored in pure lactic acid. The specimens were observed under a stereo-microscope. Only PIXE results from the roots in which the mycorrhizal structures were observed (Fig. 1), were used in further evaluation; then six cross-sections of the main root of different plants and six cross-sections of the thinnest roots were used for the final analysis.

2.5. Statistics

Data were analyzed with the non-parametric Kruskal–Wallis and the Mann–Whitney *U*-test ($p < 0.05$). The analyses were performed using Statistica (StatSoft).

3. Results

3.1. Morphology and anatomy of Berkheya coddii roots

The root system of *Berkheya coddii* was characterized by one primary root, several adventitious roots and thin lateral roots (Fig. 1a). AMF-inoculated plants had shorter primary roots but